Effects of silymarin on gossypol toxicosis in divergent lines of chickens

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ABSTRACT Gossypol, a pigment of cotton, is a hepatic toxin for chickens. Thus, despite its high protein content, inclusion of cottonseed meal in poultry diets is problematic. Silymarin, an extract from milk thistle, has hepatoprotective qualities and could potentially serve as a feed additive to offset the toxicity of gossypol. The objective of this study was to determine if silymarin could counteract gossypol toxicosis. Cockerels (n = 144) from lines divergently selected for humoral immunity were used. Three individuals from each line were randomly assigned to a cage and fed a corn-soybean meal (control) diet for 14 d. Six cages per line were then randomly assigned 1 of 4 dietary treatments (1,000 mg/kg of gossypol, 1,000 mg/kg of silymarin, 1,000 mg/kg of both gossypol and silymarin, or a control diet). Body weight and feed intake data were collected for 21 d, with chickens bled weekly to collect plasma and determine hematocrits. Chickens were then killed, and livers were collected for subsequent histology and enzymatic activity analyses. Endpoints measured weekly were analyzed with repeated measures and regression methodologies. Plasma and liver enzyme activities, and histological measures, were analyzed using ANOVA. No significant interactions between diets and lines were observed. Chickens assigned to the gossypol and gossypol-silymarin diets stopped gaining weight at d 14 (P < 0.001) and lost weight by d 21 (P < 0.001). Gamma glutamyltransferase was also elevated in these chickens at d 14; activities increased further by d 21 (P < 0.001). Histological examination of liver slices indicated substantial lipidosis (P < 0.001). Furthermore, quinone reductase activity was higher in gossypol- and gossypol-silymarin-treated chickens than in control and silymarin-treated chickens (P < 0.001). Silymarin did not alleviate any clinical effects of gossypol toxicosis.

Key words: chicken, gossypol, silymarin, quinone reductase, gamma glutamyltransferase

INTRODUCTION

Corn prices have increased substantially over the past few years (Stillman et al., 2009), with poultry producers seeking alternative feeds. One potential feed alternative is cottonseed meal (CSM). Cottonseed meal has inadequate energy content to be substituted for corn. However, CSM is a good source of protein, similar to soybean meal (Rahman et al., 2001; Nagalakshmi et al., 2007) and could be used to replace soybean meal to offset corn costs. Unfortunately, CSM contains the toxin gossypol, a pigment produced by the cotton plant (Kenar, 2006). Gossypol in diets fed to chickens is associated with growth depression, lameness, decreased egg size and hatchability, and yolk and albumen discoloration (Nagalakshmi et al., 2007).

In mammalian species, gossypol is considered a cardiac toxin, causing labored breathing, jugular vein dis-
to improve glycemic control for type 2 diabetes (Detaille et al., 2008). Milk thistle extracts are also used to successfully treat hepatitis patients (Bares et al., 2008; Ferenci et al., 2008).

Silymarin has also been used to control another hepatic toxicosis, aflatoxicosis, in chickens (Tedesco et al., 2004). In both in vitro and in vivo studies, cellular damage caused by aflatoxin has been attributed to increased free-radical production (Amstad et al., 1984; Rastogi et al., 2001). Gossypol toxicity is of similar origin (Kovací et al., 2003). Rastogi et al. (2001) found that silymarin reversed cellular damage in rats caused by aflatoxin. Silymarin could therefore potentially alleviate gossypol toxicosis and, if included as a feed additive, thereby allow CSM to be fed to chickens.

Genetic differences in chickens in response to toxicants have been documented, including gossypol (Lordeo et al., 2007). Such is also the case for disease resistance (Cheng, 2010). White Leghorn chickens were divergently selected for humoral immune response (Gross and Siegel, 1980). At generation 9 of selection, Ubosi et al. (1985a,b) tested the sensitivity of the lines to aflatoxin. They hypothesized that differences in humoral immunity may affect an animal's general capacity to respond to environmental challenges. Although, there was a clear effect of aflatoxin on liver function, there were no differences in response between lines. An additional 26 generations of divergent selection have since occurred. Therefore, the lines are more distinct genetically in humoral immunity (Kuehn et al., 2006), perhaps introducing greater sensitivities to environmental challenges.

This study had 2 objectives. The first objective was to determine if silymarin would reduce the effects of gossypol toxicosis in chickens. Our second objective was to determine if genetically distinct lines of chickens would differ in their sensitivity to gossypol and silymarin in their diets.

**MATERIALS AND METHODS**

**Birds and Experimental Design**

Lines of White Leghorn chickens have been developed at Virginia Tech based on antibody response to a 0.1-mL i.v. injection of a 0.25% solution of SRBC 5 d postinjection. Through this selection, a low-antibody (LA) and a high-antibody (HA) line have been developed (Gross and Siegel, 1980; Martin and Dunnington, 1990; Kuehn et al., 2006).

Chicks from generation 35 parents of both lines were reared in Petersime starter batteries (model 2SD 24, Petersime Incubator Co., Gettysburg, OH) and had ad libitum access to feed (corn-soybean meal mash diet) and water with 24 h of light exposure. At 35 d of age, 72 males from each line were moved from the Petersime batteries to larger Harford batteries (Harford Metal Products, Aberdeen, MD). Each battery contained 12 cages (4 cages per row; 30 × 15 × 16.5 cm). Five batteries were used. Within a line, 3 chickens were randomly assigned to a cage and each cage to a location in a battery. Each battery contained an equal number of cages with HA and LA males. In total, there were 24 cages per line.

All chickens were moved into their assigned cage 14 d before the beginning of the experiment (i.e., 35 d of age). Throughout this acclimation, and the subsequent experimental period, chickens were given free access to water and feed and were exposed to continuous light. At 49 d of age, the chickens were given access to their assigned experimental treatment.

The Virginia Tech Institutional Animal Care and Use Committee approved all housing and experimental procedures.

**Treatments**

In a preliminary trial, the effect of different concentrations of gossypol and silymarin in the diet on growth and food intake was evaluated. The doses considered were based on free gossypol concentrations that have been reported to cause toxicosis (Nagalakshmi et al., 2007). Two concentrations of silymarin and gossypol were used: 500 and 1,000 mg/kg. There were no differences in food intake or BW gain for chickens fed diets with either concentration of silymarin (P > 0.10). However, food intake and growth of chickens fed the higher concentration of gossypol (1,000 mg/kg) were depressed (P < 0.01), with relatively little consequence of the lower concentration (500 mg/kg). The higher concentration of both compounds was used in the current study.

Four dietary treatments were used: gossypol (G), silymarin (S), gossypol and silymarin combined (G-S), and a control (C). For the treatments with gossypol and silymarin singly, the respective compound was added at a concentration of 1,000 mg/kg to C, a corn-soybean meal in mash form. The G-S diet had both gossypol and silymarin added to C at a concentration of 1,000 mg/kg.

Gossypol was purchased from TimTec LLC (Newark, DE). It was extracted from plants of the genus *Gossypium* of the family *Malvaceae* and was certified as 98% pure. Silymarin was purchased from Sigma-Aldrich (St. Louis, MO). Six cages within a line were randomly assigned to each dietary treatment.

Feed was mixed on Friday of each week. Both silymarin and gossypol are light-sensitive; therefore, the diets were stored in plastic buckets with lids and then placed inside a black plastic bag. On Monday of each week, chickens were individually weighed, and the feed intake of each cage was recorded. Residual feed was discarded.

**Blood Collection and Processing**

After weighing, blood from the brachial vein was collected in a 4-mL lithium-heparin Vacutainer tube.
(BD Vacutainer, Franklin Lakes, NJ) and placed on ice. On the same day, 2 capillary tubes of whole blood were drawn from the Vacutainer tubes and centrifuged (model 335, Fisher Scientific, Pittsburgh, PA) for 4 min to determine hematocrits. Plasma was collected and stored at −20°C for subsequent analyses.

**Aspartate Aminotransferase.** As an indicator of liver function, aspartate aminotransferase (AST) activity was measured using a spectrophotometric kit purchased from Pointe Scientific (Canton, MI). Protocols were modified so that the kit could be used and were modified for a 96-well microtiter plate measuring absorbance on 100 µL of sample and 200 µL of reagent.

**Gamma Glutamyltransferase.** Gamma glutamyltransferase (GGT) activity, another indicator of hepatic function, was measured using a spectrophotometric kit (Bio-Rad, Hercules, CA). Bovine serum albumin was used to establish a standard curve.

**Plasma Protein.** Total plasma protein was determined using the BioRad method (Bio-Rad Laboratories; Bradford, 1976). Bovine serum albumin was used to establish a standard curve.

**Tissue Collection**

After 21 d of treatment, all chickens were killed via cervical dislocation. A section of the left lobe of the liver was removed and subdivided into 3 smaller samples. Each sample was frozen in liquid nitrogen and then stored at −80°C until analyzed for enzyme activity. Two slices from the left lobe of the liver were also collected from each chicken and stored in 10% buffered formalin for histologic evaluation.

**Enzyme Activity Assays**

All chemicals were purchased from Sigma-Aldrich.

**Sample Preparation.** Liver samples were weighed and a 25% homogenate was prepared in 0.1 M phosphate buffer (pH 7.4, 1.15% KCl) using a Polytron blender (Brinkman Instruments, Westbury, NY). Homogenates were centrifuged for 10 min at 15,000 × g at 5°C. Supernatant was recentrifuged for 40 min at 50,000 × g at 5°C. The supernatant, cytosol, was transferred into a microtube and the pelleted, microsomal portion was resuspended in an equal volume of 10 mM Tris-acetate buffer (pH 7.4, 0.1 mM EDTA, 20% glycerol). After 15 min of incubation, 20 µL of each microsomal sample was added to the plate in triplicate. Eighty microliters of the respective standards or 100 µL of the 80 µM 7-BQ substrate was added to each of the microplate wells. The plate was incubated at 37°C for 15 min. To prevent background interference, opaque 96-well plates were used.

The microsomal fraction of each sample was diluted 1:4 with 10 mM Tris-acetate buffer (pH 7.4, 0.1 mM EDTA, 20% glycerol). After 15 min of incubation, 20 µL of each microsomal sample was added to the plate in triplicate. Eighty microliters of the NAD phosphate-generating system, as described by Crespi et al. (1997), was added to each well. The reaction progressed for 30 min at 37°C and was stopped with 75 µL per well of a solution of 80% acetonitrile and 20% 0.5 M Tris buffer (pH 9). The plate was read with a microplate spectrophotometer (SpectraMax M5, Molecular Devices Corp.). Enzyme activity was reported as micromoles of 7-BQ oxidized/min per milligram of protein.

**Protein.** Cytosolic and microsomal proteins were determined using protein dye-binding (Bio-Rad Protein Assay, Bio-Rad Laboratories; Bradford, 1976). Bovine serum albumin was used to establish a standard curve.

**Histologic Evaluation**

Liver slices were removed from formalin and embedded in paraffin blocks. The blocks were sent to Histo-Scientific Research Laboratories (Mt. Jackson, VA), where 5-µm slices were cut from each block and fixed to glass slides. Slides were stained with hematoxylin and eosin. One slide per chicken was reviewed by a board-certified veterinary pathologist and received a score for lipidosis, heterophils, and lymphoid nodules on a 0 (no abnormality) to 3 (extensive abnormality)
scale (Table 1). The pathologist was blinded to treatment when evaluating slides.

**Feed Analyses**

Samples from all diets were analyzed for DM, CP, crude fat, crude fiber, and amino acid content (Cumberland Valley Analytical Services, Hagerstown, MD). Samples from G and G-S diets were analyzed for free gossypol content (Pope Testing Laboratories Inc., Irving, TX).

**Statistical Analyses**

All analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary, NC). Cage within line and diet was the experimental unit. Where measurements were collected on individual chickens, these were averaged by cage. For consistency, feed intake for a cage was expressed as the average weekly intake for an individual chicken.

The same chickens within a cage were measured each week over the length of the experiment. Therefore, a repeated measures analysis using the MIXED procedure was conducted. The linear mixed model fitted was as follows:

\[ Y_{ijkl} = \mu + W_i + L_j + D_k + (WL)_{ij} + (WD)_{jk} + (LD)_{jk} + (WLD)_{ijk} + e_{ijk} + e_{ik} + e_{jk}. \]

where \( Y_{ijkl} \) was the response variable recorded every week \( W [i = 0 (d 0), 1 (d 7), 2 (d 14), or 3 (d 21)] \) from line \( L (j = 1 or 2 for HA or LA, respectively) \) randomly assigned to diet \( D (k = 1, ..., 4, for G, S, G-S, or C, respectively) \) by cage \((l = 1, ..., 6)\) and \( \mu \) was the overall mean. Week, line, and diet were fitted as fixed effects, along with their interactions. Random terms were cage nested within the line \( \times \) diet \( e_{ijk} \) and residual \( e_{ij} \).

Because the time interval between successive measurements was constant (a single week), an autoregressive covariance structure among weeks with homogenous variances was assumed. Differences among lines and diets, singly and in combination, were tested across all levels of time (week). The error term used to form these test statistics was \( e_{ijk} \).

In addition, for those measurements collected weekly (i.e., BW, feed intake, plasma enzymes, hematocrit), a linear mixed model was fitted using the MIXED procedure as follows:

\[ Y_{ijkl} = \beta x + L_j + D_k + (LD)_{jk} + e_{ijkl}. \]

where the vector \( \beta \) was regression coefficients (intercept and linear and quadratic terms) for the covariate week \((x)\). To model differences in the values of these coefficients by line, diet, and line \( \times \) diet, their interaction with these effects was fitted. The error term used to test the significance of the regression coefficients, in conjunction with line, diet, and the line \( \times \) diet interaction, was \( e_{ijkl} \).

Liver enzyme measurements were analyzed using ANOVA. The model fitted was as follows:

\[ Y_{ijkl} = \mu + L_j + D_k + (LD)_{jk} + e_{ijkl}. \]

where \( Y_{ijkl} \) was the average enzyme activity level in cage \( l \) within a line \( j \) and diet \( k \) and \( e_{ijkl} \) was the residual. Comparisons of means for line, diet, and their interaction were based on orthogonal contrasts.

Histologic evaluations were analyzed in 2 ways. First, scores for each histological evaluation (lipidosis, heterophils, and lymphoid nodules) were averaged for each cage and analyzed using ANOVA with model [3]. Second, the scores on individual chickens were analyzed using a \( \chi^2 \) test.

**RESULTS**

**Feed Analyses**

All diets were similar for DM [891 (SD 13) g/kg], CP [240 (SD 39) g/kg of DM], crude fat [38 (SD 1) g/kg of DM], crude fiber [50 (SD 5) g/kg of DM], and ash [59 (SD 2) g/kg of DM] contents. There were also no differences in lysine [0.120 (SD 0.001) g/kg], methionine [0.032 (SD 0.001) g/kg], or cysteine [0.037 (SD 0.001) g/kg] contents. Furthermore, free gossypol content was similar in the G (0.040%) and G-S (0.043%) diets. Gossypol was added to the diet at a concentration of 1,000 mg/kg. Approximately 50% was therefore bound in the diet, which is common for gossypol (Nagalakshmi et al., 2007).

There were no significant interactions among diets and lines \((P > 0.05)\). Therefore, the effects of diet and line on weights and other biological measures are presented separately.

<p>| Table 1. Scoring systems for histological analyses |
|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Score</th>
<th>Lipidosis</th>
<th>Heterophils</th>
<th>Lymphoid nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>25% of cells with cytoplasm vacuolated</td>
<td>Few in some portal areas</td>
<td>1 per 4× magnification</td>
</tr>
<tr>
<td>2</td>
<td>50% of cells with cytoplasm vacuolated</td>
<td>Few in half of the portal areas</td>
<td>2 to 3 per 4× magnification</td>
</tr>
<tr>
<td>3</td>
<td>100% of cells with cytoplasm vacuolated</td>
<td>Few in all portal areas</td>
<td>&gt;3 per 4× magnification</td>
</tr>
</tbody>
</table>
Dietary Effects

**BW and Feed Intake.** On average, chickens on all treatments gained weight during the first 14 d of the experimental period. Thereafter, G and G-S did not increase in BW. Based on the repeated measures analysis (model [1]), by d 21 ($P < 0.001$) these chickens had lower BW than C and S chickens (Table 2).

Slope of the regression of BW on week (model [2]) for G and G-S chickens was less than that of C and S chickens ($P < 0.001$), further substantiating that these chickens weighed less than C and S chickens. Furthermore, BW gains in C and S chickens were relatively constant over time (only the value of the slope differed from zero ($P < 0.001$)), whereas the BW of G and G-S chickens did not increase after d 14, necessitating the fit of a quadratic term in the model ($P < 0.001$).

During the first 14 d, there were no differences in feed intake among diets ($P = 0.891$). However, by d 21, based on the repeated measures analyses, chickens fed diet G and G-S had consumed less feed (on average, 0.74 times as much; $P = 0.001$) than chickens fed C and S diets (Table 2). With the fit of the regression, there was a difference between the G and G-S chickens as compared with S and C chickens in the shape of their food intake curves; for the pairs of treatments, both the values of their linear and quadratic coefficients differed ($P < 0.001$). Differences in slope reflected chickens fed diets G and G-S eating less than those fed S and C diets in general over the length of the experiment. Differences in quadratic terms coincided with more substantial reductions in feed intake of G and G-S chickens.

**Liver Enzymes.** There were no differences among diets (model [3]) for GST or CYP450 3A4 activities ($P = 0.126$ and $P = 0.647$, respectively). Quinone reductase activity (Table 3) was higher ($P < 0.001$) in chickens fed the G and G-S diets than those fed the S and C diets.

**Histologic Evaluations.** Lipidosis in chickens fed G (1.61 ± 0.12) and G-S (1.80 ± 0.12) diets was approximately 1.7 times greater than that of chickens fed diets S (1.07 ± 0.12) and C (0.97 ± 0.12) ($P < 0.001$). However, there was no histological evidence that damage in the G and G-S chickens was irreversible. Diet had no effect on lymphoid nodules [mean (SD) 1.23 (0.36); $P = 0.070$] or heterophils [mean (SD) 1.30 (0.63); $P = 0.079$].

A $\chi^2$ analysis was also performed on these data. More chickens fed G and G-S diets (n = 44) had category 2 and 3 lipidosis scores than those fed S and C diets (n = 10) ($P < 0.001$). As with the ANOVA, there were no differences in heterophils ($P = 0.336$) or lymphoid nodules ($P = 0.415$) among diets.

**Clinical Chemistry.** Chickens fed the G and G-S diets had numerically higher GGT levels than those fed C and S diets beginning at d 14 (Table 4). Based on the repeated measures analysis (model [1]), this difference became substantially larger by d 21 ($P < 0.001$). In-

### Table 2. Least squares means for weekly BW and feed intake (per bird) across lines for chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>BW (g) by day on treatment</th>
<th>Feed intake (g) by day on treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Gossypol</td>
<td>446a</td>
<td>538a</td>
</tr>
<tr>
<td>Gossypol + silymarin</td>
<td>444a</td>
<td>527a</td>
</tr>
<tr>
<td>Silymarin</td>
<td>440a</td>
<td>547a</td>
</tr>
<tr>
<td>Control</td>
<td>436a</td>
<td>546a</td>
</tr>
</tbody>
</table>

$^{ab}$Means in the same column with different superscripts are different ($P < 0.001$).

$^1$There were no interactions between line and diet on treatment ($P > 0.5$).

$^2$SEM = 11.7, error df = 51.

$^3$SEM = 7.6, error df = 73.

### Table 3. Least squares means for hepatic quinone reductase (QR), glutathione S-transferase (GST), and cytochrome P450 (CYP450 3A4) activities across lines on chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>QR activity $^2$ (nmol of NADH oxidized/min per mg of protein)</th>
<th>GST activity $^3$ (mmol of GSH$^3$ conjugated/min per mg of protein)</th>
<th>CYP450 3A4$^4$ activity (µmol of 7-BQ$^5$/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gossypol</td>
<td>3.29a</td>
<td>0.017a</td>
<td>0.239a</td>
</tr>
<tr>
<td>Gossypol + silymarin</td>
<td>3.45a</td>
<td>0.015a</td>
<td>0.246a</td>
</tr>
<tr>
<td>Silymarin</td>
<td>2.82b</td>
<td>0.016a</td>
<td>0.261a</td>
</tr>
<tr>
<td>Control</td>
<td>2.75b</td>
<td>0.014a</td>
<td>0.281a</td>
</tr>
</tbody>
</table>

$^{ab}$Means in the same column with different superscripts are different ($P < 0.001$).

$^1$SEM = 0.144, error df = 40.

$^2$SEM = 0.009, error df = 40.

$^3$Glutathione.

$^4$SEM = 0.025, error df = 40.

$^5$7-Benzylxyquinoline.
including both the linear and quadratic coefficients in the regression (model [2]) of GGT on week improved the fit of the statistical model. For chickens fed G and G-S diets, GGT activities increased consistently with time throughout the experiment. The shape of that relationship appeared curvilinear in chickens fed C and S diets, and the linear (P < 0.001) and quadratic coefficients (P = 0.093) differed among these pairs of treatments.

Similar to GGT, including both linear and quadratic coefficients in the regression of AST on week improved the fit of model [2]. Although AST activities increased consistently (linearly) with week throughout the experiment for chickens fed C and S diets, the relationship for chickens fed G and G-S diets was curvilinear. Although, for chickens fed C and S diets, only the values of the slope differed from 0 (P < 0.001); however, for those fed G and G-S diets, fitting a quadratic term was also justified (P < 0.001).

Diet had no effect on hematocrits [mean (SD) 32.4% (3.4)]. There was no relationship between hematocrits and week among diets (linear term: P = 0.164; quadratic term: P = 0.935).

**Line Effects**

**BW and Feed Intake.** At d 21, LA chickens weighed less than HA chickens (P < 0.001) regardless of diet (Table 5). During the last week of treatment, both lines of chickens fed G and G-S diets consumed less feed (on average, 0.74 times as much; P < 0.001) than those consuming C and S diets (data not shown). However, there were no differences in feed intake between C and S treatments for HA and LA chickens.

**Liver Enzyme and Histologic Evaluations.** Line had no effect on QR (P = 0.615), GST (P = 0.333), or CYP450 3A4 activity (P = 0.078). Line had no effect on lipidosis score [mean (SD) 1.37 (0.56); P = 0.413], although LA chickens had more (1.69 ± 0.097) heterophils than HA chickens (0.90 ± 0.097; P < 0.001). Line LA chickens had more lymphoid nodules (1.45 ± 0.054) than LA chickens (1.00 ± 0.054; P < 0.001). A χ² analysis revealed that more LA chickens (n = 35) had category 2 and 3 heterophil scores than HA chickens (n = 6; P < 0.001), and there were more HA chickens (n = 30) with category 2 and 3 lymphoid nodule scores than LA chickens (n = 2; P < 0.001). However, as found with ANOVA, there were no differences in lipidosis between lines (P = 0.842; data not shown).

**Clinical Chemistry.** Irrespective of diet, GGT level in HA chickens was, on average, 1.2 times that of LA chickens (P = 0.002; Table 5). By d 21, there were differences between lines among diets (P < 0.001), although that was largely associated with C. The values of the intercept (P < 0.001) and quadratic coefficient (P = 0.019) differed between lines.

At d 7 of treatment, LA chickens had substantially higher (on average, 2.0 times as much) AST levels than HA chickens (P < 0.001). This difference became smaller by d 14 (P = 0.16) and was no longer evident by d 21 (P = 0.65). Hematocrits declined as the ex-

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**Table 4.** Least squares means for plasma gamma glutamyltransferase (GGT) and aspartate aminotransferase (AST) across lines for chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>GGT (U/L) by day on treatment¹</th>
<th>AST (U/L) by day on treatment²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Gossypol</td>
<td>7.6a</td>
<td>6.0a</td>
</tr>
<tr>
<td>Gossypol + silymarin</td>
<td>7.6a</td>
<td>6.0a</td>
</tr>
<tr>
<td>Silymarin</td>
<td>7.8a</td>
<td>7.0b</td>
</tr>
<tr>
<td>Control</td>
<td>7.8a</td>
<td>6.2a</td>
</tr>
<tr>
<td>SEM</td>
<td>0.39</td>
<td>0.72</td>
</tr>
</tbody>
</table>

¹Means in the same column with different superscripts are different (P < 0.01).
²Maximum SEM = 0.72, error df = 40.

**Table 5.** Least squares means for weekly BW, feed intake, gamma glutamyltransferase (GGT), and aspartate aminotransferase (AST) for high-antibody (HA) and low-antibody (LA) chickens across diets

<table>
<thead>
<tr>
<th>Line</th>
<th>BW (g) by day on treatment¹</th>
<th>Feed intake (g) by day on treatment²</th>
<th>GGT (U/L) by day on treatment³</th>
<th>AST (U/L) by day on treatment⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>HA</td>
<td>446a</td>
<td>545a</td>
<td>625a</td>
<td>687a</td>
</tr>
<tr>
<td>LA</td>
<td>438a</td>
<td>535a</td>
<td>611b</td>
<td>643b</td>
</tr>
</tbody>
</table>

¹Means in the same column with different superscripts are different (P < 0.05).
²Maximum SEM = 8.3, error df = 51.
³Maximum SEM = 5.4, error df = 73.
⁴Maximum SEM = 0.51, error df = 40.
⁵Maximum SEM = 1.5, error df = 40.
experiment progressed. Still, across week, hematocrits for HA chickens were, on average, 1.1 times those for LA chickens \((P < 0.001)\).

**DISCUSSION**

**Dietary Effects**

Reduced BW and feed intake are common signs of gossypol toxicosis (Henry et al., 2001b; Nagalakshmi et al., 2007). Gossypol binds essential amino acids such as lysine (Henry et al., 2001a). Although reduced feed intake could reflect insufficiencies in essential nutrients, chemical analysis did not reflect differences in amino acid composition among diets. Thus, the reduction in feed intake was a consequence of health status, with the depression in BW in chickens fed the G diets reflecting voluntary reductions in feed intake rather than nutrient composition of the diet. Addition of silymarin to the diet did not offset the effects of gossypol on BW or feed intake.

Gamma glutamyltransferase and AST are physiological markers of liver health. High plasma GGT has been associated with biliary hyperplasia and bile duct carcinoma (Harr, 2002). Significantly higher GGT values in G and G-S chickens implied that gossypol was causing damage to biliary ducts. Furthermore, average plasma GGT concentrations during the final week for G and G-S chickens were 12.9 and 13.8 U/L, respectively. These values were above the normal range of 0 to 10 U/L for birds (Harr, 2002).

Plasma AST increased in chickens fed diets G and G-S after 14 d of treatment, which indicated that gossypol was eliciting a toxic response. Surprisingly, AST levels decreased precipitously in these chickens during the last 7 d of treatment, which coincided with lower feed intake and may have resulted in reduced exposure to gossypol allowing liver recovery. There was no histological evidence of permanent liver damage; therefore, liver recovery may have begun once gossypol exposure was reduced.

The AST values observed in this study do not coincide with those reported by Hrubec et al. (2002). Chickens fed G and G-S diets had AST values 10-fold lower than those reported as normal in mammalian species. Although the spectrophotometric kit used to assay AST has been validated in mammalian species, it had not been validated in birds (Biotron Diagnostics). Therefore, it is possible that chicken plasma contains matrix constituents not encountered by the kit’s manufacturer that interfere with absorbance. Although this may preclude comparison of AST values in this experiment with those for mammals, nonetheless those for G and G-S birds were significantly different than those for their counterparts.

Tedesco et al. (2004) reported that a silymarin concentration of 600 mg/kg in the diet reduced plasma GGT and AST in broilers with aflatoxicosis. Furthermore, silymarin significantly reduced these enzymes in humans with liver disease (Wellington and Jarvis, 2001). However, in this experiment, plasma AST and GGT levels were not improved (e.g., lower level compared with G chickens) in the G-S group.

Gossypol causes erythrocyte fragility and has been repeatedly reported to decrease hematocrits (Henry et al., 2001b; Matondi et al., 2007; Adeyemo, 2008). Although the lack of differences in hematocrits observed among diets in this study is contrary to some literature, they are consistent with those of Kalla and Chadha (1990), who fed chickens gossypol by oral intubation.

Quinone reductase is a cytosolic enzyme responsible for the reduction of quinones produced through the metabolism of dietary phenols, aromatics, and polycyclic aromatic compounds (Talalay and Dinkova-Kostova, 2004). Gossypol is a polycyclic compound, making it a prime substrate for QR. Therefore, it is not surprising that QR activity increased in chickens fed G and G-S diets to compensate for increased quinone production. Silymarin is hypothesized to be an inducer of enzymes for detoxification (Zhao and Agarwal, 1999). However, QR activity in chickens fed the S diet was not different from C. Furthermore, QR activity in chickens fed the G-S diet was not greater than G-fed chickens.

Glutathione-S-transferase is an important phase II enzyme. Gossypol reportedly binds to GST preventing it from binding with normal substrates. This causes a reduction in GST activity (Sugiyama et al., 1984). Kiruthiga et al. (2007) reported that silymarin prevented a reduction in GST activity. Because GST activity was similar for G and C chickens in this experiment, there is little reason to expect a therapeutic effect of silymarin on GST activity in G-S chickens.

Cytochrome P450 3A4 is an important phase I enzyme in xenobiotic metabolism. Several studies have shown that gossypol and silymarin both inhibit CYP450 activity (Ma and Black, 1984; Johansen and Misra, 1990; Kiruthiga et al., 2007). An in vitro study with silybin concentrations of 25, 50, 100, and 250 \(\mu\)M indicated that CYP450 3A4 inhibition depended on silymarin concentration and length of exposure (Sridar et al., 2004). Cytochrome P450 3A4 was not reduced in this experiment. There were no differences in CYP450 3A4 among chickens fed the various diets. The concentration of silymarin may have been insufficient to elicit such a response. Additionally, Kosina et al. (2005) reported that silymarin did not reduce CYP450 3A4 in human hepatocytes in culture.

Lipidosis scores were higher in chickens fed G and G-S diets than those fed the other diets, which are indicative of lipid storage and thus liver malfunction (Plaa and Charbonneau, 2008). Lipidosis is reversible and there were no histological indicators of permanent liver damage. Thus, it can be hypothesized that liver health would be improved in G and G-S chickens if they were switched to a gossypol-free diet. However, if exposed to a high enough dosage of gossypol for a substantial time period, irreversible damage is probable.
Silymarin has a polyphenolic structure, which is the reason it has antioxidant properties. Its hydroxyl groups have the potential to scavenge for free radicals (Miranda et al., 2000). Gossypol also has a polyphenolic structure. Potentially, the combined concentrations of gossypol and silymarin used exposed the chickens to an excessive amount of polyphenols. As a consequence, silymarin could lose its antioxidant properties. Furthermore, antioxidants can become prooxidants when exposures are increased (Miranda et al., 2000). Such results were observed when chickens were fed butylated hydroxytoluene, a feed antioxidant. Sudhakar et al. (2007) found that exposing chickens to large doses (i.e., 520, 1,040, and 2,080 mg/kg) of butylated hydroxytoluene resulted in congestion and hyperemia of the liver and kidney, hemorrhaging in the liver, and death. Furthermore, Mizutani et al. (1987) reported that butylated hydroxytoluene exacerbated liver damage in mice when dosed with buthionine sulfoximine, an inhibitor of glutathione synthesis.

**Line Effects**

There was no overwhelming evidence that the HA and LA chickens responded differently to the diets used in this experiment. Low-antibody chickens fed G and G-S diets consumed less feed and weighed less than HA chickens. This is contradictory to a toxicology study with these lines in which LA chickens weighed more than HA chickens (Ubosi et al., 1985b). However, in the cited study, husbandry conditions were different and the experiment studied aflatoxin, an established immunotoxin (Bondy and Pestka, 2000). Therefore, it is not unreasonable to assume that chickens selected for immune response would respond differently to an immunotoxin than to gossypol.

In terms of clinical chemistry, LA chickens had numerically higher AST and lower GST concentrations than HA chickens. These differences, however, were not statistically significant. Therefore, there does not appear to be a greater sensitivity to gossypol toxicity in one line than the other. Differences in lymphoid nodules and heterophils between HA and LA chickens were independent of diet. Therefore, these hematopoietic differences appear simply a correlated response to the long-term selection for antibody response to SRBC (Gross and Siegel, 1980).

Silymarin did not alleviate the effects of gossypol toxicosis. None of the criteria used to assess liver health were improved in chickens on the G-S diet. Furthermore, silymarin alone did not affect liver function as reported previously in the literature. It is also possible the concentration of gossypol used in the diets was sufficiently high, so that no matter the level of silymarin fed, damage would occur. Perhaps, gossypol fed at a level between 500 and 1,000 mg/kg in the diet would cause toxicosis yet still respond to silymarin treatment. Future studies should test various concentrations of gossypol in the diet. In addition, there was no substantial difference in response to gossypol in the HA and LA lines. Long-term selection for humoral immune response appears to offer no advantage in coping with gossypol toxicosis.

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