Original Article

Multigeneration Reproductive Study of Hydroxyprogesterone Caproate (HPC) in the Rat: Laboratory Results and Clinical Significance

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To demonstrate reproductive safety of a new commercial product for reducing the risk of preterm birth, HPC (17α-hydroxyprogesterone caproate, Makena; manufactured by Baxter Pharmaceutical Solutions, Bloomington IN for Ther-Rx Corporation, St. Louis, MO) was administered intramuscularly in Charles River Laboratory CD strain rats. HPC was given at intervals equal to the half-life measured in rats during three phases of embryo-fetal development: during the period of ovarian development (RP1, days 8, 14, and 20), following implantation of the embryo (TP, days 6, 12, and 18), and, corresponding to the start of the drug in week 16 or later in humans, after gonadal formation including differentiation of the testes (RP2, day 17). Dose levels up to 30× the human therapeutic doses were utilized including 0 (vehicle), 5, 25, and 150 mg/kg (volume 0.6 ml/kg). Four groups of 25 time-mated rats each were used for each phase. In addition, equal numbers of naïve (untreated) rats of opposite gender were used for F_1 breeding studies. HPC did not produce any consistent test-article–related findings in the treated F_0 dams, their developing F_1 fetuses and did not affect the ability of the latter to produce a viable F_2 generation. The F_1 offspring did not evidence any adverse effects during their behavioral, sensory, and developmental assessments, including teratogenicity. Based on the cumulative data obtained from rats treated over two generations and during development in this study, the No-observable-effect-level (NOEL) was established as 150 mg/kg. This study supports the absence of reproductive toxicity with HPC in published studies in animal models and in human clinical trials. *Birth Defects Res (Part B)* 95:160–174, 2012. © 2012 Wiley Periodicals, Inc.

Key words: ***hydroxyprogesterone caproate (HPC); product for preterm birth; two-generation reproduction study; developmental toxicity***

INTRODUCTION

Progestational agents (progestins) are useful therapeutic agents, especially in matters of reproductive function. They act by binding to a cytosoluble/nuclear receptor (progesterone receptor) and, in some tissues such as endometrium, by down-regulating tissue estrogen receptors and stimulating pathways of estrogen metabolism (Schindler, 1996). The progestin 17α-hydroxyprogesterone caproate (HPC), when administered weekly by intramuscular injection, has been shown to reduce the risk of preterm birth before 37 weeks in humans (Meis et al., 2003). The study results from that publication included a statistically nonsignificant increased rate of miscarriages and stillbirths. Nonclinical animal studies in mice, rats, rabbits, guinea pigs, horses, and nonhuman primates conducted with HPC demonstrated developmental or reproductive toxicity in only one species. Resorption and/or abortion occurred in rhesus monkeys at equivalent human doses (10 mg/kg) according to several reports (Courtney and Valerio, 1968; Hendrickx et al., 1987), but another subhuman primate, cynomolgus monkey, was unaffected when given the same dosage in the same gestation interval (Hendrickx et al., 1987). A small and limited study, in which the drug was given intraperitoneally rather than intramuscularly, was performed in Wistar rats and suggested the possibility of dose-related decreases in sperm counts, sperm motility, and testosterone levels with dose-related increases in follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Pushpalatha et al., 2004). The significance of these results is not clear however because very limited information was available in the publication and studies performed in men with benign prostatic hypertrophy treated

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with HPC showed no changes in LH and FSH over time (Meiraz et al., 1977). A review of the subject concluded that absence of complete information from rat toxicology studies and human pharmacokinetic studies made it difficult to ascribe the animal and human findings to a single root cause. This was due in part to the potential for interspecies metabolic differences and in part to the use of the drug in women at higher risk of both preterm birth and adverse pregnancy outcomes (Christian et al., 2007). Therefore, a study was conducted to determine if these isolated reports of toxicity would be seen in a prospectively designed well-controlled laboratory study. The results of that multigenerational reproductive study in the rat under two reproductive phase scenarios plus a developmental toxicity phase are presented in this manuscript.

MATERIALS AND METHODS

Test Article and Vehicle Preparation

The test article, 17α-HPC injection 250 mg/ml (Makena) manufactured by the commercial supplier (Baxter Pharmaceutical Solutions, Bloomington IN), was used without adjustment for purity (99.81%). The dosing formulation at nominal concentration of 250 mg/ml was administered neat (undiluted) to animals receiving the highest dose. Low and mid dose test article formulations were prepared on each day of use at nominal concentrations of 8.3 and 41.6 mg/ml, respectively, by dilution with vehicle. On a per kilogram basis, the low, mid, and high doses represent approximately 1×, 5×, and 30× the human dose. The 150 mg/kg dose represents 5× the human dose on a per square meter basis. A preliminary pharmacokinetic study showed that at the time of peak plasma concentration (24 hr) there was also approximately a 30-fold difference in the plasma concentration of HPC between a 5 mg/kg and 150 mg/kg dose. Prepared formulations were stored at room temperature and were stirred using a magnetic stir bar and stir plate. The contents of the vial were mixed and filtered through a Millipore Millex GV 0.22 μm PVDF Durapore syringe filter (Millipore, Billerica, MA) into a sterile amber glass serum bottle. Fresh vehicle (placebo), comprised of Benzyl benzoate, USP (46% v/v), Benzyl alcohol, NF (2% v/v), and Castor oil, USP (Q.S. to volume) (Baxter Pharmaceutical Solutions) was used each day of administration. Vehicle was filtered in an identical manner to the test article. The possibility of test article loss through the sterile filtration process was not considered to have been significant, as there were no consistent patterns of loss observed in the recovery rates reported for the preparations analyzed.

Analysis of Dosing Formulations

Homogeneity and stability analyses were performed in previous studies. The results showed test article homogeneity of the vehicle at the diluted low-dose concentration of 8.3 mg/ml (nominal concentration) and 24-hr stability at ambient conditions for the low (8.3 mg/ml) and high (250 mg/ml) dose concentrations. Concentration analyses were performed from duplicate or triplicate samples (0.5 ml each) from the preparations at each concentration used including the vehicle. Samples were collected from the middle of the container, using a sterile needle and syringe, and placed into sterile amber glass serum bottles for later analyses. The samples were analyzed using a HPLC system with UV detection.

Animals

Sprague Dawley (SD) (CD [CRL:CD (SD)]) strain rats were obtained from Charles River Laboratory (CRL) for all study phases. The animals were acclimated from the time of arrival to the time of dosing on gestational day (GD) 8 (reproductive phase 1 [RP1]), GD 6 (teratology phase [TP]), and GD 17 (reproductive phase 2 [RP2]). During this time, all rats were observed daily for any clinical signs of disease and given a detailed clinical examination prior to study selection for dosing. All animals were observed for mortality, morbidity, injury, and availability of food and water twice daily throughout the duration of the study. Animals assigned to each study phase had body weight within ±20% of the mean body weight. The vehicle and test article were administered in all phases using an appropriately sized plastic disposable syringe attached to a 26-gauge hypodermic needle. Each dose was split approximately equally between the right and left hindlimb muscle, resulting in an equivalent dose volume of about 0.3 ml/kg per site. All animals were individually housed in suspended, stainless steel, wire-mesh–type cages, except for the reproductive phases, during pairing, near parturition, and during lactation, in an environmentally controlled room. During pairing of the reproductive phases each F_1 female was housed in the cage of a naïve male at a ratio of 1:1. On approximately GD 20, reproductive phase F_0 females were individually housed in plastic solid bottom cages containing wood chip bedding. Females were housed in these solid bottom cages with the pups during the 21-day lactation period. Pups were housed as littermates by sex at the time of weaning (lactation day [LD]) 21) until selection for the next generation or euthanasia (post natal day [PND] 28). Fluorescent lighting was provided for approximately 12 hr/day. Temperature and humidity were continuously monitored and recorded. The protocol-designated ranges were 64 to 79° Fahrenheit (F) and 30 to 70% relative humidity, respectively. Meal Lab Diet (Certified Rodent Diet #5002, PMI Nutrition International, Richmond, Indiana) was available ad libitum. Tap water was available ad libitum via an automatic watering system or water bottles affixed to the cage and was monitored for specified contaminants at periodic intervals. Dams were checked twice daily for parturition. Based on the date of parturition (LD 0) and the day of positive evidence of copulation (Gestation Day 0), gestational length was calculated for each individual female.

Study Phases

Reproductive phase 1 (RP1). A total of 116 time-mated female rats were received for this phase from CRL. The females were approximately 8 to 10 weeks of age at arrival (GD 0). A total of 110 males and 110 females were received from CRL as naïve animals to be mated with the in utero-treated F_1 animals. The naïve animals were acclimated for 1 week before selection for pairing. Animals were randomly assigned to study at arrival using a standard, by weight, block randomization procedure based on GD 0 body weights. A total of 100 female rats

(weighing 172–225 gm, at randomization) were assigned to the control and treatment groups of 25 females each at dose levels of 0, 5, 25, and 150 mg/kg given as a dosing volume of 0.6 ml/kg (Groups 1–4). The vehicle (placebo) and test article were administered once on GD 8, 14, and 20 via intramuscular injection, which is the intended route of the drug in humans. The dosing was designed to cover most of the period of female reproductive organ development. The interval corresponds to the half-life of HPC derived from a preliminary pharmacokinetic study, conducted in the same species, which demonstrated a half-life of approximately 6 days (Data on file). The control group received the vehicle (placebo) in the same manner and dosing regimen as the treated groups. Individual doses were based on the most recent body weights.

Teratology phase (TP). A total of 118 time-mated female rats were received from CRL at approximately 8 to 10 weeks of age at arrival (GD 0). Animals were randomly assigned to study at arrival using a standard, by weight, block randomization procedure based on GD 0 body weights. A total of 100 female rats (weighing 163–220 gm at randomization) were assigned to control and three treatment groups of 25 females each at dose levels of 0, 5, 25, and 150 mg/kg given as a dosing volume of 0.6 ml/kg (Groups 5–8). The vehicle (placebo) and test article were administered once on GD 6, 12, and 18 via intramuscular injection, the dosing interval corresponding to the critical period of embryo-fetal development in this species. The control group received the vehicle (placebo) in the same manner and dosing regimen as the treated groups. Individual doses were based on the most recent body weights.

Reproductive phase 2 (RP2). A total of 121 time-mated female rats were received from CRL at approximately 8 to 10 weeks of age at arrival (GD 0). A total of 110 male and 110 female SD rats were received from the supplier as naïve animals to be mated with the in utero-treated F_1 animals. Animals were randomly assigned to study at arrival using a standard, by weight, block randomization procedure based on GD 0 body weights. A total of 100 female rats (weighing 197–263 gm at randomization) were assigned to control and three treatment groups of 25 females each at dose levels of 0, 5, 25, and 150 mg/kg given as a dosing volume of 0.6 ml/kg (Groups 9–12). The vehicle (placebo) and test article were administered once on GD 17 via intramuscular injection, a timepoint after testicular differentiation (GD 13–15) in the rat. The control group received the vehicle (placebo) in the same manner and dosing regimen as the treated groups. Individual doses were based on the most recent body weights.

EXPERIMENTAL DESIGN AND PROCEDURES

Observations

A tabulated summary of observations and procedures performed during the study is in Table 1.

Statistical Analysis

The set of comparisons used in the statistical analyses is as follows: Control group 1 versus RP1 treatment groups 2, 3, and 4; control group 5 versus TP treatment groups 6, 7, and 8; control group 9 versus RP2 treatment groups 10, 11, and 12. Depending on sample size and individual test of significance F_0 parental body weights, F_0 lactation body weights, gestational length (F_0, F_1), copulatory interval (F_1), male reproductive organ weights, sperm concentration, litter size, stillborn and viability (F_0 and F_1 offspring), developmental indices (F_1), behavioral tests (F_1) were analyzed by group pair-wise comparisons (Welch, 1937; Dunnett, 1955; Snedecor and Cochran, 1989; Milliken and Johnson, 1992). Male and female fertility indices, mating indices, fecundity indices, and gestational indices were analyzed for significance with the use of Fisher's exact test (Zar, 1999). The number of sperm abnormalities and motility values, pup sex ratio, stillborn index, pup survival, and auditory response testing results were evaluated by the arcsin-square-root transformation method followed by group pair-wise comparisons (Steel and Torrie, 1980). For endpoints that describe categories rather than numerical measures, a test of association (Agresti, 1990) was conducted if variability occurred among the groups. Appropriate results are reported at the 0.05 significance levels and all endpoints were analyzed using two-tailed tests.

RESULTS

Analysis of Dosing Formulations

Concentration analysis revealed group mean recovery rates from the daily preparations were all within the acceptance criteria of ±15%, with the exception of the low-dose group in the RP1 (GD 20) and the TP (GD 6) phases. The group recovery rates at these timepoints and dose levels were 77.1 and 76.4%, respectively. During the course of the study, concentration evaluations showed group recovery rates in the RP1 that ranged from 77.1 to 110.6%, in the TP that ranged from 76.4 to 106.6%, and in the RP2 that ranged from 94.2 to 100.4%. Overall, the concentration evaluations showed good correlation between the target and actual concentration administered.

RP1 In-life Examinations

All F_0 females survived to scheduled necropsy, except for one 5 mg/kg female that was found dead on Day 25. The cause of death could not be positively determined at necropsy. The only remarkable macroscopic necropsy finding in the animal was a distended urinary bladder. The F_0 gestation and lactation body weights were comparable among the groups.

The parturition and litter data for the F_0 females demonstrated 19, 23, 24, and 20 females for the control-, low-, mid-, and high-dose groups, respectively, that produced live offspring resulting from pregnancy rates of 76, 92, 96, and 80%, respectively (Table 2). Of the females that delivered, there were no adverse test-article–related effects observed. This included the following parameters: number of females delivering stillborns, gestational length, gestational index, stillborn index (number of stillborn pups divided by the number of pups born), litter size (live-born and total live plus dead pups at birth), and implantation scars per dam. For the F_0 females that delivered, there were no test-article–related effects on F_1 pup survival or sex ratios during the lactation period from parturition to weaning (LD 0–21).

Table 1
Observations and Procedures Performed During Study

Schema		Gestational day														
Study phase	Rationale	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
RP1	Cover female reproductive period			X						X						X
TP	Important embryo-fetal development	X						X						X		
RP2	Post testicular differentiation												X			

Observation/procedure	Schedule
Detailed clinical observation	RP1—GD8—euthanasia TP = GD 6 – 20 RP2—GD 8 and 14
Body weight	RP1—every 3 days GD 8–20, LD 4–21 TP—variable 2–4 days, GD 6–20 RP2—3–6 days GD 8–20 LD 3–4 days 4–21
Food consumption F_0 females	RP1, TP, RP2 at body weight intervals
Necropsy F_0 females not delivering	RP1 and RP2 on GD 25 (Salewski, 1964)
Parturition F1/F2	Toward end of gestation -> birth Duration of gestation (LD 0) Litter size Number stillborn/live-born Body weight as above Gross abnormalities F1
F1 pup selection, pups culled, RP1, RP2	Eight each/litter selected (four male, four female) F1 behavior and developmental indices performed LD2 righting response, unfolding of pinna LD11 cliff aversion LD13 eye opening LD16 righting reflex (Hard and Larsson, 1975) LD21 Irwin test (Irwin, 1968) PND22 Preyer's response (Vernier and Alleva, 1968) Pups weighed LD 0, 4, 7, 14, 21 F0/F1 LD 4, 21 euthanized, uterine implantation scars Dead pups dissected (Stuckhardt and Poppe, 1984). PND28 F1 landmark assessment, vaginal opening females PND35 preputial separation males PND±35 motor activity testing PND 75–80 learning/memory testing
F1 breeding RP1, RP2	F1 at 80 days of age and step through passive avoidance testing 20 day matings: exposed females to naïve males and naïve females to exposed males
Postmortem evaluation	GD20 TP Sectioning method (Wilson, 1965) and skeletal staining (Dawson, 1926) RP1, RP2 F1 organ weights, sperm analyses

During lactation there were several different clinical observations in the F_1 pups, but as most were sporadic in nature, few in number, and did not show dose-related effects, these findings were not considered to be toxicologically meaningful. Preweaning, 25 of 903 (2.7%) of total pups had clinical findings. The most common observation was decreased activity seen in seven animals. Other observations included skin discoloration, abrasions, scabbing, and swelling of various body areas. Post weaning, there were no clinical observations. Statistically significant increases ($p < 0.05$) in mean pup body weight were noted at the high-dose group during the lactation and postweaning periods but an increase in pup body weight was not considered to be an adverse toxicological finding. The macroscopic necropsy observations for the F_1 pups (stillborn, died during lactation, culled LD 4, and unselected to continue on study PND 28) showed no adverse effects that could be attributed to treatment. There were no statistically significant changes in the F_1 pup reflex, sensory, or developmental indices recorded during lactation, as compared to the controls. These parameters included static righting reflex, pinna detachment, cliff aversion, eye opening, air drop righting reflex, and auditory response (Supplemental Table 1). In addition, the neuropharmacological evaluation did not reveal any treatment-related effects in any group.

A statistically significant decrease ($p < 0.05$) in body weight was observed in the low-dose females at confirmation of vaginal opening, and a statistically significant increase ($p < 0.05$) in the days to reach preputial separation was seen in the males in the mid-dose group (Supplemental Table 1). However, these values were not

Table 2
Reproductive Phase 1 Summary of F0 Natural Delivery and F1 Litter Data[a]

Endpoint		0 mg/kg	5 mg/kg	25 mg/kg	150 mg/kg
No. females on study		25	25	25	25
No. females pregnant		19	23	24	20
Females delivering litters	*N*	19	23	24	20
	%	76.0	92.0	96.0	80.0
With stillborn pups	*N*	1	2	3	0
	%	5.0	9.0	12.5	0.0
With all stillborn	*N*	0	0	0	0
	%	0.0	0.0	0.0	0.0
Gestational length (days)	(Mean ± SD)	21.9 ± 0.2	22.0 ± 0.2	21.8 ± 0.4	22.0 ± 0.3
No. of pups at day 0					
Total pups born/litter	(Mean ± SD)	11.1 ± 2.6	10.3 ± 2.0	11.0 ± 2.7	10.4 ± 2.5
Live-born/litter	(Mean ± SD)	11.0 ± 2.6	10.2 ± 1.5	10.9 ± 2.7	10.4 ± 2.5
Stillborn/litter	(Mean ± SD)	0.1 ± 0.2	0.2 ± 0.7	0.1 ± 0.3	0.0 ± 0.0
Gestational index	%	100.0	100.0	100.0	100.0
Stillborn index (%/litter)	(Mean ± SD)	0.40 ± 1.8	1.37 ± 5.0	1.26 ± 3.5	0.00 ± 0.0
Total implantation scars/litter	(Mean ± SD)	12.1 ± 1.3	11.3 ± 2.0	12.0 ± 1.9	11.5 ± 2.2
No. live pups/litter					
Day 4 (preculling)	(Mean ± SD)	10.4 ± 2.3	9.9 ± 2.2	10.9 ± 2.2	10.3 ± 2.3
Day 4 (postculling)	(Mean ± SD)	7.6 ± 1.4	7.7 ± 1.0	7.9 ± 0.7	7.7 ± 1.0
Day 7	(Mean ± SD)	7.6 ± 1.4	7.7 ± 1.0	7.8 ± 0.6	7.7 ± 1.0
Day 14	(Mean ± SD)	7.6 ± 1.4	7.6 ± 1.0	7.7 ± 0.6	7.7 ± 1.0
Day 21	(Mean ± SD)	7.6 ± 1.4	7.6 ± 1.0	7.7 ± 0.6	7.7 ± 1.0
Sex ratio (% males/animals)					
Pups day 0	Mean%/litter (±SD)	57.03 ± 17.5	48.23 ± 17.2	49.00 ± 17.0	47.84 ± 16.7
Pups day 4 (preculling)	Mean%/litter (±SD)	57.51 ± 17.6	49.15 ± 18.7	46.61 ± 13.9	47.24 ± 16.7
Pups day 4 (postculling)	Mean%/litter (±SD)	55.26 ± 14.0	51.98 ± 14.1	48.66 ± 8.9	49.38 ± 13.1
Pups day 21	Mean%/litter (± SD)	55.26 ± 14.0	51.90 ± 14.0	49.79 ± 9.6	49.38 ± 13.1
Pup survival indices					
Viability index (0–4 days)	Mean%/litter (±SD)	95.71 ± 13.4	88.07 ± 28.4	92.91 ± 21.7	99.17 ± 2.6
Lactation index (4–21 days)	Mean%/litter (±SD)	100.00 ± 0.0	98.81 ± 3.8	98.15 ± 6.7	100.00 ± 0.0

[a]No statistical differences noted.

considered test article related, as they did not show a typical dose response effect, and the values at the high-dose level were comparable to the control values for each gender.

There was a slightly increased rate in rearing for the F_1 males at the 25 and 150 mg/kg doses during the study interval of 15 to 20 min that was statistically significant ($p < 0.05$). However, these values were not statistically significant over the entire period of 20 min, so they were dismissed as not toxicologically meaningful. There was a general trend of slightly increased level of motor activity (basic movement, fine movement, rearing, and total distance traveled) in male and female animals at these two dose levels as well (Supplemental Tables 2 and 3). However, these changes were not statistically different from the controls, and therefore not considered of toxicological significance. The results of the learning and memory evaluation indicated that the males at the 150 mg/kg were passive (i.e., passing the test) at a frequency of 76 versus 60% in the controls; the females at the 150 mg/kg were passive at a frequency of 80 versus 64% in the controls. Due to the slight variability in the passive frequency in the control animals, the data indicate that there were no adverse effects on learning and memory in any treatment group (Supplemental Tables 2 and 3). All F_1 in utero exposed and naïve animals survived to scheduled necropsy. There was no evidence of adverse clinical observations that could be attributed to treatment for the F_1 female animals during the premating, mating, gestation, or lactation periods. Mean body weight in the in utero exposed F_1 males (premating, mating, and postmating) and F_1 females (premating, gestation, and lactation) did not show any statistically significant changes, as compared to the controls. Likewise, naïve females that were used for mating with the in utero exposed F_1 males did not show any statistically significant changes in mean body weight during gestation or lactation. Mean body weight gain was statistically increased in the in utero exposed F_1 females at the mid-dose level during study interval weeks 1 to 2. Other statistically significant decreases in body weight gain were not considered to be toxicologically meaningful, as they did not follow a clear dose–response pattern and the mean body weights at these dose levels as a group were unaffected by treatment.

The reproductive and fertility indices for the F_1 in utero exposed females mated to naïve males, and naïve females mated to in utero exposed F_1 males, did not show any significant adverse changes that could be attributed to treatment (Tables 3 and 4). These parameters included mating, fertility and fecundity indices, and copulatory intervals. The parturition and litter data for the in utero exposed F_1 females did not show any adverse effects that could be attributed to treatment (Table 5). These parameters included the number of females delivering litters, the number of females with stillborns, gestational length, mean litter size (live-born pups and total live plus dead pups), as well as

Table 3
Reproductive Phase 1 Summary of F1 Reproductive and Fertility Parameters in Exposed Females Mated to Naïve Male Rats[a]

Endpoint	0 mg/kg	5 mg/kg	25 mg/kg	150 mg/kg
No. females on study	25	25	25	25
No. females paired	25	25	25	25
No. females mated	25	24	24	24
No. pregnant	25	23	23	24
Female mating index, %	100.0	96.0	96.0	96.0
Female fertility index, %	100.0	92.0	92.0	96.0
Female fecundity index, %	100.0	95.8	95.8	100.0
Females with confirmed mating day, N	24	22	20	17
Copulatory interval (days) (mean ± SD)	3.0 ± 1.4	2.8 ± 1.2	3.4 ± 3.4	3.0 ± 1.2
No. males on study	25	25	25	25
No. males paired	25	25	25	25
No. males mated	25	24	23	24
No. males impregnating a female	25	23	22	23
Male mating index, %	100.0	96.0	92.0	96.0
Male fertility index, %	100.0	92.0	88.0	96.0
Male fecundity index, %	100.0	95.8	95.7	100.0

[a]No significant differences.

Table 4
Reproductive Phase 1 Summary of F1 Reproductive and Fertility Parameters in Naive Females Mated to Exposed Male Rats[a]

Endpoint	0 mg/kg	5 mg/kg	25 mg/kg	150 mg/kg
No. females on study	25	25	25	25
No. females paired	25	25	25	25
No. females mated	25	25	25	25
No. pregnant	25	25	24	25
Female mating index, %	100.0	100.0	100.0	100.0
Female fertility index, %	100.0	100.0	96.0	100.0
Female fecundity index, %	100.0	100.0	96.0	100.0
Females with confirmed mating day, N	23	24	24	21
Copulatory interval (days) (mean ± SD)	3.0 ± 2.2	2.7 ± 1.2	3.5 ± 2.8	2.2 ± 1.2
No. males on study	25	25	25	25
No. males paired	25	25	25	25
No. males mated	24	25	24	24
No. males impregnating a female	24	25	23	24
Male mating index, %	96.0	100.0	96.0	96.0
Male fertility index, %	96.0	100.0	92.0	96.0
Male fecundity index, %	100.0	100.0	95.8	100.0

[a]No significant differences.

gestation, stillborn, and viability indices. The parturition and litter data for the naïve females mated to the in utero exposed F_1 males showed an insignificant increase in the stillborn rate at 150 mg/kg (four females vs. one female in the control) (Table 6). Even though this was slightly higher than the controls, the number of affected animals and the remaining parameters in this dose group, such as the number of live-born pups and total pups per litter and their survival, was comparable to the other treatment groups as well as the controls. Further the high standard deviation for this value indicates wide variation in this case. The gestational index and the number of implantation scars per dam were also comparable among the groups. Gestational length was statistically significant at the low dose, being very slightly shortened, but this effect was not observed at higher doses and was thus not considered toxicologically significant. The F_2 pups born to the in utero exposed F_1 females, and to the naïve females mated to the in utero exposed F_1 males, did not show any test-article–related effects on survivability during lactation up to LD 4. A statistically identified increase in the viability index for the F_2 pups born to the mid dose naïve females (97.83 vs. 89.48% in the controls) was not considered toxicologically significant, as the value showed no adverse toxicological effect as the value was not replicated at the high dose.

No adverse effects were observed in the sex ratios for the F_2 pups treated with the test article born to the F_1 in utero treated females, or naïve females mated to in utero exposed males, as compared to the controls. No significant clinical observations were observed either in the F_2 pups born to the F_1 in utero exposed females or the naïve females mated to in utero exposed males. The mean F_2 pup weights for the F_1 in utero exposed

Table 5
Reproductive Phase 1 Summary of F1 Natural Delivery and Litter Data in Exposed Females Mated to Naïve Male Rats[a]

Endpoint		0 mg/kg	5 mg/kg	25mg/kg	150 mg/kg
No. females on study		25	25	25	25
No. females pregnant		25	23	23	24
Female fertility index		100.0	92.0	92.0	96.0
Females delivering litters	*N*	25	23	23	24
	%	100.0	92.0	92.0	96.0
With stillborn pups	*N*	2	0	2	1
	%	8.0	0.0	8.7	4.2
With all stillborn	*N*	0	0	0	0
	%	0.0	0.0	0.0	0.0
Gestational length (Days)	(Mean ± SD)	21.8 ± 0.8	22.0 ± 0.2	21.9 ± 0.7	21.8 ± 0.6
No. of pups at day 0					
Total pups born/litter	(Mean ± SD)	14.7 ± 2.6	14.9 ± 2.0	14.0 ± 4.5	14.1 ± 2.6
Live-born/litter	(Mean ± SD)	14.4 ± 2.6	14.9 ± 2.0	13.9 ± 4.4	14.1 ± 2.6
Stillborn/litter	(Mean ± SD)	0.3 ± 1.0	0.0 ± 0	0.1 ± 0.5	0.0 ± 0.2
Gestational index	%	100.0	100.0	100.0	100.0
Stillborn index (%/litter)	(Mean ± SD)	1.71 ± 6.0	0.0 ± 0	0.77 ± 2.6	0.32 ± 1.6
Total implantation scars/litter	(Mean ± SD)	15.8 ± 1.9	15.7 ± 1.6	15.7 ± 3.5	15.1 ± 2.1
No. live-born pups/litter (day 4)	(Mean ± SD)	13.5 ± 3.3	14.0 ± 3.3	13.0 ± 4.5	13.6 ± 2.9
Sex ratio (% males/animal)					
Pups day 0	Mean%/litter (±SD)	48.59 ± 15.8	50.14 ± 14.7	52.47 ± 14.8	47.34 ± 17.1
Pups day 4	Mean%/litter (±SD)	48.85 ± 16.2	50.66 ± 15.2	53.63 ± 18.0	47.11 ± 17.6
Pup survival indices					
Viability index (0–4 days)	Mean%/litter (±SD)	94.00 ± 16.2	94.23 ± 17.8	89.90 ± 22.2	96.53 ± 8.9

[a]No statistical differences noted.

Table 6
Reproductive Phase 1 Summary of F1 Natural Delivery and Litter Data in Exposed Males Mated to Naïve Female Rats

Endpoint		0 mg/kg	5 mg/kg	25mg/kg	150 mg/kg
No. females on study		25	25	25	25
No. females pregnant		25	25	24	25
Female fertility index		100.0	100.0	96.0	100.0
Females delivering litters	*N*	25	25	24	25
	%	100.0	100.0	96.0	100.0
With stillborn pups	*N*	1	2	2	4
	%	4.0	8.0	8.3	16.0
With all stillborn	*N*	0	0	0	0
	%	0.0	0.0	0.0	0.0
Gestational length (days)	(Mean ± SD)	22.1 ± 0.4	21.8 ± 0.4[a]	22.0 ± 0.5	22.0 ± 0.4
No. of pups at day 0					
Total pups born/litter	(Mean ± SD)	14.7 ± 2.9	14.8 ± 2.1	14.5 ± 2.5	15.0 ± 3.9
Live-born/litter	(Mean ± SD)	14.6 ± 2.9	14.7 ± 2.1	14.3 ± 2.6	14.6 ± 4.1
Stillborn/litter	(Mean ± SD)	0.1 ± 0.4	0.2 ± 0.6	0.2 ± 0.8	0.4 ± 1.0
Gestational index	%	100.0	100.0	100.0	100.0
Stillborn index (%/litter)	(Mean ± SD)	0.47 ± 2.4	1.03 ± 3.6	1.37 ± 5.5	5.94 ± 20.5
Total implantation scars/litter	(Mean ± SD)	16.5 ± 2.7	16.0 ± 2.1	15.8 ± 1.7	16.3 ± 2.8
No live-born pups/litter (day 4)	(Mean ± SD)	13.8 ± 3.1	14.2 ± 1.9	14.0 ± 2.5	14.8 ± 2.9
Sex ratio (% males/animal)					
Pups day 0	Mean%/litter (±SD)	55.49 ± 14.4	47.97 ± 13.5	49.73 ± 11.1	51.37 ± 14.7
Pups day 4	Mean%/litter (±SD)	53.64 ± 14.4	48.33 ± 14.5	50.13 ± 10.9	51.55 ± 15.0
Pup Survival Indices					
Viability index (0–4 days)	Mean%/litter (±SD)	89.48 ± 21.7	96.75 ± 5.0	97.83 ± 5.6[a]	96.92 ± 5.3

[a]Significantly different ($p < 0.05$) from control.

females were comparable among the groups on LD 0 and 4. A slight decrease in the LD 4 mean pup weight was statistically significant ($p < 0.05$) but of no biological significance for the 150 mg/kg F_2 pups born to naïve females mated to in utero exposed males. These values were probably the result of slightly larger control pups than usual. The mean combined F_2 pup weight from control F_1 in utero exposed females was 9.47 versus 10.22 gm from control naïve females. In addition, a true dose–response relationship could not be established, as the mid-dose (25 mg/kg) pups had mean pup weights that were higher than those from the control group. Therefore, this

apparent decrease in pup body weight at the 150 mg/kg does not indicate a test-article–related effect.

The macroscopic findings in the F_2 pups from the F_1 in utero exposed and naïve females were sporadic in nature, few in number, and did not show an apparent dose–response effect and therefore were not considered to be test article related. A statistically significant ($p < 0.05$) but toxicologically insignificant increase in the epididymis to body weight ratio was calculated for the low-dose level but this was not considered to be test article related, as the values for the organ to body weight ratios for the epididymis at the higher dose levels were comparable to the controls and not statistically different (Supplemental Table 4).

The sperm parameters for the F_1 in utero exposed males selected for mating did not show evidence of any test-article–related effects. The increase in the number of abnormal sperm in the mid-dose animals (10.2 vs. 2.2% in the controls) was not considered toxicologically meaningful, as the value for the high-dose males was lower (2.8%) and similar to that of the controls.

TP In-Life Examination

All F_0 females survived to scheduled necropsy on GD 20. There were no clinical observations that showed any relationship to treatment at any dose level tested. No statistically significant changes in mean body weight were observed at any dose level tested during the gestation period, as compared to the controls. There were some slight increases in mean body weight change during the gestation period at the lower dose levels, which were not considered biologically significant.

Mean food consumption was statistically increased at the mid dose over GD 6 to 18. Similarly, mean food consumption was also statistically increased at the high dose during GD interval 15 to 18. These changes were not considered toxicologically meaningful, as there were no corresponding statistically significant changes in group mean body weight at any dose level.

There were no test-article–related macroscopic changes observed in the F_0 females at any dose level tested. Two animals, one in the control and one in the high-dose, were not pregnant and this resulted in pregnancy rates of 96, 100, 100 and 96% for the control, 5, 25, and 150 mg/kg groups, respectively (Table 7). As the fetal external and visceral examinations were negative, the specimens for skeletal evaluation were not required to be examined in accordance with the protocol.

The fetuses in the TP were examined viscerally using the standard Wilson's sectioning technique, which includes a mid-coronal section of the brain region to again look for evidence of malformations. There was no evidence of any abnormalities.

The data obtained from the uterine examination did not show any results that could be attributed to test article exposure. This included evaluation of the number of corpora lutea, implantations, viable fetuses, and litter size per litter. An increased postimplantation loss was seen at the 150 mg/kg but this was not considered toxicologically meaningful as the value (11%) was within the historical control range of the laboratory (range 2.1–11.3%) and there was no statistical significance. Gravid uterine weight, adjusted body weight, and adjusted body weight gain among the groups were unaffected by treatment. The calculated mean sex ratios for the fetuses examined did not show any statistically significant change in the treated animals, as compared to the controls. Mean fetal body weight was comparable among the groups and did not show a pattern that could be attributed to treatment. During the fetal external examination, there was one fetus at the low-dose that had a malformation (absent tail). There were no fetal visceral malformations observed at any dose level tested, but there were a few visceral variations noted and only one occurred at the highest dose. It had increased renal pelvic cavitation and dilated ureter and was not considered to be test article related.

RP2 In-Life Examinations

All F_0 females survived to scheduled necropsy, with the exception of one low-dose animal that was found dead on day 23 and the cause could not be determined at necropsy.

There were no adverse clinical observations that showed any dose responsive pattern that would be indicative of a test-article–related effect at any dose tested. There were some minor findings of sparse hair in the forelimb/foot at all dose groups at a frequency ranging from 2 to 15 animals per group during gestation and lactation, but these findings were sporadically distributed among the groups and did not show a relationship to treatment. The mean gestation and lactation body weights and body weight gains for the F_0 females were comparable among the groups. Mean food consumption in the F_0 females during gestation and lactation was unaffected by treatment. No F_0 animals examined at necropsy showed any abnormal findings.

All F_0 females were pregnant and delivered live offspring. There were no test-article–related effects on any parameter evaluated (Table 8). These included females with stillborn pups, gestational length, stillborn index, mean number of pups (live-born pups and total live plus dead pups), or total implantation scars per dam. F_1 pup survival was unaffected by treatment during the lactation period. Survivability indices were comparable among the groups on LD 0 to 4 and 4 to 21. F_1 pup sex ratios did not show any test-article–related effects in the treatment groups compared to the controls. The only remarkable clinical observations noted in the F_1 pups from birth to weaning were increased traumatic lesions of the feet (and infrequently limbs) in the mid- (25 mg/kg/day) and high-dose (150 mg/kg/day) groups. These were not treatment related, but were considered to have been caused by a traumatic injury to the limbs as the pups were being tattooed for postnatal identification. The findings were all first observed on the same day during the lactation period. No other abnormal limb findings were observed in the F_2 pups during RP2, nor were any similar limb findings documented in either the F_1 or F_2 pups in RP1.

Statistically significant increases ($p < 0.05$) in F_1 pup body weights were documented for the first week (LD 0, 4 [pre- and postcull], and 7) at the low-dose level but were not considered to be toxicologically meaningful, as there were no corresponding changes in mean pup body weights at the two higher dose levels.

Table 7
Teratology Phase Summary of Maternal and Developmental Observations at Uterine Examination[a]

Endpoint		0 mg/kg	5 mg/kg	25 mg/kg	150 mg/kg
No. females on study		25	25	25	25
No. not pregnant		1	0	0	1
No. pregnant		24	25	25	24
Pregnancy index (%)		96.0	100.0	100.0	96.0
No. died pregnant		0	0	0	0
No. abortions		0	0	0	0
No. early deliveries		0	0	0	0
No. females with all resorptions		0	0	0	0
No. females with viable fetuses day 20 gestation		24	25	25	24
Corpus lutea no. per animal	(Mean ± SD)	11.8 ± 1.8	11.9 ± 2.2	12.4 ± 20.0	13.0 ± 2.2
Implantation sites no. per animal	(Mean ± SD)	10.6 ± 1.6	10.5 ± 1.8	11.6 ± 1.7	11.1 ± 1.5
Preimplantation loss % per animal	(Mean ± SD)	9.96 ± 11.3	10.66 ± 12.3	5.90 ± 7.9	13.32 ± 11.7
Viable fetuses no. per animal	(Mean ± SD)	10.0 ± 2.3	9.9 ± 2.7	10.9 ± 1.5	9.9 ± 1.9
Fetal sex ratio % males per animal	(Mean ± SD)	51.5 ± 14.3	57.0 ± 16.8	49.4 ± 17.4	47.3 ± 21.9
Fetal weight (gm)mean	(Mean ± SD)	4.01 ± 0.19	3.91 ± 0.19	3.94 ± 0.30	3.98 ± 0.21
Postimplantation loss % per animal	(Mean ± SD)	7.11 ± 14.8	6.44 ± 18.0	5.86 ± 6.9	11.17 ± 12.7
Nonviable fetuses no. per animal		0	0	0	0
Litter size no. per animal	(Mean ± SD)	10.0 ± 2.3	9.9 ± 2.7	10.9 ± 1.5	9.9 ± 1.9
Resorptions: early + late no. per animal	(Mean ± SD)	0.6 ± 1.1	0.6 ± 1.8	0.7 ± 0.8	1.3 ± 1.5
Resorptions: early no. per animal	(Mean ± SD)	0.6 ± 1.1	0.6 ± 1.6	0.7 ± 0.8	1.3 ± 1.5
Resorptions: late no. per animal	(Mean ± SD)	0.0 ± 0.0	0.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0

[a]No statistical differences noted.

At necropsy, there were no macroscopic findings in the F_1 pups (stillborn, deaths during lactation, culled LD 4, and unselected to continue on study PND 28) that could be attributed to treatment.

During lactation, the F_1 pups showed a slight but statistically significant decrease ($p < 0.05$) in the days to pinna detachment for the mid dose animals (2.2 vs. 2.5 days in the controls). However, this was not considered toxicologically meaningful, as the values for the low- and high-dose levels were not statistically different from the control value. The remaining parameters were comparable among the groups and consisted of static righting reflex, cliff aversion, eye opening, air drop righting reflex, and auditory response. In addition during the neuropharmacological evaluation, there were no effects that could be attributed to treatment (Supplemental Table 5). The results of the sexual maturation testing did not reveal any test-article–related effects. Vaginal opening and preputial separation results for the female and male pups, respectively, were comparable among the groups. There were some statistical increases in the motor activity parameters documented at all treatment levels at varying study intervals. In the males, this was most pronounced at the mid-dose level, and in the females, it was sporadically distributed among the treatment groups. However, there was no consistent dose–response pattern among the animals, and therefore, it was difficult to consider the data to be a test-article–related effect. The results of the learning and memory evaluation showed that the males at 150 mg/kg were passive at a frequency of 64 versus 76% in the controls and the females at 150 mg/kg were passive at a frequency of 72 versus 60% in the controls. Due to the slight variability in the passive frequency in the control animals (males to females) in this study, the data indicate that there were no test-article–related effects on learning and memory in the treatment groups (Supplemental Tables 6 and 7).

All F_1 in utero exposed and naïve animals survived to scheduled necropsy, with the exception of one control F_1 female that was found dead on day 85; the cause of death could not be determined at necropsy.

There were no significant clinical observations noted that could be attributed to treatment for the in utero exposed F_1 males or females during the observation periods evaluated.

There were no statistically significant changes in mean body weight or body weight changes for the F_1 in utero exposed males during the premating, pairing, and postmating periods, and F_1 in utero exposed females during the premating, gestation, and lactation periods. For the naïve females, there were no statistically significant changes in mean gestation or lactation body weight. There was a statistical decrease ($p < 0.05$) in lactation body weight gain for the females mated to the in utero exposed males treated at the 150 mg/kg (study interval days 0–4), but this was not considered toxicologically meaningful, as they occurred in the exposed males group only.

There were no test article effects noted in the male and female mating, fertility, or fecundity indices, including no significant changes in the copulatory intervals for the F_1 in utero exposed females mated to naïve males (Supplemental Table 8), or naïve females mated to F_1 in utero exposed males, as compared to the controls (Supplemental Table 9). At the 150 mg/kg dose, the number of females with confirmed mating (GD 0 identified by vaginal plug or sperm) was slightly lower for the F_1 in utero exposed females (19 vs. 22 in the controls) and naïve females mated to in utero exposed males (20 vs. 23 in the controls). These values were not considered toxicologically meaningful as the pregnancy rate in each instance was comparable to

Table 8
Reproductive Phase 2 Summary of F0 Natural Delivery and F1 Litter Data[a]

Endpoint		0 mg/kg	5 mg/kg	25 mg/kg	150 mg/kg
No. females on study		25	25	25	25
No. females pregnant		25	25	25	25
Females delivering litters	*N*	25	25	25	25
	%	100.0	100.0	100.0	100.0
With stillborn pups	*N*	2	1	0	0
	%	8.0	4.0	0.0	0.0
With all stillborn	*N*	0	0	0	0
	%	0.0	0.0	0.0	0.0
Gestational length (days)	(Mean ± SD)	21.8 ± 0.5	22.0 ± 0.4	21.9 ± 0.4	21.8 ± 0.5
No. of pups at day 0					
Total pups born/litter	(Mean ± SD)	12.6 ± 1.4	12.3 ± 2.4	13.3 ± 1.9	12.7 ± 1.5
Live-born/litter	(Mean ± SD)	12.5 ± 1.4	12.2 ± 2.4	13.3 ± 1.9	12.7 ± 1.5
Stillborn/litter	(Mean ± SD)	0.1 ± 0.4	0.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Gestational index	%	100.0	100.0	100.0	100.0
Stillborn index (%/litter)	(Mean ± SD)	0.88 ± 3.2	0.27 ± 1.3	0.00 ± 0.0	0.00 ± 0.0
Total implantation scars/litter	(Mean ± SD)	13.3 ± 1.6	13.2 ± 2.0	13.8 ± 1.5	13.2 ± 1.7
No. live pups/litter					
Day 4 (preculling)	(Mean ± SD)	12.4 ± 1.5	11.8 ± 3.1	13.1 ± 1.9	12.5 ± 1.5
Day 4 (postculling)	(Mean ± SD)	8.0 ± 0.0	7.7 ± 1.4	8.1 ± 0.6	8.0 ± 0.0
Day 7	(Mean ± SD)	8.0 ± 0.0	7.6 ± 1.4	8.0 ± 0.2	8.0 ± 0.2
Day 14	(Mean ± SD)	8.0 ± 0.0	7.6 ± 1.4	8.0 ± 0.2	8.0 ± 0.2
Day 21	(Mean ± SD)	8.0 ± 0.0	7.6 ± 1.4	8.0 ± 0.2	7.9 ± 0.3
Sex ratio (% males/animals)					
Pups day 0	Mean%/litter (±SD)	48.69 ± 17.1	47.98 ± 16.0	50.93 ± 13.7	52.07 ± 14.8
Pups day 4 (preculling)	Mean%/litter (±SD)	49.14 ± 17.1	46.05 ± 18.8	50.60 ± 13.4	52.49 ± 14.3
Pups day 4 (postculling)	Mean%/litter (±SD)	48.50 ± 7.5	48.51 ± 14.2	49.32 ± 5.7	50.50 ± 4.4
Pups day 21	Mean%/Litter (± SD)	48.21 ± 7.6	48.81 ± 14.3	49.21 ± 5.8	51.07 ± 4.8
Pup survival indices					
Viability index (0–4 days)	Mean%/Litter (±SD)	99.00 ± 2.8	91.05 ± 26.6	98.53 ± 3.0	98.42 ± 3.3
Lactation index (4–21 days)	Mean%/Litter (±SD)	99.50 ± 2.5	99.48 ± 2.6	98.41 ± 5.9	99.00 ± 3.5

[a]No significant differences.

the other treatment groups and the controls. There were 24, 25, 24, and 24 females pregnant for the F_1 in utero exposed females in all groups. And, there were 25, 24, 25, and 25 females pregnant for the naïve females mated to the in utero exposed males in all groups.

The only remarkable findings in the F_1 parturition and litter data were a statistically significant decrease ($p < 0.05$) in gestational length for the 150 mg/kg groups. This included 21.9 versus 22.4 days in the controls for the F_1 in utero exposed females (Table 9), and 22.0 versus 22.4 days in the controls for the naïve females mated to the F1 in utero exposed males (Table 10). While flagged as statistically significant ($p < 0.05$), these values were not considered biologically significant as they fell within the expected delivery days for this laboratory, were directly comparable to all other values for this parameter in the other reproductive phase in the complete study, and were of minimal extent (less than one-half day shorter than control values). Further, a probable major contributor to the perceived significance are the mean values in the controls (22.4 days), which are at the maximum published mean control value for this rat strain, 22.42 ± 0.53 based on 209 studies (Hood, 1997), making any lesser values appear of greater difference, as in the 150 mg/kg groups in Tables 9 and 10. The remaining litter parameters for the F_1 in utero exposed females and naïve females were comparable among the groups and included the number of females delivering, the number of females with stillborn pups, gestational and stillborn indices, mean number of pups at birth (live-born and total live plus dead pups), and total implantation scars per litter on LD 4. F_2 pup survival was unaffected by treatment as evidenced by the survivability indices during lactation (LD 0–4) that were comparable among the treatment groups, and similar to the controls for the pups born to F_1 in utero exposed and naïve females mated to in utero exposed males. The sex ratios calculated for the F_2 pups born to F_1 in utero exposed females and naïve females mated to in utero exposed males did not show any test-article–related effects.

There were no clinical observations noted that could be attributed to treatment in the F_2 pups born to either the F_1 in utero exposed females, or naïve females mated to in utero exposed males. Mean F_2 pup body weights were directly comparable among the groups for both the F_1 in utero exposed females and the naïve females mated to in utero exposed males.

During the macroscopic evaluation of the F_2 pups born to F_1 in utero exposed females (mated to naïve males), there were no necropsy findings in the stillborns, dead, or pups on LD 4, that indicated a relationship to treatment. Similarly, macroscopic findings in F_2 pups born to naïve females (mated to F_1 in utero exposed males) did not show any effects that were toxicologically

Table 9
Reproductive Phase 2 Summary of F1 Natural Delivery and Litter Data in Exposed Females Mated to Naïve Males

Endpoint		0 mg/kg	5 mg/kg	25 mg/kg	150 mg/kg
No. females on study		25	25	25	25
No. females pregnant		24	25	24	24
Female fertility index		96.0	100.0	96.0	96.0
Females delivering litters	*N*	24	25	24	24
	%	100.0	100.0	100.0	100.0
With stillborn pups	*N*	1	2	3	0
	%	4.2	8.0	12.5	0.0
With all stillborn	*N*	0	0	0	0
	%	0.0	0.0	0.0	0.0
Gestational length (days)	(Mean ± SD)	22.4 ± 0.6	22.1 ± 0.5	22.0 ± 0.4	21.9 ± 0.6[a]
No. of pups at day 0					
Total pups born/litter	(Mean ± SD)	13.9 ± 1.9	13.8 ± 2.5	14.6 ± 2.0	14.6 ± 2.6
Live-born/litter	(Mean ± SD)	13.8 ± 1.9	13.6 ± 2.5	14.5 ± 1.9	14.6 ± 2.6
Stillborn/litter	(Mean ± SD)	0.0 ± 0.2	0.2 ± 0.6	0.1 ± 0.3	0.0 ± 0.0
Gestational index	%	100.0	100.0	100.0	100.0
Stillborn index (%/litter)	(Mean ± SD)	0.30 ± 1.5	1.11 ± 4.2	0.78 ± 2.1	0.0 ± 0.0
Total implantation scars/litter	(Mean ± SD)	15.3 ± 1.7	15.0 ± 2.0	15.5 ± 1.7	15.6 ± 2.3
No live-born pups/litter (day 4)	(Mean ± SD)	13.4 ± 1.6	13.4 ± 2.4	14.1 ± 1.6	14.1 ± 2.3
Sex ratio (% males/animal)					
Pups day 0	Mean%/litter (±SD)	46.69 ± 11.6	44.01 ± 12.5	50.99 ± 12.5	50.19 ± 13.2
Pups day 4	Mean%/litter (±SD)	45.98 ± 12.9	43.70 ± 12.3	51.44 ± 12.3	50.68 ± 12.6
Pup survival indices					
Viability index (0–4 days)	Mean%/litter (±SD)	97.31 ± 5.2	98.95 ± 2.5	97.67 ± 4.6	97.40 ± 4.0

[a]Significantly different ($p < 0.05$) from control.

Table 10
Reproductive Phase 2: Summary of F2 Natural Delivery and Litter Data in Naïve Females Mated to Exposed Male Rats

Endpoint		0 mg/kg	5 mg/kg	25 mg/kg	150 mg/kg
No. females on study		25	25	25	25
No. females pregnant		25	24	25	25
Female fertility index		100.0	96.0	100.0	100.0
Females delivering litters	*N*	25	24	25	25
	%	100.0	100.0	100.0	100.0
With stillborn pups	*N*	0	3	3	2
	%	0.0	12.5	12.0	8.0
With all stillborn	*N*	0	0	0	0
	%	0.0	0.0	0.0	0.0
Gestational length (days)	(Mean ± SD)	22.4 ± 0.5	22.2 ± 0.5	22.1 ± 0.4	22.0 ± 0.3[a]
No. of pups at day 0					
Total pups born/litter	(Mean ± SD)	14.1 ± 2.6	14.3 ± 2.1	14.3 ± 2.2	14.6 ± 2.7
Live-born/litter	(Mean ± SD)	14.1 ± 2.6	14.0 ± 2.1	14.2 ± 2.2	14.5 ± 2.7
Stillborn/litter	(Mean ± SD)	0.0 ± 0.0	0.3 ± 1.0	0.1 ± 0.3	0.1 ± 0.3
Gestational index	%	100.0	100.0	100.0	100.0
Stillborn index (%/litter)	(Mean ± SD)	0.0 ± 0.0	1.87 ± 6.6	0.82 ± 2.3	0.53 ± 1.8
Total implantation scars/litter	(Mean ± SD)	15.2 ± 2.3	15.2 ± 2.1	15.5 ± 2.0	15.6 ± 1.9
No. live-born pups/litter (day 4)	(Mean ± SD)	13.8 ± 2.5	13.6 ± 2.1	13.6 ± 3.3	14.2 ± 2.7
Sex ratio (% males/animal)					
Pups day 0	Mean%/litter (± sd)	49.20 ± 12.1	47.86 ± 15.1	51.57 ± 11.3	50.73 ± 15.2
Pups day 4	Mean%/litter (± sd)	49.12 ± 12.0	47.90 ± 14.9	53.43 ± 14.3	50.36 ± 14.6
Pup survival indices					
Viability index (0–4 days)	Mean%/litter (± sd)	97.98 ± 3.5	97.70 ± 3.9	95.29 ± 16.3	97.48 ± 3.9

[a]Significantly different ($p < 0.05$) from control.

meaningful. There were a few observations of renal pelvic cavitation and distended ureters in both male and female animals from both the F_1 in utero exposed and naïve females that were distributed sporadically throughout the dose groups. However, these findings were not considered to be test article related, as they were relatively few in number and were also documented in the control animals. F_1 macroscopic observations revealed a few animals with mild renal pelvic dilatation in the treated groups, but these findings were not considered toxicologically meaningful as this finding has previously been observed at low incidence in F_1 control animals.

For the F_1 males, there were no statistically significant changes in organ weights for the cauda epididymis, epididymides, prostate, or the testes. A decreased seminal vesicle weight and organ to body weight ratio at 5 and 150 mg/kg was observed. These changes were not considered to be test article related, as the organ weight, and organ to body weight ratio for the seminal vesicles at the middose level (25 mg/kg) was equivalent to that of the controls. Therefore, no dose–response relationship could be established. For the F_1 females, there were no statistically significant changes in the ovarian weights, as compared to the controls (Supplemental Table 10). The sperm evaluations for the F_1 in utero exposed males were comparable among the groups and did not show any test-article–related effects.

DISCUSSION

This large combined multigeneration and developmental toxicity study in rats was conducted with HPC given over an extended interval during three phrases: fetal development (reproductive phase 1), following testicular differentiation (reproductive phase 2), and over the "critical period of organogenesis" (teratology phase). These treatment periods were chosen to cover the entire reproductive scenario in the rat, a species flagged for reproductive dysfunction in a previous but limited study in males (Pushpalatha et al., 2004), and to include the gestational period analogous to the recommended timing for use of the drug in pregnant women with a singleton pregnancy who have a history of singleton spontaneous preterm birth.

There are no published data to provide a rationale for the observed small difference in miscarriage and stillbirth rates and thus further results will be required from larger clinical trials that are currently ongoing.

Even though the maintenance of pregnancy requires a balance of hormone levels, the mechanism of action of HPC in maintaining pregnancy is largely unknown. It is known that sex steroids are able to affect cells and tissues that contain specific receptors to allow them to initiate various metabolic processes (Brent, 2005). Progesterone receptor B (PR-B) functions as a transcriptional activator of progesterone-responsive genes while progesterone receptor A (PR-A) functions as a transcriptional inhibitor of all steroid hormones (Wen et al., 1994). Through PR-A, all progestins, including HPC, down regulate target tissue estrogen receptors and stimulate pathways of estrogen metabolism (Schindler, 1996).

Early on in pregnancy, there are high levels of progesterone, initially supplied by the corpus luteum and subsequently the placenta. In late pregnancy as labor proceeds, progesterone and the progesterone receptors participate in the pharmacologic regulation of labor onset, by acting together to permit the process to proceed (Berghella, 2010). While in some animals parturition is triggered by a drop in circulating progesterone levels and an increase in circulating estrogen levels, this is not observed in humans and nonhuman primates. In Rhesus monkeys, there has been shown to be rather a functional change in progesterone receptors with an increase in PR-A and a decrease in PR-B (Haluska et al., 2002). HPC, however, has not been demonstrated to have a greater affinity for either PR-A or PR-B (Attardi et al., 2007). Given the relatively low levels of HPC relative to the progesterone increase from pregnancy and the greater affinity of progesterone compared to HPC for both PR-A and PR-B, it seems unlikely that the interaction with receptors would lead to either developmental or reproductive toxicity.

There were few adverse findings in any of the reproductive phases in rats with either gender. Adverse outcomes for the most sensitive parameters of therapeutic relevance to humans, namely viability at term or during lactation, length of gestation, and survival of offspring (in F_1 and F_2) litters were rare in this animal species. In the RP1 phase, treatment on GD 8, 14, and 20, yielded an increased but nonstatistically significant number of stillborns in the group treated with 150 mg/kg compared to the controls (Table 6). There was a nonstatistically significant increase in postimplantation loss with the same treatment in the teratology phase (Table 7), but in neither case did these effects alter the number of viable fetuses per litter, and fit within the historical control data of the laboratory. With regard to the effect of HPC in the rat model on the length of gestation, this parameter was statistically significantly shorter for the 150 mg/kg groups in RP2 for both the female- and male-treated subgroups mated to naïve animals (Tables 9 and 10). However, neither value was considered related to HPC treatment, representing only biological variation, as explained earlier. The third sensitive parameter, survival of the offspring, did not exceed any control value at any timepoint including livebirth, or on GD 4 (viability index) or GD 21 (lactation index or weaning). In addition to absence of significant reproductive effects on the most sensitive parameters as cited above, there were no biologically adverse findings of any type in the three-phase reproductive outcomes.

One potential limitation of the study was the marked difference in litter size and associated outcomes in the control animals. The supplier provided time-mated F_0 females for each phase, which were specified by the protocol to be within 8 to 10 weeks at the time of pairing at the supplier and weigh between 190 and 240 gm at arrival. Individual birth dates for each animal are not provided by the supplier, but animals were mated between 56 and 70 days of age for all three phases. It is unlikely that the difference in the litter size was correlated to the age of the parental females, particularly as the body weight range at arrival was similar between the time-mated females received for each phase. The differences in litter size identified are most likely a result of normal variability within this strain of rat when age is considered.

The published literature on HPC in animal reproductive studies supports the absence of toxicity at nonmaternally toxic doses and lack of toxicity to either reproduction in general or fetal development in laboratory animal studies. Studies in mice (Johnstone and Franklin, 1964; Seegmiller et al., 1983), rats (Lerner et al., 1962), squirrel monkeys (Aksel et al., 1991), and cynomolgus monkeys (Hendrickx et al., 1987) all demonstrated absence of toxicity when given in various situations during the reproductive cycle. Further, this extended study failed to corroborate the adverse effects reported in the limited study in male rats referred to earlier (Pushpalatha et al., 2004).

Since the publication of the National Institute Child Health and Human Development (NICHD) study of

HPC, the question of whether there is or is not a safety signal has been controversial (Meis et al., 2003). In that study, with a 2:1 randomization of HPC to placebo, there were five miscarriages and six stillbirths among 310 subjects in the HPC arm (3.6%) compared to zero miscarriages and two stillbirths in 153 patients on the placebo arm (1.3%). This was offset by the fact that the neonatal mortality rate was higher in the placebo arm (9/151, 6.0%) compared to in the HPC arm (8/295, 2.7%). None of these differences were statistically significant. The miscarriages and stillbirths were not classified by the investigators as related to HPC but were rather related to underlying obstetrical or maternal complications. Notably, having zero miscarriages in the placebo arm is below the expected baseline rate, particularly in this high-risk patient population, which may account for some of the apparent difference between treatment arms. Data presented at the Food and Drug Administration (FDA) Advisory Board for HPC compared the results seen in the placebo arm to other observational studies conducted by NICHD in subjects with a prior preterm birth that demonstrated miscarriage rates of 1.4 and 3.1% (Adeza Biomedical, 2006). Other randomized studies comparing HPC to placebo, performed in subjects with multiple gestation pregnancies, have failed to demonstrate any consistent trend suggesting increased fetal loss for subjects receiving HPC (Rouse et al., 2007; Caritis et al., 2009; Combs et al., 2010).

Progesterone receptors are expressed widely throughout the female reproductive tract, the male reproductive tract, and nonreproductive tissues such as the thymus, muscle, bone, central and peripheral nervous systems, lung, and islet cells of the pancreas. This in part has raised concerns about congenital anomalies being increased with the use of progesterone especially during the first trimester of pregnancy. This study has demonstrated in a rat model that there is no evidence of an increase in any anomaly in either the male of female reproductive tract or in any other organ system. Further, as described below, large case-control and cohort studies in humans have not demonstrated a pattern of an increase in malformations with the use of HPC.

A meta-analysis of studies reporting on almost 4000 human pregnancies demonstrated a negative association between the use of various hormones in pregnancy and the induction of congenital defects (Schardein, 1980). This review found that some synthetic progestins, such as ethisterone or norethindrone, have the potential for androgenic activity that could result in virilization of the female fetus, and that a few reported cases (at least 10 cases) have associated natural progesterone with virilization. The critical time for exposure appears to be in the first 10 weeks of gestation. A later meta-analysis reviewed 186 published articles and included 14 studies (seven cohort and seven case-control) involving 65,567 women. This meta-analysis also showed no association between exposure to sex hormones and external genital malformations (Raman-Wilms et al., 1995).

In the published NICHD study, there were nine babies found to have congenital anomalies and these were equally divided between groups. This included one HPC subject with a micropenis and testes; testicular infarction secondary to intrauterine torsion and possible hypogonadism (Meis et al., 2003). Further, in a follow-up study performed in 278 subjects (194 randomized to HPC and 84 to placebo) followed at a mean age of 48 months (range 30–64), there was no evidence of a difference in the rate of developmental delays in subjects receiving HPC (Northen et al., 2007).

Special labeling was required by FDA for synthetic and natural progestins between 1977 and 1999 because it was believed that there was an association between progestins and nongenital malformations (congenital heart and limb-reduction defects). This link has not been confirmed in subsequent studies and therefore FDA removed this requirement in 1999 (Brent, 2005).

A cohort study of 13,643 pregnancies in Germany found no increase in major malformations in 460 cases of first trimester exposure to HPC compared to controls (Michaelis et al., 1983). Another cohort of 24,000 pregnancies delivered in Olmstead County, Minnesota in 1936 to 1974 found that the 649 offspring exposed to HPC had no increase in congenital anomalies compared to controls. For 501 infants exposed to only HPC (i.e., no other progestin), the median total exposure was 1625 mg (range: 125–11,250 mg). A notable feature of this study was the long period of follow-up of the children, with a mean of 11.5 years (Resseguie et al., 1985).

The teratogenic potential of HPC was evaluated in pregnant women in Hungary (Dudas et al., 2006). Cases with congenital anomalies were selected from the Hungarian Case-Control Surveillance of Congenital Abnormalities (HCCSCA) and controls matched for sex, birth week in the year when the case was born, and the district of parents' residence were selected from the national Birth Registry of the Central Statistical Office for the Hungarian Case-Control Surveillance of Congenital Abnormalities. There was no difference in the number of HPC-treated women between cases and controls. Further, no association was found between any type of congenital anomaly and HPC treatment given during the second and/or third month of pregnancy. When HPC treatment was examined "any time during the pregnancy," a difference was seen in the limb deficiency congenital anomaly group compared to the matched control group. However, this finding was not replicated in the HPC treatment period of the second and/or third months, the critical period of major congenital anomalies. The authors concluded daily HPC administration did not indicate any teratogenic potential during early pregnancy.

The findings of this study in a laboratory animal under controlled conditions also show no adverse effects on development. There were no observed effects in the F_1 offspring and no evidence of a decreased ability of either males or females to produce the next generation. There were similar sperm counts observed in treated and untreated males. Likewise, there was no evidence of effects seen in the F_2 generation indicating a carryover from in utero exposure of the F_1 animals. While numerical increases in some parameters in individual studies with small numbers of events were seen, these may be entirely due to chance. No animal study can rule out the possibility of rare events in humans and so further clinical studies in humans are required. A large ongoing study enrolling over 1700 subjects to look for relatively rare events is currently ongoing.

The body of scientific evidence indicates no reproductive or teratogenic activity of HPC when administered after the first trimester, as indicated for the reduction of the risk of preterm birth.

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SUPPORTING INFORMATION

Table S1 Reproductive Phase 1 Summary of F_1 Physical Development and Sexual Maturity

Table S2 Reproductive Phase 1 Summary of F_1 Behavioral Observation (Motor Activity) and Passive Avoidance—Male

Table S3 Reproductive Phase 1 Summary of F_1 Behavioral Observation (Motor Activity) and Passive Avoidance—Female[a]

Table S4 Reproductive Phase 1 Summary of F_1 Organ Weight Values

Table S5 Reproductive Phase 2 Summary of F_1 Physical Development and Sexual Maturity

Table S6 Reproductive Phase 2 Summary of F_1 Behavioral Observation (Motor Activity) and Passive Avoidance—Male

Table S7 Reproductive Phase 2 Summary of F_1 Behavioral Observation (Motor Activity) and Passive Avoidance—Female

Table S8 Reproductive Phase 2 Summary of F1 Reproductive and Fertility Parameters in Exposed Females Mated to Naïve Male Rats

Table S9 Reproductive Phase 2 Summary of F1 Reproductive and Fertility Parameters in Naïve Females Mated to Exposed Male Rats

Table S10 Reproductive Phase 2 Summary of F_1 Organ Weight Values