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Effects of Dose and Glycosylation on the Transfer of Genistein into the Eggs of the Japanese Quail (*Coturnix japonica*)

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Soy isoflavones have been associated with several beneficial effects of soy in human diets. However, most soy is consumed by livestock in the Western countries. It is possible that isoflavones could be transferred and/or accumulated into animal products, which could become additional sources of dietary isoflavones for humans. Our objectives were to determine whether dietary isoflavone genistein could be transferred and/or accumulated into the eggs of Japanese quail (*Coturnix japonica*) and how the supplementation dosage and glycosylation of the isoflavone would affect this transfer. Adult reproductive female Japanese quail were randomly assigned to treatment groups that received encapsulated 50 or 100 mg genistein or 80 mg genistin per day (four quail per treatment) for 5 days. A control group (two quail) received placebo capsules. Eggs were collected prior to treatment and then daily for 15 days. The egg, separated into yolk and white, and pulverized quail diet were extracted in 80% methanol for 2 h and either centrifuged or filtered before evaporation of the solvent. The extracts were redissolved in 16% acetonitrile for high-performance liquid chromatography (HPLC) analyses. Genistein and genistein metabolites were detected in the egg yolks of treated quail. Trace concentrations of genistein were detected in the control group, due to the presence of genistein derivatives in the diet. Neither genistein nor its metabolites were found in egg white. Levels of genistein in the eggs increased significantly from the 3rd day of supplementation and reached the maximum about 2 days after the supplementation stopped. The higher dose of genistein supplementation resulted in higher genistein concentrations in egg yolks. Glycosylation decreased the transfer and accumulation of genistein into the egg yolks.

KEYWORDS: Genistein; genistin; egg yolk; Japanese quail; transfer; accumulation

INTRODUCTION

Interest in soy isoflavones has exploded in the past decade and continues to increase due to a wealth of data suggesting that these soy ingredients possess potent and wide-ranging biological activities (*1*). Soy isoflavones, also known as phytoestrogens, have a spatial conformation similar to that of native estrogens (*1*). These phytoestrogens can bind to the estradiol receptor (ER), especially to the ERβ receptor subtype (*2*), but with lower affinity than estradiol. Moreover, soy phytoestrogens are believed to exert both estrogenic and antiestrogenic activities. Isoflavones also possess nonhormonal activities, such as antioxidant (*3*, *4*) and antiproliferative (*5*, *6*) activities. Some beneficial effects that have been associated with isoflavones include prevention of breast, prostate, and colon cancers (*7*−*9*), lowering the risk of cardiovascular disease (*10*, *11*), relief of menopausal symptoms (*12*, *13*), and improvement of bone health (*14*, *15*).

With the awareness of potential benefits of isoflavones, efforts have been made to seek ways to incorporate isoflavones into the diet of people in Western countries that are unaccustomed to traditional soyfoods. However, currently, soy consumption is still very limited in the United States (*16*), due to the limited acceptability of the beany flavor of soyfoods and the dietary habits of the U.S. people (*17*). It is interesting to note that most soy meal is consumed by livestock, especially by poultry. The poultry industry consumed half of the U.S. soy meal produced in 2001 (*18*). It is possible that isoflavones in animal feed could be transferred and/or accumulated into the resulted animal products, such as meat and eggs. In this way, people could benefit from these phytochemicals through foods of animal sources, and the consumption of isoflavones could be increased without altering deeply rooted food habits. Eggs have been used for this strategy to increase the consumption of various fatty acids, minerals, and vitamins by modifying the diet of laying hens (*19*). Eggs could also be possibly used as the carrier of isoflavones. At the same time, soy isoflavones, which have been

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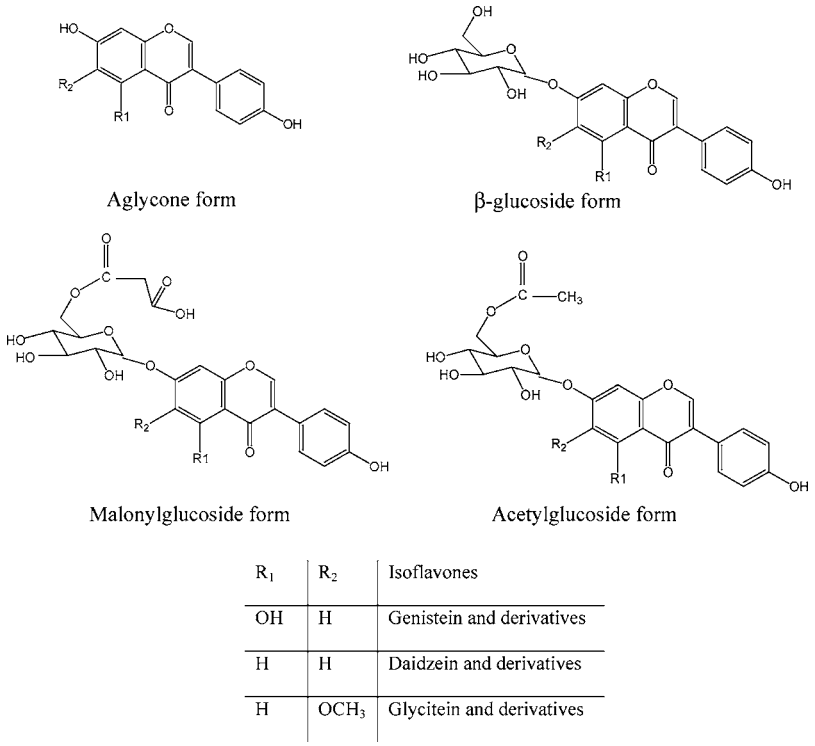

R_1	R_2	Isoflavones
OH	H	Genistein and derivatives
H	H	Daidzein and derivatives
H	OCH_3	Glycitein and derivatives

Figure 1. Four chemical forms of soy isoflavones.

reported to reduce plasma cholesterol (*20*) and LDL oxidation (*21*), may help increase the consumption of eggs, which has been reduced significantly due to their high cholesterol concentration (*22*). Eggs may become more appealing with the added protection effect of isoflavones, or the cholesterol concentration in the eggs may be directly reduced by the high content of isoflavones in the bird diet.

This study investigated the transfer and/or accumulation of isoflavones into the egg through dietary supplementation using Japanese quail (*Coturnix japonica*) as the animal model (*23*, *24*). The Japanese quail provides an advantageous avian model for studying the transfer of dietary additives into eggs because quail are prolific egg layers and regulation of the hypothalamic−pituitary−gonadal axis is similar to other domestic and wild birds (*25*). Soy isoflavones exist in the forms of aglycones (genistein, daidzein, and glycitein) or their β-glucoside derivatives (genistin, daidzin, and glycitin), acetylglucoside derivatives (6″-*O*-acetylgenistein, 6″-*O*-acetyldaidzin, and 6″-*O*-acetylglycitin), and malonylglucoside derivatives (6″-*O*-malonylgenistin, 6″-*O*-malonyldaidzin, and 6″-*O*-malonylglycitin) (**Figure 1**) (*26*). Because soy meal contains isoflavones in diversified structure forms, known concentrations of purified standards were used to determine the manner of transfer and/or accumulation of isoflavones into the eggs. Genistein has been most extensively studied and has the most potent estrogenic activity (*27*, *28*). However, isoflavones exist mainly in their glycosylated and acylated forms in soybean and soy products (*29*). Hydrolysis by stomach acid and conversion to the more active genistein by gut microflora or gut glucosidases appears to occur before absorption (*30*, *31*). However, recent studies suggest that isoflavones in their glycosylated forms are much less effectively absorbed than those in aglycone forms (*32*). Maternal deposition of lipophilic dietary components is a fundamental pathway for embryonic exposure. In mammals, this exposure often occurs during lactation. In birds, lipophilic chemicals, including estradiol, are maternally deposited in the egg yolk (*33*). Therefore, we tested transfer of both genistein of different doses as well as genistin, the β-glucoside derivative of genistein in Japanese quail.

The objectives of this study were (i) to determine if the soy isoflavone genistein is transferred and/or accumulated into the eggs of the Japanese quail; (ii) to ascertain if the dose of genistein affects transfer into the eggs; and (iii) to establish whether the glycosylation affects the transfer and/or accumulation of genistein into the eggs.

MATERIALS AND METHODS

Chemicals. Genistein and genistin standards for the supplementation were purchased from Toronto Research Chemicals (Toronto, Canada), and isoflavone standards (genistein, daidzein, glycitein, genistin, daidzin, glycitin, and acetylgenistin) for HPLC analyses were purchased from LC Laboratories (Woburn, MA). All of the isoflavone standards used had a purity greater than 99%, except for acetylgenistin, which had a purity greater than 98%. Acetonitrile, acetic acid, methanol, and flavone were HPLC grade reagents from Fisher Scientific (Fair Lawn, NJ). Sodium acetate trihydrate was from J. T. Baker Inc. (Phillipsburg, NJ).

Experimental Animals. Japanese quail (*C. japonica*) maintained at the Animal and Avian Sciences department, UMCP, were randombred and sexually mature in 8−9 weeks. The environment was temperature and light (15L:9D) controlled. Animals were maintained, and the experiment was conducted under the UMCP IACUC approved protocols.

Feeding Trial. To precisely administer certain amounts of genistein or genistin to Japanese quail, these isoflavone standards were put into small gel capsules. Japanese quail were randomly assigned to receive encapsulated 50 or 100 mg genistein or 80 mg genistin per day for 5 days, with four quail in each treatment group. A control group with two quail received placebo capsules. One small capsule (10 mm length $\times$ 0.4 mm diameter) containing the complete dose for each treatment (or empty for the control group) was given to each bird as a single dose per day. All of the quail had free access to Purina wild bird diet and water. The bird diet was mixed prior to use to ensure uniformity. Eggs were harvested daily from the first day of treatment for 15 days.

Isoflavone Extraction. Isoflavone extraction was based on the procedures described by Fukutake et al. (*34*). Isoflavones were extracted

from pulverized bird feed using 10 mL of aqueous methanol solution (80%), in a 1:5 (w:v) ratio, and using flavone as internal standard. The sample was homogenized for 2 h at room temperature and vacuum filtered through Whatman No. 41 filter paper (Whatman International Ltd., England) using a Buchner funnel. The filtrate then was evaporated using a rotary evaporator with a 40 °C water bath (Büchi, Switzerland). The extract was redissolved in 5 mL of a 16% aqueous acetonitrile solution and refrigerated for no more than 4 h before HPLC analysis.

A modification of the procedure was developed in our laboratory to extract isoflavones from eggs. Direct use of 80% methanolic solution caused denaturation of the protein and reduced solvent−sample interaction. To overcome this problem, the separated egg yolk sample or egg white sample was first homogenized with deionized water 1:1 (w:v) ratio, followed by addition of methanol to a final concentration of 80% methanol solution. This simple modification allowed more uniform homogenization of the samples, small particle size, and more efficient recovery of isoflavones from egg. After 2 h of stirring under room temperature, the samples were centrifuged (Beckman centrifuge J2-21M, Beckman Instrument Inc., Palo Alto, CA) at 33.6*g*, 10 °C for 10 min. The supernatant was collected, and the solvent was evaporated using a rotary evaporator with a 40 °C water bath. The sample was redissolved in 5 mL of 16% aqueous acetonitrile solution and refrigerated until HPLC analysis or for β-glucuronidase hydrolysis.

***β*-Glucuronidase Hydrolysis.** The isoflavone extract (1 mL) obtained from the egg sample was mixed with 5 mL of 0.1 M sodium acetate buffer (pH 5.0) and 100 μL β-glucuronidase (from *Helix pomatia*, with 131 000 β-glucuronidase units/mL and 3.180 sulfatase units/mL, Sigma Chemical Co., St. Louis, MO), and the mixture was incubated at 37 °C for 5 h. After the enzyme reaction, the mixture was passed through Sep-Pak C18 cartridge (Waters Co., Milford, MA), previously activated with methanol. Isoflavones were bound to the cartridge while the enzymes and sodium acetate were washed away using 16% acetonitrile. Isoflavones were then recovered with 80% methanol. The eluent was concentrated in a rotary evaporator with a 40 °C water bath. The concentrate obtained was redissolved in a 1 mL of 16% acetonitrile solution and refrigerated until HPLC analysis.

HPLC Analysis. Analysis of isoflavones was carried out using reversed-phase separation of the compounds on a C18 column (Waters Corp.) and using a linear gradient composed of acidified water (A: 0.1% acetic acid and 5% acetonitrile in water) and acidified acetonitrile (B: 0.1% acetic acid in acetonitrile) as the eluent. A high-pressure liquid chromatography (Waters Delta 600 system) equipped with a photodiode array detector (Waters 996), autosampler (Waters 717plus), and Millennium^{32} software (Waters Corp.) was used. Elution was monitored at 259 (egg samples) or 254 nm (diet samples), and spectral data from 200 to 450 nm were recorded and stored over the time of the run on all samples.

Separation of Isoflavones in the Diet. After the injection of 50 μL of the diet extract sample, the linear gradient started from 10% B to 14% over 10 min, then increased to 20% over 2 min, maintained at 20% for 8 min, continued to increase to 70% over 10 min, maintained at 70% for 3 min, and returned to 10% at the end of the 34 min running time. The flow rate was maintained at 1 mL/min throughout the running of the sample.

Separation of Isoflavones in the Egg. After the injection of 150 μL of the egg (yolk or white) extract sample, the linear gradient started from 16% B to 70% over 15 min, maintained at 70% for 3 min, and returned to 16% at the end of the 20 min running time. The flow rate was maintained at 1 mL/min.

Isoflavones were identified by comparing spectral data and retention times to those of pure standards. Calibration curves, prepared by using different concentrations of pure isoflavone standards (genistein, daidzein, glycitein, genistin, daidzin, glycitin, and acetylgenistin) and flavone (used as internal standard), were used for quantitation of the isoflavones in the samples. Losses of internal standard during sample preparation and analysis are expected to correlate with losses of the compounds of interest. Quantification of isoflavones was based on the calibration curves and corrected with the peak area ratio of flavone and isoflavones. Because of a lack of reference standards for isoflavones in malonylglucoside form, malonylglucosides were calculated from the β-glucoside standards, since the molar extinction coefficients of the malonylglucoside conjugates approximate those of the β-glucoside conjugates (*26*).

Statistical Analysis. The experiment was carried out according to a CRD with four replicates for treatment groups (daily supplementation of capsulated genistein 100 mg, genistein 50 mg, and genistin 80 mg) and two replicates for the control group (placebo capsules). Each Japanese quail was considered as an experimental unit. It was assumed that data from different experimental unit were independent. Statistical analysis was done by using the SAS/STAT package (version 8.1, 1999, SAS Institute Inc., Cary, NC). Logarithmic data transformation was applied to the concentrations to minimize correlation between the mean and the variance of the data. ANOVA and repeated measure analysis using the MIXED procedure were conducted.

RESULTS AND DISCUSSION

Detection of Genistein in the Egg Yolk. Genistein was detected in the egg yolks of both genistein- and genistin-treated groups (**Figures 2** and **3**) and in some egg yolk samples of the control quail, which could be due to the presence of isoflavones in the diet (**Figure 4**). There were no detectable isoflavones found in any egg white samples. The chromatograms obtained from the egg yolks of birds fed genistein aglycone, at the two different doses, were very similar in pattern and reflected the two doses of the compounds. However, the chromatograms of the egg yolk samples from the birds fed the glucoside form, genistin, showed a slight difference in pattern (**Figure 2**). In both of the chromatograms shown in **Figure 2**, peak 1 and peak 2 were not identified as isoflavones based on their spectrum characteristics. In addition, peaks 1 and 2 were not affected by the feeding trials and appeared in all egg yolk samples at about the same concentrations when comparing within quail. Peak 3 was identified as genistein, because its retention time and spectrum matched those of the genistein reference standard. Peak 4 and peak 4′ both had spectrum characteristics similar to that of genistein but different retention times (**Figure 3**), suggesting that they might be genistein-related compounds with different chemical structures, possibly originated from the metabolite products of genistein or genistin. However, genistin was not detected in any egg samples, even in the egg samples from the bird supplemented with genistin.

Isoflavones in the Feed. Isoflavones were found in the bird diet, due to the use of soy meal as an ingredient in the diet (**Table 1**). Because each quail consumed about 14 g of feed daily (*35*), an animal consumed on average about 2.7 mg of total genistein, 2.1 mg of total daidzein, and 0.4 mg of total glycitein from the feed, in addition to the supplementation. The detected genistein in the eggs from control group may be due to genistein and its derivatives present in the diet. However, no soy isoflavones other than genistein were found in the eggs, even though the diet contained about the same total amount of genistein and daidzein. This suggests that genistein transferred more efficiently into the eggs as compared to daidzein. Other studies have shown more genistein than daidzein retained in the plasma, while more daidzein than genistein was excreted in urine (*36*). The relatively lower hydrophilicity of genistein as compared to daidzein could partially explain the difference between genistein and daidzein relative to transfer into the eggs (*36*). Further study is needed to confirm this hypothesis.

Egg Production Rate. Egg production was monitored during isoflavone supplementation, since isoflavones have been reported to interfere with reproduction in sheep (*37*), pig and cattle (*38*), and California quail (*39*). Furthermore, maintenance of egg production rate would limit dietary supplementation of isoflavones through poultry diets. In the current study, egg production rates per quail per day were as follows: genistein,

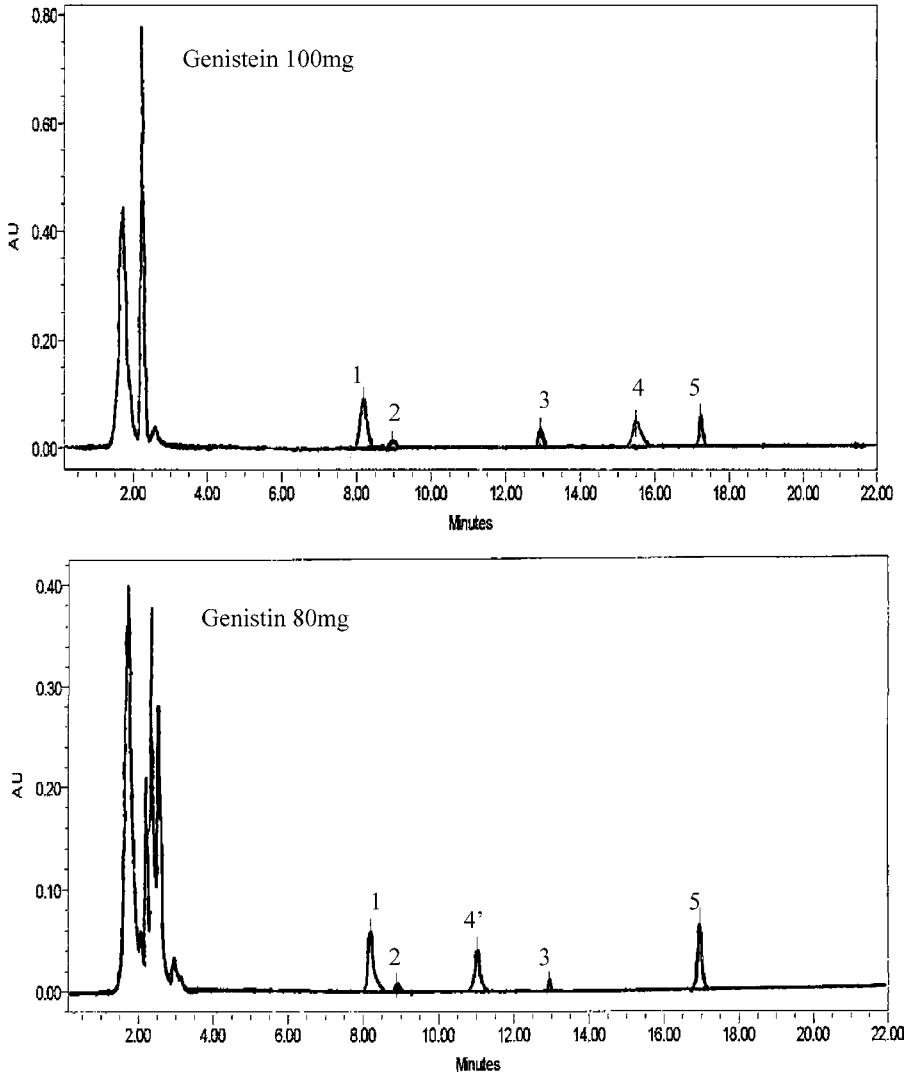

Figure 2. HPLC chromatograms of the egg yolk samples from genistein 100 mg group and genistin 80 mg group at day 6 of supplementation.

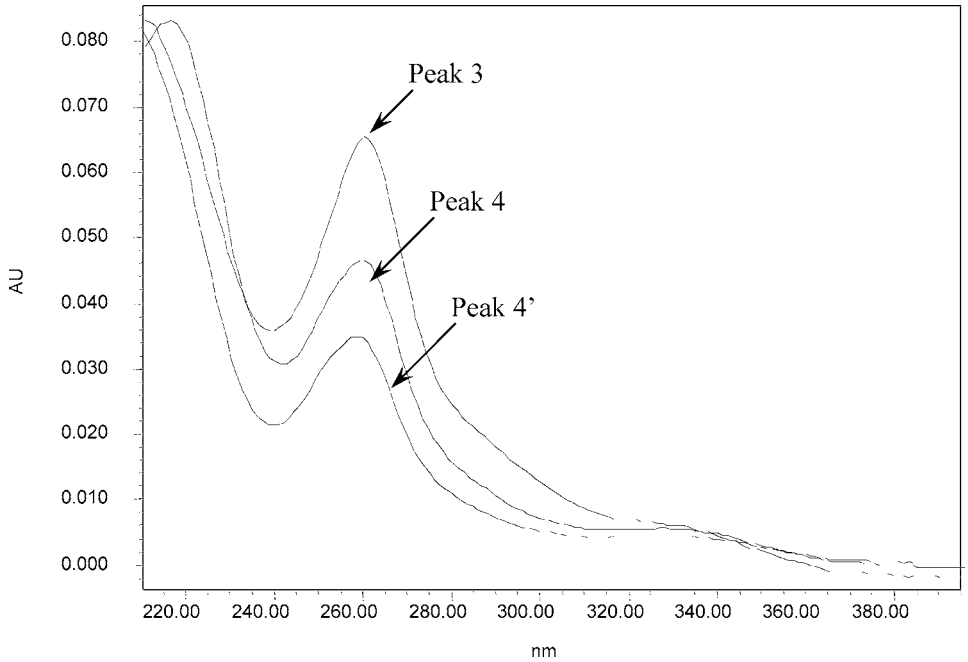

Figure 3. Spectra of genistein and genistein-related peaks in the egg yolk samples.

50 mg = 0.88 ± 0.032; genistein, 100 mg = 0.88 ± 0.074; genistin, 80 mg = 0.88 ± 0.069; and control = 0.88 ± 0.069, with no significant difference ($P = 0.8774$) among treatment and control groups. No abnormality was observed in the health

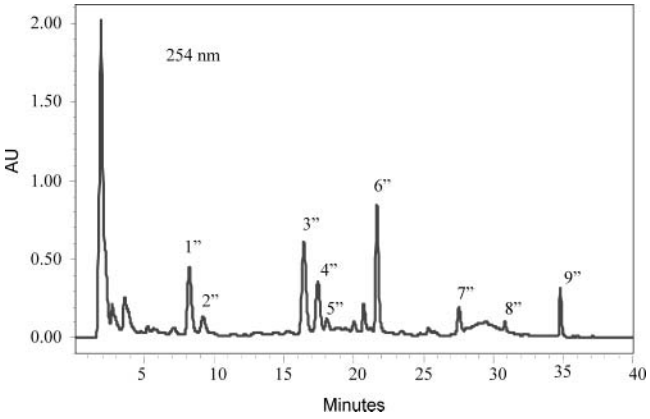

Figure 4. HPLC chromatograms of the Purina bird diet sample.

Table 1. Isoflavone Contents in Purina Bird Diet (mg/g)[a]

	genistein and derivatives	daidzein and derivatives	glycitein and derivatives
aglycone	0.004	ND	ND
β-glucoside	0.088	0.079	0.016
acetylglucoside	0.018	ND	ND
malonylglucoside	0.084	0.069	0.010
total	0.194	0.148	0.026

[a] Means from three replicates. ND, not detected. Individual isoflavones concentrations were adjusted for molecular weight difference, i.e., normalized to the aglycone form.

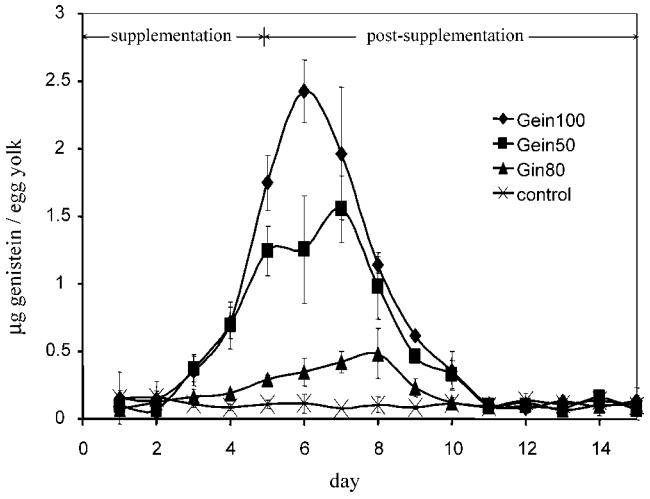

Figure 5. Changes of the amount of genistein in egg yolk of all groups over the experiment period.

status and other behaviors of the laying quail, in either the treated or the control groups, during the period of the experiment.

Changes of the Genistein Concentration in Egg Yolks. **Figure 5** shows the means of genistein concentrations in the egg yolks for each treatment or control group over the 5 days of treatment and 10 days posttreatment. No significant differences were detected between control and treatment groups by univariate ANOVA for the initial days of treatment (days 1 and 2) and over the last 5 days of egg collection (days 11−15) because formation of the yolk requires several days and yolks in eggs laid early in the treatment would not be expected to contain high levels of genistein. Similarly, yolks formed late in the trial would develop at a time in which there were diminishing stores of genistein from the treatments. Isoflavones had been depleted after treatment had stopped.

The concentrations of genistein in the yolk of treatment groups were significantly higher than that of the control group from day 3 to day 10 ($P < 0.05$). On the basis of these results, treatment and control groups were compared by repeated measure analysis using data only from day 3 to day 10. Repeated measure analysis was employed since the data from eggs laid by the same quail were correlated. The ARH(1) model was chosen as the variance/covariance structure model, based on the model fitting statistics. The statistical analysis showed a significant day effect ($P < 0.0001$) and confirmed the observed increase and then decrease trend of genistein concentration in the egg yolk of the treated group over the time.

Genistein concentrations in the egg yolk of all treatment groups increased gradually from day 3, reached the maximum about 2 days after the treatment stopped, and then gradually decreased to the baseline level after day 10. This pattern suggests that genistein was not only transferred into the eggs but also accumulated in the yolk during growth of the follicle and associated yolk deposition during egg formation. In this study, the highest amount of genistein in the egg yolk obtained was less than 3 μg/egg yolk, which is relatively low as compared to the amount of supplementation (100 mg/day). We would expect to find higher accumulation if the period of genistein supplementation were prolonged and the supplementation were increased, as we did not observe a plateau in concentration. The trace amounts of genistein in the control group remained unchanged over the experiment period, indicating that genistein in the bird diet containing soybean meal was also transferred into the egg yolk. However, the unchanged genistein level suggests that consistent exposure to low dose of genistein supplementation reached a plateau concentration of a relatively low level in the egg yolks.

Effects of Dose and Glycosylation. Repeated measure analysis showed that the genistein 100 mg treatment differed significantly from that of genistein 50 mg ($P = 0.0281$), indicating a dose effect on the transfer and accumulation of genistein into the egg yolks. The highest amount, 1.6 ± 0.2 μg/egg yolk, was reached at day 7 in the 50 mg genistein group, and 2.4 ± 0.2 μg/egg yolk was reached at day 6 in the 100 mg genistein. The exact relationship between the dietary dosage and the transfer into the egg yolks cannot be clearly determined yet because only two doses were examined. However, the data demonstrate that the concentration of genistein in the eggs increased with an increasing dose of genistein. Again, studies are needed to determine the maximum transfer level associated with a dietary dose of genistein.

Genistein aglycone and the glucoside counterpart genistin were administered at equivalent mole amounts. If genistin were readily converted to its aglycone genistein and absorbed as suggested by some studies (*30*, *31*), we would expect to see no difference in the egg accumulation rate between the genistin 80 mg group and the genistein 50 mg group. In this study, supplementation with the glucoside form resulted in a significantly lower genistein concentration in the egg yolk than supplementation with the aglycone form ($P = 0.001$). In the genistin 80 mg group, the highest amount, 0.5 ± 0.2 μg/egg yolk, was reached at day 8. This result was consistent with the finding of Izumi et al. (*30*) that isoflavone aglycones were absorbed faster and in higher amounts than their glucosides. Genistin was not detected in the egg yolks of the genistin-treated group. Until now, no evidence has been found to support direct absorption of the conjugated forms of isoflavones (*40*, *41*).

Genistein-Related Compounds. In the egg yolks of both genistein- and genistin-treated groups, we found compound(s)

with spectral characteristics matching those of genistein and were therefore suspected as the metabolite(s) of genistein or genistin. We found that the concentrations of these compounds also had a similar temporal pattern to genistein over the experiment period according to the peak area on the HPLC chromatograms. This pattern was in agreement with the hypothesis that these compounds are formed as a result of the genistein or genistin supplementation. The genistein-related peak of the genistin-treated group (peak 4′) appeared earlier than that of the genistein-treated group (peak 4) on the HPLC chromatograms (**Figure 2**). It could be speculated that the metabolite(s) from genistin are more hydrophilic than those from genistein. Selected egg samples were submitted to β-glucuronidase (with some sulfatase activity) hydrolysis, since the major metabolites of isoflavones found in urine and plasma were glucuronides and sulfates (*42*, *43*). After the enzyme treatment, the total amount of genistein was drastically increased, with a clear reduction on the incidence of the genistein-related peaks. This suggested that the metabolite(s) of genistein or genistin might be in the form of glucuronide or sulfide conjugates. The increase of the genistein concentration after enzyme hydrolysis was always observed, but high variability was obtained in the magnitude of the increase. It has been reported (*44*) that intestinal microflora may play an important role in the isoflavone metabolism. This large variability may be due to the interindividual differences in gut microflora. We found up to a 3-fold increase of genistein concentration in the egg yolk samples from the quail on the genistein aglycone treatment and up to a 4-fold increase in samples from the quail on the genistin glucoside treatment.

CONCLUSION

In summary, genistein was transferred and accumulated into the egg yolks of Japanese quail by dietary supplementation of genistein or genistin. The higher dietary dose resulted in a higher genistein concentration in egg yolks during the peak period, indicating a dose effect. Glycosylation in genistin decreased the transfer and accumulation of genistein into the egg yolks. These results suggest the possibility and utility of developing isoflavone-enriched eggs. Even though the genistein concentrations found in this study were low, it may be possible to increase accumulation by increasing the length of the supplementation period. The concentrations of isoflavones in the egg yolk could also be altered by changing the concentration of isoflavones in the diets. Aglycone isoflavones would be more effectively absorbed and transferred to the egg yolk than their glycosylated conjugates.

ABBREVIATIONS USED

ANOVA, analysis of variance; ARH(1), heterogeneous first-order autoregressive; CRD, completely randomized design; HPLC, high-performance liquid chromatography; IACUC, Institutional Animal Care and Use Committee; LDL, low-density lipoprotein; UMCP, University of Maryland, College Park.

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