Lycopene Incorporation into Egg Yolk and Effects on Laying Hen Immune Function

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ABSTRACT Carotenoids are partially responsible for the colors of plants and when consumed by humans and animals are deposited into tissues (e.g., skin and egg yolk in laying hens) and may have health benefits. Because carotenoids are more available when consumed from egg yolk sources than vegetables, this research examined the ability of the laying hen to deposit dietary lycopene, a carotenoid that imparts red color in tomatoes, into the egg yolk and to investigate effects on immune function. All birds were housed in commercial cages, had ad libitum access to water, and were fed 100 g/bird per day. Experiment 1 consisted of 4 dietary concentrations of lycopene (0, 65, 257, and 650 mg of lycopene/kg of diet). High-performance liquid chromatography analysis confirmed that dietary lycopene was incorporated into egg yolks. Experiment 2 was a completely randomized design, with 3 concentrations of lycopene (0, 420, and 840 mg of lycopene/kg of diet) and 6 concentrations of α-tocopherol (0, 84, 164, 200, 284, and 364 mg of α-tocopherol/kg of diet). Egg yolk lycopene (P < 0.05) and vitamin E (P < 0.05) were increased with increasing dietary concentrations, whereas lutein and zeaxanthin concentrations remained constant. Immune responses (inflammatory, cutaneous basophil hypersensitivity, 1° and 2° antibody response) were induced but were not affected by dietary lycopene or vitamin E. These data indicate that lycopene can be incorporated into egg yolks, and at these dietary concentrations, α-tocopherol and lycopene may not affect the immune system of the laying hen.

Key words: carotenoid, lycopene, egg yolk, immune, α-tocopherol

INTRODUCTION

The color of an egg yolk is affected by several different components in the laying hen diet. For instance, different lipid profiles of feed can affect egg yolk color as seen with the addition of conjugated linoleic acid to diets (Suksombat et al., 2006). Egg yolk color is also influenced by the type and concentration of carotenoids present (Leeson and Caston, 2004; Karadas et al., 2006). Carotenoids are pigments found naturally in the plant-based feedstuffs that laying hens consume; once consumed, some of the carotenoids are transferred to egg yolk.

Consumption of several carotenoids by humans has been associated with health benefits. One example is lutein, an oxygenated carotenoid found in egg yolk, which has been shown to prevent macular degeneration and cataracts (Olmedilla et al., 2003). Research demonstrates that carotenoid absorption in the human intestine is increased when consumed with lipids, suggesting that eggs are a good delivery system for some carotenoids. This is the case with lutein, which is more bioavailable from egg yolk compared with spinach or supplements (Chung et al., 2004). In addition to lutein, lycopene is another carotenoid with health benefits in humans, and lycopene consumption is associated with decreased cancer risk. Lycopene functions as an antioxidant, effectively lowering oxidative damage to DNA in cancerous cells and specifically prostate cancer cells (Wertz et al., 2004; Stacewicz-Sapuntzakis and Bowen, 2005). Lycopene can slow cell cycle progression by down-regulating proteins important in cell growth (Wertz et al., 2004) and by increasing gap-junction communication thus controlling cell proliferation (Campbell et al., 2004); however, these mechanisms are likely multifactorial.

The objective of the present study was to determine the ability of the laying hen to incorporate lycopene into egg yolk. Lycopene is fat-soluble and may compete with other fat-soluble compounds for deposition into egg yolk. Therefore, the effect of lycopene on vitamin E (α-tocopherol) yolk deposition was also examined.
Vitamin E is a general term for a group of 8 different isomers including 4 tocopherols and 4 tocotrienols. The α-tocopherol form is readily absorbed by the human body and is the common form of vitamin E in supplements. Vitamin E has many human health benefits associated with it, most notably its antioxidant capabilities, which help prevent cardiovascular disease by preventing oxidation of low-density lipoprotein (Chu and Liu, 2004). Along with the health benefits to humans, there are also potential benefits for the laying hen. For example, α-tocopherol supplementation can help counteract the egg quality deterioration that occurs as a result of heat stress (Kirunda et al., 2001) and can modulate several components of the avian immune response (Leschinsky and Klasing, 2001). Additionally, egg α-tocopherol concentrations can be enriched by addition of this compound to the diet of a laying hen (Frigg et al., 1992; Grobas et al., 2002), which provides an opportunity to simultaneously improve the human food supply as well as the health of the laying hen.

The primary objective of this research was to examine the ability of the laying hen to deposit lycopene into egg yolk and to determine optimal dietary concentrations. The second objective was to examine the possibilities of incorporating both lycopene and α-tocopherol into the egg yolk when feeding the two in the same diet and to explore any effects of these dietary treatments on the immune system of the hen.

MATERIALS AND METHODS

For each of 2 trials, chickens (Hyline W36, Single Comb White Leghorn) were fed a basal diet formulated to meet or exceed all nutrient requirements for laying hens (NRC, 1994; Table 1). For each of the 2 experiments, the basal diet was supplemented with different concentrations of lycopene. Dietary concentrations of lycopene were chosen based on the amount of lycopene contained in an uncooked 100-g tomato (2.57 mg of lycopene, USDA, 2005; Nutrient Database Number 11529), as well as data concerning the absorption and egg yolk deposition of lutein by laying hens, which deposited ~10% of lutein intake for diets with 125 mg/kg of lutein but only ~3% with 500 mg/kg of lutein (Leeson and Caston, 2004). Lycopene was supplied in a 5% powder form on a gelatin carrier that also contained 1% dl-α-tocopherol (DSM Nutritional Products Inc., Basel, Switzerland). Previous experiments in our laboratories demonstrated that in this form, lycopene was not absorbed by the laying hen (unpublished data), and therefore, water (60°C) was used to solubilize the lycopene before dietary addition. α-Tocopherol was added in the form of α-tocopherol acetate (500 IU/g, DSM Nutritional Products Inc.). The California Polytechnic State University Animal Care and Use Committee approved all protocols.

Experiment 1

Using a completely randomized design, 23-wk-old first-cycle White Leghorn laying hens from the California Polytechnic State University flock were randomly assigned to 1 of 4 dietary lycopene concentrations. Birds were fed diets containing 0, 65, 257, or 650 mg of lycopene/kg of diet. Hens were housed in commercial-type cages (n = 3 cages/diet, 4 birds/cage, 871 cm²/bird) with ad libitum access to water. At d 15, eggs were collected, yolk color scores were determined, and HPLC analyses were conducted.

Experiment 2

Using a completely randomized design, 39-wk-old first-cycle White Leghorn laying hens (not previously used in lycopene trials) from the California Polytechnic State University flock were randomly assigned to 1 of 6 dietary treatments. Dietary treatments were as follows (mg of lycopene/kg of diet, mg of α-tocopherol/kg of diet): diet 1 (0, 0), 2 (420, 84), 3 (840, 164), 4 (0, 200), 5 (420, 284), and 6 (840, 364). Supplemental α-tocopherol was added to diets 4 to 6 at 200 mg/kg; however, final α-tocopherol concentrations were greater, because the lycopene supplement contained α-tocopherol as well. Hens were housed in commercial cages (n = 4 cages/
diet, 4 birds/cage, 871 cm²/bird) with ad libitum access to water. Whole egg, albumin, and yolk weights were determined for eggs collected on d 15.

Subsequent to egg parameter measurement, several parameters of the immune response were measured for samples collected at 15 d of dietary treatment. Two birds per cage were used to examine inflammatory immune response to lipopolysaccharide (LPS), 1 bird per cage was used to examine cutaneous basophil hypersensitivity, and 1 bird per cage was used to examine antibody responses.

Inflammatory immune response was measured following stimulation with LPS (intraabdominal injection, 1 bird/cage, 4 birds/diet, at 1 mg of LPS/kg of BW; L7261, Sigma-Aldrich, St. Louis, MO). Control birds were not injected, because saline injection does not induce an inflammatory immune response (Laurin and Klasing, 1987). At 24 h postinjection, blood was collected from the LPS-injected bird and the control bird from each pen, and then birds were killed via cervical dislocation. Body, spleen, and liver weights were collected, and spleen and liver weights were expressed as a percentage of total BW. The acute phase protein that sequesters iron during a bacterial invasion, haptoglobin, was measured in plasma (LPS birds and respective control birds) with a phase haptoglobin assay from Tridelta Diagnostics (Morris Plains, NJ). This kit examines iron binding by colorimetric detection. There is new evidence that chickens have a haptoglobin analog that is similar in function to mammalian haptoglobin but not in sequence (Wicher and Fries, 2006). The kit used in this experiment does not use antibody-specific binding but rather iron binding, so it is likely that the haptoglobin analog is being measured.

Cutaneous basophil hypersensitivity, as a measure of heterophil and macrophage-mediated immune responses (Koutousov et al. 2007), was measured in 1 bird per cage (4 birds/diet) after stimulation with phytohemagglutinin-P (PHA, L8754, Sigma-Aldrich) at 200 μg/bird (0.1 mL of 2 mg of PHA/mL of PBS stock solution). This dose was chosen based upon previous research (El-Lethey et al., 2003). Two birds per pen were injected with PHA into the right toe web of the right foot, and PBS was injected into the right toe web of the left foot as a control. Thickness of left (control) and right (PHA-injected) toe webs were measured at 6 and 24 h postinjection using a digital micrometer (Fisher Scientific, Pittsburgh, PA). The degree of swelling was determined by subtracting the thickness of the PHA-injected toe from the PBS-injected toe.

Humoral immunity was measured in 1 bird per cage (4 cages/diet) with a series of 2 vaccinations of 2,4-dinitrophenyl conjugated with keyhole limpet hemocyanin (324121, Calbiochem, La Jolla, CA) at 3.33 mg of keyhole limpet hemocyanin/kg of BW i.v. in the wing web (1 bird/cage, same bird for primary and secondary injection). The dose concentration was based on previous research by Hangalapura et al. (2003). Primary antibody response was measured in plasma at 10 d post primary injection (i.e., d 25 of dietary treatment), and secondary antibodies were measured at 15 d (5 d post secondary injection; i.e., d 30 of dietary treatment) by ELISA assay as described previously (Arias and Koutousov, 2006).

Hens in both experiments were fed their assigned diets at 100 g/bird per day for 15 d because a laying hen requires approximately 14 d for a follicle to develop. Eggs were collected daily, and preliminary analysis of lycopene incorporation was completed using the Roche yolk color score fan (standardized set of color swatches corresponding to a number from 1 to 15). Whole yolks were separated and matched to the most appropriate color swatch by the same individual (treatments were blinded) for each experiment. High-performance liquid chromatography analysis (DSM Nutritional Products Inc.) quantified the concentration of lycopene (experiments 1 and 2), lutein, zeaxanthin, and α-tocopherol in the same yolks used for color scoring. The HPLC samples consisted of 2 homogenized egg yolks from the same pen on the same date (d 15); homogenized samples were placed into 15-mL tubes, wrapped with foil, and held at −20°C until analyzed. Whole livers were obtained from birds in experiment 2 and frozen individually at −20°C. High-performance liquid chromatography analysis was conducted to determine the concentration of α-tocopherol in the livers.

The analysis for lycopene was conducted using HPLC according to DSM Nutritional Products protocol (Schierle et al., 2003a). Briefly, yolk samples were mixed with maxatase and demineralized water, followed by ethanol and dichloromethane. The residue of the evaporated solution was resuspended in ethanol:tetrahydrofuran (9:1). An aliquot was injected into the HPLC system with a Suplex pKb-100, 5 μm, 250 cm × 4.6 mm column (Supelco, St. Louis, MO). The run time was 25.6 to 25.8 min, and lycopene was detected at 445 nm using a standard of all-trans-lycopene. The HPLC procedures for lutein and zeaxanthin were carried out according to DSM Nutritional Products protocol (Schierle et al., 2003b). Briefly, yolk samples were mixed with maxatase and demineralized water, followed by ethanol and dichloromethane. The extract was purified on silica gel using solid phase extraction columns and 300 mg of silica gel, and carotenoids were eluted using n-hexane:diethyl ether (1:1) and then evaporated. Samples were suspended in n-hexane:acetone (81:19) before being injected into the HPLC system with a LiChrosorb Si60, 5 μm, 250 × 4 mm column (Merck, Darmstadt, Germany). The run time for zeaxanthin was 12 and 13.3 to 13.5 min, lutein was 12.8 to 13.6 min, and carotenoids were detected at 450 nm using a standard of all-trans-zeaxanthin. High-performance liquid chromatography analysis of α-tocopherol was completed according to DSM Nutritional Products protocols (DSM Nutritional Products, 2005). Yolk or liver samples were used for analysis. Egg yolks were diluted with deionized water and then mixed with ethanol and petroleum ether. Liver samples were prepared with ascorbic acid, etha-
nol, and petroleum ether. For each sample, isooctane was added, and then the isooctane layer was removed. This fraction was injected into the HPLC system with a Chromegasphere, 5 μm, spherical silica 15.0 × 4.6 mm column (ES Industries, Marlton, NJ). The run time was 15 min; α-tocopherol was detected at 325 nm using α-tocopherol acetate standards.

Statistical analysis was performed using 1-way ANOVA for experiment 1 (main effect of diet) and a 2-way ANOVA for experiment 2 (main effect of diet, immune parameter, and their interaction) with JMP statistical software version 4 (SAS Institute Inc., Cary, NC). When the main effects or interaction were significant (**P < 0.05**), Tukey’s least squares means was used to determine differences between means. Egg yolk lycopene concentrations were also examined using regression analysis to determine optimal yolk lycopene incorporation relative to dietary lycopene concentrations.

**RESULTS**

**Experiment 1**

The Roche yolk color score fans can be used to assign a numeric value to describe the relative color of the egg yolk; a darker yolk corresponds to a greater numerical score. After 15 d of feeding the assigned diets, egg yolk color was significantly affected by dietary lycopene concentration (**P < 0.01**). Eggs from hens fed 65, 257, or 650 mg of lycopene/kg of diet had significantly greater yolk color score (mean = 11.5, 12.0, 12.0, respectively) than that of eggs from hens fed 0 mg of dietary lycopene (mean = 8.5; **P < 0.05** for each).

High-performance liquid chromatography analysis of egg yolks also showed a significant effect of diet (**P < 0.05**). Eggs from hens fed 0 mg of lycopene had no detectable lycopene concentrations (<20.00 μg/kg), whereas eggs from hens fed 257 or 650 mg of lycopene/kg of diet had significantly greater lycopene concentrations as compared with those fed 65 mg of lycopene/kg of diet (**P < 0.05** for each; Figure 1). The regression analysis results predict that optimal lycopene incorporation into egg would occur at 420 mg of lycopene/kg of diet (Figure 2).

**Experiment 2**

Similar to experiment 1, yolk lycopene concentrations were influenced by diet lycopene concentrations. Lycopene fed at 420 and 840 mg/kg of diet resulted in yolk lycopene concentrations significantly greater (**P < 0.05**) than diets with no added lycopene (Figure 3). Birds fed diets containing supplemental α-tocopherol (from either source) had egg yolks with greater (**P < 0.01**) α-tocopherol concentrations (Figure 4A). Liver α-tocopherol concentrations increased with each increase in dietary α-tocopherol content (Figure 4B). Liver mass as a percentage of BW was greater (**P <
0.01) in hens fed α-tocopherol without added lycopene, as opposed to those fed diets without added lycopene or α-tocopherol (mean = 0.020 ± 0.001%, 0.024 ± 0.001% respectively; \( P < 0.01 \)). Whole egg, albumin, and yolk weights were unaffected by dietary treatments (60.63 g ± 0.38, 33.97 g ± 0.29, 18.90 g ± 0.18, respectively; \( P > 0.35 \) for all). Lutein and zeaxanthin concentrations were also unaffected by dietary treatments (\( P = 0.21 \) and 0.16; mean = 9.97 mg/kg of egg yolk ± 0.53 and 8.03 mg/kg of egg yolk ± 0.40, respectively).

The immune parameters measured in this trial were not affected by dietary treatment (\( P > 0.05 \) for all), although immune responses were induced. At 24 h postinjection, LPS-stimulated birds had increased liver weight (17% increase; \( P < 0.01 \)), spleen weight (26.5% increase; \( P < 0.01 \)), and haptoglobin concentrations (107% increase; \( P < 0.05 \)), compared with control birds. Similarly, the cutaneous basophil hypersensitivity reaction was induced by PHA, based upon increased swelling (6 h of swelling = 93% increase; 24 h of swelling = 50% increase: \( P < 0.01 \)). Finally, antibody responses were stimulated by keyhole limpet hemocyanin vaccination (primary titers 7.02 ± 4.64 and secondary titers 8.20 ± 5.44, \( \log_2 \) units).

DISCUSSION

The primary goal of this research was to establish a method to enrich eggs with lycopene and to determine optimal concentrations of dietary inclusion. These experiments demonstrate that supplementation of laying hen diets with at least 65 mg of lycopene/kg of diet resulted in detectable egg yolk lycopene deposition. The inclusion of lycopene in egg yolk was first evaluated subjectively with yolk color as an indicator. Carotenoids contribute to egg yolk color, and therefore, changes in dietary carotenoids should modify the color of egg yolk. In these trials, yolk color scores were greater from eggs laid by hens fed lycopene-enriched diets compared with those fed control diets. This response was expected, because lycopene is a red pigment and is darker than the yellow lutein and zeaxanthin pigments normally present in egg yolk. However, there was no difference in color score across the lycopene-enriched groups. This is an important consideration, because consumers judge the quality of an egg by the interior color (Karunajeewa et al., 1984). Color score is a good visual indication of the yolk profile being changed, but HPLC analysis was completed to quantify lycopene incorporation into egg yolks.

High-performance liquid chromatography results demonstrated that when birds were fed \( \geq 65 \) mg of lycopene, yolk lycopene deposition was \( \sim 2.1 \) to 3.4 mg of lycopene/kg of egg yolk, which is equivalent to \( \sim 0.09 \) mg of lycopene in a standard large egg (17 g of yolk). In comparison, a 100-g, ripe, uncooked tomato contains 2.57 mg of lycopene (USDA, 2005, Nutrient Database Number 11529); thus, the lycopene-enriched egg yolk...
contains ~16% of the lycopene in a tomato on a per-gram basis. Despite the lower concentration of yolk lycopene as compared with tomatoes, the availability of lycopene from egg yolk may be greater. In humans, absorption of lutein from eggs was 2.12 times that of the absorption from vegetable sources (Chung et al., 2004). Lutein and lycopene are both lipophilic carotenoids, so it may be hypothesized that lycopene will be more readily absorbed from an egg than from a tomato. This suggests that lycopene-enriched yolks, even at low concentrations, may be a good source of lycopene. However, additional research is necessary to support this hypothesis.

Regression analysis predicted that 420 mg of dietary lycopene would result in optimal egg yolk inclusion, and diets that contained lycopene concentrations above 420 mg/kg resulted in numerically lower yolk lycopene. The reasons for this are not clear; however, the saturation of the intestinal absorption mechanisms probably accounts for some lower efficiency of uptake. The amount of lycopene deposited into egg yolk was approximately 4.5% at low dietary concentrations (65 mg/kg of diet) and decreased to 0.6% when fed high-lycopene diets (650 mg/kg of diet). Similarly, lutein deposition in egg yolks declined by 5-fold (from about 10 to 2%) as dietary lutein increased (Leeson and Caston, 2004). Quantitatively, lutein deposition in that study was greater than lycopene deposition in the current trial. The difference in deposition concentrations between lutein and lycopene is probably due to structural differences; lutein has 2 oxygenated rings, making it more lipophilic than the nonoxygenated lycopene and potentially more easily absorbed in the gastrointestinal tract of hen. Additionally, the chemical structure of the carotenoid may affect its transport through the circulation and ultimately its deposition into egg yolk, and it is likely that all of these factors play a role in the decreased deposition of lycopene as compared with previous research examining lutein egg yolk deposition.

Many components of eggs are beneficial to human health, and it is important that any modifications to egg yolk nutrient composition are not made at the expense of compounds with beneficial effects on human health. Because lycopene is fat-soluble, there is concern that high dietary lycopene may decrease the absorption or deposition, or both, of other lipid-soluble compounds such as lutein. However, contrary to that hypothesis, the deposition of lutein and zeaxanthin into egg yolk was unaffected by dietary treatment. This finding was in accordance with research by Karadas et al. (2006), in which there was no difference in lutein or zeaxanthin concentrations in quail eggs from birds fed a tomato powder-enriched diet. Additionally, there was no negative effect of high dietary α-tocopherol or

Figure 3. Diet lycopene affects yolk lycopene concentrations. High-performance liquid chromatography analysis was conducted on yolks to determine lycopene content (n = 4 replications/diet) from hens fed diet 1 (0, 0), 2 (420, 84), 3 (840, 164), 4 (0, 200), 5 (420, 284), and 6 (840, 364), presented as: (mg of lycopene/kg of diet, mg of α-tocopherol/kg of diet). Yolk lycopene concentrations for diets 1 and 4 were not detectable (ND, <20 μg of lycopene/kg of egg yolk). Data are presented as mean lycopene ± SEM. Bars with different letters are significantly different (P < 0.05).
lycopene on the yolk deposition of either compound, and supplemental dietary α-tocopherol increased egg yolk α-tocopherol concentrations. Thus, it is possible to enrich an egg yolk with α-tocopherol and lycopene, with no detrimental effect on lutein and zeaxanthin concentration.

Figure 4. Diet α-tocopherol affects (A) yolk and (B) liver α-tocopherol concentrations. High-performance liquid chromatography analysis was conducted on yolks and livers to determine α-tocopherol content (n = 4 replications/diet) from hens fed diet 1 (0, 0), 2 (420, 84), 3 (840, 164), 4 (0, 200), 5 (420, 284), and 6 (840, 364), presented as: (mg of lycopene/kg of diet, mg of α-tocopherol/kg of diet). Data are presented as mean yolk α-tocopherol ± SEM. Bars with different letters are significantly different (P < 0.05).
Finally, it was important to determine if the dietary additions affected parameters of the hen immune system. Three immune system parameters were measured in this trial. The inflammatory immune response was stimulated using LPS, based on increased liver and spleen weights and induction of acute phase protein synthesis. The cutaneous basophil hypersensitivity response was stimulated using PHA-P, based on characteristic swelling response (Koutsos et al., 2007). The primary and secondary antibody response was stimulated using 2,4-dinitrophenyl conjugated with keyhole limpet hemocyanin, based upon detectable antibody titers. However, there were no effects of dietary additions on any of these parameters. Because each of these parameters were measured only at 1 or 2 time points, and because the responses were not examined at the cellular level, it is possible that differences in the time course, duration, or cellular response to each of these antigens varied due to dietary treatment but were not measured in the current trial. However, no systemic changes in these immune parameters were noted in the current trial. Therefore, further research is needed to better understand any effects of these nutrients on the immune system of the hen, but our data support that there is no negative effect of feeding dietary lycopene or α-tocopherol on the immune system of laying hens under these conditions.

This research demonstrates that lycopene can be incorporated into table eggs at concentrations that are detectable (3.5 mg of lycopene/kg of egg yolk). The bioavailability of lycopene in humans from an egg yolk as compared with dietary plant sources remains to be determined. The addition of lycopene and α-tocopherol to the hen diet does not affect the concentrations of other carotenoids present in the egg yolk. Dietary lycopene and α-tocopherol at the concentrations tested did not affect parameters of the immune system, including inflammatory immune response, cutaneous basophil hypersensitivity, or antibody responses. Therefore, it is possible to create a lycopene and α-tocopherol-enriched egg without affecting other carotenoid concentrations or hen health.

**REFERENCES**


