Carotenoids from In Ovo or Dietary Sources Blunt Systemic Indices of the Inflammatory Response in Growing Chicks (Gallus gallus domesticus)\textsuperscript{1,2}

Elizabeth A. Koutsos,*\textsuperscript{4} Juan Carlos García López,\textsuperscript{13} and Kirk C. Klasing\textsuperscript{1}

\*Department of Animal Science, California Polytechnic State University, San Luis Obispo, CA 93407 and \textsuperscript{1}Department of Animal Science, University of California, Davis, CA 95616

ABSTRACT Lutein, a dihydroxycarotenoid, has antioxidant and immunomodulatory potential. Two 2 × 2 × 2 factorial designs examined effects of carotenoids during in ovo embryogenesis and, in the diet posthatch, on the systemic inflammatory response to lipopolysaccharide (LPS). In both trials, breeder hens were fed a carotenoid-replete (40 mg lutein/kg) or a carotenoid-deplete diet; eggs were collected, and chicks were hatched from carotenoid-deplete or carotenoid-replete eggs. Meat-type chicks (n = 160 and n = 144, respectively) were then fed diets containing 0 or 40 mg lutein/kg diet and either injected or not injected with LPS. LPS injection increased plasma haptoglobin and Zn (P < 0.01) and reduced plasma Fe and Cu (P < 0.01). Chicks hatched from carotenoid-deplete eggs had greater changes in plasma Fe and S post-LPS than chicks hatched from carotenoid-replete eggs (P < 0.05 for each). Compared with chicks fed 40 mg lutein/kg diet, chicks fed 0 mg lutein had greater body weight losses and higher plasma haptoglobin and relative thymus, bursa, and spleen weights post-LPS (P < 0.05). Data suggest that a lack of carotenoid exposure, either in ovo or posthatch, increases parameters of systemic inflammation. J. Nutr. 136: 1027–1031, 2006.

KEY WORDS: \textbullet\ carotenoid \textbullet\ lutein \textbullet\ egg yolk \textbullet\ inflammation

Carotenoids are postulated to have a variety of immunomodulatory functions; however, experimental results are often conflicting and may reflect differences in the specific carotenoid tested, its route of administration, the species used, and/or the immune parameter measured. In avian species, the primary carotenoids of economical and ecological interest are the oxygenated carotenoids, including lutein, its isomer zeaxanthin, and canthaxanthin (1). Lutein, the second most abundant carotenoid in nature (2), is consumed by wild birds and is routinely fed to commercial poultry flocks (often in combination with canthaxanthin) to pigment skin and eggs in order to optimize consumer product acceptance (3). Lutein is also consumed and absorbed by mammals (1). In addition to pigmentation properties, there is considerable interest in the role of this carotenoid in avian and mammalian immune responses. In mammals, dietary lutein affects antibody production, B-cell proliferation and differentiation, the delayed-type hypersensitivity response, and T-cell subset proportions (4,5), whereas data are less clear in avian species (6,7). In addition to acquired immune responses, the proposed antioxidant function of carotenoids (8) suggests a role for carotenoid-based modulation of innate immune responses, as shown for other antioxidant nutrients (9).

The innate immune response is the first line of defense against pathogen exposure. Pathogen exposure induces transcription and translation of genes for cytokines and other immune mediators that promote inflammatory responses and induce acute-phase protein production and redistribution of trace minerals and other nutrients in the body (10,11). For example, plasma haptoglobin is a positive acute-phase protein, and plasma levels increase after inflammatory stimulation (12). Other acute-phase proteins are associated with reduced plasma Zn and Fe and increased plasma Cu (13). Additionally, expression of inducible nitric oxide synthase is increased, resulting in production of nitric oxide (NO)\textsuperscript{5} and, subsequently, other reactive oxygen and nitrogen species (14).

Regulating the expression of cytokines and other immune mediators is an essential mechanism by which nutrients modulate immune function (15) and is one way that carotenoids can exert immunomodulatory effects (16). However, and importantly, the level of carotenoids to which the developing embryo is exposed (i.e., in ovo carotenoid exposure) affects subsequent deposition of dietary carotenoids in tissues of the posthatch chick (17). Specifically, chicks hatched from carotenoid-deplete (C−) eggs have a more reduced ability to deposit dietary carotenoids into their tissues than chicks hatched from carotenoid-replete (C+) eggs. Thus, the effects of dietary carotenoids on the avian immune system may be confounded by in

\textsuperscript{1}Presented in part in Experimental Biology 2003 in abstract form (Koutsos EA, Calvert CC, Humphrey BD, Klasing KC. Interactions between carotenoids and the inflammatory response in chickens (abstract). FASEB J. 2003;17:A7295

\textsuperscript{2}Supported by U.S. Department of Agriculture National Research Initiative Competitive Grant 2002-02048 and California Agricultural Research Initiative 04-018.

\textsuperscript{3}Current address: Instituto de Investigacion de Zonas Desertas, Universidad Autonoma de San Luis Potosi, Altair 200 fracc. del Llano, San Luis Potosi, SLP, Mexico CP 78377.

\textsuperscript{4}To whom correspondence should be addressed. E-mail: ekoutsos@calpoly.edu.

\textsuperscript{5}Abbreviations used: BW, body weight; C−, carotenoid-deplete; C+, carotenoid-replete; L+Z, lutein and zeaxanthin; NO, nitric oxide.

0222-3166/06 $5.00 © 2006 American Society for Nutrition.

1027
Staphylococcus aureus zeaxanthin 562 (0.01), but was not affected by in ovo lutein.

The effect of in ovo carotenoid exposure and dietary lutein level on the inflammatory response to LPS was examined in growing chickens. Systemic inflammation was examined by measuring plasma haptoglobin, NO and minerals (Fe, Zn, Cu, and S), and body weight gains.

MATERIALS AND METHODS

Designs for the following experiments were similar, and exceptions are noted below. Two experiments were designed with a 2 × 2 factorial arrangement of treatments consisting of 2 in ovo lutein levels, 2 dietary lutein levels, and 2 LPS levels. To establish 2 egg carotenoid levels, hens were fed either a carotenoid-deplete diet or a diet supplemented with 40 mg lutein/kg diet, as previously described [resulting in C+ eggs containing ~125 nmol lutein + zeaxanthin (L+Z)/egg or C− eggs containing no detectable carotenoids]. The level of carotenoids in the C+ egg is similar to that of a typical domesticated chicken egg (17). On the day of hatch, chicks from each in ovo lutein treatment were randomly assigned to 1 of 2 diet lutein levels: each pen of chicks received ad libitum access to basal diet (Table 1) plus either 0 mg diet lutein/kg diet or 40 mg diet lutein/kg diet (Oroglo Dry, Kemin Industries). Diet lutein levels were chosen to be similar to those fed to commercial poultry. All chicks were housed in identical brooder battery cages (Petersime Inc.) in a temperature-controlled room (25°C) under 24 h light. At 14 d (Expt. 1) or 21 d (Expt. 2) posthatch, chicks were randomly assigned to 1 of 2 LPS treatments. Chicks were either not injected (controls) or injected with LPS from Salmonella typhimurium (Sigma L7261). Saline injection does not induce an acute-phase response (18), therefore the control group was not injected. The University of California Davis Animal Care and Use Committee approved all procedures for Experiments 1 and 2.

Experiment 1. Broiler chicks (Cobb × Cobb strain, n = 160 at egg yolk level) were randomly assigned to 1 of 20 pens (n = 8/pen) and each pen was randomly assigned to diet lutein treatment as described above. At 14 d posthatch, chicks within pens were randomly assigned to 1 of 2 LPS treatments: chicks were either not injected (control) or injected with 1 mg LPS/kg BW intra-abdominally. At 24 h post-LPS injection, chicks were bled via cardiac puncture into heparinized tubes for plasma isolation and then euthanized. The left liver, lobe, bursa, thymic lobes, and spleen were removed and weighed. The dependent variables measured included performance parameters (body weights and feed intake), plasma minerals (Zn, Fe, and Cu), and plasma lutein and zeaxanthin (L+Z).

Measurement of inflammatory status. Plasma haptoglobin (Expt. 1) was measured according to manufacturer instructions, using a commercial kit (Phase Haptoglobin kit, Tridelta Diagnostics, TP801). Plasma Zn, Fe, and Cu (Expt. 2) were measured by inductively coupled plasma mass analysis (University of Arkansas Central Analytical Lab). The concentration of the NO metabolite, nitrite, was measured in plasma (Expt. 1) using the Griess reagent method (Sigma, G4410) as previously described (19). Phagocytosis (Expt. 1) was measured in whole blood diluted 1:10 in sterile RPMI 1640 containing 5% fetal bovine serum and 1% penicillin/streptomycin. BODIPY-coated Escherichia coli and Staphylococcus aureus (Molecular Probes E-2864 and S-2854, respectively) were added at 1:100 cell to particle ratio, and cells were incubated in the dark for 15 min at 41°C and then placed on ice for 5 min. Plates were gently washed, rinsed with methanol, and then read at 505/513 excitation/absorption spectrum using a fluorescent microscope. Macrophages were designated as having phagocytosed particles or not, and values are expressed as the percentage of total macrophages that had phagocytosed particles.

Measurement of carotenoids (Expt. 2). Tissues and diets were thawed, weighed, homogenized, and L+Z were extracted as previously described (17). L+Z were analyzed at 464 nm using a UV/vis spectrophotometer for Experiment 2.

Statistical analysis. Data were analyzed by general linear model (JMP software, SAS), using a 3-way ANOVA. Variances were all confirmed to be homogeneous prior to ANOVA. Three-way interactions that did not approach significance (P > 0.25) were removed from the model and 2-way ANOVA was used to examine data. Dependent variables were examined for the main effect of in ovo lutein level, diet lutein level, LPS treatment, and their interactions. When main effects or interactions were significant, differences between means were identified using Tukey’s LSMEANS comparisons. Differences between means were considered significant at P < 0.05. Values are means ± SEM.

TABLE 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean meal</td>
<td>343.0</td>
</tr>
<tr>
<td>Rice flour</td>
<td>550.0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>5.6</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>20.0</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>47.5</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>17.5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>2.5</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>2.5</td>
</tr>
<tr>
<td>l-Methionine</td>
<td>3.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.5</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.4</td>
</tr>
<tr>
<td>Choline</td>
<td>1.0</td>
</tr>
</tbody>
</table>

1 Diet was offered ad libitum for the duration of the trial, and was formulated to meet or exceed all requirements for growing chicks (32).

2 Vitamin mix contained (per kg final diet): 1.8 mg thiamin, 3.6 mg riboflavin, 11.5 mg pantothenic acid, 35 mg niacin, 3.5 mg pyridoxine, 0.6 mg folic acid, 0.2 mg biotin, 10 μg vitamin B-12, 0.9 mg retinyl palmitate, 50 μg cholecalciferol, 36.8 mg all-rac-α-tocopheryl acetate, and 5 mg menaquinone. Mineral mix contained (per kg final diet) 0.2 mg selenium, 8.1 mg copper, 40.7 mg zinc, 62 mg manganese, 105.4 mg iron, and 0.35 mg iodine.

RESULTS

Experiment 1. In ovo lutein level and diet lutein level did not affect performance parameters, including body weight or feed intake from 1 to 14 d (P > 0.30; 14-d BW = 520 ± 18 g; 14-d feed intake = 562 ± 14 g/bird). Feed intake was reduced by ~3% at 6 h post-LPS in all treatment groups except for chicks hatched from C+ eggs fed 0 mg lutein, which had an ~53% reduction (in ovo lutein × diet lutein × LPS interaction; P = 0.04).

Plasma NO was increased by LPS treatment at 6 h postinjection (P < 0.01), but was not affected by in ovo lutein or diet lutein (P > 0.10). Similarly, ex vivo macrophage phagocytosis was not affected by in ovo lutein or diet lutein (P > 0.10), but was reduced in LPS-injected chicks (P < 0.01;
controls, 82.7 ± 1.0%; from LPS-injected chicks, 17.9 ± 0.9%).

Plasma haptoglobin was increased by LPS treatment at 24 h postinjection (P < 0.01). The LPS-induced increase in plasma haptoglobin was greater (P < 0.05) for chicks fed 0 mg lutein than for those fed 40 mg lutein (diet lutein × LPS, P = 0.01) (Fig. 1A). Chicks that were hatched from C+ eggs and that were fed 0 mg lutein had a greater plasma haptoglobin (P < 0.05) than chicks from the other treatment combinations (in ovo lutein × diet lutein interaction, P = 0.05; Fig. 1B). This difference is attributed primarily to LPS-injected chicks from the C+ eggs that were fed 0 mg lutein; plasma haptoglobin levels in this group (0.216 ± 0.022 g/L) were approximately double that of other LPS-injected birds (0.122 ± 0.018 g/L).

**Experiment 2.** Chicks from C− eggs hatched at a higher BW (53.5 ± 0.2 g) than chicks hatched from C+ eggs (50.9 ± 0.1, P < 0.01). However, in ovo lutein (P > 0.10) and diet lutein (P > 0.10) did not affect chick BW on d 7, 14, or 21 posthatch. At 24 h post-LPS, chicks fed 0 mg lutein had lower BW (mean = −52.6 g) than chicks fed 40 mg lutein (mean = +30.6 g; diet lutein × LPS, P = 0.02). In addition to overall changes in BW, changes in tissue weights (as a percentage of BW) at 24 h post-LPS treatment were evident. Relative bursa, spleen, and thymus weight were increased by LPS treatment (P < 0.01 for each main effect), but a diet lutein × LPS interaction (P < 0.01; Fig. 2 A, B, and C) indicates that the changes were greater for chicks fed 0 mg lutein than chicks fed 40 mg lutein. Finally, liver weight was increased by LPS (3.8 ± 0.1%) compared with controls (2.9 ± 0.1%; P < 0.01), but was not affected by diet lutein or in ovo lutein (P > 0.60 for each).

Plasma L+Z were increased by feeding lutein (P < 0.01). Additionally, an in ovo lutein × LPS interaction (P = 0.02) demonstrates that chicks hatched from C+ eggs had a reduction in plasma L+Z (44.7% reduction) following LPS treatment (P < 0.05), whereas chicks hatched from C− eggs did not (18.1% increase).

Plasma mineral levels reflect metabolic changes induced by an inflammatory acute-phase response. Plasma Cu was higher in chicks hatched from C+ eggs (1.08 ± 0.12) than chicks hatched from C− eggs (0.84 ± 0.08 μmol/L; P = 0.04). Compared with controls, LPS treatment increased plasma Cu (P = 0.02; 35% increase) and reduced plasma Zn (P < 0.01; 38% reduction). Chicks from C− eggs had lower plasma Fe

**FIGURE 1** Plasma haptoglobin in growing broiler chickens is affected by diet lutein and LPS challenge (A), and by in ovo lutein and diet lutein (B). Broiler chicks were hatched from carotenoid-deplete or carotenoid-replete egg yolks (n = 160/egg type), fed either 0 or 40 mg of diet lutein/kg diet (n = 40/diet × egg type), and injected with LPS (2 mg/kg BW intra-abdominally) or not injected at 14 d posthatch (n = 20/injection × diet × egg type). Plasma haptoglobin was measured at 24 h post-LPS challenge. Within graphs, bars with different letters are significantly different (P < 0.05). Values are means ± SEM.

**FIGURE 2** Diet lutein level affects the change in bursa (A), spleen (B), and thymus weights in growing broiler chickens (C). Broiler chicks were fed either 0 or 40 mg of diet lutein/kg diet (n = 144/diet type), and were injected with LPS (1 mg/kg BW intra-abdominally) or not injected at 21 d posthatch (n = 72/injection × diet type). Tissue weights were examined at 24 h post-LPS treatment, and are expressed as a percentage of BW. Within graphs, bars with different letters are significantly different (P < 0.05). Values are means ± SEM.

**FIGURE 3** Plasma Fe (A) and plasma S (B) are affected by egg yolk carotenoids and LPS challenge. Broiler chicks were hatched from carotenoid-deplete or carotenoid-replete egg yolks (n = 144/egg type) and were injected with LPS (1 mg/kg BW intra-abdominally) or not injected at 21 d posthatch (n = 72/injection × egg type). Plasma mineral levels were measured at 24 h post-LPS. Within graphs, bars with different letters are significantly different (P < 0.05). Values are means ± SEM.

**DISCUSSION**

In these experiments, the effect of lutein on growth and the acute-phase response to inflammation were measured. Additionally, we examined the relative influence of lutein exposure in ovo compared with exposure posthatch. In general, diet lutein and in ovo lutein had minimal effects on the growth of chicks. Chicks hatched from C− eggs were larger than chicks hatched from C+ eggs in Experiment 2, but there was no detected difference in BW at 14–21 d posthatch. Previous research confirms no effect of in ovo lutein on chick hatching weights (17).

The LPS challenges in these experiments resulted in inflammatory immune responses, as indicated by changes in NO, plasma haptoglobin, plasma minerals, plasma liver carotenoids, and feed intake. LPS treatment resulted in increased plasma Cu and reduced plasma Zn, which was expected, because the acute-phase response induced by LPS increases plasma Cu (20) as a result of increased ceruloplasmin synthesis and secretion (21) and reduces plasma Zn (13) due to increases in hepatic metallothionein synthesis (22). LPS treatment also...
reduced plasma and liver carotenoids, but this effect was limited to chicks exposed to carotenoids in the egg. These data support previous observations of reductions in carotenoids post-LPS (23) and suggest that carotenoid status plays a major role.

Dietary lutein blunted indices of systemic inflammation. Compared with chicks fed dietary lutein, chicks fed no dietary lutein had greater BW reduction post-LPS, had greater increases in bursa, thymus, and spleen weights post-LPS, and had greater plasma haptoglobin responses post-LPS, which indicates that these chicks underwent a greater systemic inflammatory response. The effect of dietary lutein on inflammatory response has not been previously characterized, although lutein has been demonstrated to affect several aspects of acquired immunity (4,5). The current data suggest that dietary lutein modulates the systemic inflammatory response insofar as chicks fed 0 mg lutein had greater systemic inflammatory responses.

Egg yolk carotenoid exposure also affected parameters of systemic inflammatory immune responses. Birds hatched from carotenoid-deplete eggs had more dramatic reductions in plasma Fe after the LPS challenge. A reduction in plasma Fe during an acute-phase response occurs in birds because of increased hepatic ferritin synthesis (13,24) and in mammals because of reduced hepatic transferrin synthesis (25). Observed reductions in plasma S after LPS challenge are likely related to reductions in plasma methionine as demonstrated in chickens (13), and increased glutathione synthesis in the liver, spleen, kidneys, and other tissues, as demonstrated in septic rats (26). Together, these data indicate that chicks hatched from C−eggs had greater systemic inflammatory responses than did chicks hatched from C+ eggs.

The basis by which embryonic carotenoids can affect post-hatch immune responses is not known. However, other studies have demonstrated that the manipulation of egg carotenoids can affect posthatch chick immune responses, and inoculating barn swallow eggs with lutein resulted in increased wing web swelling in response to phytohemagglutinin (27). The mechanism for embryonic carotenoid effects later in the chick’s life may be related to developmental effects of embryonic carotenoids or due to the persistence of altered carotenoid concentrations in critical tissues in the posthatch chick. Most yolk-derived carotenoids are deposited into the embryonic liver (28), in contrast to the profile of diet carotenoid deposition in the posthatch chick, where carotenoids are more broadly distributed (29). Additionally, the liver carotenoid concentration in newly hatched chicks is significantly higher than could be achieved by feeding a diet containing high levels of carotenoids, demonstrating the significant impact that maternal diet has on avian progeny carotenoid deposition (30). Since the liver is critically involved in the acute-phase component of the inflammatory immune response, it may be that embryonic carotenoid exposure, by affecting liver carotenoid concentration, thus affects the hepatic component of the inflammatory immune response. Alternatively, it may be that the enrichment of immune cells with carotenoids affects the immune response. For example, monocytes incorporate ~10-fold higher levels of β-carotene compared with those of nonimmune tissues (31). Thus, enrichment of immune cells with carotenoids from dietary or embryonic origin may affect the inflammatory immune response.

Finally, interactions between in ovo lutein and diet lutein were evident during these trials. Specifically, the plasma haptoglobin response was significantly greater in chicks hatched from C+ eggs and fed 0 mg dietary lutein compared with all other treatments. Because both dietary-lutein and embryonic-carotenoid exposures affected aspects of the systemic inflammatory response (in particular, the lack of carotenoids tended to enhance this response), it may be that a lack of carotenoids either in ovo or in the posthatch diet tends to increase parameters of systemic inflammatory immune responses.

In summary, in ovo and dietary exposures to lutein affected components of the systemic inflammatory immune response in chicks. Systemic inflammatory responses were enhanced in birds without embryonic carotenoid exposure, because lutein depletion in ovo resulted in enhanced acute-phase responses. Similarly, a lutein-free diet consumed by hatchlings was associated with an enhanced systemic inflammation, based upon BW losses, increased plasma haptoglobin, and increased bursa, thymus, and spleen weights after LPS injection. Overall, these data suggest that a lack of carotenoid exposure, either in the egg or in the diet, results in enhanced systemic inflammation. The combination of exposure to in ovo lutein and diet lutein appears to result in the smallest magnitude of systemic inflammation induced by LPS.

ACKNOWLEDGMENTS

We would like to thank Guochen Hu for his assistance with animal care at the University of California Davis; Jenny Bennett for assistance with phagocytosis assay; and Joel Judge, Kristina Bourn, Angela Amato, Annika Hoffman, and Elizabeth Gillingham for their assistance with animal care and/or sample analyses at California Polytechnic State University.

LITERATURE CITED