Regulatory Toxicology and Pharmacology 70 (2014) S105–S113

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

90-Day dietary toxicity study with esterified propoxylated glycerol (EPG) in Micropigs

John Wedig [a,1], David H. Bechtel [b,*]

[a] T.P.S, Inc., 10424 Middle Mt. Vernon Rd, Mt. Vernon, IN 47620, United States
[b] Best Foods, Division of CPC International, 150 Pierce St., Somerset, NJ 08873, United States

ARTICLE INFO

Article history:
Available online 8 December 2014

Keywords:
Esterified propoxylated glycerol
EPG
Fat substitute
Subchronic oral toxicity

ABSTRACT

The subchronic (90-day) toxicity of esterified propoxylated glycerol (EPG) was assessed in micropigs. Animals (5/sex/group) received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively. Corn oil served as the vehicle control (0 g/kg bw/day). Subsets of animals were evaluated at Week 6; the remainder between Weeks 12 and 14. With the exception of liver and serum vitamin levels, statistically significant difference between control and EPG groups were seen sporadically, and with no apparent connection to treatment and/or no consistency across time intervals. EPG intakes of 3 and 5 g/kg bw/day, but not at 1.5 g/kg bw/day were associated with significantly lower serum 25-OH vitamin D levels. Serum total vitamin D levels were significantly lower across all EPG groups. There were also trends toward lower levels of liver vitamins A and E among EPG-treated animals, but the effects were less consistent. The effects on vitamin levels observed in EPG-treated animals were not accompanied by any signs of vitamin deficiency (*e.g.*, effects on growth, clinical signs, or clinical pathology), and might have been related to the larger mass of EPG acting as a lipid "sink" during transit in the gastrointestinal tract.

1. Introduction

Esterified propoxylated glycerols (EPGs) represent a family of fat- and oil-like substances, resembling triglycerides in structure and appearance, but modified to prevent or limit their digestion when consumed in food. They consist of multiple propylene glycol units inserted between the glycerol and fatty acid moieties of fats and oils. Their poor absorption results in a low- to no-calorie profile when substituted for fat in the diet.

The present study examined safety of a hydrogenated version of EPG that is considered the "core" version (H-EPG-05-HR/SO 9:1)[2] in micropigs (*Sus scrofa*) following administration in the feed for up to 90 days (13 weeks). The micropig is considered an appropriate model species because like humans, they are true omnivores and share many important features of anatomy, physiology, and function of the gastrointestinal systems (Bode et al., 2010).

2. Materials and methods

This study was sponsored by ARCO Chemical Company, Newton Square, Pennsylvania and Best Foods, Somerset, New Jersey. It was conducted at T.P.S., Inc., Mt. Vernon, Indiana, from February to May, 1993 (in-life portion), in compliance with the principles of Good Laboratory Practice (GLP) regulations of the United States Food and Drug Administration (FDA).

2.1. Animals

Young healthy male and female Yucatan Micropigs® were obtained from Charles River Laboratories, Windham, ME. Twenty male micropigs weighing 12.808–18.189 kg and twenty female micropigs weighing 10.991–19.384 were started on the study following an acclimation period of approximately 5.5 months. Following the acclimation period, animals (8–10 months old; 5/sex/group) of adequate body weight and in good health

* Corresponding author at: Intertek Scientific & Regulatory Consultancy, 1011 US Highway 22, Suite 200, Bridgewater, NJ 08807, United States. Fax: +1 908 429 9260.
E-mail address: david.bechtel@intertek.com (D.H. Bechtel).

[1] Now retired.

[2] The nomenclature used to identify specific EPG version is based on the total number of propylene glycol units attached to the glycerol back bone, the source or identity of the fatty acids attached to the propylene glycol units, and the hydrogenation status of the final product. For example; H-EPG-05 HR/SO 9:1 is an EPG in which 05 represents the mean number of propylene glycol units per glycerol, HR/SO represents high-erucic acid rapeseed oil/soybean oil in a 9:1 ratio. The initial "H" indicates that the product is hydrogenated converting all fatty acids into their fully saturated counterpart. For example, erucic acid is converted to behenic acid.

http://dx.doi.org/10.1016/j.yrtph.2014.11.012
0273-2300/

(determined by a detailed physical and ophthalmic examination, including slit lamp biomicroscopy, urinalysis, and clinical pathology) were stratified by weight and assigned to each of the study groups (Table 1) based on a computer-generated randomization schedule.

2.2. Test article and dosing

The test material, esterified propoxylated glycerol [H-EPG-05 HR/SO 9:1; EPG (stabilized with tocopherols, including α-tocopherol), Lot Nos. 753485 and 850201], an off-white solid, was provided by the sponsor. Bulk (55-gallon drum containers) material was stored at −20 °C until thawed, repackaged (5-gallon), and stored at −25 to −3 °C. Prior to use, working aliquots were thawed and stored refrigerated (0–11 °C).

Animals received feed containing up to 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively. The vehicle, corn oil, served as a control (0 g/kg bw/day).

The carrier was Certified Agway® Prolab® Minipig Diet Meal (Lots No. Jul 16 92 W2 and Dec 9 92 W1); corn oil (Mazola® 100% pure corn oil, Lot No. 2321) was added at 4% (w/w). The fat content in of the diets (with corn oil) was approximately 7% by weight; the remaining major components of the feed were approximately 17% protein, 13% crude fiber, 10% moisture, and 7% ash.

For each diet, the appropriate amount of EPG (corn oil only for control) was placed in a glass beaker with corn oil; the mixture was stored at 55 °C for at least 1 h, followed by stirring on a magnetic stir plate until dissolved. The EPG/corn oil blend and a fraction of the total feed was placed in a mixer (Univex M-20 or Hobart V-1401 mixer) for approximately 5 min; any residue remaining on the beaker and stir bar was rinsed into the mix with approximately 100 g of additional corn oil, followed by feed that was set aside. This mixture was blended in a ribbon mixer with the remaining feed for approximately 10 min.

Each diet was prepared the day before study initiation and weekly thereafter. Prepared diets were stored protected from light in plastic containers lined with food-grade plastic bags at 32–52 °C. Analysis of the (weekly) feed for EPG revealed the following range of concentrations for each of the 5%, 10%, and 17% diets, respectively: 4.5–5.1%; 8.4–9.9%; and 13.3–15%.

Animals were fed three times per day (morning, noon, and evening) for at least 90 days (13 weeks), with approximately three hours between feedings. The EPG portion of the diet was divided, weighed, and provided in the morning and evening feedings. Any supplemental feed (control feed) that might be needed to provide enough calories[3] was administered during the noon feeding. EPG-containing feed was weighed before and after the feeding period, unless none remained (*i.e.*, marked as "consumed").

2.3. Housing

Animals were housed individually in adjacent runs (approximately 3′ × 6′) with chain-link wire sides, epoxy coated floors, and hardwood chip bedding, in an isolated temperature – (54–81 °F) and humidity – (20–86%) controlled animal room with filtered air supply (10–15 changes/h) and cycled lighting (12-h light/12-h dark). Minimum and maximum room temperatures and humidity were recorded daily. The runs were cleaned daily and sanitized approximately every 2 weeks. Fresh tap water was available *ad libitum* from automatic water nipples. Concentrations of contaminants in the feed and drinking water were considered to be below levels capable of compromising the study.

Table 1
EPG concentration and group composition.

Group	Treatment		Number of animals/sex[b]
	EPG[*] g/kg/day	Dietary concentration[+] % (w/w)	
Control (AVI1)[**]	0	0	5
Low EPG (AVI2)	1.5	5	5
Mid EPG (AVI3)	3	10	5
High EPG (AVI4)	5	17	5

[*] Approximate levels; animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.
[**] The basic diet was supplemented with 4% (w/w) corn oil; test diets contained EPG and 4% (w/w) corn oil as vehicle.
[+] % (w/w) = weight of EPG per weight of basal diet including corn oil.

2.4. Observations

2.4.1. Mortality and clinical signs

All animals were observed twice daily throughout the test period for mortality, moribundity, general health, physical appearance, and pharmacological, toxicological, or behavioral effects. This included visual inspection of the feces for appearance, consistency and any evidence of phase separation (*i.e.*, appearance of oily layers or deposits in the stools).

2.4.2. Body weight and weight gain

Individual body weights were obtained on a single beam balance (Nordic Forge 4600) during pre-test, day-1, and weekly thereafter. Body weights used for calculating the relative organ weights at necropsy were obtained after an overnight fast immediately prior to necropsy.

2.4.3. Feed consumption and EPG intake

Food consumption was recorded daily during the study. Feed efficiency [weight gain (g)/feed consumed (g)] was calculated on a weekly basis.

2.4.4. Physical and ophthalmic examination

Each animal was subjected to a detailed physical examination by the attending veterinarian at pre-test and during Weeks 6 and 13; a general physical examination was conducted weekly otherwise by a technician. Animals were tested for the presence of fecal parasites at pre-test and during Weeks 7 and 14.

Ophthalmic examinations (including slit-lamp biomicroscopy) were performed by a Board-certified veterinary ophthalmologist prior to study initiation and during Weeks 6 and 13. Prior to examination, animals were sedated with 20–25 mg/kg of Ketamine HCl (Ketaset®, Aveco, Fort Dodge, IA, Lot Nos. 440164 and 440176,) *via* intramuscular (i.m.) injection. The eyes were dilated with Tropicamide Ophthalmic Solution, USP 1% (Schein, Port Washington, NY, Lot Nos. 91K410 and 92J800).

2.4.5. Hematology, clinical chemistry, and urinalysis

Blood samples for hematology and serum chemistry were collected from the anterior vena cava after an overnight fast. Collections were made at pre-test and during Weeks 6 and 13. RBC count, hematocrit, MCV, MCH, MCHC, WBC count, hemoglobin, and platelet count were measured using a Cell-Dyn® 900 Hematology (Sequoia-Turner). ProT and APTT were measured using a BBL Fibrometer. Accustain™ (Sigma) was used on bone marrow smears

[3] Additional amounts of the control diet (containing 4% corn oil) were administered across all groups, based on an algorithm, to ensure each animal received approximately 500 to 600 g of feed per day. The levels ranged from 280 g/day in an animal weighing 9 kg to 15 g/day in a 19.5-kg pig; no supplementation would have been required once body weight reached 20 kg.

and differential WBC count, while reticulocytes were viewed using the new methylene blue stain. Clinical chemistry parameters were measured by the Beckman Synchron CX5 System (Beckman Instruments, Inc., Brea, California.)

A 24-h urine sample was collected in stainless steel metabolism cages from all animals (hydrated with tap water at 20 mL/kg) pre-test and during Week 6, and Week 12 or 13. Urinalysis and urine chemistry were performed using Chemstrip® 8 (Boehringer-Mannheim Diagnostics, Inc.) and Beckman Synchron CX5 System (Beckman Instruments, Inc., Brea, California.), respectively.

2.4.6. Liver and serum vitamins

At scheduled necropsy, a separate aliquot of blood was drawn before pentobarbital administration. The clotted sample was centrifuged and the serum stored at −20 °C until analysis for 25-hydroxyvitamin D and total vitamin D. Analyses for 25-hydroxyvitamin D and total vitamin D were performed by radioimmunoassay detailed in Hollis et al. (1993) and HPLC according to the procedure described in Liel et al. (1988) with minor modifications, respectively.

For liver vitamins A and E (control and 5 g/kg bw/day animals only), a representative sample of liver from each lobe was harvested at necropsy, weighed, frozen in liquid nitrogen, and stored at −70 °C until analyzed. Duplicate samples were taken from adjacent sites. Analyses for liver vitamins A and E were performed by HPLC and gas chromatography (GC) using flame ionization detector (FID), respectively.

2.4.7. Tissue EPG

At scheduled necropsy, duplicate representative samples of the liver (each lobe), kidneys, spleen, and adipose tissue were harvested from each animal, weighed, and stored at approximately −20 °C until analyzed. EPG was determined in samples from all control and 5 g/kg bw/day EPG animals. Tissue samples were analyzed using Waters 845 HPLC and data system with a reverse phase, Beckman Ultrasphere ODS (25 cm × 4.6 mm ID), Dupont Zorbax Rx-C8 guard cartridge.

2.4.8. Fecal analysis

During Week 6 and Weeks 12 or 13, animals (5/sex) from the control and 5 g/kg bw/day EPG groups were housed in stainless steel metabolism cages in order to collect 24-h fecal samples. The samples were weighed, divided in half, and stored at −20 ± 10 °C. One set of samples was used for cholesterol, total free fatty acids, and bile acids analysis. The other half was sent to RTI (Research Triangle Park, NC) for EPG (triester) and EPG metabolites (mono- and diester) analysis.

Feces were analyzed for fatty acids, cholesterol, calcium, fat (ether extract), and total bile acids. Fatty acids and cholesterol were measured using gas chromatography. The concentration of calcium in the feces was calculated by comparing the absorbance of atomized calcium to a standard reference curve, following the digestion of the feces by a 600 C ash procedure. Fat is extracted by ethyl ether on a Tecator Soxtec (a fat extraction apparatus), ethyl ether is evaporated, and then the residual fat is weighed. Concentration of bile acids is quantified by measuring the generation of NADH (spectrophotometrically or spectrofluorimetrically) from NAD^+ during the oxidation of 3-α-hyroxysteroids.

2.4.9. Bowel transit times

Bowel transit time was determined for each animal from the control and 5 g/kg bw/day EPG groups during pre-test, Week 6, and Weeks 12 or 13. Wood chips were removed from the runs 3–4 days before this procedure. Radio-opaque markers (Epidural Catheter, Cat. No. 4912-18, Concord Portex, Keene, NH, Lot Nos. 205124 and 212220) were cut into 20 sections (~2-mm each) and subsequently placed in a No. 13 Torpac gelatin capsule (Torpac, Ltd., Toronto, Canada). Prior to placement into metabolism cages, each animal received a capsule orally after feeding. During pre-test, feces were collected at approximately 0–4, 4–8, 8–12, and 12–24 h post-dosing. During Week 6, feces were collected at 0–24, 24–30, 30–26, and 26–48 h post-dosing. During Week 12 or 13 feces were collected separately at 0–24, 24–48, 48–60, and 60–72 h post-dosing. Each sample was packaged in a labeled plastic bag and frozen until X-rayed to determine the number of markers per fecal sample.

2.4.10. Necropsy, organ weights, and histopathological examination

Following at least 13 weeks of treatment and an overnight fast, animals were sacrificed in the order designated by a computer-generated randomization schedule. All animals were anesthetized with sodium pentobarbital, weighed, exsanguinated, and necropsied. The necropsy included gross examination of: the external surfaces of the body; thoracic, abdominal, and pelvic, cavities and enclosed organs; and the head and cervical organs and tissues.

The adrenals, brain, heart, pituitary, kidneys, liver, spleen, cecum, thyroid, prostate and gonads (testes and ovaries) were removed and weighed (paired organs separately).

All lesions identified during gross examination were examined microscopically, along with the following major organs and tissues: adrenals; aorta; bone with marrow (costochondral junction/rib); brain; cecum; cervix; colon; duodenum; epididymides; esophagus; eyes (both); gallbladder; heart; ileum; jejunum; kidneys; liver (all lobes); lung (with mainstem bronchi); mammary gland (females only); mandibular lymph node; mesenteric lymph node; ovaries; pancreas; peripheral nerve (with skeletal muscle); pituitary; prostate; rectum; salivary gland; seminal vesicles; skin; spinal cord (cervical, mid-thoracic, lumbar); spleen; stomach (cardiac, fundic, and pyloric regions); testes; thymus; thyroid (with parathyroid if in thyroid section); tongue; trachea; urinary bladder; uterus; and vagina. Tissues were preserved in 10% neutral buffered formalin (or other suitable fixative), processed, embedded, sectioned, stained with hematoxylin and eosin, and examined by a qualified pathologist.

2.4.11. Statistical analyses

Statistical methods used in the evaluation of numerical data were those of Dunnett, an analysis of variance (ANOVA) model to compare one or more treatment groups to a control group (Dunnett, 1955, 1964). Statistical significance was assessed through p values equal to or less than 0.05 or 0.01.

3. Results

3.1. Mortality and clinical signs

All micropigs survived to the end of the study.

There was evidence of pneumonia across all groups upon necropsy and histopathological examination (see below), but no signs otherwise. Clinical signs seen in animals receiving EPG were limited to: "distended abdomen" in one male receiving 1.5 g/kg bw/day, first seen at Week 1 and last seen at Week 14; "appears thin" in one female from each the 1.5 g/kg bw/day (first seen at Week 1, last seen at Week 14) and 3 g/kg bw/day (first seen at Week 6, last seen at Week 8) groups; and "lameness–right front leg" seen first at Week 12 and last at Week 14 in one 5 g/kg bw/day female. These isolated observations are not regarded as effects of EPG treatment.

Visual inspection of the feces showed no phase separation (*i.e.*, appearance of oily layers or deposits in the stools).

Table 2
Summary of body weights (kg).

Week	Male EPG in diet[a] (g/kg bw/day)				Female EPG in diet[a] (g/kg bw/day)			
	0	1.5	3	5	0	1.5	3	5
0	15.5 ± 1.77	15.5 ± 1.80	15.5 ± 1.66	15.2 ± 1.67	14.4 ± 1.93	14.6 ± 2.80	14.8 ± 2.22	15.1 ± 2.88
1	17.1 ± 2.02	17.6 ± 1.89	17.7 ± 1.39	17.5 ± 1.75	15.9 ± 2.24	15.8 ± 3.13	16.6 ± 2.47	16.8 ± 3.18
2	17.5 ± 2.04	17.6 ± 1.84	17.4 ± 1.55	17.5 ± 1.78	16.8 ± 2.12	15.7 ± 3.00	17.2 ± 2.77	17.2 ± 3.00
3	17.2 ± 1.84	17.6 ± 1.57	16.9 ± 1.63	17.5 ± 1.57	16.8 ± 1.72	15.6 ± 3.23	16.7 ± 2.66	17.0 ± 3.16
4	18.7 ± 2.09	18.5 ± 1.42	18.2 ± 1.88	19.0 ± 1.82	18.0 ± 2.05	16.4 ± 3.47	17.6 ± 3.30	18.5 ± 3.02
5	19.5 ± 2.39	19.5 ± 1.77	18.6 ± 1.80	19.9 ± 1.73	19.1 ± 2.02	17.0 ± 3.66	18.5 ± 3.45	18.7 ± 3.63
6	18.7 ± 2.28	19.3 ± 1.87	18.3 ± 1.95	19.3 ± 1.65	17.5 ± 2.20	15.3 ± 3.68	17.5 ± 3.62	17.8 ± 4.00
7	20.3 ± 2.93	20.3 ± 1.89	19.5 ± 1.62	20.6 ± 1.72	19.8 ± 2.15	17.4 ± 3.61	19.5 ± 3.82	19.9 ± 4.02
8	20.6 ± 2.87	20.9 ± 1.77	20.3 ± 1.72	21.4 ± 1.92	20.9 ± 2.30	18.0 ± 4.03	20.6 ± 4.04	20.5 ± 4.14
9	21.4 ± 3.33	21.5 ± 2.04	20.8 ± 1.85	21.7 ± 2.59	21.6 ± 2.62	18.0 ± 4.02	21.0 ± 4.70	20.9 ± 3.97
10	21.9 ± 3.29	22.4 ± 2.12	21.0 ± 2.12	22.6 ± 2.53	22.0 ± 2.48	18.3 ± 4.33	21.9 ± 4.44	21.8 ± 4.50
11	23.2 ± 4.15	23.2 ± 2.34	21.6 ± 2.13	23.4 ± 2.77	22.9 ± 2.48	18.6 ± 4.62	22.5 ± 5.02	22.0 ± 4.18
12	23.1 ± 4.42	23.7 ± 2.14	22.1 ± 2.44	23.6 ± 2.99	23.7 ± 2.71	19.2 ± 4.88	23.3 ± 5.62	22.7 ± 4.60
13	24.0 ± 4.34	24.0 ± 2.67	22.3 ± 2.59	24.1 ± 3.45	23.3 ± 2.97	18.8 ± 4.96	23.2 ± 5.92	22.2 ± 5.36

Values represent mean ± SD, unless otherwise specified.
Number of animals (N) = 5/group.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.

Table 3
Summary of feed consumption (g/day).

Week	Male EPG in diet[a] (g/kg bw/day)				Female EPG in diet[a] (g/kg bw/day)			
	0	1.5	3	5	0	1.5	3	5
1	602.1 ± 1.2	601.3 ± 0.4	602.3 ± 1.0	599.6 ± 7.2	597.3 ± 8.6	594.0 ± 12.9	593.9 ± 16.0	598.7 ± 7.3
2	604.5 ± 6.5	603.7 ± 5.8	597.7 ± 0.7	598.9 ± 0.8	601.2 ± 1.9	595.9 ± 9.2	598.0 ± 1.3	604.6 ± 12.8
3	607.6 ± 13.3	604.4 ± 6.8	602.4 ± 0.8	601.2 ± 0.7	601.6 ± 0.9	597.1 ± 9.9	605.5 ± 6.3	608.8 ± 16.4
4	605.0 ± 6.1	604.5 ± 6.6	601.6 ± 2.0	604.7 ± 0.5	601.6 ± 0.7	595.7 ± 12.8	597.5 ± 8.7	615.9 ± 27.1
5	616.5 ± 33.7	608.7 ± 13.3	605.4 ± 6.8	612.6 ± 15.9	607.8 ± 13.0	600.0 ± 14.4	614.3 ± 19.5	621.1 ± 46.5
6	615.3 ± 52.3	615.0 ± 33.1	605.0 ± 12.6	620.5 ± 28.9	610.7 ± 38.1	608.6 ± 29.4	622.3 ± 38.2	629.6 ± 58.6
7	616.2 ± 32.8	615.6 ± 33.4	607.1 ± 12.5	618.5 ± 24.5	607.5 ± 13.1	593.1 ± 17.9	609.9 ± 28.8	624.2 ± 53.2
8	637.8 ± 72.1	625.8 ± 45.5	615.3 ± 26.4	636.4 ± 39.2	626.0 ± 45.5	611.3 ± 28.7	642.6 ± 52.2	651.0 ± 78.1
9	643.4 ± 69.3	638.4 ± 48.8	631.1 ± 43.9	661.1 ± 57.2	646.3 ± 63.7	616.5 ± 43.1	667.3 ± 80.6	667.2 ± 93.2
10	659.2 ± 87.4	656.5 ± 59.3	634.0 ± 47.8	657.2 ± 78.1	647.3 ± 64.6	616.3 ± 43.7	671.9 ± 90.7	660.4 ± 97.3
11	670.1 ± 90.3	681.8 ± 59.4	642.7 ± 54.3	686.7 ± 71.1	667.0 ± 76.0	618.2 ± 49.9	690.3 ± 99.0	680.6 ± 113.5
12	705.9 ± 115.9	705.5 ± 74.8	662.1 ± 65.5	713.1 ± 85.1	694.0 ± 74.0	624.5 ± 62.6	712.8 ± 112.7	683.2 ± 114.5
13	706.8 ± 115.0	722.1 ± 64.3	671.2 ± 69.5	723.8 ± 91.5	721.6 ± 83.9	637.3 ± 67.2	734.2 ± 128.9	699.0 ± 125.6

Values represent mean ± SD, unless otherwise specified.
Number of animals (N) = 5/group.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.

3.2. Body weight and weight gain

As Table 2 illustrates, there were no differences among groups in body weights. However, body weight change among females receiving 1.5 g/kg bw/day was significantly ($p \leqslant 0.01$ or $p \leqslant 0.05$) lower than control from Weeks 2–14 (data not shown). This might have been related to pneumonia that became evident upon necropsy and histopathological examination (see below). Statistical analysis of the data without two animals (AVI2F01 and AVI2F04) from this group that had the lowest body weight changes showed no statistical significant differences in body weight change.

As expected, some effects on body weights and body weight changes were seen when animals were placed in metabolism cages, possibly related to stress.

3.3. Feed consumption and EPG intake

As shown in Table 3, there were no differences among groups in feed consumption. Weekly feed efficiency was generally positive; negative numbers were seen sporadically, such as during Week 6 (all male and female groups) and Week 13 (all females), but this might have been related to the stress of housing in metabolism cages.

The mean weekly achieved intake of EPG/kg body weight/day among treatment groups, was1.5, 3.1, and 5.1 g/kg bw/day and comparable to the target value for each of the 1.5, 3, and 5 g/kg bw/day groups.

3.4. Physical and ophthalmic examination

Fecal examinations for parasites at pre-test indicated arachnid ova and strongyle eggs to be present. The micropigs were treated with 1 mL/34 kg SQ IVOMEC 1% (Merck, #TBC-184). Fecal examinations for parasites during Weeks 7 and 14 were negative. The weekly general physical exams during the study indicated some of the animals were thin.

Ophthalmic examination did not reveal any findings related to treatment.

3.5. Hematology, clinical chemistry, and urinalysis

The results of the clinical pathology and urinalysis are summarized in Tables 4–9. Isolated differences were observed between the control and EPG groups, but with no evidence of a relationship to EPG.

Table 4
Hematology values for males.

EPG[a] (g/kg bw/day)	N	RBC (10^6/mm^3)	HGB (g/dL)	HCT (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dL)	PLT (10^3/mm^3)	PT (s)
Week 6									
0	5	7.73 ± 0.40	14.4 ± 0.3	42.8 ± 2.8	55.6 ± 5.0	18.6 ± 1.4	33.7 ± 2.2	387 ± 72	12.5 ± 0.3
1.5	5	7.47 ± 0.45	14.1 ± 0.4	42.5 ± 1.8	57.0 ± 2.7	18.9 ± 1.1	33.3 ± 1.4	438 ± 85	13.1 ± 0.3
3	5	7.07 ± 0.43	13.9 ± 0.8	41.7 ± 3.0	59.0 ± 2.2	19.7 ± 0.7	33.4 ± 1.7	452 ± 65	12.9 ± 0.3
5	5	7.39 ± 0.87	14.1 ± 0.7	42.7 ± 2.5	58.4 ± 3.9	19.3 ± 1.4	33.1 ± 1.5	454 ± 61	13.0 ± 0.7
Week 13									
0	5	7.75 ± 0.55	15.4 ± 0.5	42.1 ± 1.7	54.6 ± 4.2	19.9 ± 1.5	36.6 ± 0.3	458 ± 59	13.9 ± 0.4
1.5	5	7.19 ± 0.43	14.7 ± 0.7	41.2 ± 2.3	57.4 ± 3.6	20.5 ± 1.1	35.8 ± 0.5*	389 ± 38	13.9 ± 0.3
3	5	7.28 ± 0.56	15.2 ± 0.9	42.2 ± 2.7	58.2 ± 3.4	20.9 ± 0.9	36.0 ± 0.5	417 ± 120	13.5 ± 0.6
5	5	7.36 ± 0.44	15.2 ± 0.6	43.1 ± 2.3	58.6 ± 4.2	20.6 ± 1.4	35.2 ± 0.4**	388 ± 74	13.8 ± 0.3

EPG[a] (g/kg bw/day)	N	APTT (s)	WBC (10^3/mm^3)	NEUTR (%)	LYMPH (%)	MONO (%)	EOSIN (%)	BASO (%)
Week 6								
0	5	15.7 ± 0.5	11.7 ± 2.7	60.6 ± 10.1	37.2 ± 11.5	1.0 ± 1.4	1.2 ± 1.3	0
1.5	5	16.4 ± 1.2	14.8 ± 2.0	55.0 ± 11.3	41.0 ± 8.0	1.2 ± 1.1	2.8 ± 3.0	0
3	5	16.2 ± 1.3	17.1 ± 2.0**	58.8 ± 9.7	32.6 ± 5.4	1.6 ± 1.3	7.0 ± 4.9	0
5	5	16.5 ± 1.4	12.0 ± 2.2	53.0 ± 7.5	42.2 ± 6.8	1.0 ± 1.0	3.8 ± 2.9	0
Week 13								
0	5	16.9 ± 0.8	13.9 ± 2.3	60.0 ± 7.4	34.6 ± 9.6	4.2 ± 3.5	1.2 ± 1.3	0
1.5	5	16.0 ± 0.6	13.8 ± 1.5	53.0 ± 8.9	43.0 ± 8.0	3.0 ± 1.2	1.0 ± 1.0	0
3	5	16.7 ± 1.3	15.2 ± 2.1	60.2 ± 11.0	36.2 ± 12.4	1.8 ± 2.9	1.8 ± 1.8	0
5	5	16.7 ± 0.7	10.8 ± 0.9*	49.8 ± 2.2	44.2 ± 3.6	3.4 ± 1.9	2.6 ± 1.1	0

Values represent mean ± SD, unless otherwise specified.
RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet count; PT: prothrombin time.
APTT: activated partial thromboplastin time; WBC: white blood cell count; NEUTR: neutrophils LYMPH: lymphocyte count; MONO: monocyte count; EOSIN: eosinophil count; BASO: basophil count.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.
* Statistically significant at $p \leqslant 0.05$ by Dunnett's test.
** Statistically significant at $p \leqslant 0.01$ by Dunnett's test.

Table 5
Hematology values for females.

EPG[a] (g/kg bw/day)	N	RBC (10^6/mm^3)	HGB (g/dL)	HCT (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dL)	PLT (10^3/mm^3)	PT (s)
Week 6									
0	5	7.10 ± 0.66	13.3 ± 1.1	41.6 ± 2.3	58.8 ± 3.7	18.8 ± 0.8	32.0 ± 1.5	369 ± 95	12.4 ± 0.4
1.5	5	7.38 ± 0.51	13.7 ± 0.8	41.5 ± 2.7	56.2 ± 4.2	18.6 ± 1.1	33.1 ± 1.1	424 ± 116	12.5 ± 0.3
3	5	7.34 ± 0.46	13.7 ± 0.6	40.9 ± 2.5	55.8 ± 4.0	18.7 ± 1.3	33.6 ± 0.9	453 ± 86	12.5 ± 0.7
5	5	7.23 ± 0.48	13.8 ± 0.3	41.1 ± 2.1	57.0 ± 1.9	19.1 ± 1.0	33.6 ± 1.2	456 ± 131	12.6 ± 0.6
Week 13									
0	5	7.10 ± 0.36	14.8 ± 0.2	41.0 ± 1.4	58.0 ± 4.2	20.9 ± 1.4	36.1 ± 1.0	422 ± 106	13.5 ± 0.4
1.5	5	7.48 ± 0.56	15.1 ± 1.0	42.2 ± 4.1	56.4 ± 5.2	20.2 ± 1.3	35.9 ± 1.4	436 ± 111	13.2 ± 1.2
3	5	7.40 ± 0.57	15.0 ± 0.7	42.1 ± 2.1	57.2 ± 5.0	20.3 ± 1.6	35.6 ± 0.4	414 ± 55	13.8 ± 0.4
5	5	7.18 ± 0.51	14.6 ± 0.9	41.2 ± 2.8	57.4 ± 1.1	20.4 ± 0.4	35.6 ± 0.9	355 ± 102	14.1 ± 0.5

EPG[a] (g/kg bw/day)	N	APTT (sec)	WBC (10^3/mm^3)	NEUTR (%)	LYMPH (%)	MONO (%)	EOSIN (%)	BASO (%)
Week 6								
0	5	15.9 ± 0.9	12.6 ± 3.8	55.6 ± 11.0	42.6 ± 10.0	0.8 ± 0.8	1.0 ± 1.0	0
1.5	5	16.5 ± 0.4	13.9 ± 2.9	58.4 ± 14.8	39.6 ± 14.2	1.2 ± 0.8	0.8 ± 1.3	0
3	5	15.8 ± 1.0	15.2 ± 3.3	57.8 ± 12.4	38.6 ± 11.2	1.0 ± 0.7	2.6 ± 2.7	0
5	5	15.7 ± 1.2	15.8 ± 7.6	54.0 ± 6.4	39.6 ± 11.7	0.8 ± 0.8	5.6 ± 6.6	0
Week 13								
0	5	16.5 ± 1.6	11.1 ± 1.9	44.0 ± 6.4	51.2 ± 6.7	2.6 ± 2.4	2.2 ± 0.8	0
1.5	5	16.2 ± 1.9	12.3 ± 1.5	45.6 ± 17.5	51.6 ± 17.7	1.4 ± 1.5	1.4 ± 1.5	0
3	5	16.2 ± 1.4	13.2 ± 3.2	48.4 ± 12.7	47.0 ± 14.0	1.6 ± 1.8	3.0 ± 1.6	0
5	5	17.6 ± 1.1	11.3 ± 1.5	48.0 ± 8.1	47.8 ± 8.9	2.8 ± 1.5	1.4 ± 0.5	0

Values represent mean ± SD, unless otherwise specified.
RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet count; PT: prothrombin time.
APTT: activated partial thromboplastin time; WBC: white blood cell count; NEUTR: neutrophils LYMPH: lymphocyte count; MONO: monocyte count; EOSIN: eosinophil count; BASO: basophil count.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.

Table 6
Clinical chemistry values for males.

EPG[a] (g/kg bw/day)	N	AST (IU/L)	ALT (IU/L)	GGT (IU/L)	GLU (mg/dL)	BUN (mg/dL)	CREAT (mg/dL)	CHOL (mg/dL)	TRIG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Week 6												
0	5	41.6 ± 5.1	119.9 ± 13.1	49.4 ± 10.0	76.0 ± 10.1	12.5 ± 3.0	0.86 ± 0.24	83.7 ± 17.4	45.3 ± 18.9	49.3 ± 8.6	25.4 ± 12.4	9.1 ± 3.8
1.5	5	46.8 ± 13.7	104.1 ± 32.2	48.8 ± 5.8	73.1 ± 13.7	14.5 ± 3.1	0.97 ± 0.17	76.8 ± 18.5	59.3 ± 20.5	45.5 ± 12.7	19.5 ± 3.0	11.9 ± 4.1
3	5	39.2 ± 4.6	93.3 ± 14.2	49.7 ± 10.2	74.4 ± 8.7	16.9 ± 2.9	0.90 ± 0.18	87.1 ± 24.2	38.6 ± 16.2	51.8 ± 16.1	27.7 ± 9.9	7.7 ± 3.2
5	5	35.1 ± 10.3	85.4 ± 18.0*	52.0 ± 8.0	76.2 ± 12.3	15.8 ± 2.7	0.98 ± 0.16	79.0 ± 13.8	40.8 ± 15.4	50.1 ± 6.5	20.7 ± 9.2	8.2 ± 3.1
Week 13												
0	5	33.8 ± 4.6	78.9 ± 11.2	51.9 ± 14.0	75.1 ± 10.6	16.6 ± 3.2	1.04 ± 0.11	86.0 ± 27.7	30.2 ± 11.0	47.1 ± 13.6	32.9 ± 15.0	6.0 ± 2.2
1.5	5	44.3 ± 14.0	91.0 ± 24.5	52.3 ± 8.5	84.7 ± 13.4	13.3 ± 2.1	1.01 ± 0.12	80.0 ± 10.4	47.2 ± 14.2	47.1 ± 7.2	23.5 ± 6.9	9.4 ± 2.8
3	5	44.7 ± 8.5	101.0 ± 7.6	51.5 ± 7.9	78.6 ± 9.5	15.4 ± 0.7	0.93 ± 0.04	92.1 ± 18.9	42.2 ± 17.9	53.5 ± 9.2	30.2 ± 10.4	8.4 ± 3.6
5	5	35.0 ± 10.3	74.7 ± 2.3	56.5 ± 5.8	70.7 ± 9.8	19.1 ± 1.4	1.02 ± 0.12	90.4 ± 14.7	37.8 ± 13.5	53.3 ± 10.2	29.5 ± 6.9	7.6 ± 2.7

		T BILI (mg/dL)	PHOS (mg/dL)	T PRO (g/dL)	ALB (g/dL)	GLOB (g/dL)	A/G RATIO	CA (mg/dL)	MG (mg/dL)	CL (mEq/L)	NA (mEq/L)	K (mEq/L)
Week 6												
0	5	0.16 ± 0.02	7.0 ± 0.9	7.0 ± 0.2	3.8 ± 0.3	3.1 ± 0.1	1.2 ± 0.1	11.3 ± 0.4	2.5 ± 0.2	105.3 ± 2.0	141.3 ± 2.1	4.86 ± 0.85
1.5	5	0.16 ± 0.02	7.0 ± 0.2	6.9 ± 0.4	3.3 ± 0.4*	3.5 ± 0.4	1.0 ± 0.2	11.2 ± 0.3	2.4 ± 0.2	103.8 ± 4.3	138.8 ± 2.5	5.07 ± 0.66
3	5	0.14 ± 0.02	7.3 ± 0.4	6.9 ± 0.4	3.5 ± 0.3	3.4 ± 0.3	1.1 ± 0.1	11.1 ± 0.3	2.5 ± 0.2	104.4 ± 3.5	139.9 ± 2.8	4.67 ± 0.57
5	5	0.14 ± 0.02	7.2 ± 0.6	6.9 ± 0.7	3.6 ± 0.2	3.3 ± 0.8	1.1 ± 0.3	11.0 ± 0.4	2.5 ± 0.3	106.2 ± 1.9	141.0 ± 1.4	5.33 ± 0.52
Week 13												
0	5	0.14 ± 0.02	7.2 ± 0.4	6.8 ± 0.3	3.8 ± 0.3	3.0 ± 0.2	1.3 ± 0.2	11.2 ± 0.4	2.4 ± 0.1	105.6 ± 2.5	143.6 ± 2.8	4.38 ± 0.48
1.5	5	0.17 ± 0.02	7.3 ± 0.6	6.7 ± 0.2	3.6 ± 0.3	3.1 ± 0.3	1.2 ± 0.2	10.9 ± 0.4	2.4 ± 0.1	107.6 ± 4.8	142.0 ± 2.5	4.92 ± 0.27
3	5	0.15 ± 0.04	6.9 ± 0.5	6.9 ± 0.3	3.7 ± 0.1	3.2 ± 0.3	1.2 ± 0.2	11.0 ± 0.3	2.5 ± 0.2	104.2 ± 3.2	142.3 ± 0.8	4.60 ± 0.75
5	5	0.14 ± 0.02	7.6 ± 0.4	6.9 ± 0.2	3.8 ± 0.2	3.1 ± 0.3	1.3 ± 0.2	11.4 ± 0.2	2.6 ± 0.3	107.8 ± 2.3	146.4 ± 2.2	5.30 ± 0.84

Values represent mean ± SD, unless otherwise specified.
AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyl transferase; GLU: glucose; BUN: urea nitrogen; CREAT: creatinine; CHOL: cholesterol; TRIG: triglyceride; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; VLDL: very low-density lipoprotein cholesterol.
T BILI: total bilirubin; PHOS: inorganic phosphorus; T PROT: total protein; ALB: albumin; GLOB: globulin; A/G RATIO: albumin/globulin ratio; CA: calcium; MG: magnesium; CL: chloride NA: sodium; K: potassium.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.
* Statistically significant at $p \leqslant 0.05$ by Dunnett's test.

Table 7
Clinical chemistry values for females.

EPG[a] (g/kg bw/day)	N	AST (IU/L)	ALT (IU/L)	GGT (IU/L)	GLU (mg/dL)	BUN (mg/dL)	CREAT (mg/dL)	CHOL (mg/dL)	TRIG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Week 6												
0	5	64.4 ± 17.8	100.8 ± 22.6	56.3 ± 2.6	75.0 ± 22.1	17.2 ± 4.4	1.05 ± 0.23	114.2 ± 18.1	41.3 ± 6.0	61.4 ± 9.0	44.5 ± 14.2	8.3 ± 1.2
1.5	5	46.1 ± 10.0	112.3 ± 19.8	53.9 ± 6.0	65.4 ± 7.9	13.7 ± 4.8	0.80 ± 0.20	94.9 ± 22.9	25.8 ± 10.1	59.9 ± 9.3	29.8 ± 14.7	5.2 ± 2.0
3	5	37.7 ± 11.4*	103.8 ± 46.0	45.1 ± 8.4	66.0 ± 7.2	14.5 ± 3.0	0.92 ± 0.41	94.7 ± 17.2	44.1 ± 21.0	58.5 ± 12.2	27.4 ± 8.2	8.8 ± 4.2
5	5	43.1 ± 10.3*	92.0 ± 26.9	49.3 ± 8.8	67.6 ± 7.8	12.6 ± 2.5	0.79 ± 0.21	88.8 ± 19.8	36.5 ± 19.4	54.1 ± 18.3	27.5 ± 4.6	7.3 ± 3.9
Week 13												
0	5	54.4 ± 14.6	99.5 ± 28.1	52.3 ± 5.8	76.2 ± 13.4	16.7 ± 3.8	1.04 ± 0.11	111.7 ± 13.9	44.1 ± 8.8	60.2 ± 9.1	42.7 ± 8.0	8.8 ± 1.8
1.5	5	67.2 ± 50.7	111.6 ± 22.8	59.4 ± 8.7	79.4 ± 19.0	14.2 ± 2.8	0.83 ± 0.15	97.9 ± 22.2	27.9 ± 14.0	62.2 ± 7.8	30.1 ± 14.4	5.6 ± 2.8
3	5	40.6 ± 4.9	96.1 ± 21.0	46.3 ± 8.7	65.7 ± 8.9	13.0 ± 1.5	0.94 ± 0.24	99.4 ± 21.6	35.0 ± 18.6	62.6 ± 10.3	29.8 ± 10.4	7.0 ± 3.7
5	5	47.0 ± 8.4	104.5 ± 22.2	51.3 ± 9.8	67.9 ± 5.3	11.8 ± 1.1*	0.89 ± 0.20	90.4 ± 19.3	31.6 ± 12.3	56.8 ± 13.3	27.3 ± 6.9	6.3 ± 2.5

		T BILI (mg/dL)	PHOS (mg/dL)	T PRO (g/dL)	ALB (g/dL)	GLOB (g/dL)	A/G RATIO	CA (mg/dL)	MG (mg/dL)	CL (mEq/L)	NA (mEq/L)	K (mEq/L)
Week 6												
0	5	0.18 ± 0.04	7.3 ± 0.2	7.1 ± 0.5	3.7 ± 0.1	3.4 ± 0.4	1.1 ± 0.1	10.8 ± 0.1	2.4 ± 0.1	105.4 ± 2.2	138.8 ± 2.2	4.74 ± 0.33
1.5	5	0.14 ± 0.02	7.1 ± 0.8	6.5 ± 0.4*	3.4 ± 0.2	3.0 ± 0.3	1.1 ± 0.1	10.8 ± 0.6	2.2 ± 0.2	99.1 ± 7.0	137.6 ± 8.2	4.77 ± 0.58
3	5	0.14 ± 0.02	7.3 ± 1.1	6.7 ± 0.3	3.4 ± 0.3	3.3 ± 0.3	1.0 ± 0.2	10.8 ± 0.6	2.4 ± 0.4	98.1 ± 9.5	135.8 ± 9.5	4.82 ± 0.71
5	5	0.15 ± 0.04	7.1 ± 0.5	6.7 ± 0.3	3.4 ± 0.3	3.3 ± 0.4	1.0 ± 0.2	10.9 ± 0.5	2.4 ± 0.3	101.0 ± 3.6	138.9 ± 2.9	5.12 ± 0.74
Week 13												
0	5	0.15 ± 0.02	7.1 ± 0.4	7.1 ± 0.3	3.7 ± 0.2	3.4 ± 0.1	1.1 ± 0.1	10.7 ± 0.5	2.3 ± 0.3	102.7 ± 4.9	140.2 ± 5.4	5.22 ± 0.99
1.5	5	0.17 ± 0.04	7.2 ± 0.5	6.5 ± 0.5	3.5 ± 0.2	3.0 ± 0.4	1.2 ± 0.2	10.6 ± 0.7	2.2 ± 0.2	102.3 ± 7.4	140.0 ± 8.9	5.15 ± 0.86
3	5	0.16 ± 0.02	7.5 ± 0.9	6.9 ± 0.4	3.7 ± 0.3	3.2 ± 0.4	1.2 ± 0.2	11.1 ± 0.7	2.5 ± 0.4	100.3 ± 6.3	140.2 ± 7.4	5.77 ± 0.86
5	5	0.19 ± 0.03	7.1 ± 0.2	6.8 ± 0.2	3.6 ± 0.2	3.2 ± 0.2	1.1 ± 0.2	10.6 ± 0.4	2.2 ± 0.1	102.0 ± 3.2	136.1 ± 2.8	5.24 ± 0.74

Values represent mean ± SD, unless otherwise specified.
AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyl transferase; GLU: glucose; BUN: urea nitrogen; CREAT: creatinine; CHOL: cholesterol; TRIG: triglyceride; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; VLDL: very low-density lipoprotein cholesterol.
T BILI: total bilirubin; PHOS: inorganic phosphorus; T PROT: total protein; ALB: albumin; GLOB: globulin; A/G RATIO: albumin/globulin ratio; CA: calcium; MG: magnesium; CL: chloride; NA: sodium; K: potassium.
* Statistically significant at $p \leqslant 0.05$ by Dunnett's test.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.

Table 8
Urinalysis values for males.

EPG[a] (g/kg bw/day)	N	VOL (mL)	PHOS (mg/dL)	CA (mg/dL)	MG (mg/dL)	CL (mEq/L)	NA (mEq/L)	K (mEq/L)
Week 6								
0	5	756 ± 451	9.2 ± 14.0	8.6 ± 4.3	20.2 ± 10.6	151.0 ± 26.4	71.5 ± 41.4	87.36 ± 31.71
1.5	5	595 ± 341	2.4 ± 1.4	13.2 ± 6.2	18.9 ± 11.4	119.7 ± 30.5	51.6 ± 25.5	71.40 ± 38.76
3	5	995 ± 256	2.2 ± 0.8	9.0 ± 4.1	17.8 ± 10.3	130.0 ± 67.0	54.1 ± 44.9	73.82 ± 32.19
5	5	722 ± 578	4.0 ± 4.8	6.7 ± 4.1	9.7 ± 8.6	120.7 ± 51.1	60.2 ± 51.1	74.08 ± 42.44
Week 12								
0	5	1352 ± 902	15.4 ± 18.3	9.0 ± 3.0	22.8 ± 13.5	64. 4 ± 28.4	60.0 ± 24.8	61.01 ± 23.58
1.5	5	1235 ± 991	6.0 ± 6.2	8.8 ± 4.9	20.1 ± 7.4	71.7 ± 35.4	59.9 ± 38.4	83.82 ± 43.13
3	5	1726 ± 492	4.4 ± 4.3	7.0 ± 1.1	16.0 ± 7.8	54.5 ± 23.9	45.9 ± 18.2	51.15 ± 25.71
5	5	1053 ± 340	24.9 ± 53.7	9.3 ± 3.2	18.2 ± 20.3	62.3 ± 63.1	48.6 ± 48.3	61.93 ± 67.23

		PHOS CHEM (mg/kg/h)	CA CHEM (mg/kg/h)	MG CHEM (mg/kg/h)	CL CHEM (mE/kg/h)	NA CHEM (mE/kg/h)	K CHEM (mE/kg/h)
Week 6							
0	5	0.062 ± 0.039	0.109 ± 0.050	0.329 ± 0.279	0.254 ± 0.192	0.107 ± 0.089	0.128 ± 0.078
1.5	5	0.026 ± 0.015	0.137 ± 0.071	0.188 ± 0.056	0.145 ± 0.086	0.062 ± 0.050	0.078 ± 0.038
3	5	0.049 ± 0.020	0.213 ± 0.122	0.411 ± 0.229	0.275 ± 0.111	0.115 ± 0.076	0.162 ± 0.062
5	5	0.039 ± 0.025	0.083 ± 0.043	0.086 ± 0.037	0.163 ± 0.105	0.073 ± 0.053	0.093 ± 0.058
Week 12							
0	5	0.183 ± 0.108	0.209 ± 0.138	0.409 ± 0.112	0.126 ± 0.053	0.123 ± 0.061	0.126 ± 0.063
1.5	5	0.105 ± 0.103	0.144 ± 0.077	0.355 ± 0.163	0.130 ± 0.072	0.107 ± 0.070	0.137 ± 0.064
3	5	0.140 ± 0.150	0.233 ± 0.080	0.500 ± 0.169	0.171 ± 0.054	0.146 ± 0.050	0.156 ± 0.034
5	5	0.223 ± 0.455	0.169 ± 0.073	0.253 ± 0.139	0.092 ± 0.053	0.073 ± 0.043	0.089 ± 0.053

Values represent mean ± SD, unless otherwise specified.
VOL: volume; PHOS: phosphorous; CA: calcium; MG: magnesium; CL: chloride; NA: sodium; K: potassium.
CHEM: chemistry; PHOS: phosphorous; CA: calcium; MG: magnesium; CL: chloride; NA: sodium; K: potassium.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.

Table 9
Urinalysis values for females.

EPG[a] (g/kg bw/day)	N	VOL (mL)	PHOS (mg/dL)	CA (mg/dL)	MG (mg/dL)	CL (mEq/L)	NA (mEq/L)	K (mEq/L)
Week 6								
0	5	629 ± 284	9.6 ± 5.0	6.1 ± 2.7	13.5 ± 7.8	83.0 ± 15.1	61.6 ± 18.7	87.19 ± 16.67
1.5	5	361 ± 64	11.9 ± 9.6	12.5 ± 4.7	30.9 ± 17.4	139.6 ± 79.4	99.1 ± 66.9	124.00 ± 55.27
3	5	937 ± 390	12.5 ± 9.0	8.0 ± 5.6	15.3 ± 15.0	76.3 ± 38.2	54.1 ± 34.6	72.05 ± 35.68
5	5	774 ± 490	11.1 ± 7.6	6.9 ± 3.0	12.7 ± 11.0	60.7 ± 50.0	40.2 ± 43.7	59.04 ± 45.28
Week 13								
0	5	1072 ± 560	1.8 ± 0.4	5.3 ± 1.5	9.1 ± 4.6	42.4 ± 12.1	33.6 ± 10.2	49.23 ± 16.79
1.5	5	1136 ± 486	2.2 ± 2.0	7.8 ± 1.3	8.0 ± 3.8	33.3 ± 14.7	29.3 ± 9.8	36.10 ± 16.08
3	5	1275 ± 855	7.8 ± 6.6*	5.7 ± 2.0	17.4 ± 5.8*	53.6 ± 18.5	39.3 ± 15.0	58.49 ± 20.58
5	5	1155 ± 453	2.5 ± 2.1	5.8 ± 2.8	9.7 ± 4.6	37.4 ± 20.2	31.1 ± 21.9	36.77 ± 17.89

		PHOS CHEM (mg/kg/h)	CA CHEM (mg/kg/h)	MG CHEM (mg/kg/h)	CL CHEM (mE/kg/h)	NA CHEM (mE/kg/h)	K CHEM (mE/kg/h)
Week 6							
0	5	0.129 ± 0.076	0.090 ± 0.061	0.223 ± 0.213	0.123 ± 0.079	0.093 ± 0.062	0.123 ± 0.065
1.5	5	0.136 ± 0.165	0.126 ± 0.082	0.291 ± 0.165	0.131 ± 0.076	0.093 ± 0.062	0.117 ± 0.058
3	5	0.309 ± 0.334	0.209 ± 0.230	0.345 ± 0.359	0.151 ± 0.065	0.107 ± 0.052	0.143 ± 0.060
5	5	0.208 ± 0.272	0.138 ± 0.142	0.191 ± 0.209	0.099 ± 0.094	0.066 ± 0.075	0.095 ± 0.084
Week 13							
0	5	0.037 ± 0.028	0.108 ± 0.077	0.159 ± 0.085	0.077 ± 0.039	0.061 ± 0.033	0.085 ± 0.037
1.5	5	0.048 ± 0.050	0.199 ± 0.093	0.203 ± 0.136	0.085 ± 0.056	0.076 ± 0.045	0.089 ± 0.055
3	5	0.230 ± 0.283	0.146 ± 0.125	0.415 ± 0.306	0.125 ± 0.083	0.101 ± 0.075	0.129 ± 0.074
5	5	0.045 ± 0.032	0.133 ± 0.094	0.181 ± 0.032	0.068 ± 0.026	0.055 ± 0.028	0.069 ± 0.021

Values represent mean ± SD, unless otherwise specified.
VOL: volume; PHOS: phosphorous; CA: calcium; MG: magnesium; CL: chloride; NA: sodium; K: potassium.
CHEM: chemistry; PHOS: phosphorous; CA: calcium; MG: magnesium; CL: chloride; NA: sodium; K: potassium.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.
* Statistically significant at $p \leqslant 0.05$ by Dunnett's test.

3.6. Liver and serum vitamins

Liver and serum vitamin data are summarized in Table 10.

The mean liver vitamin A level of females receiving 5 g/kg bw/day of EPG was significantly ($p \leqslant 0.01$) lower compared to control. In males, the mean liver vitamin A was also lower, but the difference was not statistically significant, except when male and female values were analyzed together ($p \leqslant 0.01$).

The effect on liver vitamin E was the reverse: in females from the 5 g/kg bw/day group, there was no apparent effect, whereas

Table 10
Liver and serum vitamin levels.

EPG[a] (g/kg bw/day)	N	Male				Female			
		Liver Vitamin A[b] (μg/g)	Liver Vitamin E[c] (μg/g)	Serum Vitamin D (ng/mL)	Serum 25-OH-Vitamin D (ng/mL)	Liver Vitamin A[b] (μg/g)	Liver Vitamin E[c] (μg/g)	Serum Vitamin D (ng/mL)	Serum 25-OH-Vitamin D (ng/mL)
0	5	60.4 ± 11.3	7.8 ± 0.8	1.9 ± 0.7	20.2 ± 1.4	73.4 ± 11.0	7.2 ± 1.1	2.8 ± 0.5	23.1 ± 1.8
1.5	5	ND	ND	0.8 ± 0.2**	17.7 ± 2.0	ND	ND	1.3 ± 1.0**	20.1 ± 4.1
3	5	ND	ND	0.9 ± 0.4**	13.3 ± 2.7**	ND	ND	0.8 ± 0.3**	16.1 ± 3.1**
5	5	49.0 ± 12.8	6.8 ± 0.4*	<0.5[d]	10.0 ± 1.2**	50.9 ± 8.5**	7.0 ± 0.7	<0.5[d]	10.9 ± 2.1**

ND: Not done.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.
[b] Trans-retinol.
[c] α-Tocopherol.
[d] Below the limit of detection: 0.5 ng/mL.
* Statistically significant at $p \leqslant 0.05$ by Dunnett's test.
** Statistically significant at $p \leqslant 0.01$ by Dunnett's test.

Table 11
Fecal assay dataa.

EPG[a] (g/kg bw/day)	N	Male					Female				
		Fatty acid (g/100 g)	Cholesterol (mg/100 g)	Calcium (mg/100 g)	Fat (ether extract) (g/100 g)	Total bile acids (mM/100 g)	Fatty acid (g/100 g)	Cholesterol (mg/ 100 g)	Calcium (mg/100 g)	Fat (ether extract) (g/100 g)	Total bile acids (mM/100 g)
Week 6											
0	5	1.7 ± 0.3	87 ± 20	3704 ± 260	3.4 ± 0.8	0.588 ± 0.249	2.4 ± 0.2	75 ± 8	4452 ± 282	4.5 ± 0.9	0.457 ± 0.259
5	5	11.8 ± 2.3**	66 ± 15	2464 ± 288**	26.7 ± 2.3**	<0.005[b]	9.5 ± 1.4**	52 ± 20*	2750 ± 293**	25.4 ± 1.6**	0.061 ± 0.125*
Week 12 or 13											
0	5	1.7 ± 0.3	79 ± 09	4456 ± 389	3.2 ± 0.8	1.032 ± 0.143	2.0 ± 0.4	60 ± 10	3214 ± 468	4.4 ± 0.7	0.458 ± 0.311
5	5	10.4 ± 1.7**	44 ± 02**	2846 ± 153**	25.9 ± 4.4**	0.143 ± 0.287**	9.7 ± 1.8**	73 ± 11	2394 ± 278**	23.7 ± 2.5**	0.609 ± 0.327

Values represent mean ± SD, unless otherwise specified.
[a] Animals received feed containing 5%, 10%, and 17% EPG, mixed accordingly throughout the study to deliver 1.5, 3, and 5 g/kg bw/day of EPG, respectively.
[b] Below the limit of detection: 0.005 mM/100 g.
* Statistically significant at $p \leqslant 0.05$ by Dunnett's test.
** Statistically significant at $p \leqslant 0.01$ by Dunnett's test.

in males, it was slightly but significantly ($p < 0.05$) lower. When the male and female values were combined, there was no significant EPG-related effect on the vitamin E content of the liver (data not shown).

Serum 25-OH-vitamin D levels were significantly ($p \leqslant 0.01$) lower among animals receiving the two highest concentrations of EPG (3 and 5 g/kg bw/day); the effect was not evident in animals receiving 1.5 g/kg bw/day of EPG. Compared to control animals, serum total vitamin D levels were significantly ($p \leqslant 0.01$) lower across all groups receiving EPG. Values from all males and females receiving 5 g/kg bw/day were below the assay limit of detection, 0.05 ng/mL; in females especially, the effect appeared to be related to EPG concentration.

3.7. Tissue EPG

No EPG (triester) was found in the liver, kidneys, spleen, or adipose tissue of animals from the control and 5 g/kg bw/day EPG groups.

3.8. Fecal analysis

The results of fecal analyses are summarized in Table 11.

EPG was present, primarily as the triester, in the fecal samples of all animals receiving 5 g/kg bw/day. Two control animals (1 male; 1 female) appeared to have traces of EPG diester and/or triester in the feces, but this was considered possibly artifactual by the laboratory, since the fecal samples were no longer frozen when received.

Fecal total fat and fatty acid levels were significantly ($p \leqslant 0.01$) greater among animals receiving EPG at 5 g/kg bw/day than control animals at all time points. A major increase was noted in the percent of C18:0 and C22:0 saturated fatty acids in the feces of EPG group males and females (data not shown).

Fecal calcium was significantly ($p \leqslant 0.01$) lower at all time points among animals (males and females separately and combined) receiving 5 g EPG/kg bw/day of EPG *vs.* the controls. This might have been a volume-related effect, since no statistically significant differences were observed when the results on a mg/24-h basis were compared (data not shown).

There was no clear effect of EPG treatment on the excretion of cholesterol or bile acids. In males receiving EPG, fecal cholesterol was lower, but reached statistically significance only at the end of the study. In females, fecal cholesterol was significantly lower only at Week 6.

In general, total bile acid was lower among animals receiving EPG; in the case of males at Week 6, the levels were below the limit of detection (0.005 mM/100 g). However, the relationship to EPG is uncertain. By the end of the study, the difference for males was less extreme (*i.e.*, above the limit of detection) and there was no statistically significant difference for females.

3.9. Bowel transit time

Bowel transit times, as assessed by the time taken for radioopaque markers to travel the length of the gut over a 24-h period, were comparable between the control group and the 5 g/kg bw/day EPG group (data not shown).

3.10. Necropsy, organ weights, and histopathological examination

Gross findings in the lungs of subsets of animals from all groups, including the control, were suggestive of pneumonia; the incidence was greater among females. Histopathological examinations confirmed the finding of minimal to moderate chronic pneumonia with infiltration of primarily lymphoid cells and macrophages. The incidence among the groups was 5, 6, 6, and 3 animals each from the 0, 1.5, 3, and 5 g/kg bw/day groups, respectively. Other findings occurred across all groups or in individual animals, with no evidence of a relationship to EPG (data not shown).

Evaluation of organ weight data showed no statistically significant differences, with the exception of the following: greater ($p \leqslant 0.05$) relative heart weight in females receiving 1.5 g/kg bw/day; greater ($p \leqslant 0.05$) relative thyroid weight in females receiving 1.5 g/kg bw/day and 5 g/kg bw/day; and greater ($p \leqslant 0.05$) relative spleen weight in females receiving 5 g/kg bw/day.

4. Discussion

Dietary administration of EPG at concentrations of 1.5, 3, and 5 g/kg bw/day to micropigs for up to 13 weeks was associated with some differences that reached statistical significance. In general, with the exception of the liver and serum vitamin levels, these effects were seen sporadically and with no apparent connection to EPG dose (not concentration-related) and/or no consistency across time intervals. Effects on body weights might have been the result of chronic pneumonia (otherwise asymptomatic) discovered upon necropsy and histopathology, and/or stress from periodic housing in metabolism cages.

Fecal excretion of total fat and fatty acids was significantly greater among animals receiving EPG. However, this effect was not unexpected. Aside from the fat and fatty acids already present in the feed (same as control), animals receiving EPG would have consumed (and excreted) the fatty acids that are part of the EPG molecule.

Serum 25-OH vitamin D levels were unaffected by EPG at 1.5 g/kg bw/day; at 3 and 5 g/kg bw/day, EPG was associated with significantly lower levels. Serum total vitamin D levels were significantly lower across all EPG groups; in females, in a concentration-dependent manner. There were also trends toward lower levels of liver vitamins A and E among EPG-treated animals, but the effects were less consistent.

The biological significance of these findings is uncertain. EPG is intended to replace fats and oils in food products, and similar substances have been shown to interfere with the absorption of lipid-soluble nutrients. For example, pigs fed olestra for 4–26 weeks had lower liver and serum concentrations of vitamins A and E, and lower serum 25-OH vitamin D, in a dose-dependent manner (reviewed by Tulley et al., 2005). Olestra has also been associated with lower absorption of fat-soluble vitamins in humans (Schlagheck et al., 1997). However, it seems unlikely that the apparent effects of EPG of vitamin D in the present study would have been due to interference with the metabolic conversion of vitamin D in the liver to the biologically active metabolite (*i.e.*, 25-OH-vitamin D), since there was no indication that EPG was absorbed. More importantly, the reductions in vitamin levels observed in EPG-treated animals were not accompanied by any signs of vitamin deficiency, as assessed by growth, clinical observations, clinical pathology, and anatomical pathology endpoints.

The decrease in serum vitamin D and 25-OH-vitamin D seen in these pigs is significantly greater than any observed in other species tested (mouse, rat, dog, humans) and may be due to the relatively greater mass of EPG (up to approximately 125 g/day) administered in this study. The highest EPG concentration in this study was 5 g/kg bw/day, while in other studies the greatest concentration was approximately 2 g/kg bw/day. Importantly, we observed that the serum concentration of 25-OH-vitamin D did not decline in proportion to the decline observed in the parent vitamin and, in fact, a NOAEL for serum 25-OH-vitamin D was established at the 1.5 g EPG/kg bw/day concentration. It seems reasonable to conclude that the pigs efficiently converted available vitamin D to maintain physiologically active concentrations of the 25-hydroxy metabolite.

With respect to the role of dose mass, it seems useful to consider that EPG, as a nondigestible lipid-like substance, might act as a lipid "sink" or additional "compartment" for the distribution of lipid-soluble substances, including fat-soluble vitamins present in the gut lumen. However, EPG is not severely hydrophobic, exhibiting a Kow value in the range of 3.2–3.4 similar to the triglyceride fats it is intended to replace. Accordingly, lipid-soluble substances partitioning into EPG are not strongly held and might readily repartition back into the aqueous phase, establishing an equilibrium state. While lipid-soluble nutrients are continuously absorbed from the gut lumen by the usual physiological processes, the solubility equilibrium would be expected to draw more of the nutrient from the EPG compartment. As the unabsorbable EPG progresses down the gastrointestinal tract, beyond the area of active nutrient absorption, it would still retain a certain amount of the lipid-soluble nutrient. The amount retained would be in part proportional to the mass of EPG present. This consideration may in great part account for the observed significant decrease in vitamin D in the pigs, which received considerably greater amounts of EPG than other animal species tested, and, importantly, amounts considerably greater than the likely maximum daily intake by human consumers.

The results of a subsequent 1-year study in pigs (data not shown) confirmed a no-observable-adverse-effect level (NOAEL) for total 25-OH-vitamin D between 1 and 2 g EPG/kg bw/day, with no indication of effects potentially attributable to vitamin D deficiency.

Funding sources statement

This study was sponsored by ARCO Chemical Company (Newton Square, PA). Choco Finesse, LLC, who has acquired the rights to develop and commercialize EPG, hired Intertek Scientific & Regulatory Consultancy (Bridgewater, NJ) to prepare this manuscript.

Conflict of interest statement

The authors are unaware of any conflicts of interest.

References

Bode, G., Clausing, P., Gervais, F., Loegsted, J., Luft, J., Nogues, V., Sims, J.under the auspices of the Steering Group of the RETHINK Project, 2010. The utility of the minipig as an animal model in regulatory toxicology. J. Pharmacol. Toxicol. Methods 62, 196–220.

Dunnett, C.W., 1955. A multiple-comparison procedure for comparing several treatments with a control. JASA 50, 1096–1121.

Dunnett, C.W., 1964. New tables for multiple comparisons with a control. Biometrics 20, 482–491.

Hollis, B.W., Kamerud, J.Q., Selvaag, S.R., Lorenz, J.D., Napoli, J.L., 1993. Determination of vitamin D status by radioimmunoassay with ^{125}I-labeled tracer. Clin. Chem. 39, 529–533.

Liel, Y., Ulmer, E., Shary, J., Hollis, B.W., Bell, N.H., 1988. Low circulating vitamin D in obesity. Calcif. Tissue Int. 43, 199–201.

Schlagheck, T.G., Riccardi, K.A., Zorich, N.L., Torri, S.A., Dugan, L.D., Peters, J.C., 1997. Olestra dose response on fat-soluble and water-soluble nutrients in humans. J. Nutr. 127, 1646S–1665S.

Tulley, R.T., Vaidyanathan, J., Wilson, J.B., Rood, J.C., Lovejoy, J.C., Most, M.M., Volaufova, J., Peters, J.C., Bray, G.A., 2005. Daily intake of multivitamins during long-term intake of olestra in men prevents declines in serum vitamins A and E but not carotenoids. J. Nutr. 135, 1456–1461.