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Safety studies on epigallocatechin gallate (EGCG) preparations. Part 3: Teratogenicity and reproductive toxicity studies in rats ☆

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Abstract

Green tea and its principal active ingredient, epigallocatechin gallate (EGCG), have been demonstrated to have anticancer properties through interactions with multiple biochemical processes. Since these processes are often crucial in normal fetal development it is important to evaluate the potential effects of EGCG on the fetus. EGCG preparations of >91% purity were administered to pregnant rats during organogenesis and development in order to define the safety of Teavigo™, a high-concentration EGCG extract produced by the same novel method. In an initial preliminary study using subcutaneous and gavage routes, there was no evidence of any direct embryo–fetal toxicity, although some maternal toxicity was seen. In the main teratogenicity study, feeding pregnant rats diets supplemented at 1400, 4200 or 14,000 ppm during organogenesis was non-toxic to dams or fetuses. A two-generation study in rats fed 1200, 3600 or 12,000 ppm EGCG preparation showed no adverse effects on reproduction or fertility. The highest dose reduced the growth rate of offspring, and there was a slight increase in pup loss. A growth effect among pups was also seen at 3600 ppm, but in the second generation only. The lowest dose was considered the overall no-observed adverse effect level (NOAEL). As dams consumed twice the amount of feed during the crucial lactation period, the NOAEL was equivalent to 200 mg/kg/day EGCG preparation.
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1. Introduction

Green tea made from *Camellia sinensis* leaves has a long and safe history of use and studies have suggested that regular consumption may reduce the risk for cancer and promote cardiac health (for reviews, see Higdon and Frei, 2003; Ioannides and Yoxall, 2003). Research into these effects has shown that epigallocatechin gallate (EGCG), a principal component of green tea, has measurable anticancer properties and likely acts through multiple biochemical pathways (Bode and Dong, 2004; Hou et al., 2004). EGCG has been demonstrated to promote apoptosis in cultured cancer cells, inhibit vascular endothelial growth factor activity, reduce angiogenesis, and inhibit neoplastic transformation. Recently, EGCG was also reported to inhibit chicken and bovine liver dihydrofolate reductase activity and results from cell culture experiments suggested this was a mechanism by which EGCG inhibited cancer cell growth (Navarro-Peran et al., 2005). These biochemical pathways and events are not unique to cancerous cells but also hold important biological functions in normal physiological conditions. Under certain circumstances, such as organogenesis, their regulation is crucial for normal development of the fetus. Although green tea

Abbreviations: AUC, area under the curve; C_{max}, maximum concentration; EGCG, epigallocatechin gallate; NOAEL, no-observed adverse effect level.

☆ Portions of the two-generation study have previously been reported in abstract form at the 2005 Society of Toxicology Annual Conference.

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consumption is generally not associated with adverse outcomes in birth, it is important to ensure that the safety of green tea-derived compounds for ingestion is fully investigated, especially in an age where such food factors are being purified and consumed at levels which may be greater than would occur from consuming the food itself.

A unique patented process for purifying EGCG from a hot water extract of *C. sinensis* leaves has been developed by DSM Nutritional Products Ltd, to produce Teavigo™, which contains greater than 90% EGCG. In prior reports we confirmed that EGCG isolated using this process was non-genotoxic based on data from both in vitro and in vivo assay systems (Isbrucker et al., in press-a). The acute, short-term (90-day), and dermal safety of this EGCG product has also been investigated (Isbrucker et al., in press-a). Here we report on the safety of EGCG exposure during organogenesis and development in the rat. These results are further confirmed in a multi-generation study in the rat, in which other aspects of reproductive function were also investigated.

2. Methods

2.1. Materials

EGCG was prepared from hot water extracts of *C. sinensis* leaves as described previously (Isbrucker et al., in press-a). Briefly, the initial extract was further enriched for catechins by a fractionation with ethylacetate, followed by chromatographic separation of EGCG in ethanol/water. The resultant product was spray-dried prior to use. This novel purification method is the basis for the production of Teavigo™, a preparation from *C. sinensis* leaves comprising greater than 90% EGCG. Although different EGCG preparations were used in this study, all had EGCG concentrations of at least 91%, and isolates were kept consistent within each assay. Water was present in all EGCG preparations, but comprised less than 4% of the final product. In addition, epicatechin gallate (⩽3.01%), gallocatechin gallate (⩽0.12%) and other, unidentified, catechins (⩽0.54%) were also detected, but their combined totals were below 3.7% in all EGCG preparations. Residual solvents from the extraction process (mainly ethanol, isopropanol and methanol) were detected in some preparations but remained under 30 ppm. Caffeine was also detected in some batches, but at less than 0.01% in all preparations. Although other impurities were detected (⩽1.2%), they were not identified. These impurities would occur naturally in green tea and, at the concentrations detected, are not expected to influence the outcome of the assays.

2.2. Animals

Wistar (SPF) rats were purchased from Biological Research Laboratories (Fuellinsdorf, Switzerland) and Sprague–Dawley rats were purchased from Iffa Credo (l'Arbresle, France). All rats were acclimatized for a minimum of 1 week prior to entering the experiments, and housed at 22 ± 2 °C with 12 h light/dark cycles. Animals were provided with standard rat or rodent diet and fresh drinking water ad libitum. EGCG-supplemented diets replaced the standard feed where indicated. These animal studies were conducted under the respective national ethical guidelines of the countries in which they were conducted.

2.3. Preliminary teratogenicity study

An initial teratogenicity study was conducted in Wistar (SPF) rats but was not in accord with standardized industry guidelines for such assays (Schmitt and Aebischer, 1997). Because EGCG is poorly absorbed following its oral administration and subject to rapid metabolism (Unno and Takeo, 1995) a subcutaneous route for administration of the EGCG preparation was chosen to avoid a first-pass effect and to maximize plasma concentrations. Forty-two (42) male and female Wistar rats were placed in pairs overnight for mating. The presence of a vaginal plug on the following morning indicated that mating had occurred and was designated as day 0 of gestation. On day 6 of gestation the mated females were divided randomly among 5 test groups. Groups 1–4 consisted of 8 animals/group, which were administered EGCG (91.9% purity) subcutaneously at doses of 0 (control, group 1), 40 (group 2), 200 (group 3) or 500 (group 4) mg/kg/day. Group 5 comprised 10 mated females administered 1000 mg EGCG/kg/day by oral gavage. The EGCG preparation was diluted in sterile injectable water and administered at a volume of 5 ml/kg/day, whereas the oral dose was given at a volume of 10 ml/kg/day. Control animals received an equivalent amount of water. Dosing continued to day 17 of gestation. Due to observed toxicities, group 4 was terminated shortly after the first dose and group 3 was terminated after the third dose. Mortality, clinical symptoms, body weights, food consumption, maternal macroscopic observations and reproduction parameters at cesarean section on day 17 were recorded for all animals. The recovered fetuses were assessed for external, visceral and skeletal alterations. Two rats from groups 2 and 5 were bled on gestation day 16 at 0.5, 1, 2, 6, and 24 h after dosing for determination of plasma EGCG kinetics.

2.4. OECD guideline teratogenicity study

A guideline-conforming developmental toxicity study (Pfannkuch et al., 2002b) was conducted in Sprague–Dawley rats according to US Food and Drug Administration principles (FDA, 2000) and Organization for Economic Co-operation and Development (OECD) guideline (No. 414) for developmental toxicity studies (OECD guidelines are available at URL: http://www.oecd.org). Timed-mated female rats (25/group) were randomly assigned to one of four dose groups and fed standard powdered diet admixed with EGCG (91.2% purity) at concentrations of 0 (control), 1400, 4200, or 14,000 ppm. These concentrations were chosen to deliver nominal average doses of 100, 300 and 1000 mg EGCG/kg/day. Animals were fed the test diets from day 6 to cesarean section on day 20 of gestation. An additional 3 rats were included in each group for assessment of EGCG absorption on days 7 and 16 of gestation.

Clinical condition, and reaction to treatment were recorded twice daily. Body weights were recorded on days 0, 6, 11, 15, 18 and 20 of gestation. Food and water consumption were calculated for each period between weighings. All females were killed by carbon dioxide inhalation on assumed day 20 of gestation for examination of their uterine contents. At necropsy, the females were examined macroscopically for pathological changes in maternal organs. The ovaries and uterus were removed and examined, including the placentae. Pregnancy status, number of corpora lutea, number and distribution of live fetuses and embryonic/fetal deaths, individual pup weights and sex were recorded for each dam. Embryonic deaths were classified as either early (only placenta visible at termination) or late (both the placenta and embryonic tissue were visible at termination). The uterus of all dams were placed in ammonium sulfide solution to stain for any previously undetected implantation sites. Each fetus was examined for external defects and half of each litter was processed further to examine for visceral anomalies. The other fetuses in each litter were eviscerated, fetal carcasses fixed in ethanol and processed for skeletal examination. The skeletal examinations were performed following digestion of the soft tissues with aqueous potassium hydroxide and staining of the skeleton with Alizarin red. The remaining fetuses were preserved in Harrisson's fluid for fixed visceral examination by the modified Wilson–Barrow technique. These fixed-fetal examinations were performed under low-power magnification.

Fetal abnormalities were categorized as either malformations (structural defect which were rare in the control population and thought to be life threatening or of major physiological consequence), anomalies (minor abnormalities or defects which are relatively rare in the control population

and not considered to be of major physiological consequence), or variations (minor abnormalities, defect or alternative forms which are either common in the control population or are of no known physiological consequence). For the cesarean data, group mean values were calculated per litter. The data were checked for homogeneity of variance across groups using Bartlett's test. Homogenous data were then analyzed by analysis of variance (ANOVA) followed by Dunnett's test if the ANOVA was significant. Non-homogenous data, including the numbers of resorptions and all litter-based percentages, were analyzed by the non-parametric Kruskal–Wallis test followed by Dunn's test if the Kruskal–Wallis was significant. Selected incidence data were analyzed using a chi-square test for all groups followed by Fisher's two-tailed test with Bonferroni correction for each treated group versus the control if the chi-square was significant.

2.5. Two-generation reproductive toxicity study

A two-generation reproductive toxicity study was conducted in Sprague–Dawley rats according to US FDA guidelines for reproduction studies (FDA, 2000; Pfannkuch et al., 2002a). Rats, at 6-weeks of age, were randomly assigned to one of four treatment groups (30/sex/group) and fed diets containing 0, 1200, 3600 or 12,000 ppm EGCG (91.2% purity). These concentrations were estimated to deliver nominal doses of 0, 100, 300 and 1000 mg EGCG/kg/day, respectively. After 10 weeks treatment, all rats were paired, one male to one female within a group, for mating. The rats were maintained on their designated diets during mating, gestation and through to necropsy after weaning. Pups from each litter (F_1 generation) were randomly culled to 25/sex/group and continued on the test diets at the same dose. These F_1 rats were reared for 8 weeks, mated and the females were allowed to rear their young (F_2 generation) to weaning.

Vaginal smears were taken daily from all F_0 and F_1 females for approximately 3 weeks to determine their estrous cycle. Mating was confirmed by the presence of sperm in the vaginal smear or by vaginal plug. Fertility and reproductive performance of the F_0 and F_1 generations were assessed by mating performances, duration of gestation, parturition and viability, growth and development of offspring.

Daily observation of physical appearance, behavior and reaction to treatment were recorded. Body weight and food consumption were monitored during the premating, gestation and lactation periods. All animals dying during the study were necropsied. Surviving F_0 and F_1 animals were killed by carbon dioxide inhalation followed by exsanguination and submitted to a macroscopic examination. Abnormal organs or tissues were sampled for possible histopathological examination. For both F_0 and F_1 adult animals, the following organs were weighed: adrenal glands, brain, coagulating glands with seminal vesicles, epididymides, kidneys, liver, ovaries, pituitary, prostate (dorsolateral), spleen, testes, uterus (with cervix). All organs and tissues sampled were fixed and preserved in 10% neutral formalin, except the testes and epididymides which were fixed in Bouin's solution. Organ and tissue sections were stained with hematoxylin and eosin, and testes were also stained by the periodic acid Schiff's reaction. Histological examination was made of these organs from randomly selected parental animals (10/sex/group) from the control and the high dose groups of both F_0 and F_1 generation. A histopathological examination was performed on all gross lesions, except where the diagnosis was judged unnecessary for the outcome of the study. A similar examination of the reproductive organs was performed for all animals suspected of having reduced fertility.

Examination of the testes was conducted in order to identify gross lesions or other effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen. Histopathological evaluation of the intertubular cell compartment of the testes included an assessment of the Leydig cells, the blood vessels and other cell types. The general appearance of the seminiferous tubules and lumen was noted. The epididymis was examined by longitudinal section to include the caput, corpus and cauda in order to identify any lesions including sperm granulomas, leukocytic infiltration, aberrant cell types within the lumen or the absence of clear cells in the caudal epithelium. Sperm counts and motility were determined from all adult F_0 and F_1 males and assessed using automated equipment (Hamilton Thorne Biosciences, Inc., Beverly, MA). The left cauda epididymis was used for the assessment of sperm motility and the sperm count was derived from the left testis following removal of the tunica albuginea. For each male, 200 epididymal sperm were also examined for morphology changes.

Blood sampling for proof of absorption of EGCG was performed at terminal necropsy from F_1 adults and their F_2 weanlings. Blood samples were collected from five F_1 adults/sex/group and their F_2 weanlings to derive their average plasma EGCG concentration per dose group. For pups, the blood was pooled from two male and two female pups per litter and averaged from five litters per dose group.

Body weights, body weight changes, organ weights, maternal food consumption and cesarian section data were tested for homogeneity of variance using Bartlett's test. Homogenous data were analyzed using analysis of variance (ANOVA), while non-homogenous data were analyzed using non-parametric tests such as Kruskal–Wallis test and Dunn's test. Organ weights were also analyzed in terms of percentage of total body weight. The number of resorptions, dead fetuses and all litter-based percentages were analyzed by non-parametric methods. Litter incidence were analyzed using a chi-square test for all groups followed by Fisher's two-tailed test with Bonferroni correction for each treatment group versus the control. For the pre-mating estrus cycle data, the group mean cycle length was calculated as the arithmetic mean of the individual mean values. Cycle irregularity was noted in the form of the standard error of the mean cycle length.

3. Results

3.1. Preliminary teratogenicity study

Rat dams showed a poor tolerance to the subcutaneous injection of EGCG at doses of 200 and 500 mg/kg/day. Clear signs of toxicity (poor general condition, piloerection, crouched posturing) were noted after the administration at these doses, and six of eight animals dosed at 500 mg/kg/day were found dead after the first administration. All rats from the mid and high dose groups were moribund and were sacrificed before the scheduled date. Large areas of necrotic tissue were observed in the injection region of these animals. Clay-colored livers were noted in five animals of the 200 mg/kg/day group, and histology revealed severe liver cell necrosis and renal tubular degeneration. Plasma EGCG concentration and fetal examinations were not determined from animals in these dose groups.

Pregnant rats dosed subcutaneously with 40 mg/kg/day EGCG showed a reduction in food consumption accompanied by a marginal reduction in body weight gain. No such adverse effects were noted in dams given 1000 mg/kg/day EGCG by gavage. There was no indication of adverse effects of treatment on the developing fetus in either of these dose groups. Numbers of corpora lutea, implantations, live fetuses and number of resorbed fetuses in treated animals were comparable to controls (Table 1). Fetal body weights, sex ratio, and physical or skeletal development were not affected by the maternal subcutaneous treatment with 40 mg EGCG/kg/day or oral dosing with 1000 mg/kg/day. Although some fetuses showed abnormal or variant ureters and/or blood vessels, there was no clear pattern of occurrence and the observed deviations were also seen in control animals. Therefore, these abnormalities

Table 1
Pregnancy and embryo–fetal data in Wistar rats administered EGCG subcutaneously from gestation days 6 to 20 (preliminary study)

	Control	Dose EGCG	
		40 mg/kg/day, s.c.	1000 mg/kg/day, p.o.
Dams entering study	8	8	10
Litters	6	6	7
Corpora lutea/animal	13.8 ± 1.6	14.3 ± 1.9	14.1 ± 2.0
Implant sites/animal	9.9 ± 5.6	14.0 ± 1.9	11.6 ± 4.9
Total resorptions/animal	1.0 ± 2.1	0.8 ± 0.8	1.3 ± 1.6
Live fetuses			
Total	69	79	72
Male	38	42	34
Female	31	37	38
No. per animal	8.6 ± 5.6	13.2 ± 2.0	10.3 ± 4.3
Mean fetal body weight (g):			
Litter	4.8 ± 0.2	4.6 ± 0.4	5.0 ± 0.8
Male	4.9 ± 0.2	4.7 ± 0.4	5.1 ± 0.8
Female	4.6 ± 0.2	4.5 ± 0.3	4.6 ± 0.2

s.c. = subcutaneous; p.o. = per os (oral gavage).

were not considered to be compound-related. Plasma EGCG concentrations were slightly higher following the subcutaneous administration of 40 mg EGCG/kg/day (C_{max} = 191 μg/ml; $AUC_{0-24\,h}$ = 2670 μg h/ml) than after the oral administration with 1000 mg/kg/day (C_{max} = 139 μg/ml; $AUC_{0-24\,h}$ = 1840 μg h/ml).

3.2. OECD guideline teratogenicity study

Feeding pregnant rats a diet containing EGCG at levels of 1400, 4200 or 14,000 ppm resulted in the administration of EGCG at average doses of 111, 337 or 1079 mg/kg/day, respectively. Mean free-EGCG plasma concentrations showed a nearly linear dose relationship (Fig. 1), with higher levels after 10 days of dosing.

All rats survived to the end of the study without any mortalities and no treatment-related clinical signs. There was a slight, but statistically significant, transient reduction in mean food consumption (data not shown) in the high dose group as compared with controls during the first 5 days of treatment (days 6–11 of gestation). Although this reduction in food consumption was considered to be an effect of treatment, it was not considered to constitute an adverse effect as there was no associated reduction in body weight gain among these animals. Food consumption in the low and mid dose groups were unaffected by treatment. There was a slight increase in the mean water consumption in the 4200 and 14,000 ppm groups during days 11–20 of gestation when compared to controls, but these did not reach statistical significance and there was no obvious evidence of a dose relationship.

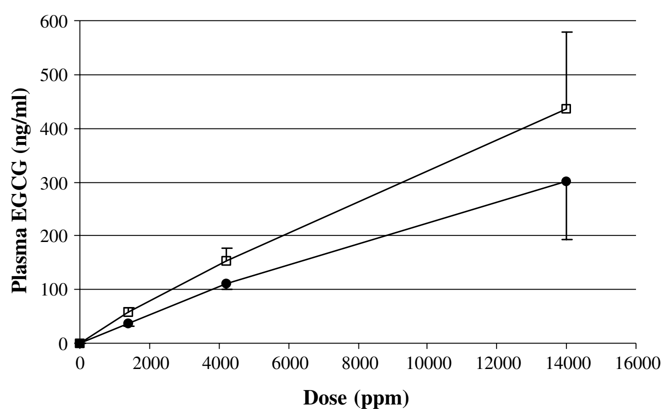

Fig. 1. Free-EGCG plasma concentrations of pregnant Sprague–Dawley rats 1 day (closed circles) and 10 days (open squares) after initiation of feeding with EGCG-supplemented diets. Each point represents the average from 3 animals; error bars indicate standard deviation.

No treatment-related macroscopic findings were noted in any of the dams at the terminal necropsy examination. Although gravid uterine weight varied with litter size, this was not related to the treatment regimen. No adverse effects of EGCG administration were apparent on embryo–fetal survival, postimplantation losses or mean live litter size (Table 2). The mean fetal weights and sex ratios were comparable among all groups.

Only one fetus with malformations (absent lower jaw and caudal displacement of the ears) was observed and occurred in a fetus from the control group. No other external, visceral, or skeletal malformations were noted that could be attributed to the treatment. There was a higher incidence of fetuses with renal pelvic dilatation (Table 2) in the 4200 ppm group ($n = 13$) compared with the control ($n = 2$); however, in the absence of a similar effect in the 14,000 ppm group ($n = 1$) this was considered to be incidental and not treatment-related. The incidences of other, less severe, visceral changes observed among all four groups included convoluted or slightly dilated ureters, and slightly dilated renal pelvis; however there was no difference in their frequency or occurrence between any dose group and controls. There was one incidence of a hemorrhage within the cerebellum of a pup from the mid dose group. The skeletal anomalies and variations observed were unaffected by EGCG treatment, as there was no significant difference in

Table 2
Pregnancy and embryo–fetal data in Sprague–Dawley rats fed EGCG in their diet from gestation days 6 to 20

	Control 0 ppm	Low dose 1400 ppm	Mid dose 4200 ppm	High dose 14,000 ppm
Dams entering study	25	25	25	24[a]
Litters	25	25	23[b]	23[b]
Net body weight gain (g; mean ± s.d.)	143.5 ± 27.3	151.0 ± 20.7	149.7 ± 19.8	138.3 ± 25.9
Gravid uterine weight (g; mean ± s.d.)	67.9 ± 26.4	77.7 ± 18.4	76.4 ± 17.6	71.9 ± 18.4
Corpora lutea	364	377	346	356
Preimplantation loss	51	45	38	65
Postimplantation loss	38	19	18	19
Live fetuses				
Total	275	313	290	272
Male	137	155	125	125
Female	138	158	165	147
No. per animal (mean ± s.d.)	11.0 ± 4.5	12.5 ± 3.0	12.6 ± 2.8	11.8 ± 3.1
Mean fetal body weight (g; mean ± s.d.)				
Total	4.0 ± 0.5	4.1 ± 0.4	4.0 ± 0.4	4.0 ± 0.3
Male	4.2 ± 0.5	4.2 ± 0.4	4.1 ± 0.4	4.2 ± 0.2
Female	3.9 ± 0.4	3.9 ± 0.4	3.9 ± 0.4	3.9 ± 0.3
Dilated renal pelvis (% fetal incidence)	1.6	2.0	9.2	0.8

[a] One animal was removed from the study due to mistimed pregnancy.
[b] Two rats from group 3 and one from group 4 had false pregnancies.

incidence between dosed animals and controls. These skeletal observations were predominantly incomplete, partial, bipartite or advanced ossifications of the skull, cranial structures, paws, sternebrae and vertebrae.

3.3. Two-generation reproductive toxicity study

Calculations based on food consumption showed that the achieved intakes during the pre-mating period were close to the nominal doses of 100, 300 and 1000 mg EGCG/kg/day, and slightly higher for the F_1 generation (Table 3). These differences were likely due to the age at which the F_0 generation started on the diet. The achieved intakes during the lactation phase for each generation were at least double the target doses due to the marked increase in food consumption by the nursing dams. There was no significant effect of treatment on water consumption in any generation. Plasma EGCG levels in male F_1 rats at termination were lower than for corresponding F_1 females in all dose groups (Table 4), whereas F_2 pups had high plasma concentrations on day 21 postpartum.

Mean body weights of the F_0 male (Fig. 2) and female (Fig. 3) rats were slightly reduced in the 12,000 ppm EGCG group. This occurred mainly during the first few weeks of treatment and was accompanied by a transient reduction in food consumption for the males only in week 1. Total body weight gain for F_0 male and female rats was not significantly different between treated and control groups. Mean body weight gain of the high dose F_1 males and females was reduced after birth and their absolute mean body weights were significantly reduced throughout the course of the study (Figs. 2 and 3). There was no significant decrease in the mean body weights of the low dose or mid dose male or female rats of either generation as compared to controls (results not shown).

The slight reduction in weight among the high dose F_0 generation rats was accompanied by a significant reduction in spleen, ovary and uterine weights of the female animals

Table 3
Achieved mean EGCG consumption (mg/kg/day) among F_0 and F_1 Sprague–Dawley rats fed diets supplemented with EGCG at concentrations of 1200, 3600, and 12,000 ppm

	Male			Female		
	1200 ppm	3600 ppm	12,000 ppm	1200 ppm	3600 ppm	12,000 ppm
F_0 Generation						
Weeks 1–10	102.0	310.0	1086.5	107.8	322.1	1090.0
Days G0–G20[a]	–	–	–	95.0	283.4	955.1
Days L1–L21	–	–	–	206.9	648.5	2163.4
F_1 Generation						
Weeks 1–8	163.5	495.2	1807.5	163.0	496.4	1797.9
Days G0–G20	–	–	–	107.4	318.4	1137.2
Days L1–L21	–	–	–	233.7	704.1	2672.0

[a] G0–G20 refers to the gestation period; L1–L21 refers to the lactating (nursing) period.

Table 4
Free-EGCG plasma concentrations (ng/ml) in F_1 male (10 weeks) and female rats (15 weeks) and their pups (21 days of age) following the administration of EGCG-supplemented diets

		Control 0 ppm	Low dose 1200 ppm	Mid dose 3600 ppm	High dose 12,000 ppm
F_1 Male	Mean	<5 ng/ml[a]	<5	30.10	43.51
	Range	–	–	18.22–60.11	33.60–54.92
F_1 Female	Mean	<5	12.22	66.42	173.3
	Range	–	<5–19.02	19.41–180.6	78.04–336.7
F_2 pups[b]	Mean	<5	30.03	253.5	306.6
	Range	–	10.12–51.46	199.8–352.0	126.8–478.1

[a] 5 ng/ml was the limit of detection.
[b] Blood samples were pooled from 2 male and 2 female pups per litter and averaged from five litters per dose.

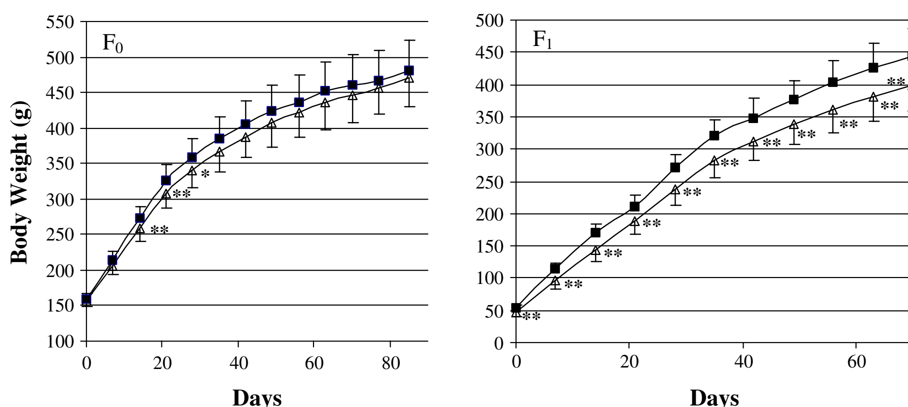

Fig. 2. Growth curves for F_0 and F_1 generation male rats. Animals were untreated (solid squares) or administered diets supplemented with 12,000 ppm EGCG (open triangles). F_1 data commences on the first day postweaning. For simplification, the data from low and mid dose animals was omitted, but were not significantly different from the untreated controls. Each point represents the average from 30 (F_0) or 25 (F_1) animals; error bars indicate standard deviation. Significant difference from control (ANOVA/Dunnett test) $^{*}p < 0.05$; $^{**}p < 0.01$.

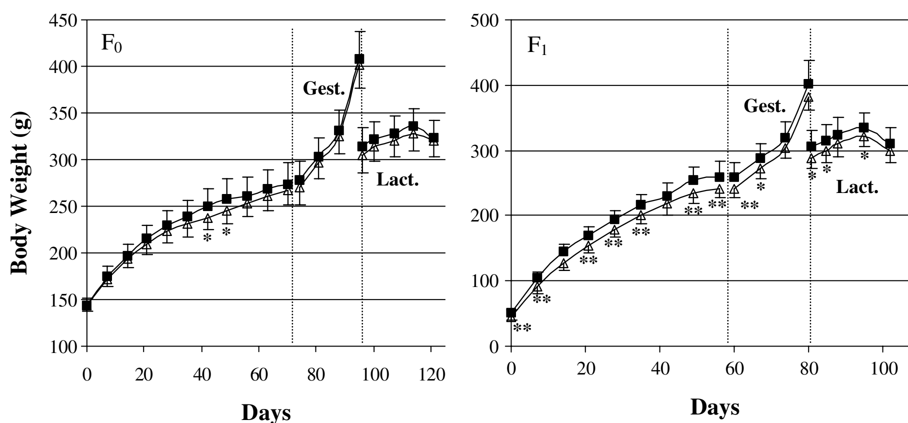

Fig. 3. Growth curves for F_0 and F_1 generation female rats. Animals were untreated (solid squares) or administered diets supplemented with 12,000 ppm EGCG (open triangles). F_1 data commences on the first day postweaning. For simplification, the data from low and mid dose rats was omitted, but were not significantly different from the untreated controls. Each point represents the average from 26 to 30 (F_0) or 22 to 25 (F_1) animals; error bars indicate standard deviation. Gest. = gestation period, Lact. = lactation period. Significant difference from control (ANOVA/Dunnett test) $^{*}p < 0.05$; $^{**}p < 0.01$.

(Table 5). Male F_0 rats had no weight reduction of any organ or tissue measured among any treatment group. Both sexes of the F_1 generation fed 12,000 ppm EGCG showed significant reductions in absolute kidney and liver weight (Table 5), but in terms of percent body weight these organs were not significantly affected (data not shown). The males from this group had reduced spleen and prostate weights, but in females there was no significant effect on spleen, ovary or uterine weights as seen in the F_0 generation. F_2 female pups in the low dose group showed a slight increase in brain weight, but because this was not dose-dependent it was considered incidental and not related to

Table 5
Absolute organ weights of male and female of Sprague–Dawley rats administered EGCG-supplemented diets over two successive generations

		Control 0 ppm	Low dose 1200 ppm	Mid dose 3600 ppm	High dose 12,000 ppm
F_0 Generation					
Brain (g)	M[a]	2.23 ± 0.09	2.27 ± 0.12	2.26 ± 0.12	2.24 ± 0.10
	F	1.99 ± 0.11	2.00 ± 0.10	2.01 ± 0.13	1.99 ± 0.10
	PM	1.54 ± 0.07	1.54 ± 0.07	1.51 ± 0.08	$1.49 \pm 0.08^{**}$
	PF	1.49 ± 0.06	1.49 ± 0.06	1.48 ± 0.07	$1.45 \pm 0.06^{**}$
Kidneys (g)	M	3.38 ± 0.39	3.56 ± 0.39	3.50 ± 0.41	3.35 ± 0.28
	F	2.18 ± 0.20	2.22 ± 0.22	2.14 ± 0.21	2.11 ± 0.21
Liver (g)	M	15.68 ± 2.78	17.27 ± 2.75	16.55 ± 2.47	14.53 ± 1.75
	F	12.14 ± 1.88	12.33 ± 1.46	12.20 ± 1.57	12.11 ± 1.39
Spleen (g)	M	0.74 ± 0.11	0.79 ± 0.10	0.80 ± 0.11	0.77 ± 0.13
	F	0.60 ± 0.08	0.61 ± 0.09	0.60 ± 0.08	$0.54 \pm 0.07^{**}$
	PM	0.25 ± 0.04	0.25 ± 0.05	0.24 ± 0.04	$0.18 \pm 0.05^{**}$
	PF	0.25 ± 0.05	0.24 ± 0.05	0.23 ± 0.04	$0.18 \pm 0.05^{**}$
Thymus	PM	0.27 ± 0.05	0.27 ± 0.04	0.26 ± 0.05	$0.22 \pm 0.04^{**}$
	PF	0.29 ± 0.04	0.28 ± 0.06	0.27 ± 0.06	$0.23 \pm 0.04^{**}$
Right testis (g)	M	1.86 ± 0.16	1.87 ± 0.15	1.85 ± 0.12	1.89 ± 0.15
Prostate gland (g)	M	1.75 ± 0.28	1.80 ± 0.32	1.68 ± 0.42	1.79 ± 0.33
Ovaries (g)	F	0.13 ± 0.02	0.13 ± 0.03	0.13 ± 0.02	$0.11 \pm 0.02^{**}$
Uterus (mg)	F	0.56 ± 0.17	0.53 ± 0.15	0.54 ± 0.22	$0.44 \pm 0.18^{**}$
F_1 Generation					
Brain (g)	M	2.18 ± 0.10	2.20 ± 0.08	2.18 ± 0.10	2.17 ± 0.10
	F	2.00 ± 0.10	2.01 ± 0.16	1.99 ± 0.08	2.02 ± 0.09
	PM	1.51 ± 0.08	1.54 ± 0.09	1.53 ± 0.08	1.49 ± 0.09
	PF	1.46 ± 0.07	$1.50 \pm 0.09^{*}$	1.48 ± 0.07	1.43 ± 0.09
Kidneys (g)	M	3.27 ± 0.35	3.26 ± 0.35	3.14 ± 0.39	$2.90 \pm 0.45^{**}$
	F	2.33 ± 0.19	2.28 ± 0.20	2.24 ± 0.20	$2.18 \pm 0.20^{*}$
Liver (g)	M	13.13 ± 1.61	12.85 ± 1.63	13.07 ± 1.64	$11.13 \pm 1.86^{**}$
	F	12.60 ± 1.72	11.98 ± 1.41	12.10 ± 1.35	$11.40 \pm 1.39^{*}$
Spleen (g)	M	0.76 ± 0.10	0.76 ± 0.09	0.78 ± 0.13	$0.68 \pm 0.12^{*}$
	F	0.58 ± 0.07	0.58 ± 0.12	0.59 ± 0.11	0.51 ± 0.08
	PM	0.27 ± 0.21	0.25 ± 0.06	0.21 ± 0.04	$0.18 \pm 0.05^{**}$
	PF	0.23 ± 0.06	0.25 ± 0.06	0.21 ± 0.05	$0.17 \pm 0.05^{**}$
Thymus	PM	0.27 ± 0.05	0.27 ± 0.06	0.26 ± 0.06	$0.21 \pm 0.04^{**}$
	PF	0.29 ± 0.06	$0.26 \pm 0.06^{*}$	$0.25 \pm 0.04^{**}$	$0.22 \pm 0.05^{**}$
Right testis (g)	M	1.89 ± 0.12	1.89 ± 0.09	$1.78 \pm 0.17^{*}$	1.82 ± 0.15
Prostate gland (g)	M	1.43 ± 0.26	1.46 ± 0.23	1.41 ± 0.20	$1.23 \pm 0.18^{*}$
Ovaries (g)	F	0.13 ± 0.02	0.14 ± 0.03	0.13 ± 0.02	0.12 ± 0.02
Uterus (mg)	F	0.52 ± 0.26	0.65 ± 0.24	0.58 ± 0.26	0.48 ± 0.20

Values are the mean ± standard deviation from 28 to 30 F_0 and 24 to 25 F_1 adults or pups from the F_0 ($n = 48$–58) and F_1 ($n = 42$–50) litters. Significant difference from control (Dunn's or Dunnett's tests) $^{*}p < 0.05$; $^{**}p < 0.01$.
[a] M = adult male; F = adult female; PM = male pup; PF = female pup. Pup organs weights were determined on day 21 of lactation.

EGCG exposure. There was also a slight, but significant, decrease in the mean thymus weights of the F_2 female pups in all dose groups. However, the individual thymus weights from all, but one, of the low dose F_2 female pups were within the normal biological range of control animals. In male pups this effect was only noticed at the highest dose. Because of the minimal severity and isolated nature of this finding at the lowest EGCG dose, it was not considered a toxicologically adverse effect.

Despite some reduced organ weights, macroscopic observation and histopathology did not reveal any treatment-related abnormalities. Although minimal to moderate vacuolation of the adrenal cortex (zona fasciculata) was observed in some male rats of both generations in all treatment groups, this change is known to occur spontaneously in this species, and the incidence was similar between the high dose and control groups. A single F_1 male of the 12,000 ppm group had marked sperm cell granuloma; however, this finding was considered incidental. There were no remarkable histopathology findings among female rats of either generation that could be related to treatment.

There was a slight delay in the sexual maturation for both sexes in the F_1 12,000 ppm dose group (Table 6), which was linked to their reduced growth rate. However, there was no evidence of an adverse effect of treatment in any group on reproductive performance, mating performance, or fertility of the two successive generations. Gonadal function, as assessed by sperm analysis, microscopic examination of the testis and epididymis, determination of the estrous cycle and microscopic examination of the ovaries was also similar between control and treatment groups of both generations (Table 7).

Table 6
Mating performance, fertility and time to sexual maturation of Sprague–Dawley rats administered EGCG-supplemented diets over two successive generations

	Control 0 ppm	Low dose 1200 ppm	Mid dose 3600 ppm	High dose 12,000 ppm
F_0 Generation				
No. of females				
Paired	30	30	30	30
Inseminated	30	30	29	30
Pregnant	28	29	27	29
Pre-coital interval-days	2.17 ± 1.67	1.93 ± 1.10	2.38 ± 1.52	3.37 ± 3.58
Copulation/fertility index (%)	100/93	100/97	97/93	100/97
F_1 Generation				
Time to sexual maturation[a] (days; mean ± s.d.)				
Males	43 ± 2	43 ± 2	43 ± 2	46 ± 2***
Females	32 ± 2	35 ± 2	36 ± 2	38 ± 2***
No. of females				
Paired	25	25	25	24
Inseminated	24	25	25	24
Pregnant	24	25	25	23
Pre-coital interval-days	2.61 ± 1.20	2.65 ± 1.30	2.56 ± 2.47	2.08 ± 1.06
Copulation/fertility index (%)	96/100	100/100	100/100	100/96

Significant difference from control ***$p < 0.001$.
[a] Sexual maturation as indicated by Balabano-preputial skinfold cleavage or vaginal opening.

Table 7
Quantitative evaluation of sperm and ovary parameters of Sprague–Dawley rats fed EGCG-supplemented diets over two successive generations

	Control 0 ppm	Low dose 1200 ppm	Mid dose 3600 ppm	High dose 12,000 ppm
F_0 Generation				
Males (*n*)	29	30	30	30
Sperm counts ($\times 10^6$)	203.7 ± 36.6	200.9 ± 40.0	209.4 ± 52.4	191.7 ± 38.1
Motile sperm (%)	78.1 ± 20.9	77.1 ± 19.3	82.2 ± 19.4	77.2 ± 24.1
Abnormal sperm (%)	4.8 ± 2.6	5.1 ± 1.9	3.9 ± 0.9	4.7 ± 2.9
Females (*n*)	12	–	–	11
Follicles				
Primordial	22.8 ± 14.2	n.r.	n.r.	20.2 ± 16.1
Growing	6.3 ± 2.7			5.5 ± 2.0
Antral	12.7 ± 4.3			12.9 ± 5.5
Corpora lutea	17.9 ± 5.1	n.r.	n.r.	15.3 ± 5.4
F_1 Generation				
Males (*n*)	25	25	25	25
Sperm counts ($\times 10^6$)	210.0 ± 71.6	199.4 ± 66.3	218.1 ± 37.8	210.8 ± 66.0
Motile sperm (%)	80.6 ± 15.6	82.3 ± 14.6	82.5 ± 8.3	78.0 ± 18.0
Abnormal sperm (%)	5.0 ± 2.6	4.6 ± 1.6	4.3 ± 1.0	5.7 ± 4.2
Females (*n*)	11	–	–	11
Follicles				
Primordial	22.0 ± 10.5	n.r.	n.r.	26.8 ± 13.4
Growing	8.8 ± 4.9			8.0 ± 2.6
Antral	15.1 ± 5.2			15.1 ± 4.7
Corpora lutea	20.6 ± 9.1	n.r.	n.r.	18.3 ± 5.3

n.r. = not recorded.

The mean live litter size at birth, the live birth index and pup viability through to day 4 postpartum were comparable among all treatment groups of both generations (Table 8). An increase in the postnatal mortality of the offspring from days 5 to 21 postpartum in the 3600 ppm groups of both generations was principally due to a single female in each case with a complete litter loss. Since there was no pup loss among the majority of remaining litters from this dose group in either generation, the incidences were not considered treatment-related. However, there was a significant increase of pup loss between days 4 and 21 postpartum among the high dose F_0 and F_1 animals (Table 8). A statistically significant decrease in the growth rates of the offspring from the high dose F_1 generation and a very slight effect on the growth of the offspring from the F_0 generation was noted. A few treatment-associated pup observations

Table 8
Pregnancy and litter data from two successive generations of Sprague–Dawley rats administered EGCG-supplemented diet

	Control 0 ppm	Low dose 1200 ppm	Mid dose 3600 ppm	High dose 12,000 ppm
F_0 Generation				
No. litters with liveborn	26[a]	28[a]	27	29
Pups/litter (mean ± s.d.)	12.9 ± 2.6	12.8 ± 3.4	13.4 ± 2.1	13.2 ± 2.8
Total live pups delivered	332	348	354	382
No. litters with stillborn	2	6	5	1
Total stillborn delivered	3	11	7	2
No. with all stillborn	0	0	0	0
Postnatal deaths[b]				
Days 0–4	6	3	3	6
Days 5–21	1	0	19**	13**
% Male pups	53.0	49.1	50.4	48.2
Implantation sites/litter (mean ± s.d.)	15.0 ± 2.0	14.4 ± 2.9	15.2 ± 2.2	14.9 ± 2.2
Duration of gestation (mean ± s.d.)	21.8 ± 0.4	21.9 ± 0.5	22.0 ± 0.3	21.7 ± 0.5
Pup weight (g; mean ± s.d.)				
Day 1	7.3 ± 0.6	7.2 ± 0.6	7.2 ± 0.5	7.2 ± 0.7
Day 7	16.1 ± 1.2	16.4 ± 1.7	15.6 ± 1.7	15.1 ± 1.5*
Day 14	30.7 ± 2.0	31.2 ± 2.8	30.3 ± 2.2	28.5 ± 2.1**
Day 21	52.7 ± 3.2	52.8 ± 4.8	51.1 ± 3.8	45.5 ± 4.2**
F_1 Generation				
No. litters with liveborn	24	25	25	23
Pups/litter (mean ± s.d.)	13.7 ± 3.4	13.7 ± 2.7	14.6 ± 1.5	13.7 ± 1.6
Total Live Pups Delivered	324	332	359	309
No. litters with stillborn	3	7	4	4
Total Stillborn Delivered	4	10	6	5
No. with all stillborn	0	0	0	0
Postnatal deaths[b]				
Days 0–4	4	5	2	13
Days 5–21	1	2	10*	27**
% Male pups	53.4	49.2	52.9	51.3
Implantation sites/litter (mean ± s.d.)	14.9 ± 3.6	15.0 ± 2.3	15.9 ± 1.9	14.7 ± 1.7
Duration of gestation (mean ± s.d.)	22.0 ± 0.4	22.0 ± 0.4	21.8 ± 0.4	21.7 ± 0.6
Pup weight (g; mean ± s.d.)				
Day 1	7.2 ± 0.4	7.4 ± 0.6	7.2 ± 0.4	7.0 ± 0.7
Day 7	16.1 ± 1.1	16.6 ± 1.6	15.5 ± 1.9	14.6 ± 2.3**
Day 14	32.0 ± 2.5	32.4 ± 2.8	30.9 ± 1.8	28.9 ± 3.1**
Day 21	51.3 ± 3.4	51.5 ± 4.6	48.0 ± 2.5*	44.0 ± 4.7**

Significant difference from control $^{*}p < 0.05$; $^{**}p < 0.01$.
[a] Excludes one pregnant female submitted to a caesarean section in error.
[b] Postnatal deaths do not include pups culled on day 4.

were noted in the high dose group, including a thin, or unkempt, appearance, cyanosis and weakness. Consistent with the reduced growth rate in this group, the brain, spleen and thymus weights of the offspring from each generation of high dose animals were lower than in the control group (Table 5). Despite these effects, there was no adverse influence of treatment on the pre-weaning physical or functional development of the offspring. Day of pinna unfolding, incisor eruption, eye opening, and auditory or pupil reflexes were comparable among all groups. Similarly, pup necropsy findings on day 4 postpartum or at weaning were unremarkable and among pups found dead the findings of autolysis and evidence of cannibalization were incidental in nature.

4. Discussion

Tea, but not caffeine, consumption has been associated with developmental neural tube defects when maternal exposure was high during the preconceptual period (Correa et al., 2000), but the type of tea – green or black – was not specified in this epidemiology study. Recently, EGCG was demonstrated to inhibit dihydrofolate reductase activity (Navarro-Peran et al., 2005), which could explain a mechanism by which tea is linked to increased incidences of anencephaly and spina bifida. However, such published data associating tea with birth defects is remarkably sparse, especially in light of the prominence and long history of tea consumption. Nonetheless, it is important that the safety of green tea extracts, which represent a concentration of the bio-active components from tea, be verified. Prior to the current study reported here, only one other related developmental study is known, and it was published in abstract form (Faqi et al., 2001). This study reported no effects when Polyphenon E (approximately 50% EGCG) was administered to rats by oral gavage during days 6–15 of gestation at doses up to 1000 mg/kg/day.

In a guideline-directed teratogenicity study reported here, we found no indication of teratogenic effect when EGCG was administered in the diets of rats during organogenesis at doses delivering up to a nominal 1000 mg EGCG/kg/day. The preliminary study in which EGCG was administered by subcutaneous injection provided supporting evidence of the absence of teratogenicity. Although this study reported a high incidence of mortality in pregnant rats administered 200 or 500 mg/kg/day EGCG subcutaneously, this was most likely due to local intolerance consequent to the low pH of the formulation (approximate pH 4) and the protein-precipitating action of EGCG, with both factors contributing to the observed necrotized tissue surrounding the injection region. Nonetheless, the results from the pilot study provide an indication that EGCG is non-teratogenic when plasma concentrations reach levels as high as 191 μg/ml. Although the subcutaneous route of administration represents an extreme situation to maximize plasma EGCG concentrations, it is unrealistic when applied to the human situation of oral consumption. Also, based on published kinetic data in humans it is unlikely that such high plasma EGCG concentrations would be attained following the oral dosing with Teavigo™. Ullmann et al. (2003) reported a maximum plasma concentration of 963 ng EGCG/ml following a single oral administration of 800 mg Teavigo™. This increased to 2800 ng/ml following 10 days dosing with 800 mg/day (Ullmann et al., 2004), a concentration 1/70th of that measured in the injected rats. This increased plasma concentration with continual exposure to EGCG was also noted in the oral dose teratology study. Pregnant rats administered EGCG in their diet had a small, but significant increase in plasma concentrations over the 10 day period suggesting an accumulation of EGCG. However, this data should be interpreted cautiously due to the possibility of physiological changes during pregnancy influencing the plasma levels, in addition to temporal fluctuations in consumption. An EGCG accumulation effect was previously reported in Sprague–Dawley rats administered green tea polyphenols in their drinking water for 14 days (Kim et al., 2000), but continued exposure for an additional 14 days decreased the accumulation returning the plasma EGCG levels to the initial values. This finding may explain the greater plasma EGCG concentrations found after 10 days feeding in the teratology study than after chronic feeing in the two-generation study, and suggests the possible induction of an adaptive response.

Following a two-generation study of EGCG consumption in rats, dams were found to have higher plasma EGCG levels at the end of the lactation period than did male rats of the same dose groups. This difference is consistent with the greater amount of food consumed by the female rats during lactation, when plasma EGCG concentrations were determined. Because EGCG was administered in the diet and not delivered as a bolus dose, the plasma concentrations in this study do not represent the C_{max} but do confirm compound exposure and indicate relative levels in the animals. Also, the dietary concentrations of EGCG were not adjusted for the increased food intake during lactation, and, therefore, the nursing dams consumed nearly twice as much EGCG as did the adult males. Although an elevation in plasma EGCG levels in nursing dams may partially reflect altered states of absorption, metabolism and/or elimination, the doubling in these plasma concentrations as compared to the male rats are most reasonably accounted for by the comparable increase in EGCG consumption. As pups consume a much greater quantity of food per body weight than do adults, their very high plasma EGCG levels at the end of the lactation period are not unexpected, especially in light of the greater EGCG doses ingested by the dams and that the pups had started eating solid diet at the time their plasma samples were obtained. Also, the elevated plasma levels may indicate that pups have either enhanced absorption rates for EGCG and/or do not eliminate EGCG as rapidly as adults. Rat milk EGCG levels were not measured in this study and the extent to which EGCG is secreted in milk might provide some clarification to the basis of these findings. Information on the secretion of EGCG in human breast milk is also absent from the medical literature.

This study represents the first known report evaluating the reproductive and developmental toxicity of EGCG and illustrates that this green tea-derived polyphenol is not teratogenic to rats. Although the highest dose tested in the two-generation study did delay sexual maturation, this effect was likely a reflection of the reduced weight gain among these animals. Subsequent reproductive performance in these same animals was not affected by this delay. Due to reduced growth rates of the offspring in the two-generation study, a no-observable adverse effect level (NOAEL) might be set at the target dose level of 100 mg/kg/day EGCG. However, during the crucial phase of lactation the EGCG intake by the dams was at least 200 mg/kg/day and this may be considered as a more appropriate NOAEL in rats at all life stages.

In 13-week toxicity studies in the rat and dog, 500 mg/kg/day has been defined as the NOAEL for EGCG as Teavigo™ (Isbrucker et al., in press-a,b). In the two-generation study reported here, the mid dose group, nominally equivalent to 300 mg/kg/day, was without effect on adult rats and even at the high dose, nominally equivalent to 1000 mg/kg/day, the findings in adult rats primarily involved growth effects. Defining the NOAEL for the EGCG preparation in rats for all life stages, inclusive of the lactation period, as 200 mg/kg/day based on the two-generation study may be seen as conservative when the effect attributed to treatment in the mid dose group was a reduction in weight gain in the F1 generation offspring only.

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