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One-generation reproduction study of esterified propoxylated glycerol (EPG) administered in the feed to CD® (Sprague-Dawley) rats

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ABSTRACT

This one-generation study assessed the potential of esterified propoxylated glycerol (EPG) to affect reproduction and offspring development in rats. Male and female Crl:CD(SD)BR rats (30/sex/group) were exposed to EPG at 0, 0.5, 1, and 2 g/kg bw/day or at 5% (w/w) in the diet prior to (13 weeks), during, and after two consecutive matings. For dams, exposure continued through gestation and lactation; F_{1a} and F_{1b} pups were weaned to the respective diet (for up to 91 days). No consistent treatment-related effects were observed in: body weights/gains; feed consumption; clinical observations; mating indices; survival, growth and development of litters, litter sizes, body weights, sex ratios (lower % males/litter at 1 and 2 g/kg bw/day), acquisition of developmental landmarks, behavioral indices, or histology of selected organs. Lower serum vitamin D, liver vitamin A, and liver vitamin E levels were seen in some EPG-treated groups. None of the reductions were judged to be biologically significant. A/G ratio was greater among males receiving 2 g/kg bw/day and 5%. In the absence of any other related effects, the biological significance of this finding is doubtful.

1. Introduction

Esterified propoxylated glycerols (EPGs) represent a family of modified fat- and oil-like products, 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.

A one-generation reproduction study was performed in Crl:CD(SD)BR rats to evaluate the potential of a version of EPG that is considered the "core" version (H-EPG-05 HR/SO 9:1) to elicit alterations in reproductive ability, including effects during lactation, growth and development of the offspring. Vitamin status and neurobehavioral parameters were also evaluated.

2. Materials and methods

This study was sponsored by ARCO Chemical Company, Newton Square, Pennsylvania, and conducted at Research Triangle Institute (RTI), Research Triangle Park, North Carolina, from September 24, 1992 to August 10, 1993, in compliance with the Principles of Good Laboratory Practice (GLP) regulations, of the United States Food and Drug Administration (FDA).

2.1. Animals

Sprague-Dawley-derived outbred albino (Crl:CD(SD)BR rats (age: 28 days) were obtained from Charles River Laboratories, Inc. (Raleigh, NC) and allowed to acclimate to the laboratory environment for approximately 2 weeks before exposure to the test diets. At the end of the acclimation period, animals were stratified based on body weight and allocated randomly within weight classes, to five treatment groups, each consisting of 30 animals per sex per group.

2.2. Mating

Thirty (30) animals/sex/group assigned were designated as F_0 parental animals. One male to one female from each group was selected at random and housed together continuously for up to 21 days until insemination of the female was confirmed. Females presumed pregnant were observed twice daily during gestation for evidence of littering. The first litters were designated as F_{1a}. The F_0 females were allowed to rear their young to postnatal day

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(PND) 21, after which the dams were removed and the litters were weaned. As the last F_{1a} litter was weaned, F_0 females were allowed to rest for at least 10 days before a second mating with different males within the same treatment group. The second mating followed the same procedure; litters from this mating were designated as F_{1b}.

2.3. Satellite animals

Prior to weaning of the first F_{1a} and F_{1b} litters, ten (10) litters per dose group were randomly selected to create satellite groups, A, B, and C. Group A and B pups were sacrificed on PND 21 and PND 49, respectively, and subjected to necropsy, clinical pathology, vitamin status and tissue EPG. Group C pups were sacrificed on PND 91 and subjected to these procedures and to motor activity and functional observational battery on PNDs 21, 42, 63, and 84.

2.4. Housing

F_0 females were housed individually in solid bottom polycarbonate cages with stainless steel wire lids, except during mating, when each cage held one male and one female; females were housed individually during gestation and after littering until litters were weaned (PND 21). Individual weanlings were housed singly. Environmental controls for the animal room were set to maintain 68–75 °F, a relative humidity of 40–70%, and a 12-hour light/12-hour dark cycle. Variations from these conditions were documented and none were considered to have any effect on the outcome of the study. Food and water were provided *ad libitum*. There were no known contaminants in the food or water that would have interfered with this study.

2.5. Test material

The test material, esterified propoxylated glycerol [H-EPG HR/SO 9:1; EPG (stabilized with tocopherols, including α-tocopherol), lot # 753489], was provided by the sponsor. The test article was a white-colored, odorless solid received and stored frozen (−20 ± 5 °C) in the original containers at RTI.

The vehicle, Mazola® corn oil, was received from Best Foods, a Division of CPC international, Inc. 1120 Commerce Avenue, Union, NJ 07083. According to the supplier, all cases came from the same lot (Lot No. 2321) of material. The vehicle was received and stored frozen (−20 ± 5 °C). The characterization data were provided by the supplier (100% total fatty acids by GC analysis as methyl esters) and the purity was assumed to be 100% for the purpose of concentration calculations.

The carrier was Modifed NIH-07 Certified Mouse/Rat Diet No. 7722 [omitting soy oil (2.5%), decreasing corn oil by 3.5%, and factoring each ingredient level by 0.94 to allow for the addition of 6% corn oil] manufactured by Harlan Teklad Permier Lab Diets (Madison, WI), milled in Winfield, IA. The feed was stored in temperature- and humidity-regulated rooms (18 °C; 50% relative humidity).

2.6. Feed formulation

Test diets were formulated weekly in a 2-ft^3 V-shell blender with an intensifier bar (Patterson-Kelley Co., East Stroudsburg, PA). Corn oil was used as is for the control feed; EPG was blended with corn oil (40–60 °C, 20 min) at the appropriate levels and the mixture was combined with the basic feed for 0.75 to 1.25 h, depending on the size of the batch.

Samples collected showed that the feed was homogeneous and stable for at least 30 days when refrigerated (1–7 °C) and for 49 days when frozen (−12 to −18 °C). Formulations were also stable under ambient conditions (in a feed jar in a cage; exposed to air, normal room lighting, and temperatures of 22–26 °C) for at least 9 days.

2.7. Dietary level selection and study design

EPG was incorporated in the diet at levels providing 0 (vehicle control), 0.5, 1, or 2 g/kg bw/day; one group received EPG at a constant level of 5% (w/w) in the feed. The dietary concentration of 5% EPG was consistent with what is generally considered the maximum that can be given to animals without interfering with their nutrition status and caloric needs. Other EPG dose levels were administered on a g/kg bw/day/day basis to achieve constant exposure levels throughout the study. During the last week of lactation, females with litters were exposed to one-half of the target dietary EPG level to prevent potential overdosing of self-feeding pups. After weaning of their litters, F_0 females went back to the target EPG dietary levels until necropsy. F_0 males were maintained on the target EPG dietary level until necropsy. Litters were weaned (PND 21) to the respective parental diet, adjusted based on body weight.

2.8. Mortality and clinical signs

Cage-side observations for mortality and general condition were made twice daily (morning and afternoon). Any animal judged to be in moribund condition was euthanized by carbon dioxide asphyxiation and then necropsied. Any animal found dead was subjected to a complete gross pathological examination. Cage-side observations included examination for general condition, appearance, behavior, movement within the cage, availability of feed and water, presence of excreta, and appearance and consistency of the stools. Pregnant animals were examined for dystocia (difficulty in delivery); any dam showing signs of imminent abortion or premature delivery was sacrificed on the day such signs were observed. Any abnormality in nesting and nursing behaviors of the dams was recorded.

Detailed clinical examinations were conducted daily. Observations included, but were not limited to, changes in: skin and fur, eyes and mucous membranes, excretion, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, and behavior pattern.

2.9. Body weights, feed consumption, and EPG intake

F_0 male body weights were determined and recorded at initiation of treatment and weekly thereafter until scheduled sacrifice; F_0 female body weights were recorded in the same manner except as follows. During gestation, females were weighed on gestational days (GD) 0, 7, 14, and 20. Dams producing litters were weighed on lactational days (PND) 0, 4, 7, 14, and 21. For non-pregnant females showing either no signs of mating or signs of mating but no litters, and for pregnant females with all dead pups on PND 0 or with complete loss of the litter during lactation, body weights were recorded at least weekly until scheduled sacrifice. Individual body weights of live pups were measured at the time of parturition (PND 0) and were recorded for days PND 4, 7, 14 and at weaning (PND 21). All animals were weighed at sacrifice. Individual body weight gains were computed.

Feed consumption was recorded weekly for all F_0 parental animals throughout the pre-mating treatment periods until cohabitation of the sexes. For the male rats, weekly measurement of feed consumption was resumed on the Monday following the return to individual housing. During pregnancy, feed consumption of the F_0 females was recorded for GD 0–7, 7–14 and 14–20. During lactation of the F_{1a} and F_{1b} litters, maternal feed consumption was measured for PND 0–4, 4–7, and 7–14. Weekly measurement

of feed consumption of the females was resumed on the Monday following weaning of the litters. Feed consumption was not measured for F_0 males and F_0 females during the cohabitation period, since two adult animals (breeding pair) were in the same cage, or for F_0 females during the last week of the lactation periods (PND 14–21) for F_{1a} and F_{1b} litters, since maternal feed consumption after PND 14 was confounded by the contribution from the pups, which were self-feeding by this time, and the concentration of EPG in the diet for each adjusted group was reduced to 50% of its previous level to prevent inadvertent overexposure of pups at this time when they were self-feeding (as well as nursing) and ingesting more feed (and therefore EPG) relative to their body weights, than the adult animals. Upon weaning, feed consumption was recorded weekly until scheduled sacrifice for the satellite group B and C pups.

EPG intakes at the different time periods were calculated based on feed consumption, EPG concentration in the diet, and body weights.

2.10. Reproductive and offspring parameters

Reproductive and offspring indices were calculated as shown in Table 1. F_1 pups were counted, sexed, weighed, and examined grossly at birth (designated PND 0). The pups were recounted and examined grossly on PND 4 (pre-culling), 7, 14, and 21 (weaning). Anogenital distance was utilized as the method of sexing the pups on PND 0–4; anogenital distance, presence/absence of fur between anus and genital papilla, and shape of the scrotal area (flat or convex) was the method of sexing animals after PND 4.

2.11. Hematology and clinical chemistry

Blood from the retro-orbital sinus was collected from all F_0 animals immediately before initiation of treatment, before the first mating, and at the time of scheduled sacrifice. Blood from F_{1a} and F_{1b} satellite groups was collected *via* cardiac puncture immediately prior to sacrifice. The following parameters were measured: nucleated red blood cells; corrected white blood cells; white blood cell differential (segmented neutrophil, band neutrophil, lymphocyte, monocyte, eosinophil) counts; total protein; albumin; albumin:globulin (A/G) ratio; platelets; prothrombin time (PT); and activated partial prothrombin time (APTT). If not enough blood was available from a given animal (especially 21-day old F_1 satellite group A pups) to measure all of the above parameters, then only total and differential leukocyte counts were measured.

Table 1
Methods used to calculate reproductive and offspring indices.

Index	Calculation
Female fertility index (%)	No. females pregnant/No. females that mated × 100
Male fertility index (%)	No. males siring litters/No. males that mated × 100
Gestational index	No. of females with live litters/ No. of females pregnant
Pregnancy index (%)	No. pregnant females/No. males that mated × 100
Live birth index	No. of live pups at birth/ Total No. of pups born
4-Day survival index	No. of pups surviving 4 days (precull)/ Total No. of live pups at birth
7-Day survival index	No. of pups surviving 7 days/ Total No. of live pups at 4 days (postcull)
14-Day survival index	No. of pups surviving 14 days/ Total No. of live pups at 7 days
21-Day survival index	No. of pups surviving 21 days/ Total No. of live pups at 14 days
Lactational index	No. of pups surviving 21 days/ Total No. of live pups at 4 days (postcull)

2.12. Developmental landmarks

The average day of acquisition for pre-selected developmental landmarks was determined as follows. For pinna detachment, all F_{1a} and F_{1b} pups were examined on PND 1–5, with expected acquisition on PND 2–4; for lower incisor eruption, all F_{1a} and F_{1b} pups were examined on PND 8–16, with expected acquisition on PND 9–15; for auditory startle, all F_{1a} and F_{1b} pups were examined on PND 8–15, with expected acquisition on PND 10–14; for eye opening, all F_{1a} and F_{1b} pups were examined on PND 11–18, with expected acquisition on PND 12–17. For testis descent, all F_{1a} and F_{1b} males were examined on PND 17–21; after weaning on PND 21, testis descent was evaluated on F_{1a}(C) and F_{1b}(C) male pups only, with expected acquisition on PND 17–30. For vaginal patency (opening), all F_{1a}(C) and F_{1b}(C) females were examined on PND 28–43, with expected acquisition on PND 30–38. For preputial separation, all F_{1a}(C) and F_{1b}(C) males were examined on PND 30–50, with expected acquisition on PND 30–35. The actual end dates for all of the examinations were dependent on last date of acquisition of the specific developmental landmark for the population evaluated, but did not exceed the postnatal day listed above.

2.13. Functional observational battery and motor activity

A functional observational battery (FOB) test was conducted on all F_0 animals before study start and during the week before the first mating. The specific endpoints evaluated in the FOB are outlined in Table 2.

In addition, an assessment of motor activity using computer-controlled figure-eight mazes (software from San Diego Instruments, Inc., San Diego, CA) was conducted on F_0 animals during the week prior to the first mating. A test session consisted of a continuous one-hour period, divided into twelve 5-min data-collection segments. A predetermined distribution scheme was used to counter-balance animals by group across time and recording devices for the motor activity test.

The FOB and motor activity assessments were also conducted on F_{1a}(C) and F_{1b}(C) pups (as described above), on PND 21 ± 2 (weaning), PND 42 ± 2, PND 63 ± 2, and PND 84 ± 2.

2.14. Necropsy and histopathology

Parental animals and offspring on PND 21 through 91 were weighed and then sacrificed by carbon dioxide asphyxiation; offspring on PND 4 were euthanized by decapitation. All animals in all groups were subjected to a complete necropsy that included

Table 2
Functional observational battery (FOB) parameters measured.

Home cage	Posture, tremors [exertion/resting], convulsions [clonic/tonic], abnormal motor movements [writhing/circling/bizarre], gait, gait score, and wasting
Handling	Ease of removal, ease of handling, salivation, piloerection, muscle tone, fur appearance, mouth and nose deposits, and eyes [exophthalmus/deposits/conjunctivitis/exudates/opacity/lacrimation]
Open field	Posture, tremors [exertion/resting], convulsions [clonic/tonic], abnormal motor movements [writhing/circling/bizarre], gait, gait score, vocalizations, arousal, urination [No. spots], urination character, defecation [No. boli], and defecation character
Sensory and neuromuscular	Body weight, approach response, tail pinch response, pupil response, hindlimb foot splay, forelimb grip strength, hindlimb grip strength, and startle response

body weight and examination of: external surfaces; all orifices; cranial cavity; carcass; external and cut surfaces of the brain and spinal cord; thoracic, abdominal, and pelvic cavities and their viscera; and cervical tissues and organs. Special attention was paid to examination of the reproductive organs.

Tissues/organs collected for histopathological examination were preserved in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. For F_0 animals (control and 5% EPG groups), tissues examined included vagina, testes, corpus and cervix uteri, seminal vesicles, ovaries, epididymides, oviducts, prostate, pituitary gland, coagulating gland, and any tissues with gross lesions identified as being potentially treatment-related. The spleen, bone marrow, thymus, representative lymph nodes (submaxillary and mesenteric), and Peyer's patches in the small intestine were also examined microscopically for all control and 5% EPG animals, and any F_1 satellite group in which abnormal hematological results were noted (*e.g.*, abnormal monocyte count).

The following tissues (when present) were examined microscopically for any weaned pup that died or was sacrificed moribund, and from selected F_{1b} pups not assigned to satellite groups: adrenals; aorta; brain; cecum; colon; corpus and cervix uteri; duodenum; epididymides; esophagus; exorbitant lacrimal (Harderian) gland; eyes; femur with bone marrow (articular surface of the distal end); gross lesions; heart; ileum; jejunum; kidneys; liver (four lobes); lungs with mainstem bronchi; lymph nodes (submaxillary and mesenteric); mammary gland (females); any masses; muscle (skeletal); nasal turbinates; ovaries and oviduct; pancreas; Peyer's patches; pituitary; prostate; rectum; salivary gland (submaxillary); sciatic nerve; seminal vesicles; skin; spinal cord (cervical, mid-thoracic, and lumbar); spleen; sternum; stomach; testes; thymus; thyroid with parathyroid; tongue; trachea; urinary bladder; uterus; vagina; and external auditory sebaceous (Zymbal's) gland.

2.15. Liver and serum vitamin assays

At the time of scheduled sacrifice, all F_0 females and males, F_{1a} and F_{1b} PND 4 pups and F_{1a} and F_{1b} satellite groups were assessed for liver and serum fat-soluble vitamin status. Initially, only the 5% EPG and control group animals were evaluated. When it appeared that there might have been treatment-related differences in vitamin levels, the decision was made to assay samples from the remaining groups. Statistical analyses were performed to compare EPG-exposed group values to the vehicle control group, within sexes and times. However, because the samples for the remaining EPG groups were analyzed at a different time, the data were difficult to reconcile.

Selected animals were bled from the abdominal vena cava and/or *via* cardiac puncture to maximize blood volume and to minimize contamination of the sample with tissue thromboplastin. The blood was separated and at least 0.2 mL of serum was stored at approximately −20 °C until analyzed for 25-hydroxy-calciferol (vitamin D). Vitamin K status was determined indirectly by hematologic assessment of platelet count, activated partial thromboplastin time and prothrombin time for all F_0 animals, and F_1 group B and C satellite pups. Vitamin K status was determined indirectly by hematologic assessment of platelet count and prothrombin time. F_1 group A satellite pups. F_{1a} and F_{1b} PND 4 pups were subjected to vitamin D analysis only. Serum 25-OH vitamin D was analyzed by a radioimmunoassay with a commercially available kit from INCstar Corp. (Stillwater, MN).

The liver was harvested and weighed. Representative samples from each lobe were pooled, weighed, frozen using dry ice, and stored at −70 °C until analyzed for trans-retinol (vitamin A) and α-tocopherol (vitamin E) by HPLC employing UV spectrophotometric detection as adapted from the method of Milne and Botnen (1986).

Initially, liver and serum vitamin assays were limited to the 5% EPG and control groups. Further analysis was performed on the retained duplicate frozen liver samples for total vitamin A using an extraction method which incorporated a saponification step. The method involved exposure of liver homogenates to 1.8 M KOH dissolved in 50% ethanol:water at 60 °C for 2 h. This step extracts total vitamin A (both bound and free) for analysis of vitamin content, to allow comparison of vitamin A data for this study with vitamin A data from other studies which incorporated a saponification step during liver extractions.

2.16. Tissue assays for EPG

At the time of scheduled sacrifice, representative samples of the liver (all lobes), kidneys, spleen, and abdominal fat (adipose tissue) from F_0 animals and pups assigned to satellite groups (control and 5% EPG groups) were collected and stored at approximately −20 °C until analyzed for EPG concentration. Tissue samples were evaluated using Waters 845 HPLC (high-performance liquid chromatography) and data system with a reverse phase, Beckman Ultrasphere ODS (25 cm × 4.6 mm ID), Dupont Zorbax Rx-C8 guard cartridge.

2.17. Statistical analyses

The unit of comparison was the male, the female, the pregnant female or the litter. Quantitative continuous data (*e.g.*, parental and pup body weights, organ weights, food consumption, etc.) were compared among the four treatment groups and the one vehicle control group by the use of Bartlett's test for homogeneity of variances (Snedecor and Cochran, 1967). Data within groups were also evaluated for normality. If Bartlett's test indicated lack of homogeneity of variances (*i.e.*, $p < 0.001$) or the test for normality was rejected for the majority of the groups, then nonparametric statistical tests were employed for the continuous variables (Winer, 1962; see below). If Bartlett's test indicated homogeneous variances (*i.e.*, $p > 0.001$) and the data were normally distributed, then the following parametric statistical tests were employed for the continuous variables: Analysis of variance (ANOVA) was used to determine if significant differences were present among the groups with Dunnett's test for pairwise comparisons (Dunnett, 1955, 1964) to the designated control group, if the ANOVA was significant. Appropriate General Linear Models (GLM, SAS Institute, Inc., 1989a, 1989b, 1990a, 1990b, 1990c) analyses were used to determine the significance of the dose-response relationship (Test for Linear Trend).

Litter-based percentage data (*e.g.*, periodic pup survival indices) were arcsine-square root transformed prior to statistical analysis (Snedecor and Cochran, 1967) to allow use of parametric methods. For the litter-based percentage data, the ANOVA was weighted according to litter size. Nonparametric tests used for continuous data which did not have homogeneous variances included the Kruskal–Wallis test to determine if significant differences were present among the groups, followed by the Mann–Whitney *U* test for pairwise comparisons to the designated control group if the Kruskal–Wallis test was significant (Siegel, 1956). Jonckheere's test for K independent samples (Jonckheere, 1954) was used to identify significant dose-response trends for nonparametric continuous data. Frequency data such as reproductive indices (*e.g.*, mating and fertility indices) were not transformed. All indices were analyzed by Chi Square Test for Independence and Fisher's Exact Test for pairwise comparisons to the designated control group (with appropriate corrections for multiple comparisons), if Chi Square was significant (Snedecor and Cochran, 1967). For frequency data, the Cochran–Armitage Test for Linear Trend on proportions (Cochran, 1954; Armitage, 1955; Agresti, 1990) was used to identify significant dose-related linear trends for nominal data. For all

statistical tests, the significance limit of 0.05 (one- or two-tailed) was used as the criterion for significance.

A test for statistical outliers (SAS) was performed on adult body weights and feed consumption (in g/day). If examination of pertinent study data did not provide a plausible biologically-sound reason for inclusion of the data flagged as "outlier," the data were excluded from summarization and analysis and were designated as outliers. If feed consumption data in g/kg/day were negative for a given animal and day, they were designated "unrealistic" and excluded from summarization and analysis. If feed consumption data for a given observational interval (*e.g.*, GD 0–7 or PND 4–7) for a given animal were designated outliers or unrealistic, then summarized data encompassing this period (*e.g.*, GD 0–20 gestational period, or PND 0–21 lactational period) also did not include this value.

A combination of categorical, analysis of variance and parametric repeated measures techniques, survival time methods, and complex sample survey techniques for clustered or correlated data was used in the statistical analysis of the developmental landmarks/behavioral endpoints. The analyses were carried out using the FREQ, GLM, NPAR1WAY, LIFETEST, and PHGLM procedures in the SAS software package (SAS Version 6, 1990), as well as the DESCRIPT, LOGISTIC, and SURVIVAL procedures in the SUDAAN (Research Triangle Institute, Version 6.1, 1991) software package, in conjunction with a set of custom-designed analysis procedures.

For dichotomous variables in which observations arise as simple random samples from experimental strata (*e.g.*, presence/absence of a given trait in the Functional Observational Battery), contingency table techniques were employed to examine differences among the experimental groups. These analyses include Pearson's chi-square test for overall heterogeneity, followed by individual exposed vs. control group comparisons using Fisher's Exact test, and the Cochran–Armitage chi-square test (Armitage, 1955) for linear trend. Pairwise comparisons also include the estimation of an odds ratio (with a 95% confidence interval) to quantify the strength of potential toxic effects. For polychotomous data measured on a nominal or qualitative scale (*e.g.*, posture type), the Pearson chi-square test was employed for heterogeneity of the response distributions, followed by individual treated vs. control group comparisons. In addition, the contribution of each response level was evaluated to the total chi-square statistic.

For continuous analysis variables and count data (*e.g.*, body weights, rearing activity in motor activity trials), a battery of parametric (or non-parametric) tests was performed to compare the experimental groups. If the assumptions for parametric analyses were not satisfied (as determined by Bartlett's test for homogeneity of variance and Shapiro–Wilk's test for departure from normality), non-parametric analyses were employed. Parametric analyses included Dunnett's *t*-test for simultaneous exposed vs. control group comparisons ($p \leqslant 0.05$, $\leqslant 0.01$, and $\leqslant 0.001$), followed by a linear contrast for monotonic trend. Dunnett's test adjusted the overall type I error rate for multiple comparisons, eliminating the need to perform a preliminary test for overall heterogeneity. Analogously, the non-parametric analyses consisted of Fligner's (1984) test for simultaneous exposed vs. control group comparisons ($p < 0.05$, <0.01, and <0.001), followed by the Jonckheere-Terpstra (Jonckheere, 1954) test for trend. Again, since Fligner's test adjusted for multiple comparisons, the overall Kruskal–Wallis test for heterogeneity was not considered before performing the comparisons of exposed groups to control. For polychotomous endpoints measured on an ordinal scale (*e.g.*, severity scores in FOB data), nonparametric techniques as described above were employed.

A nested ANOVA model was used for analyzing continuous variables with correlated observations (*e.g.*, through clustering of responses within litters, as seen in the analysis of time to developmental landmarks). In this case, the statistical model included the regression effect of dose and litters nested within dose, the latter effect serving as the appropriate error term for testing the hypothesis of no treatment effect against various alternatives (heterogeneity, linear trend). In addition, when the design was unbalanced (*e.g.*, an unequal number of pups per litter), a Satterthwaite correction was applied to the usual *F*-test for no dose effect.

In addition to analysis of variance techniques applied at given points in time, several behavioral variables were analyzed as repeated measures on the animals over time, where "time" may actually represent trials, blocks, or 10-min periods in a 1-h session. Such response variables may include 10-min activity measures in a 1-h session on a given day, total activity, or time to habituation in motor activity trials. For continuous responses measured over time (*e.g.*, 10-min activity levels), parametric repeated measures techniques were used. The mixed analysis of variance model included exposure level as the grouping factor (fixed effect), as well as the random effects of animals nested within exposure groups, with repeated observations on the animals over time. Contrasts were constructed to examine the linear, quadratic, and cubic components of the time effect and their interaction with exposure groups. This model is useful in analyzing linear and non-linear trends in behavioral measures over time, as well as possible differences in these trends among the exposure groups (Winer, 1971). Additional covariates may also be included.

Distribution-free procedures used routinely for analyzing complex sample survey data (Cochran, 1977) were employed for two types of repeated measures data: dichotomous or incidence data (yes/no responses) and censored time-to-event data. Unlike the parametric procedures, the survey data techniques make no distributional assumptions concerning the response of interest (*i.e.*, multivariate normality) or the correlations among repeated observations (*i.e.*, compound symmetry), thereby providing flexibility for a variety of analytical settings. Consistent variance estimators for sample statistics (means, proportions, ratios, regression coefficients) are provided by applications of first-order Taylor series approximations in accordance with the hierarchical sampling design (Binder, 1993, 1992). Both descriptive statistics capabilities (*e.g.*, means, medians and their standard errors) and modeling facilities (for linear, logistic, loglinear, and proportional hazards regression) are provided in SUDAAN. Estimated design effects (or the ratio of the variance estimates for the clustered design vs. a simple random sample of independent observations) were provided for all sample statistics.

For variables which measure the time to some event on observations that are statistically independent (*i.e.*, not related through litters or repeated observations over time), standard survival analysis techniques were applied. One such example was the habituation onset time in motor activity trials (if repeated measures over days are not of concern). The logrank test (Cox, 1972; Mantel, 1966) and Tarone's (1975) test was employed to compare the event time distributions in the experimental groups. The Cox proportional hazards model (Cox, 1972) can be used to incorporate additional covariates, such as sex, if necessary. These techniques, which utilize the actual time to the event, are sensitive to differences in the overall incidence rates as well as the distribution of onset times for the event. Kaplan and Meier (1958) survival curves were used to describe the proportion of animals in each exposure group having not reached the specified event over the course of time. Non-overlapping survival time distributions indicate differences in median event times among the experimental groups. Specifically, the log rank test was used to detect overall heterogeneity in event time distributions among the experimental groups, as well as to perform individual pairwise comparisons of exposed groups to control. Tarone's test was employed to detect a dose-related trend in onset times. Median event times and censoring rates were provided.

3. Results

3.1. Mortality and clinical signs

The fate of F_0 males and females is summarized in Tables 3 and 4, respectively. A total of seven males and females died prior to scheduled sacrifice; most were found dead. None of the deaths appeared to be related to EPG.

Clinical observations were limited to occasional findings in all groups of alopecia, skin sores, and injuries to eyes from retro-orbital bleeding trauma. Alopecia is a common finding in rats in long-term housing; the sores, predominantly on the back, head and shoulders, were likely caused by rubbing against the edge of the feed jar lids.

3.2. Body weights, feed consumption, and EPG intake

No biologically relevant differences among groups for mean weekly body weights, or mean weekly body weight changes were observed. Feed consumption, in g/day or g/kg/day, also did not differ among groups. Statistically significant differences were observed occasionally, but with no apparent relationship to EPG.

Mean EPG intakes among F_0 females, F_0 males, and F_1 (weaned) pups are summarized in Tables 5–7, respectively. The adjusted diets in groups receiving 0.5, 1, and 2 g/kg/day of EPG resulted in intakes slightly below target values during the prebreed phase; intakes increased thereafter. Animals receiving a fixed intake of 5% EPG in the diet had the expected decrease in EPG intake over time; the result of feed consumption in g/day remaining relatively constant and the mean body weights increasing markedly over time so that mean feed intake in g/kg/day decreased markedly over time.

EPG intakes were at or above target values during the gestational and lactational periods. Maternal lactational EPG intake exhibited the expected increases across groups for all intervals evaluated (PND 0–4, 4–7 and 7–14). As would be expected, EPG intakes among the 0.5, 1, and 2 g/kg bw/day groups exceeded target values during lactation.

3.3. Reproductive and offspring parameters

Tables 8 and 9 summarize reproductive and offspring parameters for the each of the two consecutive litters. There were no statistically significant differences among groups, except for the percent F_{1b} male pups per litter, which was lower for the 1 and 2 g/kg bw/day EPG groups, compared to control. There were two females from the 5% EPG group whose pregnancy (as resorption sites) was evident only after necropsy. The data from these animals were not included in the summary tables, since the time of pregnancy, *i.e.*, the F_{1a} or F_{1b} mating, could not be established.

Table 3
Survival of F_0 males.

	EPG in diet (g/kg bw/day)				5%
	0	0.5	1	2	
Number started on study	30	30	30	30	30
Deaths					
Prebreeding	0	0	0	1[a]	1[b]
1st mating period	0	0	0	0	0
Holding period (before 2nd mating)	0	0	0	0	0
2nd mating period	0	0	0	0	0
Holding period (before scheduled sacrifice)	2[a,b]	1[b]	2[a,b]	0	0
Scheduled sacrifice	28	29	28	29	29

[a] Died during orbital bleeding.
[b] Found dead.

Table 4
Survival of F_0 females.

	EPG in diet (g/kg bw/day)				5%
	0	0.5	1	2	
Number started on study	30	30	30	30	30
Deaths					
Prebreeding	0	0	0	0	0
1st mating period	0	0	0	0	0
Gestation of F_{1a} litter	1[a]	0	1[b]	2[b]	1[b]
Lactation of F_{1a} litter	1[a]	0	1[a]	0	0
Holding period (before 2nd mating)	0	0	1[b]	0	0
2nd mating period	0	0	0	0	0
Gestation of F_{1b} litter	0	0	0	1[b]	0
Lactation of F_{1b} litter	0	1[a]	0	0	1[b]
Holding period (before scheduled sacrifice)	0	2[c]	1[c]	1[b]	0
Scheduled sacrifice	28	27	26	26	28

[a] Sacrificed moribund.
[b] Found dead.
[c] Died during orbital bleeding.

Table 5
Mean EPG intakes (g/kg bw/day) among F_0 females.

Period	EPG in diet (g/kg bw/day)			5%
	0.5	1	2	
Prebreeding	0.39	0.80	1.38	3.99 (range: 1.39–6.96)
Gestation [F_{1a}]	0.57	1.06	2.19	2.98 (range: 2.44–4.11)
Lactation* [F_{1a}]	0.84	1.43	3.24	4.30 (range: 2.99–5.42)
PND 0–4				
PND 4–7	1.20	1.89	4.42	6.02 (range: 4.51–7.67)
PND 7–14	1.55	2.71	5.62	8.10 (range: 6.30–9.72)
Gestation [F_{1b}]	0.54	1.00	2.02	2.79 (range: 2–3.82)
Lactation* [F_{1b}]	0.69	1.31	2.88	3.37 (range: 1.91–4.89)
PND 0–4				
PND 4–7	1.05	1.93	3.90	5.20 (range: 3.45–6.32)
PND 7–14	1.39	2.50	5.37	6.90 (range: 3.75–8.33)

PND: postnatal day.
Note: EPG intake of during mating was not calculated because male vs. female feed intake could not be determined when they were housed together.
* EPG intakes during lactation exceeded the targeted amounts due to two factors: (1) dams typically consume more feed during lactation to fuel milk production, especially during the first 2 weeks when milk production is highest (*i.e.*, increased feed intake without significant body weight change); and (2) pups begin to self-feed during the second week after birth, so that "maternal" feed consumption actually represents feed consumption for the "maternal-litter" unit from PND 10 for the last half of lactation. This latter confounder is why maternal feed consumption data were only collected for PND 0–14, and why the dietary levels of EPG were reduced by half in the formulated diets to preclude or minimize any possible overexposure of the feeding pups.

Table 6
Mean EPG intakes (g/kg bw/day) among F_0 males.

Period	EPG in diet (g/kg bw/day)			5%
	0.5	1	2	
Prebreeding	0.4	0.81	1.61	3.12 (range: 1.31–5.59)
Holding period 1 (before 2nd mating)	0.48	0.98	1.90	2.02 (range: 1.64–2.71)
Holding period 2 (before scheduled sacrifice)	0.44	0.89	1.74	1.83 (range: 1.43–2.33)

3.4. Hematology and clinical chemistry

Some statistically significant differences were observed between the control and EPG groups in hematology and clinical chemistry parameters. However, none was associated with histopathological changes or other clinical manifestation.

Table 7
Range of mean EPG intakes (g/kg bw/day) among pups after weaning.

Litter/sex	EPG in diet (g/kg bw/day)			5%
	0.5	1	2	
F_{1a} (B) ♂ PND 21–49	0.23–0.37	0.48–0.70	0.89–1.46	4.62–6.82
F_{1a} (B) ♀ PND 21–49	0.21–0.36	0.46–0.70	0.92–1.40	4.61–6.53
F_{1a} (C) ♂ PND 21–91	0.22–0.46	0.46–0.95	0.89–1.75	2.64–6.64
F_{1a} (C)♀ PND 21–91	0.24–0.56	0.46–1.05	0.95–2.22	3.98–6.41
F_{1b} (B) ♂ PND 21–49	0.23–0.39	0.47–0.71	0.87–1.51	4.73–6.52
F_{1b} (B) ♀ PND 21–49	0.21–0.43	0.46–0.76	0.90–1.70	5.15–6.62
F_{1b} (C) ♂ PND 21–91	0.24–0.46	0.46–0.88	0.86–1.93	2.57–6.60
F_{1b} (C)♀ PND 21–91	0.23–0.52	0.47–0.96	0.88–2.26	3.51–6.67

PND: postnatal day.

F_0 and F_{1b}(B) males from the 2 g/kg bw/day and 5% EPG groups had significantly greater A/G ratios; for the F_0 the difference was evident at prebreeding (after EPG treatment started but before first mating) and at necropsy. There were also trends toward greater A/G ratios in these groups and in the F_{1a}(C) and F_{1b}(A)-1 satellite group males. In at least the F_0 males (at necropsy), the effect might have been related to higher albumin levels, which showed a statistically significant trend (increase) across EPG groups.

Other effects that reached statistical significance were not consistent across groups and/or did not appear to be related to EPG concentration. Corrected white blood cells counts, for example, were greater than control in F_{1a}(A)-1 females at 0.5 g/kg bw/day and F_{1a}(B) females at 1 g, showed a significant upward trend in F_0 females at necropsy, but were lower in F_{1a}(C) males exposed to 2 g/kg bw/day or 5% EPG. Similar inconsistently higher or lower values were observed for PT, APTT, platelets, and monocytes. Total protein levels were lower than control in F_{1a}(B) females exposed to 2 g/kg bw/day or 5% EPG; there were also trends toward lower total protein levels in F_{1a}(B) females and F_{1b}(C) males.

3.5. Developmental landmarks

Some statistically significant differences in developmental landmarks were observed, but with no evidence of a relationship to EPG concentration. In particular, statistically significant differences from the control group were limited to the following: (1) the average day of pinna detachment was greater for F_{1a} females in the 1 g/kg/day EPG group, with no significant pairwise differences or trends noted for acquisition of this landmark among F_{1a} males, F_{1b} females or F_{1b} males; (2) the average day for lower incisor eruption was greater for F_{1a} males in the 1 g/kg/day EPG group and lower for F_{1b} males in the 0.5 g/kg/day EPG group; (3) eye opening occurred later for F_{1a} pups in the 1 g/kg/day EPG group and earlier for F_{1a} females in the 5% EPG group; (4) average day of testes descent exhibited an increasing trend in the F_{1a} litters, possibly related to a low control group value for the F_{1a} litters (PND 23.2 vs. PND 25.20 for F_{1b} controls).

3.6. Functional observational battery and motor activity

Some statistically significant differences in the functional observational battery (FOB) and motor activity testing were observed. However, since none showed consistency across groups (*i.e.*, offspring from both matings) or time period, the differences were considered unrelated to EPG treatment.

3.7. Necropsy and histopathological findings

For the F_0 generation, gross lesions were sporadic, occurring in a single animal, with equal frequency between the control and EPG groups, and/or with no apparent relationship to EPG concentration.

Table 8
Mean values for reproductive (F_0) and offspring (F_{1a} litter) indices.

	EPG in diet (g/kg bw/day)				5%
	0	0.5	1	2	
# Males started on study	30	30	30	30	30
# Males paired	30	30	30	29	29
# Males mated	29	29	26	26	25
# Males siring litters	24	24	22	21	20
Male fertility index	82.8%	82.8%	84.6%	80.8%	80%
# Females started on study	30	30	30	30	30
# Females paired	30	30	30	30	30
# Females mated	29	29	26	28	27
# Pregnant	24	24	22	22	21
Female fertility index	82.8	82.8	84.6	78.6	77.8
# Females with live litters	23	23	22	21	20
Gestational index	100%	95.8%	100%	100%	95.2%[†]
Pregnancy index	82.8%	82.8%	84.6%	84.6%	84%
Time to insemination[#] (days)	4.4	4.4	3.3	4	5.1
Gestational length[#] (days)	22.3	22.5	22.5	22.4	22.6
# Live pups at birth[#] (PND 0)	14.6	13.5	14	13.6	14.3
# Dead pups at birth[#] (PND 0)	0.7	0.7	1.8	0.3	0.3
# Pups born[#] (PND 0)	15.3	14.2	15.7	13.9	14.5
Stillbirth index[#]	4.5%	6.3%	13.9%	1.6%	5.5%
Live birth index[#]	95.5%	93.7%	86.1%	98.4%	94.5%
4-Day survival index[#]	95.3%	98.1%	90.7%	97.5%	93.8%
7-Day survival index[#]	94.5%	99.6%	91.6%	98.5%	100%
14-Day survival index[#]	99.5%	100%	99.4%	99%	100%
21-Day survival index[#]	100%	100%	100%	100%	100%
Lactational index[#]	94.1%	99.6%	91.1%	97.5%	100%
PND 0 (birth)					
# Live litters	23	23	21	20	19
# Pups per litter	14.6	14	14	13.6	15
Pup body weight[**] (g)	6.6	6.43	5.98	6.61	6.46
Male pup body weight[**] (g)	6.75	6.63	6.13	6.74	6.56
Female pup body weight[*] (g)	6.43	6.22	5.80	6.47	6.25
% Male pups per litter	52.4	50.1	49.7	51.8	50.5
PND 4					
# Live litters	22	23	19	20	18
# Pups per litter	13.8	13.8	13.6	13.3	14.8
Pup body weight[**] (g)	11.25	11.32	9.49	11.66	11.22
Male pup body weight[***] (g)	11.44	11.59	9.83	11.87	11.32
Female pup body weight[*] (g)	11.06	10.99	9.36	11.40	10.89
% Male pups per litter	52.2	49.9	49.5	51.5	47.7
PND 7					
# Live litters	21	23	18	20	18
# Pups per litter	9.5	9.9	8.8	8.8	9.7
Pup body weight[***] (g)	18.89	18.05	15.94	18.81	18.53
Male pup body weight[***] (g)	19.13	18.53	16.43	19.27	18.87
Female pup body weight[***] (g)	18.58	17.54	15.34	18.52	17.87
% Male pups per litter	50	50	51.9	51.1	47.9
PND 14					
# Live litters	21	23	18	20	18
# Pups per litter	9.9	9.9	9.2	8.7	9.7
Pup body weight[***] (g)	38.64	36.93	35.67	40.41	38.67
Male pup body weight[**] (g)	39.14	37.8	36.58	41.21	39.08
Female pup body weight[**] (g)	38.05	36.04	34.78	39.9	37.77
% Male pups per litter	50.3	49.6	50.9	51.1	47.9
PND 21					
# Live litters	21	23	18	20	18
# Pups per litter	9.9	9.9	9.2	8.7	9.7
Pup body weight[*] (g)	59.33	56.92	55.54	62.11	59.28
Male pup body weight[*] (g)	60.36	58.34	57.59	63.69	60.02
Female pup body weight[*] (g)	58.10	55.51	53.54	60.68	57.59
% Male pups per litter	50.3	49.6	50.9	51.1	47.9

PND: postnatal day

[†] Includes one female that was pregnant but did not deliver a live litter.

[#] Bartlett's test for homogeneity of variances was significant ($p < 0.001$) or could not be done because there was zero variance in one or more groups and/or the test for normality was rejected ($p < 0.05$) for the majority of the groups, therefore nonparametric statistical tests were employed for evaluation of the data.

[*] $p \leqslant 0.05$, ANOVA; follow-up pairwise comparison showed no statistical significance.

[**] $p \leqslant 0.01$, ANOVA; follow-up pairwise comparison showed no statistical significance.

[***] $p \leqslant 0.001$, ANOVA; follow-up pairwise comparison showed no statistical significance.

Table 9
Mean values for reproductive (F_0) and offspring (F_{1b} litter) indices.

	EPG in diet (g/kg bw/day)				5%
	0	0.5	1	2	
# Males started on study	30	30	30	30	30
# Males paired	30	30	30	29	29
# Males mated	24	23	21	20	23
# Males siring litters	20	19	16	16	21
Male fertility index	83.3%	82.6%	76.2%	80%	91.3%
# Females started on study	30	30	30	30	30
# Females paired	28	30	27	28	29
# Females mated	24	24	21	21	24
# Pregnant	20	20	16	16	21
Female fertility index	83.3%	83.3%	76.2%	76.2%	87.5%
# Females with live litters	20	20	15	16	21
Gestational index	100%	100%	93.8%	100%	100%
Pregnancy index	83.3%	87%	76.2%	80%	91.3%
Time to insemination[#] (days)[‡]	2.9	3.3	4.6	7.2	6.5
Gestational length[#] (days)	22.3	22.5	22.4	22.5	22.4
# Live pups at birth[#] (PND 0)	14.6	15.1	12.4	14.5	12.7
# Dead pups at birth[#] (PND 0)	0.4	0.5	0.8	0.1	1
# Pups born (PND 0)	15	15.5	13.2	14.5	13.7
Stillbirth index[#]	1.9%	3%	8%	0.6%	8.1%
Live birth index[#]	98.1%	97%	92%	99.4%	91.9%
4-Day survival index[#]	94.7%	97.1%	99.6%	94.5%	88.9%
7-Day survival index[#]	94.5%	98.9%	99.3%	95.3%	99.4%
14-Day survival index[#]	100%	100%	100%	93.3%	100%
21-Day survival index[#]	100%	99.5%	100%	100%	100%
Lactational index[#]	94.5%	98.4%	99.3%	88.7%	99.4%
PND 0 (birth)					
# Live litters	20	20	15	16	21
# Pups per litter[#]	14.6	15.1	13.3	14.5	12.7
Pup body weight[#] (g)	6.62	6.58	6.48	6.60	6.68
Male pup body weight[#] (g)	6.81	6.77	6.66	6.75	6.88
Female pup body weight (g)	6.36	6.33	6.30	6.41	6.34
% Male pups per litter[#,†,‡]	57.5	55.2	40.2[§§]	45[§]	51.8
PND 4					
# Live litters	20	20	15	15	18
# Pups per litter[#]	13.7	14.5	13.2	13.9	12
Pup body weight (g)	10.81	11.14	10.83	10.81	12.22
Male pup body weight (g)	11.48	11.39	11.23	11.39	12.56
Female pup body weight (g)	10.48	10.8	10.64	10.6	11.67
% Male pups per litter[#,†]	54.2	57.3	40[§]	42.4[§]	54.5
PND 7					
# Live litters	19	19	15	15	18
# Pups per litter[#]	9.1	9.5	8.9	8.5	8.8
Pup body weight (g)	18.92	18.51	17.19	18.52	19.81
Male pup body weight (g)	19.27	18.96	18.14	19.17	20.05
Female pup body weight (g)	18.56	18.02	16.76	18.35	19.26
% Male pups per litter[#,†]	51.8	50.4	41.2[§§]	40.5[§§]	54.8
PND 14					
# Live litters	19	19	15	14	18
# Pups per litter[#]	9.5	9.5	8.9	8.4	8.8
Pup body weight (g)	38.58	37.49	34.97	40.43	40.52
Male pup body weight (g)	39.24	38.2	37.33	39.93	41.1
Female pup body weight (g)	37.87	36.73	34.31	40.02	39.48
% Male pups per litter[#]	51.8[†]	50.4	41.2[§§]	43.4[§]	54.8
PND 21					
# Live litters	19	19	15	14	18
# Pups per litter[#]	9.5	9.5	8.9	9	8.8
Pup body weight[#] (g)	62.38	61.26	57.3	64.86	65.66
Male pup body weight (g)	63.62	62.88	60.9	63.75	67.03
Female pup body weight[#] (g)	61	59.59	55.94	63.94	63.17
% Male pups per litter[#]	51.8	50.7	41.2[§§]	43.4[§]	54.8

PND: postnatal day

[#] Bartlett's test for homogeneity of variances was significant ($p < 0.001$) or could not be done because there was zero variance in one or more groups and/or the test for normality was rejected ($p < 0.05$) for the majority of the groups, therefore non parametric statistical tests were employed for evaluation of the data.

[†] $p \leqslant 0.01$, Kruskal–Wallis test.

[‡] $p < 0.05$, Jonckheere's test.

[§] $p \leqslant 0.05$, Mann–Whitney U test.

[§§] $p \leqslant 0.01$, Mann–Whitney U test.

Table 10
Summary of results for vitamin analyses.

Rats	EPG in Diet (g/kg bw/day)				5%
	0	0.5	1	2	
Serum vitamin D					
F_{1a} PND 4	Ø	Ø	Ø	Ø	Ø
F_{1a}(A) PND 21	Ø	↓ m,f	↓ f	↓ m,f	↓ f
F_{1a}(B) PND 49	Ø	Ø	↑ m,f	↑ m	Ø
F_{1a}(C) PND 91	Ø	Ø	↑ m	Ø	Ø
F_{1b} PND 4	Ø	Ø	Ø	Ø	Ø
F_{1b}(A) PND 21	Ø	Ø	Ø	Ø	Ø
F_{1b}(B) PND 49	Ø	Ø	Ø	Ø	↓ f
F_{1b}(C) PND 91	Ø	Ø	Ø	Ø	Ø
F_0	Ø	↓ f	↓ f	↓ f	↓ f
Liver vitamin E					
F_{1a} PND 4	Ø	Ø	Ø	Ø	Ø
F_{1a}(A) PND 21	Ø	Ø	Ø	↓ f	↓ f
F_{1a}(B) PND 49	Ø	↓ m,f	↓ f	↓ m,f	↓ m,f
F_{1a}(C) PND 91	Ø	↓ m,f	↓ m,f	↓ m,f	↓ m,f
F_{1b} PND 4	Ø	Ø	Ø	↓ m	Ø
F_{1b}(A) PND 21	Ø	Ø	Ø	Ø	↓ f
F_{1b}(B) PND 49	Ø	↓ m,f	↓ m,f	↓ m,f	Ø
F_{1b}(C) PND 91	Ø	↓ m,f	↓ m,f	↓ m,f	Ø
F_0	Ø	Ø	Ø	↓ m	Ø
Liver vitamin A (initial assay)					
F_{1a} PND 4	Ø	Ø	Ø	Ø	Ø
F_{1a}(A) PND 21	Ø	Ø	Ø	Ø	Ø
F_{1a}(B) PND 49	Ø	↓ m,f	↓ m,f	↓ m,f	Ø
F_{1a}(C) PND 91	Ø	↓ m,f	↓ m,f	↓ m,f	↓ f
F_{1b} PND 4	Ø	Ø	Ø	Ø	Ø
F_{1b}(A) PND 21	Ø	Ø	Ø	Ø	Ø
F_{1b}(B) PND 49	Ø	↓ f	↓ m,f	Ø	Ø
F_{1b}(C) PND 91	Ø	↓ m,f	↓ m,f	↓ m,f	↓ f
F_0	Ø	↓ f	↓ m,f	↓ m,f	↓ f
Liver vitamin A (reanalysis)					
F_{1a} PND 4	Ø	Ø	Ø	Ø	Ø
F_{1a}(A) PND 21	Ø	Ø	Ø	Ø	Ø
F_{1a}(B) PND 49	Ø	Ø	↑ f	Ø	↓ m,f
F_{1a}(C) PND 91	Ø	Ø	Ø	Ø	Ø
F_{1b} PND 4	Ø	Ø	Ø	Ø	Ø
F_{1b}(A) PND 21	Ø	Ø	Ø	Ø	Ø
F_{1b}(B) PND 49	Ø	Ø	Ø	↓ m	↓ m,f
F_{1b}(C) PND 91	Ø	Ø	Ø	Ø	Ø
F_0	Ø	↓ f	↓ f	↓ m,f	↓ f

m = male; f = female
Ø = no statistically significant difference vs. control
↑ = significantly higher than control group value
↓ = significantly lower than control group value

Likewise, microscopic examinations revealed no significant differences, except for mineralization in Peyer's patches, which occurred in 1/30 control males vs. 5/30 males from the 5% EPG group. No significant differences in organ weights were noted, except for lower ($p < 0.01$) relative liver weights among males from the 2 g/kg bw/day group (vs. control), and greater thymus weights among F_0 females receiving EPG at 1 g/kg bw/day ($p < 0.01$) or 2 g/kg bw/day ($p < 0.05$).

For F_{1a} and F_{1b} pups that died during lactation (PND 0–21), necropsy findings across all groups consisted predominantly of patent (open) ductus arteriosus, no air in the lungs, empty stomach, (the pup had not yet nursed), autolysis, and cannibalization by the mother. None of the findings could be attributed to EPG exposure.

F_{1a} (B and C satellite group) pups exposed to EPG exhibited trends toward lower relative kidney weights (relative and/or absolute), but significantly ($p < 0.05$) lower values (vs. control) were seen only for male F_{1a}(B) pups exposed to 5% EPG. Similar trends toward lower liver weights were observed in F_{1a}(C) pups (significant for 2 g/kg bw/day group); however, for F_{1b} males there was a trend toward greater absolute (but not relative) liver weights.

3.8. Liver and serum vitamin assays

The results of the analyses for the fat-soluble vitamins, serum vitamin D and liver vitamins A and E are summarized in Table 10.

Serum vitamin D levels were lower in all F_0 females (but not F_0 males) receiving EPG and in F_{1a}(A) PND 21 female offspring. EPG exposure was also associated with significantly lower liver vitamin E levels in male and female offspring.

An initial analysis showed significantly lower liver vitamin A in F_0 females and offspring (male and female) across all EPG groups; in F_0 males, lower liver vitamin A levels were seen only at 1 and 2 g/kg bw/day. Reanalysis of liver vitamin A using a saponification step showed: lower levels in F_{1a}(B) and F_{1a}(C) pups (at PND 49) of both sexes exposed to 5% EPG; higher levels in F_{1a}(B) female pups exposed to 1 g/kg bw/day; and lower levels in F_{1b}(B) males exposed to 2 g/kg bw/day.

3.9. Tissue EPG assays

EPG was not detected in any liver sample analyzed (354 total from the control and 5% EPG groups). Analysis of other tissues (including some control samples) showed positive results for the presence of EPG. The results were considered artifactual, since they could not be attributed to contamination during necropsy or analysis, were evenly divided between the control and 5% EPG groups, and were not associated with other measures indicative of *in vivo* systemic exposure (*e.g.*, EPG in liver, first site after presumed intestinal absorption, which did not occur). In particular, EPG was detected in: 2/354 kidney samples (1 each at 0% and 5% EPG); 7/354 spleen samples (4 at 0% and 3 at 5% EPG); and 2/334 adipose samples (at 5% EPG).

4. Discussion

Continuous exposure to EPG up to 5% in the diet for 13 weeks of prebreed and through two breeding cycles for F_0 parental animals, and through PND 91 for F_{1a} and F_{1b} offspring, resulted in no consistent indications of reproductive or systemic toxicity. No consistent treatment-related effects were observed in F_0, F_{1a} or F_{1b} body weights, weight gains, feed consumption, clinical observations, mating indices, survival, growth and development of F_{1a} and F_{1b} litters, including litter sizes, body weights, sex ratios (except for lower percent F_{1b} male pups per litter at 1 and 2 g/kg bw/day of EPG), acquisition of developmental landmarks, behavioral indices in FOB and motor activity, or histology of selected organs.

In some groups, EPG was associated with lower serum vitamin D, liver vitamin A, and liver vitamin E. F_0 females were more severely affected for vitamins D and A, perhaps due to the increased demands from the pregnancies. However, none of the reductions were associated with other apparent consequences and were not judged to be biologically significant. In other experimental animal studies, EPG has been shown to have an effect on lipid-soluble vitamins (unpublished data). It is therefore possible that, as a nondigestible lipid-like substance, it acts as a lipid "sink" or additional "compartment" for the distribution of lipid-soluble substances, including fat-soluble vitamins present in the gut lumen. Other studies in rats and mice (data not presented) confirmed an absence of any indications of vitamin deficiencies following lifetime exposures.

Hematology and clinical chemistry showed greater (and/or trends toward greater) serum A/G (albumin to globulin) ratios in males receiving 2 g/kg bw/day and 5% of EPG. The biological significance of greater A/G ratios, if any, is doubtful, especially since the effect was not seen in all groups of males or in females.

Conflict of interest statement

The authors are unaware of any conflicts of interest.

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