Abstract. Sera from chickens that consumed a known level of (–)-gossypol (–)-GP) exhibited bioactivity against human breast cancer cells in vitro. Based on this, it is conceivable that similar anti-breast cancer activities of different magnitudes will be exhibited in biological samples harvested from (–)-GP-fed layer hens. The experimental data generated in this study may influence the fundamental thinking regarding the utilization of low cost agricultural commodities such as (–)-GP cottonseed meal to produce value-added chemopreventive animal products.

Gossypol (GP) is a naturally occurring polyphenolic pigment present in the cotton plant. Naturally occurring GP exists as a mixture of two enantiomers, (+)-GP and (–)-GP and is also found in cotton plant by-products (1) that are often consumed by humans and food-producing animals. On average, conventional cottonseeds contain 65% (+)-GP and 35% (–)-GP. However, selective breeding strategies have resulted in novel cottonseed cultivars containing 65% (–)-GP and 35% (+)-GP. In the U.S. cottonseed oil (CSO) and cottonseed meal (CSM) directly enter the human diet through their use in cooking, frying, and food processing. Previous studies demonstrated the incorporation of conjugated linoleic acid and fish oil into both adipose tissue and muscle lipid (2) as well as incorporation into the eggs (3, 4) of hens fed supplemented diets supplemented with varying levels of conjugated linoleic acid and fish oil. These authors concluded that CLA-enriched chicken poultry feeds could be an alternative vehicle for delivering health-promoting fatty acids to consumers (2, 3).

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**Materials and Methods**

Three levels of the (−)-GP were added by replacing corn oil with (−)-GP enriched cottonseed oil to a basal diet at 8 ppm, 16 ppm and 32 ppm in for the experimental diets. The basal diet (Table I) contained no added CSO and was used as a control. Each diet was fed to a group of 5 commercial hens housed in individual cages at a rate 1.25 lb per group per day. Feed was weighed weekly and recorded to determine feed intake. Body weights and feed consumption were evaluated weekly, and egg production was evaluated at the end of the 28-day production period. Serum samples were collected weekly and liver toxicity was evaluated using LDH assays on serum collected over the 28-day production period.

**Measurement of liver toxicity by LDH assay.** Hepatic LDH released into the chicken serum was measured as an indicator of (−)-GP toxicity to the chickens fed different levels of experimentally formulated GP-diets. A total of 12 serum samples (n=3 per treatment; 300 μl per sample) from chickens consuming different levels of GP for up to 28 days were randomly selected for LDH analysis by the Veterinary Clinical Pathology and Diagnostic Laboratory at The Ohio State University Veterinary Medical Teaching Hospital.

**Effect of hen sera on proliferation of MCF-7 cells.** Sera from the hens fed 32 ppm GPCSO was dialyzed against 0.2% BSA DMEM/F12 before adding to MCF-7 cells. 3x10³ MCF-7 cells were seeded in a 96-well plate, in 100 μl of DMEM/F12 supplemented with 5% FBS and incubated at 18 h. Culture medium was replaced with 0.2% BSA DMEM/F12 and incubated an additional 18 h. Culture media containing either 0% as a control, 2.5% or 12.5% treated chicken sera were incubated with MCF-7 cells for 24 h. Cell proliferation rates were quantified by using CellTiter 96 AQueous™ assay according to the manufacturer’s protocol (Promega, Madison, WI, USA).

**Gossypol binding experiment.** Column-purified chicken myosin was prepared as previously described previously (25). Purified chicken myosin (0.5 mg) and 5.18 μg of (−)-GP in 5.5 ml of PBS buffer were incubated at room temperature for 20 h. A sample of the solution was taken for subsequent SDS-PAGE and Western blot analysis. The remaining solution was dialyzed (1,000 MW cut-off) against 2x4 l of PBS at 4°C.

**Table I. Basal diet for commercial layers.**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Inclusion (%)</th>
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<tbody>
<tr>
<td>Corn</td>
<td>57.24</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>10.90</td>
</tr>
<tr>
<td>Wheat midds</td>
<td>5.00</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>11.50</td>
</tr>
<tr>
<td>Limestone</td>
<td>9.00</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.20</td>
</tr>
<tr>
<td>L-lysine</td>
<td>0.06</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.20</td>
</tr>
<tr>
<td>Corn oil</td>
<td>4.00</td>
</tr>
<tr>
<td>Salt</td>
<td>0.40</td>
</tr>
</tbody>
</table>

**Table II. Measurement of lactate dehydrogenase (LDH) from serum samples to determine gossypol toxicity in chickens fed diets containing (−)-gossypol. No significant differences were found between treatments.**

<table>
<thead>
<tr>
<th>Days on GP-containing diet</th>
<th>Concentration of hepatic LDH (IU/ml)</th>
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<tbody>
<tr>
<td></td>
<td>0 ppm</td>
</tr>
<tr>
<td>0</td>
<td>1.054</td>
</tr>
<tr>
<td>7</td>
<td>0.56</td>
</tr>
<tr>
<td>15</td>
<td>0.943</td>
</tr>
<tr>
<td>28</td>
<td>0.267</td>
</tr>
</tbody>
</table>

**SDS PAGE.** Electrophoretic analysis of the myosin-GP conjugate was performed by SDS PAGE as previously described (26). Briefly, The (−)-GP/myosin solution was mixed with an equal volume of reducing buffer sample buffer (8 M urea/2 M thiourea, 75 mM DTT, 50 mM Tris, 3% SDS, and 0.004% bromophenol blue, pH 6.8) at a ratio of 1 mg of sample per 30 μl of sample buffer and incubated on ice for 30 min. Samples were centrifuged at 10,000 × g for 10 min prior to loading onto a 1 mm 12 cm × 14 cm polyacrylamide slab gel consisting of a 10% resolving gel [30:0.8, acrylamide/(N,N'-methylenebisacrylamide)] and a 3% stacking gel containing 1% SDS. Electrophoretic separation was carried out at a constant voltage of 10 V cm⁻¹. Gels were stained with Coomassie Brilliant Blue G-250 and destained with 10% acetic acid.

**Western immunoblot analysis of (−)-GP bound myosin.** After SDS-PAGE, the proteins were electro-transferred to a nitrocellulose membrane using a GENIE electroblot transfer apparatus (Idea Scientific, Minneapolis, MN 55414) in electro-transfer buffer (40 mM TRIS, 240 mM glycine, methanol, 0.1% SDS) (Towbin et al. 1979). After transfer, the membrane was blocked with PBST (136 mM NaCl, 2 mM KCl, 6 mM Na₂HPO₄, 2 mM KH₂PO₄, pH to 7.2, 3% Tween-20) for 30 min at 37°C. The pan sarcomeric myosin heavy chain monoclonal antibody, NAA4, (27) was diluted at 1:10,000 in PBS-T and incubated on the membrane for 15 min at room temperature with gentle shaking. The membrane was washed 3 times PBS-T. Bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (H+ L) (Pierce-Endogen, Rockford, IL, USA) at a dilution of 1:2,500 in PBS-3% Tween-20 and incubated for 30 min at room temperature. The membrane was developed in StableDab solution (KPL, Inc. City State) according to manufacturer’s recommendations. Color development was stopped by rinsing the membrane in ddH₂O. The membrane was dried and the image was captured on a flatbed scanner.

**Analysis of gossypol bound myosin in cell culture.** For the cell culture experiment, the (−)-GP/myosin solution was dialyzed against 0.2% BSA DMEM/F12 before adding to cells. 3x10³ MCF-7 cells were seeded in a 96-well plate, in 100 μl of DMEM/F12 supplemented with 5% FBS and incubated for 18 h. Culture medium was replaced with 0.2% BSA DMEM/F12 and incubated an additional 18 h. The cells were then incubated with 0, 2, 4 μM of chicken myosin bound-(−)-GP, pure (−)-GP, (±)-GP or (+)-GP for 24 h at 37°C. Cell proliferation rates were quantified by using CellTiter 96 AQueous™ assay according to the manufacturer’s protocol (Promega).
Statistical analysis. Production data were analyzed using PROC MIXED in SAS v9.1.3 (SAS Institute; Cary, NC, USA). Data are reported as least square means±SE for each line. The experimental designs for the in vitro assays were based upon the advice from and consultation with the OSU Biostatistics Program and functional analysis of experimental data from cell proliferation assays were quantified and analyzed by one-way ANOVA with post-test analysis using the Boneferroni t-test.

Results

Effect of (–)-GP diets on production of commercial layers. No differences were shown between the control diets and the 8, 16 and 32 ppm (–)-GP added diets for any of the production parameters measured. There were no differences in average egg production, body weight or feed consumption in the control and (–)-GPCSM diets fed to layers (Figure 1A, 1B, and 1C, respectively).

Liver toxicity by LDH assay. The LDH data are summarized in Table I. The serum LDH for control chickens was 1.054 IU/ml. The serum LDH range from chickens consuming 8, 16, and 32 ppm of GP in (–)-GPCSM containing experimental diets for 7, 15, and 28 days were 0.185 to 0.943 IU/ml.

Effect of hen sera on proliferation of MCF-7 cells. Hens fed 32 ppm (–)-GPCSO for 28 d showed a dose-dependent decrease in the proliferation of MCF-7 cells (Figure 2). Data presented in Figure 2 demonstrate that culture medium containing 2.5% and 12.5% sera significantly (p=0.05, p=0.014) suppressed the proliferation of the ERα-positive human breast cancer cell line MCF-7 after 24 h of exposure.

(–)-GP bound to the meat protein, myosin. Data presented in Figure 3 showed a lowered electrophoretic mobility of purified myosin that was incubated with (–)-GP. Purified myosin in lane 2 migrated at its molecular mass corresponding to 223 kDa. However, after purified myosin was incubated with purified (–)-gossypol, its migration was...
inhibited (Figure 3 lane 3). In addition, Western blot presented in Figure 6 demonstrates that the myosin heavy chains were positively stained for the presence of GP.

As shown in Figure 4, treatment with (–)-GP, and (±)-GP, at 2 and 4 μM significantly suppressed the proliferation of MCF-7 cells [(–)-GP at 2 μM, \( p = 0.02 \); 4 μM, \( p = 0.0001 \); (±)-GP, 2 μM, \( p = 0.001 \); 4 μM \( p = 0.0002 \)]. However, the same concentrations of (+)-GP did not exhibit any anti-breast cancer activity in MCF-7 cells. The chicken myosin-bound (–)-GP did not have an inhibitory effect at 2 μM. However, at 4 μM there was anti-proliferative activity.

Discussion

The production data and the measure of hepatic toxicity in laying hens showed no negative effects of gossypol being added to the diets at 8, 16 or 32 ppm. These data demonstrate that short-term consumption of low levels of (–)-GP in the experimental diets formulated from the (–)-GP enriched cottonseed oil did not result in production decreases or hepatic toxicity. These results are similar to those reported by Lordelo et al. who demonstrated that commercial layers and broiler breeder hens fed 200 ppm (–)-GP and 200 ppm (+)-GP for 20 days did not exhibit any negative effects on egg production, food intake weight gain, yolk discolouration, or liver toxicity (30).

The FDA limit of free (±)-GP content in cottonseed products is 450 ppm (0.045%) (FDA, 1974) equivalent to 868.7 μM. The amount of GP used in our (–)-GP diets of 8, 6 and 32 ppm were approximately 12 to 50 fold lower than the FDA limit. Our results demonstrated decreases in proliferation of MCF-7 cells when using pure (–)-GP, (–)-GP bound to myosin and sera containing (–)-GP. The low concentrations GP enantiomers contained in the blood of chickens that consumed the (–)-GPCSM diets are biologically active and capable of inhibiting of human breast cancer cell growth in vitro.

The lowered electrophoretic mobility of the covalently bound myosin-(–)-GP complex could be due to either the prevention of the association of SDS at the proper ratio of 1.4 g per g of protein or the cross linking of the myosin heavy chains by the (–)-GP ligand, in an unknown way. It is unlikely that the myosin heavy chains shown in Figure 5 lane 3 were non-covalently cross-linked since SDS denatures proteins and disrupts all non-covalent bonds. In addition, the presence of DTT reduces any covalent disulfide bonds. Therefore, it is likely that the lowered electrophoretic mobility of the myosin heavy chains in Figure 5 lane 3 is due to the covalent binding of (–)-GP to the myosin at as yet to be determined amino acid residues.

The observation that the chicken meat protein, myosin, bound (–)-GP was biologically active at a lower potency in vitro than the pure free (–)-GP and (±)-GP is novel. This is the first evidence demonstrating that a chicken muscle protein (myosin) bound to (–)-GP is biologically active in inhibiting proliferation of a human breast cancer cell in vitro. These results provide support for our hypothesis that the (–)-GP bound to muscle proteins from the (–)-GPCSM fed layers has bioactivity in protecting the healthy consumers and human breast cancer patients.

Results from previous studies demonstrated that the GP enriched-milk is a potent aromatase inhibitor in ovarian granulosa cells (39). The 10% GP-milk, equivalent to 1.29 ppm used was about 348 times lower than the FDA limit of 450 ppm. Those investigators concluded GP-milk could be considered as a bio-active food component as an aromatase inhibitor.
In another study, GP-milk at 10% and 20% (v/v), equivalent to 2.59 ppm significantly suppressed aromatase activity when compared to the 10% and 20% control milk-treated human breast cancer cell line, MCF-7 (31). To the best of our knowledge, this was the first solid evidence demonstrating that biologically active GP present in GP-milk can exert its inhibitory action in regulating aromatase activity in MCF-7 cells as the parent compound (+)-GP does.

In another study using GP-containing serum harvested from nursing rat dams fed GP-milk exhibited a suppression of cAMP production in ovarian luteal cells (40). Using [3H]-labeled GP as a tracer it was shown that the nursing dams were able to transfer the [3H-GP through the milk to the neonatal pups (33). In the ovarian luteal cells, the [3H-GP had differential affinity for different organelles in the cell fractions (34). These results suggested that the serum protein-bound GP is biologically active in regulating the secretion of cAMP in ovarian luteal cells.

Because aromatase converts androgens into estrogens in humans, consumption of this bioactive food component could have a clinically relevant impact on human breast cancer cell proliferation. This results from the fact that aromatase inhibitors are currently clinically used and recognized as better therapeutic drugs than the anti-estrogenic drug Tamoxifen. The potential for the chemopreventive potential of meat derived from animals fed diets containing GP is highlighted by the observation that aromatase inhibitors (such as anastrozole, letrozole) have been clinically evaluated and it was concluded that they were a more effective in treatment of human breast cancer patients than the antiestrogen drug, tamoxifen for breast cancer patients (35-38).

In the current study, we show that the sera from chickens that consumed a known level of GP in the experimental diet are the first to provide relevant scientific evidence demonstrating that the bioactive GP circulated in these (–)-GPCSO fed chickens indeed possesses anti-human breast cancer activity in vivo. Based on the sera activity, it is conceivable that similar anti-breast cancer activities in a different magnitude will be presented in these biological samples harvested from the (–)-GP fed layer hens. We have confidence that the experimental data generated in this study may influence the fundamental thinking regarding utilization of low cost agricultural commodities such as (–)-GPCSO to produce value-added chemopreventive animal products to benefit the consumers in our society.

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References
