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February 23, 2004

Food and Drug Administration
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RE: PMA M020018/M5 Mentor Low-Bleed Gel-Filled Mammary Prosthesis:

Final Report: Extended One-Generation Reproductive and Developmental Study of Silicone Gel (Mentor Silicone Gel) Administered by Subcutaneous Implantation in CD® (Sprague-Dawley) Rats

Mentor Corporation is submitting the above referenced report in support of PMA M020018/M5.

We consider the existence of this submission and its contents to be confidential and exempt from public disclosure.

If additional information is needed, please contact me at (805) 879-6168.

Sincerely,

A handwritten signature in cursive script that reads "Donna Free for M. Michael".

Mack Michael, M.D.
Medical Director, Vice President
Regulatory and Clinical Submissions

2005-410101-01-02 - FINAL REPORT - MENTOR

VOLUME I OF II

FINAL REPORT

Title: Extended One-Generation Reproductive and Developmental Study of Silicone Gel (Mentor Silicone Gel) Administered by Subcutaneous Implantation in CD® (Sprague-Dawley) Rats

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Study Initiation Date: July 18, 2003

In-life Performance Dates: July 28 – December 29, 2003

Final Report Date: April 22, 2004

RTI Identification Number: 08847.000.100

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RTI Protocol No.: RTI-878

RTI Study Code: Rt03-SIL

Extended One-Generation Reproductive and Developmental Study of Silicone Gel
(Mentor Silicone Gel) Administered by Subcutaneous Implantation in CD®
(Sprague-Dawley) Rats

ABSTRACT

This study evaluated the effects, if any, of subcutaneous implantation of Mentor Silicone Gel (at 0, 3, 10, and 30 ml/kg) in female CD® (Sprague-Dawley) rats four weeks before mating with untreated males through adulthood of the F1 litters. The control group was implanted with carboxymethylcellulose (CMC) formulated into a gel with sterile saline to provide viscosity, weights, and volumes of gel material equivalent to the high-dose test gel group. The 25 F0 dams with litters/group were evaluated for survival, body weights, feed consumption, clinical observations during the prebreed, mating, gestational, and lactational periods, estrous cyclicity for two weeks prior to mating, and for reproductive (mating, gestational, and lactational) parameters. At scheduled necropsy of the F0 females (at weaning of their F1 litters), organs were weighed, examined grossly, and subjected to histopathologic evaluation (25/group, high dose and control groups). The F1 litters during lactation were evaluated for survival, growth (body weights), sex ratio, and acquisition of a series of developmental landmarks in both sexes, including anogenital distance (on postnatal day [pnd] 0 and 21), pinna detachment, surface righting reflex, incisor eruption, auditory startle, and eye opening. In addition, preweaning F1 male pups were examined for retention of nipples and/or areolae on pnd 11-13. Selected F1 males and females (approximately two/sex/litter) were necropsied at weaning on pnd 21. Anogenital distance was measured in both sexes, examination for retained nipples and/or areolae was done on the males, and organs were weighed and examined grossly.

The selected F1 postweanlings (up to three/sex/litter; 75/sex/group) were retained to adulthood, with assessment of survival, growth, clinical observations, acquisition of puberty (vaginal patency in females, preputial separation in males), auditory startle, motor activity, and learning and memory. In addition, estrous cyclicity was evaluated in F1 females for the last three weeks of the retention period. F1 females were necropsied on pnd 70 ± 5 and F1 males on pnd 85 ± 5. At the F1 necropsy, organs from both sexes were weighed, examined grossly, and subjected to histopathological evaluation (25/sex/group, high dose and control groups). F1 males

were subjected to andrological assessment, including cauda epididymal sperm motility and progressive motility (75/group; all groups), cauda epididymal sperm number and morphology, and testicular homogenization-resistant spermatid head counts, to calculate daily sperm production (DSP) and efficiency of DSP (75/group; high dose and control groups).

The summarized results of the study are as follows.

F0 Females

On study day (sd) 0, the day of test gel implantations, mean body weights were equivalent across all four groups. By sd 7, the CMC gel-implanted control females exhibited major fluid retention around the implant site, resulting in excessive weight gain (significantly greater than in the test gel groups). On sd 8, the study veterinarian aspirated the excess fluid so that the CMC control group females exhibited reduced body weights relative to the test gel groups on sd 14. From that time forward, the growth curves of the CMC control females were equivalent to the test gel group females throughout the prebreed period (ending on sd 28), gestation, and lactation. The test gel groups exhibited equivalent body weights during these periods. Weight gains during these periods were also equivalent across all groups. Maternal feed consumption was equivalent across all three test gel groups (and lower in the CMC control group). There were no treatment- or dose-related incidences in or severities of clinical observations in the F0 dams across all groups during these periods. F0 estrous cyclicity parameters during sd 14-28 were equivalent across all four groups.

During gestation, the test gel groups exhibited equivalent body weights and weight gains. The CMC control group exhibited lower body weights but equivalent weight gains to the test gel groups during this period. Feed consumption was equivalent across the three test gel groups and lower in the CMC control group. Again, clinical observations were unremarkable.

During lactation, the test gel groups exhibited equivalent body weights and weight gains. The CMC control group body weights were lower on pnd 4 and 14 but equivalent across all four groups on pnd 7 and 21. Weight gain was approximately equivalent across all four groups for pnd 0-4, 4-7, and 7-14. For pnd 14-21, the CMC control dams lost less weight than the 3 ml/kg group and lost comparable weight to the 10 and 30 ml/kg groups. Maternal lactational feed consumption was equivalent across all four groups throughout lactation. Maternal clinical observations were unremarkable.

F0 Reproduction and Lactational Indices

Precoital interval (time to insemination) and gestational length were equivalent across all four groups. The number of uterine nidation (implantation) scars, and therefore total and live pups/litter, were reduced in the CMC gel control group and equivalent across all test gel groups. Percent postimplantation loss, number of dead pups/litter, and stillbirth and live birth indices were all equivalent across the four groups, as were the F1 pup survival indices throughout lactation.

F1 Litters During Lactation

The number of live pups/litter was equivalent across the test gel groups and lower in the CMC control group for pnd 0 and 4 (precull). Once the litters were standardized to ten pups/litter on pnd 4, litter size was equivalent across all four groups. Pup body weights/litter (separately by sex or combined) were equivalent across all four groups for all lactational time points (pnd 0, 4, 7, 14, and 21), except for a slight but significant reduction in combined pup weight (but not for sexes separately) only on pnd 0 at the high dose (30 ml/kg). Sex ratio (% males)/litter was equivalent across all four groups throughout lactation.

Anogenital distance (absolute or adjusted for pup body weight) was equivalent across all four groups for both males and females on pnd 0 and 21. There were no retained nipples in any preweaning F1 males in any group. The incidence of retained areolae (no./male and no. males with ≥ 1 areolae) was also equivalent across all four groups. Acquisition of all evaluated preweaning development landmarks (pinna detachment, surface righting reflex, incisor eruption, eye opening, and auditory startle) was equivalent across all groups. Neonatal deaths were few and equivalent across all groups for pnd 0-4 and 5-21. There were no treatment- or dose-related incidences or severities of pup clinical observations or for necropsy observations of dead pups during lactation.

F1 Pup Necropsy

F1 Male Pups

There were 37, 47, 44, and 50 F1 pups necropsied on pnd 21 at 0, 3, 10, and 30 ml/kg, respectively. Anogenital distance (absolute or adjusted for body weight) was equivalent across all four groups. There were no retained nipples in any weanling male in any group. The incidence of retained areolae was equivalent across all four groups. Sacrifice body weights were

equivalent across 0, 3, and 10 ml/kg and slightly reduced ($p < 0.05$) at 30 ml/kg. Absolute organ weights were equivalent across all four groups, except for paired testis weights that were significantly reduced at 3 and 30 ml/kg when compared to the CMC control value. The values for the three test gel groups were equivalent. Organ weights relative to terminal body weights were also equivalent across all four groups, except for relative prostate weight, which was significantly reduced ($p < 0.05$) at 10 ml/kg and unaffected at 3 and 30 ml/kg. Gross necropsy findings were unremarkable.

F1 Female Pups

There were 42, 43, 46, and 48 F1 female weanlings necropsied on pnd 21 at 0, 3, 10, and 30 ml/kg, respectively. Anogenital distance (absolute and adjusted for body weight) was significantly shorter in the CMC control group and equivalent across the three test gel treatment groups. Sacrifice body weights were equivalent across all four groups. Absolute organ weights were equivalent across all four groups. Organ weights, relative to terminal body weights, were equivalent for all four groups for the brain, liver, spleen, and uterus plus cervix and vagina. Relative paired kidney weight was significantly reduced at 30 ml/kg, and relative paired ovary weights were significantly reduced at 10 and 30 ml/kg, relative to the CMC control relative organ weight values.

F1 Pup Necropsy Findings

There were no treatment- or dose-related gross necropsy findings in any group in either sex.

F0 Female Necropsy

At necropsy of F0 dams on the weaning day of their litters, gel material was retrieved in all dams in the test gel groups and in no dams in the CMC control group. Sacrifice body weights were equivalent across all four groups. Absolute organ weights were also equivalent across all four groups, except for absolute liver weight that was reduced at 30 ml/kg. Organ weights, relative to terminal body weights, were also all equivalent across the four groups, except for relative liver weight which was significantly reduced in all three test gel-implanted groups, relative to the CMC control group value, but equivalent across the three test gel groups. F0

female gross necropsy and histopathology findings exhibited no treatment- or dose-related incidence or severity.

F1 Females

During the seven-week retention period, there were no treatment- or dose-related effects on body weights, body weight changes, or in feed consumption. Acquisition of vaginal patency was delayed at 3 and 10 (but not 30) ml/kg, with body weights at acquisition increased only at 3 (but not 10 or 30) ml/kg. Adjusted age at acquisition was delayed only at 10 ml/kg. There were no treatment- or dose-related effects on auditory startle, motor activity, learning and memory, functional observational battery (FOB), or grip strength. F1 vaginal cyclicity was unaffected across groups except for the percentage of F1 females with one or more abnormal cycles, which were elevated at 3, 10, and 30 ml/kg. At necropsy, there were no effects on absolute and relative organ weights except for pituitary and uterus plus cervix and vagina that were increased at 30 ml/kg, due to the low values for these parameters in the control group. There were no treatment- or dose-related findings for the gross or histopathologic examinations.

F1 Males

During the nine-week retention period, there were no treatment- or dose-related effects on body weights, body weight changes, or feed consumption. The absolute and adjusted ages at acquisition of preputial separation were equivalent across all groups. Body weight at acquisition was increased at 10 (but not 3 or 30) ml/kg. There were no differences across groups for auditory startle, motor activity, learning and memory, or in FOB or grip strength. At necropsy, the terminal body weights were equivalent across all groups. Absolute and relative organ weights were also equivalent across all groups except for pituitary and paired testes weights that were increased with no dose-response pattern. The increased weights in the treated groups were due to the low values for these parameters in the control group. There were no treatment- or dose-related findings for the gross or histopathologic examinations.

Conclusions

Based on this study in rats, implantation of the test gel at 3, 10, or 30 ml/kg had no effect on the F0 dams' growth, estrous cycling, fertility, fecundity, pregnancy, delivery, or lactation.

The F1 offspring from test gel-implanted dams also showed no effect on survival, growth, acquisition of developmental landmarks, learning and memory, FOB, hormone-mediated endpoints (e.g., anogenital distance, retained nipples and/or areolae in preweanling males), systemic or reproductive organ weights, and gross or histopathologic lesions of organs in either F1 males or females.

OBJECTIVES

This study was intended to evaluate the potential of silicone gel (Mentor Silicone Gel) to produce effects on reproduction and development in CD® rats by subcutaneous implantation in the F0 females 28 days prior to mating. Maternal F0 animals were evaluated for effects of the test article on mating, gestation, parturition, and lactation. F1 animals were evaluated through adulthood for any potential systemic, developmental, neurobehavioral, immunologic, and/or reproductive effects from possible *in utero* and/or lactational exposure, or from any potential indirect effects of the gel on the F0 dams.

MATERIALS AND METHODS

Test Substance and Dose Formulations

The test substance, Mentor Silicone Gel (from the commercial product Siltex® low bleed, gel-filled mammary prosthesis) was received from Mentor Corporation (Santa Barbara, CA) as six sealed units on July 11, 2003. The test substance was a colorless, viscous liquid gel and was identified by the supplier as Lot No. 257949 (Reference No. 354-3007). There is no CAS No. for the test substance (test substance is the end-product gel from reaction of Gel 2167 with Gel 2168). The purity was not provided to RTI; it was provided to the FDA in the PMA (Pre-Market Approval) application. The gel from the actual commercial product was used. The control gel was carboxymethylcellulose (CMC; from Sigma Chemical Company, St. Louis, MO; 1000 centipoise), formulated as a viscous gel in sterile saline at approximately the same viscosity (3.5%) as the high dose test gel. No verification of stability of the test gel material was performed for this study, as the test substance is considered stable at room temperature (see protocol, Appendix V).

Chemical analyses of Mentor Silicone Gel were performed by the Sponsor. The Sponsor maintains documentation of chemical identity, purity, and stability data for Mentor Silicone Gel.

The density of Mentor Silicone Gel was confirmed by RTI. RTI also formulated the control CMC gel and confirmed its density.

Animals and Husbandry

One hundred twenty-two (122) virgin female rats (approximately nine weeks of age and approximately 200-225 g on arrival) and 122 male rats (approximately ten weeks old and 326-350 g on arrival) were received from Charles River Breeding Laboratories (Raleigh, NC) on July 28, 2003 (females) and August 25, 2003 (males). Females were nulliparous and nonpregnant. There were initially 120 animals (30 females/group in four groups) assigned to the study at time of implantation of the gels, with 25 females with litters/group randomly selected to continue on study once all dams had littered; two females and two males from the animals received were designated as sentinels. The actual dates of all major phases of the study are presented in Table A.

Table A. Study Schedule

Event	Dates
Animals arrived at RTI:	July 28, 2003
Implantation of test and control gels:	August 4-6, 2003
Animals paired:	September 1-3, 2003
Gestational day 0:	September 2 (first) – 15 (last), 2003
Postnatal day 0:	September 24 – October 7, 2003
F1 weaning (pnd 21):	October 15-28, 2003
F1 weanling necropsy:	October 15-28, 2003
F0 maternal necropsy:	October 15-28, 2003
F1 female necropsy:	December 1-12, 2003
F1 male necropsy:	December 15-29, 2003

Twenty-eight (28) days after implantation of the control and test gels, 120 male rats of the same strain from the RTI breeding colony (that arrived on August 25, 2003, from the same supplier) were used to generate timed-mated females. Female rats were approximately 14 weeks of age on gestational day (gd) 0. One hundred (100) female rats with litters (designated the F0 generation) were selected, once all females had littered, and were used in this study (i.e., four groups of 25 dams with litters). The remaining females not put on study, or not selected for retention, were euthanized by CO₂ asphyxiation or transferred into the IACUC training protocol.

The shipment of animals was quarantined on arrival for approximately seven days under test conditions. Animals were evaluated for endo-parasites, clinical signs, and acclimation to husbandry by the veterinarian and animal care staff. A serological health report was obtained from the supplier. They were observed daily for general health status and ability to adapt to the water bottles.

During the quarantine period, two female and two male rats were selected as sentinels. They were singly housed in the study room(s) in polycarbonate solid-bottom cages, with bedding with feed and water available *ad libitum*. The sentinels were examined once daily by cage-side observation for morbidity or mortality at the same time as clinical observations or morbidity/mortality checks for the study animals. The clinical condition of the sentinel animals were recorded only in the event that an animal was moribund or found dead. If a sentinel animal was terminated moribund, blood was collected at termination and serum samples frozen. No sentinels were found moribund or dead. At the time of the F0 female necropsy, the female sentinels were terminated. At the time of the last F1 necropsy, the male sentinels were terminated, blood samples collected, and serum samples prepared. All sentinel serum samples were submitted to BioReliance Corporation (Rockville, MD) for their Level 1 rat antibody screen. The screen evaluated the samples for the presence of antibodies against the following pathogens: Toolan H-1 virus (H-1), Sendai virus, Pneumonia virus of mice (PVM), rat coronavirus/sialodacryoadenitis (RCV/SDA), Kilham rat virus (KRV), CAR Bacillus, *Mycoplasma pulmonis* (*M. pul.*), and parvovirus.

All animals were weighed at least once during acclimation. The weight variation of the study females at initiation did not exceed $\pm 20\%$ of the mean weight. Animals were uniquely identified prior to initiation of treatment by eartag. The method of identification and animal numbers were documented in the study records.

Discomfort or injury to animals was limited in that if any animal became severely debilitated or moribund, it was humanely terminated by CO₂ asphyxiation. All necropsies were performed after terminal anesthesia with CO₂. F1 pnd 4 culled pups were euthanized by decapitation. Animals were not subjected to undue pain or distress.

The experiment was carried out under standard laboratory conditions. During the seven-day quarantine period, the males and females were singly housed in solid-bottom, polycarbonate cages (8"x19"x10.5"). The cages of the F0 animals and F1 weanlings were fitted with stainless-

steel wire lids (Laboratory Products, Rochelle Park, NJ). Sani-Chip® cage bedding (P.J. Murphy, Forest Products, Inc., Montville, NJ) was used in all cages.

All animals were housed in the RTI Animal Research Facility following arrival at RTI and for the duration of the study. RTI animal rooms are air-conditioned, and temperature and relative humidity are continuously monitored, controlled, and recorded using an automatic system (Siebe/Barber-Colman Network 8000 System with Revision 4.4.1 for Signal software (Siebe Environmental Controls [SEC]/Barber-Colman Company, Loves Park, IL). The target environmental ranges were 66-77°F (22°C ± 3°C) for temperature and 30-70% relative humidity, with a 12-hour light cycle per day (NRC, 1996). There were no temperature and/or relative humidity (RH) excursions in Animal Rooms 207 (F0 females through weaning of F1 litters) and 208 (mating of F0 females) outside the target ranges during quarantine (7/28 – 8/3/03: temperature, 71.3-72.9°F; RH, 52.4-56.1%) and prebreed (8/4 – 9/1/03: temperature, 71.1-76.3°F; RH, 37.4-59.3%). During gestation through lactation (9/2 – 10/28/03: temperature, 69.7-74.7°F; RH, 40.8-66.2%), RH exceeded the specified range for two hours on 9/4/03, with the highest reading of 80.0%. The only excursion for F0 females was therefore in RH (for two hours on one day) during gestation/lactation; it was considered minor and transient, with no effect on the study.

For the F1 female holding period in Animal Room 206 (10/18 – 12/12/03), temperature range was 69.1-75.7°F and RH range was 39.9-64.1%. For the F1 female holding period in Animal Room 404 (10/15 – 12/4/03), temperature range was 70.3-73.0°F and RH range was 43.0-61.5%. There were no temperature or RH excursions in either room. For the F1 male holding period in Animal Room 207 (10/15 – 12/29/03), temperature range was 69.6-73.5°F and RH range was 44.5-69.7% (four minor excursions: one hour at 71.5% on 11/20/03, one hour at 81.2% on 11/25/03, one hour at 73.5% on 12/3/03, and one hour at 70.3% on 12/7/03). For the F1 male holding period in Animal Room 403 (10/15 – 12/19/03), temperature range was 70.7-73.5°F and RH range was 37.0-65.0% (with no temperature excursions). For the F1 postwean offspring, there was one temperature excursion for F1 postweanling males on 11/25/03 for one hour at 77.2°F. There were four RH excursions for F1 males and one temperature excursion (only in Animal Room 207). These were few, minor, and transient with no effect on the study.

At all times, the animals were handled, cared for, and used in compliance with the NRC *Guide for the Care and Use of Laboratory Animals* (NRC, 1996).

Pelleted Purina Certified Rodent Diet® (No. 5002, PMI Feeds, Inc., St. Louis, MO) was available *ad libitum*. The analysis of each feed batch for nutrient levels and possible contaminants was performed by the supplier, examined by the Study Director, and maintained in the study records. Contaminant levels were below certified levels and did not affect the design, conduct, or conclusions of this study. The feed was stored at approximately 60-70°F, and the period of use did not exceed six months from the milling date.

Deionized water (produced at RTI from tap water; source: City of Durham, Department of Water Resources, Durham, NC) was available *ad libitum* by plastic water bottles with butyl rubber stoppers and stainless-steel sipper tubes for F0 females and F1 weanlings. The water for the males was provided by an automatic watering system (Edstrom Industries, Inc., Waterford, WI); the F0 females during the mating period (cohabited with the breeder males) were also on the automatic watering system. Study females were watched for their adaptation to water bottles. Contaminant levels of the Durham City water are measured at regular intervals by the supplier per EPA specifications and by Balazs Analytical Laboratories, Inc. (Freemont, CA). These analyses were examined by the Study Director, and the analyses (with documentation) were maintained in the study records. Contaminant levels were below the maximal levels established for potable water and did not affect the design, conduct, or conclusions of this study.

For breeding, individual females with implanted gels were placed in the home cage of singly-housed males (i.e., one male and one female). On the following morning and each morning thereafter, the females were examined for the presence of vaginal sperm or a vaginal copulation plug (Hafez, 1970). The day on which vaginal sperm or plugs were found was designated as gd 0. Those females were presumed pregnant. The initial sperm-positive females (dams), designated the F0 generation, were housed individually or with their litters until scheduled sacrifice. Sperm-negative females were retained in the same male's cage and checked for sperm or vaginal plug on successive mornings until insemination occurred or for two weeks, whichever came first. Females that remained sperm negative were retained until they delivered or until gd 26, after removal from the breeding male. When females for all treatment groups were selected, the remaining females were sacrificed by asphyxiation with CO₂. The fate of all animals was fully documented.

Mentor Silicone Gel or CMC was administered by subcutaneous implantation. On each morning (of three) of gel implantation, each female was arbitrarily selected into one of the four

groups, weighed and anesthetized (by inhalation of isoflurane to effect), and had the fur on both sides of the lower back shaved. A surgical scrub using alcohol and Betadine® swabs was applied to sterilize the insertion sites. Mentor Silicone Gel or CMC was implanted in the test animal through two small incisions in the skin of the lower back on either side of the spine (all animals had two incisions made, with implantations in both incisions). After the incisions were made, a subcutaneous pocket was formed. A prefilled sterile syringe was inserted into the pocket, the gel injected, and the syringe withdrawn. The volume and weight of each inserted gel were based on the predicted body weight of each female as 300 g on gd 0 (see Table B). The incisions were closed with wound clips. The wound clips were removed approximately seven to ten days after surgery.

Study Design

The study began with four groups of 30 females/dose group (arbitrarily chosen) in four dose groups prior to implantation of the test or control gel. After mating, all females were retained until delivery or until gd 26. Twenty-five (25) females per group with litters were randomly selected to continue on study after delivery of all litters. The remaining females (and their litters, if present) were removed from study, euthanized, and discarded. All data were collected for all dams with/without litters until selection. Only data from the 25 selected dams/group with litters were reported (all data from all F0 females are retained in the study records). See Table B for target dosage concentrations and Figure 1 for a graphical representation of the study design.

Table B. Target Dosage Concentrations

Group Number	Number of Females Implanted	Dose Level (ml/kg)	Amount of Gel Implanted ^a	
			(ml)	(g) ^b
1	25	0 (control)	9.0 (4.50 per side)	– ^c
2	25	3	0.9 (0.450 per side)	0.87 (0.44 per side)
3	25	10	3.0 (1.50 per side)	2.91 (1.46 per side)
4	25	30	9.0 (4.50 per side)	8.73 (4.36 per side)

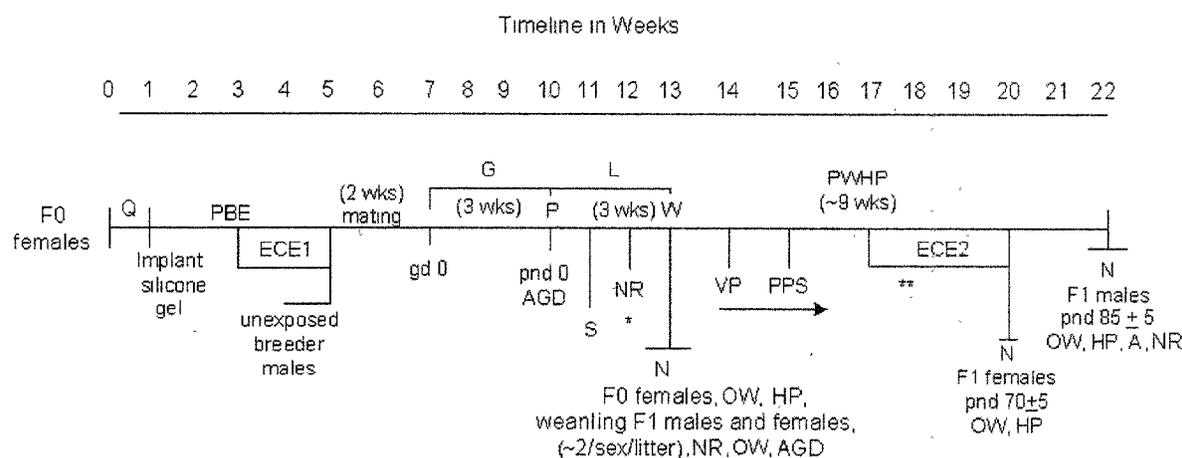
^a Based on the predicted female weight at time of mating (300 g).

^b Based on the density of the test gel: 0.97 g/ml.

^c The CMC (control) gel was at the same volume as the high dose test gel.

The doses selected by the Sponsor were 0, 3, 10, and 30 ml/kg. The 0 ml/kg was designated the control group, with the animals implanted with approximately 30 ml/kg of CMC gel of similar density to the test gel. The doses selected were the same as those employed in a previous study on a different silicone gel (Schardein, 1991; Siddiqui et al., 1994). In addition, the top dose exceeded the potential exposure of a 60 kg woman to complete rupture of two maximum-size 800 ml silicone gel implants ($1600 \text{ ml} / 60 \text{ kg} = 26.7 \text{ ml/kg}$).

Figure 1. Study Design



Key.

Q = quarantine (1 wk)
 PBE = prebreed exposure for F0 females (4 wks)
 ECE1 = F0 estrous cycle evaluation (last 2 wks of PBE)
 gd = gestational day
 G = gestation
 P = parturition (birth)
 pnd = postnatal day
 AGD = anogenital distance
 S = standardize litters to 10 pups on pnd 4 with equal sex ratio
 L = lactation
 NR = nipple and/or areolae retention in F1 males on pnd 11-13, pnd 21 and/or pnd 85 ± 5
 PWHP - postwean holding period for 3 F1 offspring/sex/litter

W = weaning on pnd 21
 N = necropsy
 VP = examination of retained F1 females (3/litter) for acquisition of vaginal patency
 PPS = examination of retained F1 males (3/litter) for acquisition of preputial separation
 * = acquisition of preweaning developmental landmarks
 ** = evaluation of postweaning motor activity, learning and memory, puberty
 ECE2 = F1 estrous-cycle evaluation (last 3 wks of holding period)
 OW = organ weights
 HP = histopathology
 A = andrology

F0 Animals

Observations for mortality were made twice daily (a.m. and p.m.), and the general condition of all animals was checked daily. Clinical observations were conducted and recorded at least once daily throughout the course of the study. This record included the day of onset and degree and duration of symptoms. These cageside observations included, but were not limited to, changes in: skin and fur, eyes, mucous membranes, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, and behavior pattern.

Observations were made for (but not limited to):

- a. Any response with respect to body position, activity, coordination, or gait
- b. Any unusual behavior such as head flicking, compulsive biting or licking, circling, etc.
- c. The presence of:
 1. Convulsions, tremors, or fasciculations
 2. Increased salivation
 3. Increased lacrimation or red-colored tears (chromodacryorrhea)
 4. Increased or decreased urination or defecation (including diarrhea)
 5. Piloerection
 6. Mydriasis or miosis (enlarged or constricted pupils)
 7. Unusual respirations (fast, slow, labored, audible, gasping, or retching)
 8. Vocalization
 9. Status of the subcutaneous implantation sites

F0 female body weights were determined and recorded initially on the day of implantation and weekly until mating. Body weights and clinical observations were determined and recorded in sperm-positive females on gd 0, 3, 6, 9, 12, 15, 18, and 20, and on pnd 0, 4, 7, 14, and 21. Feed consumption collection corresponded with the collection of the animals' body weights and was not measured during mating (when two animals were in the same cage).

For the last two weeks of the prebreed exposure period, estrous cyclicity was determined for all of the surgically implanted F0 females by daily vaginal lavage. Each vaginal smear was placed on a clean microscope slide (approximately six smears/slide), dried, stained with Toluidine blue (performed at EPL, Inc.), and examined by RTI staff to ascertain the presence or

absence of estrous cycling, the mean length of the estrous cycles per female, and any abnormalities in the cycles.

Beginning on gd 20, each F0 female was examined twice daily (a.m. and p.m.) for evidence of littering. Females who littered at morning and afternoon checks had this information recorded on the gestational sheet. Signs of dystocia or other signs of difficulty at parturition were recorded. Dams that did not produce a litter by calculated gd 26 were necropsied. Apparently nonpregnant uteri were stained with potassium ferricyanide to confirm pregnancy status. Any dams whose whole litters were born dead or died prior to pnd 21 were sacrificed and the number of uterine implantation scars recorded.

F1 Progeny During Lactation

All pups were counted, sexed, weighed, and examined as soon as possible on the day of birth (designated as pnd 0) to determine the number of viable and stillborn for each litter. Thereafter, litters were evaluated for survival, sex, gross observations, and body weights on pnd 4, 7, 14, and 21. After weaning, the F1 animals were weighed weekly, with feed consumption recorded weekly until scheduled sacrifice. Any pup that appeared moribund or that died during lactation was necropsied, when possible, to investigate the cause of death and internal status of the reproductive system. No organs were weighed or saved.

On pnd 4, the size of each litter was adjusted to ten pups, with five males and five females, if possible. Natural litters with ten or fewer pups were not culled. The culled pups were examined visceraally to confirm the internal sex. The F0 dams were allowed to rear their remaining F1 young to pnd 21.

Prewaning pups were evaluated until acquisition (or until culling on pnd 4) for surface righting reflex and pinna detachment starting at pnd 1, incisor eruption starting at pnd 8, auditory startle starting at pnd 10, and eye opening starting at pnd 11.

Anogenital distance was recorded with the individual pup weight on pnd 0 using an ocular reticule calibrated to a stage micrometer (precision to 0.1 mm) and at pnd 21 weaning necropsy using Vernier calipers (precision to 0.1 mm). The presence or absence of retained nipples and areolae on the ventrum was recorded for all F1 males at approximately pnd 11-13. Any males with one or more nipples or areolae on pnd 11-13 were uniquely marked within the litter (dye on tail) until weaning.

When each F1 litter reached pnd 21, three/sex/litter/dose were randomly selected for retention for a total of up to 75/sex/group. Retained F1 animals were individually identified by eartag. Up to two remaining F1 weanlings/sex/litter/group were necropsied.

F1 Postwean Observations and Procedures

Selected animals of the F1 generation (three/sex/litter, up to 75/sex/dose group) were held for a minimum of 49 days until the youngest selected F1 female pups were at least 70 days old, and for a minimum of 59 days until the youngest selected male pups were at least 80 days old. They were weighed, and feed consumption was measured weekly. F1 animals were examined once daily for documentation of clinical signs and observed twice daily (a.m. and p.m.) for mortality checks. For the evaluations listed below, ten different pups/sex/group (if possible) were arbitrarily selected and used for auditory function, motor activity, learning and memory, and FOB to prevent any confounders from prior experience (selected pups for different tests were marked on their tails to prevent reuse).

Auditory function was assessed by observation of the startle reflex in ten arbitrarily chosen F1 pups/sex/group that were at least 21 days old (26 ± 5 days of age). Startle reflex was measured by placing the animals individually in an isolation cabinet with a force detector attached to the bottom (San Diego Instruments, Inc., San Diego, CA). The startle reflex was automatically monitored by a Gateway 2000 P5-90 #000183 computer (startle response measurement system, San Diego Instruments, Inc., Version 6500-0076 [2000], San Diego, CA). After a five-minute acclimation period, an approximately 120 dB tone was presented to the animal for five milliseconds. The computer system monitored response for an additional 50 milliseconds after cessation of the tone and then paused for an eight-second intertrial interval. The procedure was repeated 50 times per session, one session per animal.

Each F1 female pup was observed daily beginning at 22 days of age for vaginal opening. The number of female pups with vaginal opening was recorded daily, and individual body weights at acquisition were recorded until all female pups had this response.

For the last 21 days (three weeks) of the F1 postwean holding period, all F1 females were evaluated for estrous cyclicity by taking vaginal smears of sloughed cells once daily. The slides containing the daily smears were stained and subsequently analyzed for the status of the estrous cycles.

Each F1 male pup was observed for daily preputial separation beginning at 35 days of age. The characteristic is present when the prepuce can be completely retracted to expose the glans penis. The number of male pups with this separation was recorded daily, and individual body weights at acquisition were recorded until all male pups had this response.

Ten arbitrarily chosen F1 pups/sex/group were observed in a motor activity maze for one hour on day 35 ± 2 of age. Residential cages equipped with sensors were used to determine animal activity. The apparatus detects and records ambulations and fine movements of the test animals in a home cage-like environment. Movement is detected when the animal breaks photocell beams that are spaced, such that not more than one beam can be broken at a time. For each animal, a continuous one-hour test period was used, broken into 12 five-minute data collection segments (Polycarbonate Cage Photobeam Activity System, Software Version 2052-0026 [02/09/98], San Diego Instruments, Inc., San Diego, CA).

Ten different, arbitrarily chosen F1 pups/sex/group were tested in a water-filled M (Morris) maze (Morris, 1981; Wishaw, 1985; Nanry et al., 1989; Tilson et al., 1990) when at least 40 days of age (45 ± 5 days of age). Each rat was given eight runs/day in the maze for two consecutive days. The inter-trial interval was a maximum of 15 minutes.

Once after weaning (pnd 28 ± 5) and at pnd 60 ± 5 , an FOB (Moser, 1989; Moser et al., 1991) was administered to the same ten F1 pups/sex/group. The FOB included, but was not limited to, the following endpoints:

Home Cage Observations

Abnormal motor movements	Posture
Convulsions	Total gait score
Gait	Tremors
Palpebral closure	Wasting

Handling Observations

Ease of removal	Mouth and nose deposits
Ease of handling	Muscle tone
Eyes	Piloerection
Fur appearance	Salivation

Open Field Observations

Abnormal motor movements	Defecation character	Tremors
Arousal	Gait	Urination
Convulsions	Posture	Urination character
Defecation	Total gait score	Vocalizations

Sensory and Neuromuscular Observations

Approach response	Hindlimb grip strength (Meyer et al., 1979)
Startle response	Pupil size and response
Forelimb grip strength (Meyer et al., 1979)	Tail pinch response
Hindlimb foot splay	

Physiological Observations

Body weight

Necropsy of F0 Females

At weaning of the F1 litters, F0 females were subjected to a complete gross necropsy, with selected organs weighed and retained or discarded, as appropriate (see below). The gross necropsy included examination of the external surfaces; all orifices; carcass; the thoracic, abdominal, and pelvic cavities and their viscera; and cervical tissues and organs. Gross lesions were recorded. Gel implants were retrieved, weighed, and stored in 10% neutral buffered formalin, and uterine nidation scars were counted. No F0 females appeared moribund and were euthanized or died during the study.

The following organs were weighed and retained, or retained only, in 10% neutral buffered formalin from all F0 females at scheduled necropsy. Organ weights were reported as absolute and relative to terminal body weight.

Ovaries (pair)	Kidneys (pair)
Liver	Uterus (plus cervix and vagina)
Spleen	
Lymph nodes (one cervical near implant, and one mesenteric distant from the implant) – retained only	

All retained organs were trimmed, embedded in paraffin, sectioned, and stained (hematoxylin and eosin). Full histopathology was done on all retained organs from all 25 F0 females/group for the high dose and control groups. In addition, all gross lesions from all groups

were examined histopathologically. If apparent treatment-related changes were observed in any organs in the high-dose group, then pathologic examinations on these organs were to be extended to the mid- and, if necessary, low-dose group animals. There were no treatment-related changes in the tissues from F0 females in the high-dose group.

Necropsy of F1 Male and Female Weanlings on Pnd 21

Two male and two female F1 weanlings per litter (if possible), not selected for retention, were euthanized by CO₂ asphyxiation. Individual anogenital distance and terminal body weight were recorded for all necropsied F1 weanling males and females. Any F1 weanling males selected for necropsy with nipples and/or areolae on pnd 11-13 were shaved on the ventrum and the presence of areolae and/or nipples confirmed. The following organs were weighed and retained, or retained only, in 10% neutral buffered formalin or Bouin's fixative (paired testes) for possible subsequent histopathology:

- Brain
- Cowper's glands (pair)
- Epididymides (pair)
- Esophagus (retain only)
- Kidneys (pair)
- Levator ani bulbocavernosus (LABC) complex
- Liver
- Lymph nodes (one cervical and one mesenteric) – retained only
- Ovaries (pair)
- Prostate
- Seminal vesicles with coagulating glands
- Spleen
- Testes (pair)
- Uterus (plus cervix and vagina)

F1 Adult Necropsy

At pnd 70 ± 5, F1 females (three/sex/litter) and at pnd 85 ± 5, F1 males (three/sex/litter) were subjected to a complete gross necropsy, with selected organs weighed and retained (see below). The gross necropsy included examination of the external surfaces; all orifices; carcass; the thoracic, abdominal, and pelvic cavities and their viscera; and cervical tissues and organs.

Any F1 adult males with nipples and/or areolae on pnd 11-13 were shaved on the ventrum and the presence of nipples and/or areolae confirmed. Gross lesions were recorded.

Any animal that appeared moribund and was euthanized or that died during the study was necropsied, when possible, to investigate the cause of death. Organs were not weighed or retained for these animals.

The following organs were weighed and retained, or retained only, in 10% neutral buffered formalin or Bouin's fixative (testis).

- Adrenal glands (pair)
- Brain
- Cowper's glands (pair)
- Epididymides (pair; one fixed with cauda removed)
- Esophagus (retain only)
- Kidneys (pair)
- Levator ani bulbocavernosus (LABC) complex
- Liver
- Lymph nodes (one cervical and one mesenteric) – retained
- Mammary glands (pair; in F1 adult females only) – retained
- Ovaries (pair)
- Pituitary (fixed weight)
- Prostate
- Seminal vesicles with coagulating glands
- Spleen
- Testes (pair; one fixed in Bouin's fixative, one frozen)
- Uterus (plus cervix and vagina)

Organ weights were reported as absolute and relative to terminal body weight. All retained organs were trimmed, embedded in paraffin, sectioned, and stained (hematoxylin and eosin). The most anterior (axillary) pair of mammary glands in F1 adult females was retained in buffered neutral 10% formalin (stapled to file cards to keep the preparation flat). The retained mammary glands from 25 randomly selected F1 adult females/group in the high dose and control groups were initially subjected to histopathologic examination. The procedure was as follows. Both nipples (from the most anterior two mammary glands) were identified. Transverse, whole-section trimming cuts were made on either side of both nipples. Each skin section was placed on edge in the tissue cassette. Each paraffin block, containing two skin sections, was microtomed at 4-6 microns, including the nipple or as close to the nipple as possible. Each section was stained

with hematoxylin and eosin. The examination followed the parameters of growth and differentiation of the mammary glands established by Odum et al. (1999a,b). If any possible treatment-related effects were observed, the retained mammary glands from the rest of the F1 females in the affected group(s) were to be evaluated. If evidence for a possible treatment-related effect persisted, then retained mammary glands from 25 F1 females at the next lowest group were evaluated, etc., until a no observable effect level (NOEL) was determined. Full histopathology was performed on all of the retained organs from 25/sex/group for high dose and control groups. In addition, all gross lesions from all groups were examined histopathologically. If apparent treatment-related changes were observed in any organs in the high-dose group, then pathologic examinations on these organs were extended to the mid- and, if necessary, low-dose group animals. There were no treatment-related lesions in any tissues from the initial 25 F1 females in the high-dose group.

F1 Andrology

At the time of sacrifice, one testis from each F1 male was frozen at approximately -20°C for subsequent enumeration of testicular homogenization-resistant spermatid heads for high-dose and control males. If treatment-related changes in the number of testicular homogenization-resistant spermatid heads were observed in the high-dose group, then these evaluations were extended to the mid- and low-dose groups (from retained frozen testes). In addition, one cauda epididymis from each F1 male was immediately removed and weighed. Seminal fluid from the cauda was assessed for sperm number, motility, and morphology. Sperm motility (motile and progressively motile) was assessed immediately after necropsy for all males in all groups. The epididymal sperm number and morphology (at least 200 sperm per male, if possible) were evaluated at a later date using appropriately retained sperm samples initially from the high-dose and control males. If treatment-related andrological changes were observed in the high-dose group, then these evaluations were extended to the mid- and low-dose groups (from retained sperm samples). Sperm motility, epididymal number, and spermatid head counts were assessed using an HTM-IVOS (Version 12.1c; Hamilton-Thorne Research, Beverly, MA). Sperm morphology (at least 200/male, if possible) was performed manually under a microscope. There were no treatment-related, andrological effects in the high dose F1 males initially examined.

Statistical Analysis

The unit of comparison was the pregnant F0 female, the F1 litter, or the postweanling F1 male or female, as appropriate. Treatment groups were compared to the concurrent control group using either parametric ANOVA under the standard assumptions or robust regression method (Zeger and Liang, 1986; Royall, 1986; Huber, 1967), which do not assume homogeneity of variance or normality. The homogeneity of variance assumption was examined via Levene's Test (Levene, 1960). If Levene's Test indicated lack of homogeneity of variance ($p < 0.05$), robust regression methods (available in the REGRESS procedure of SUDAAN® Release 8; RTI, 2001), which use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data, were used to test all treatment effects. They were used to test for overall treatment group differences, followed by individual tests for exposed vs. control group comparisons (via Wald Chi-Square Test) if the overall treatment effect was significant.

If Levene's Test did not reject the hypothesis of homogeneous variances, standard ANOVA techniques were applied for comparing the treatment groups. The GLM procedure in SAS® Release 8 (SAS Institute Inc., 1999a,b,c,d,e; 2000; 2001) was used to evaluate the overall effect of treatment and, when a significant treatment effect was present, to compare each exposed group to the control via Dunnett's Test (Dunnett, 1955, 1964). For the litter-derived percentage data (e.g., periodic pup survival indices), the ANOVA (SAS® Release 8) was weighted according to litter size. A one-tailed test (i.e., Dunnett's Test) was used for all pairwise comparisons to the vehicle control group, except that a two-tailed test was used for parental and pup body weight and organ weight parameters, feed consumption, percent males per litter, and anogenital distance. Student's t-test (SAS® Release 8) was used for analysis of all andrology endpoints except for epididymal sperm motility and progressive sperm motility.

Frequency data, such as reproductive indices (e.g., mating and fertility indices), were not transformed. All indices were analyzed by the Chi-Square Test for Independence for differences among treatment groups (Snedecor and Cochran, 1967). When Chi-Square revealed significant ($p < 0.05$) differences among groups, then a Fisher's Exact Probability Test, with adjustments for multiple comparisons, was used for pairwise comparisons between each treatment group value and the control group value.

Acquisition of developmental landmarks (e.g., vaginal patency and preputial separation), as well as anogenital distance, were also analyzed by Analysis of Covariance (ANCOVA; in addition to ANOVA analysis) using body weight at acquisition or measurement as the covariate.

For correlated data (e.g., body and organ weights at necropsy of weanlings, with more than one pup/sex/litter), SUDAAN® software (RTI, 2001) was used for analysis of overall significance and pairwise comparisons to the control group values.

A test for statistical outliers (SAS Institute, Inc., 1999a,b,c,d,e) was performed on retained F0 maternal and retained F1 offspring body weights, feed consumption (in g/day), and organ weights. If examination of pertinent study data did not provide a plausible, biologically-sound reason for inclusion of the data flagged as "outlier," the data were excluded from summarization and analysis and designated as outliers. If feed consumption data for a given animal for a given observational interval (e.g., sd 0-7 or 7-14, etc., during the prebreed exposure period) were designated outliers or unrealistic, then summarized data encompassing this period (e.g., sd 0-28 for the prebreed exposure period) also did not include this value. In all cases, a p -value of ≤ 0.05 was considered the appropriate indication of statistical significance.

FOB Data. FOB data measured in this study were based on relatively small numbers of animals per group, and the outcomes were likely to be sparse, skewed, or heavily tied (most or all animals had same response). Therefore, exact versions of standard asymptotic tests were used whenever possible.

Home Cage Observations. The outcomes in this section were either binary or ordinal. For the binary outcomes for home cage observations, statistical analyses were the same as for the binary outcomes under Open Field Observations (see below). For the ordinal outcome for home cage observations, the statistical analyses were the same as for the ordinal outcomes under Handling Observations (see below).

FOB: Handling Observations. The outcomes in this section were either binary or ordinal with 3-5 categories. Binary outcomes were analyzed using Fisher's Exact Test for overall heterogeneity among dose groups, followed by individual pairwise comparisons of exposed groups to control if the overall test was statistically significant. All tests were two-tailed. These tests were implemented in the FREQ procedure of SAS® 8 (SAS Institute Inc., 1999a,b,c,d,e; 2000; 2001).

Exact two-sided p -values for standard nonparametric tests were provided for ordinal outcomes. This included exact p -values (Agresti, 1990, 1992, 1996; Agresti et al., 1990) for the Kruskal-Wallis Test for overall heterogeneity among dose groups, followed by individual pairwise comparisons of exposed groups to control if the overall test was statistically significant. These tests were implemented in the FREQ and NPAR1WAY procedures of SAS® Release 8 (SAS Institute Inc., 1999a,b,c,d,e; 2000; 2001).

FOB: Open Field Observations. With the exception of the number of urinations and defecations, open field observations were analyzed as binary outcomes (categorized into normal vs. abnormal) and analyzed as above for FOB: Handling Observations. The numbers of urinations and defecations were analyzed using standard nonparametric tests as given above for FOB: Handling Observations, with ordinal outcomes.

Sensory and Neuromuscular Observations. The outcomes in this section were ordinal, with the statistical analyses for these outcomes the same as for the ordinal outcomes under Handling Observations (see above). The statistical analyses employed for forelimb and hindlimb grip strength and hindlimb foot splay (part of the sensory and neuromuscular observations) are presented below.

Grip Strength. Treatment groups were compared using either parametric ANOVA under the standard assumptions or robust linear regression methods (Zeger and Liang, 1986; Royall, 1986; Huber, 1967), as described above in the first paragraphs of the Statistical Analyses section.

Motor Activity and Auditory Startle. Univariate motor activity outcomes (total one-hour activity and activity levels in each of six consecutive ten-minute intervals) and auditory startle outcomes (maximum amplitude and time to maximum amplitude, averaged over ten trials in each of five consecutive ten-trial blocks) were first analyzed using standard parametric ANOVA methods. For each outcome, the GLM procedure in SAS® Release 8 (SAS Institute Inc., 1999a,b,c,d,e, 2000) was used to evaluate the overall effect of treatment and, when a significant treatment effect was present, to compare each exposed group to the control via Dunnett's Test (Dunnett, 1955, 1964).

Although univariate ANOVA techniques at each time point may be useful, many hypotheses can be tested more appropriately in a repeated measures framework (e.g., comparing treatment groups' response profiles). Here, time represents blocks of trials in auditory startle

tests or ten-minute intervals in a one-hour session of motor activity tests. Longitudinal data with normally distributed outcomes were analyzed simultaneously in a general linear mixed model (Laird and Ware, 1982; Diggle et al., 1994; Lindsay, 1993) via the MIXED procedure (Wolfinger, 1992; Littell et al., 1996) in SAS[®] 8. The correlation of an animal's data was taken into account by choosing an appropriate covariance structure to model the random errors (compound symmetry was used in this study). This covariance model was then incorporated into the estimating equations for regression parameters and into computations for their associated standard errors and test statistics.

Hypotheses comparing the shape of the response profiles among treatment groups were tested in the repeated measures framework. This was accomplished by fitting linear and higher order polynomials in time and testing their interaction with treatment. Significance levels for the overall treatment effect, the linear time effect, and interactions between treatment and time (linear time effect) were reported. If the treatment response profiles differ significantly over time, linear slopes were estimated and reported for each of the treatment groups, along with each of the exposed vs. control group interaction *p*-values.

A form of repeated measures analysis was included for performing a global test of the treatment effect adjusted for time. The statistical model included the effects of treatment, time (a categorical repeated factor), and their interaction, treatment*time. The goal of these analyses was to test the effect of treatment after adjusting for multiple testing over time. This assumed that the interaction between treatment and time was not statistically significant. When such interaction effects were detected, and when they were qualitative in nature, the time-adjusted analysis was eliminated and only univariate, time-specific analyses (from ANOVA runs detailed earlier) were reported. Nonqualitative interaction effects were removed from the model, and time-adjusted treatment effects evaluated (using Dunnett's test for multiple pairwise comparisons to control). In this case, significant time-specific ANOVA results were reported in conjunction with significant time-adjusted results from the repeated measures analysis. This type of analysis was implemented as a general linear mixed model in SAS PROC MIXED for continuous responses. The report on statistical analyses of motor activity and auditory startle is presented in Appendix II of this report.

Developmental Landmarks. Developmental landmarks include, for example, righting reflex, pinna detachment, incisor eruption, and eye opening. All of these parameters are

recorded on multiple pups per litter. Hence, the data are said to be cluster-correlated. In addition, time to eye opening, pinna separation, and incisor eruption may be censored (time to event not observed during the study period). For these landmarks, pups were individually tracked, so the actual day of acquisition or censoring (due to early death or termination of the observation period) was known for each animal. The data for analysis consisted of a single outcome for each pup: the day of acquisition of the landmark, with an indicator of censoring vs. complete event times. The event time was censored if, for example, the observation period terminated or the pup died naturally before the landmark was acquired.

Analysis of landmark data is complicated by a number of factors. First, the most common approach of analyzing prevalence rates (number of live pups that have acquired the landmark divided by number of live pups) at each day of observation can produce spurious associations. An analysis that detects global differences in the event time distributions is preferred to answer a single question: is onset delayed with increasing dosage? This is superior to analyzing the litter or treatment group proportions separately for each postnatal day in which landmarks were recorded.

Analyses for time-to-event data can also be biased if the animals are not tracked individually and if the censored data are omitted from the analysis. The recommended way to conduct an analysis of time-to-event data (followed in this study) requires that each animal is followed individually, and that the day at which onset was observed or the day at which the animal was removed from the study (whichever comes first) is recorded. Animals that were removed from the study because of death or study termination and that had not achieved the event by the time of their removal, were treated as censored observations in the analysis, with their censored times being the day of removal from the study. The censored data represent partial information that can be appropriately included to produce an unbiased analysis. Tracking individual animals allows an analysis based on incidence, which is the only way to truly detect delays in onset. In this study, developmental landmarks were analyzed in this manner.

A standard statistical method for comparing the event time distributions among the experimental groups is given by the Cox (1972) proportional hazards regression model. This regression technique, which uses the actual times to the event, is sensitive to differences in the overall incidence rates, as well as the distribution of onset times. The Cox proportional hazards model can be used to detect overall heterogeneity in event time distributions among the

experimental groups, perform individual pairwise comparisons of exposed groups to control, as well as testing for linear trend in dose. Median event times (the day at which half of the animals have achieved the event in each treatment group) were computed from the model. Cumulative survival distribution profiles, which describe the proportion of animals in each experimental group that have not reached the specified event over course of the postnatal period, were produced. These can be computed as predicted values from the model or by using an empirical method (Kaplan and Meier, 1958). Using this model, the relative effect of treatments was quantified on the development of the various landmarks.

The Cox Proportional Hazards Model is stated as follows:

$$\log \left[\frac{h(t|x)}{h_0(t)} \right] = X \beta$$

where t represents the day of acquisition, $X \beta$ is the linear combination of covariate effects (i.e., $a \cdot \text{DOSE}$), and $\left[\frac{h(t|x)}{h_0(t)} \right]$ is the relative risk of onset of the event at time t for animals with different sets of covariates (e.g., high dose group vs. control). The model requires the user to supply each animal's failure time (in days), an indicator for censoring (yes/no), and a set of covariate values. Animals acquiring the landmark during the observation period provided complete event times. Animals that died during the course of observation, or that were present at study termination without acquisition of the landmark of interest, were censored at their last day of observation. All event times, censored and complete, were used in the analysis.

When multiple pups from the same litter are used in an analysis, care must be taken to adjust for the resulting intralitter correlation. That is, animals from the same litter tend to respond similarly, and the data are no longer statistically independent. It is widely recognized in the statistical literature that standard analysis methods that ignore the clustering of pups within litters will tend to produce standard error estimates that are too small and test statistics that are too large (Haseman and Kupper, 1979). Hence, the chance of finding false-positive results is increased when the clustering is ignored.

To incorporate the effects of intracluster correlation and to reduce the chances of finding spurious results, a model-fitting method designed specifically for clustered data with non-normally distributed outcomes was used. Recent advances in analyzing many types of correlated data (Zeger and Liang, 1986) have led to new methods for handling time-to-event outcomes

clustered within litters. The techniques make no strict distributional assumptions about the endpoint of interest (e.g., normality) or the correlations among clustered observations.

Proportional hazards regression, described above, is used to model the marginal distribution of clustered event times as a function of covariate effects. Estimates of model parameters are obtained using methods equivalent to the univariate failure time setting (i.e., via partial likelihood), while the correlation structure within clusters is treated as a nuisance parameter. The regression parameter estimates are identical to those obtained if the data were independent, and they are known to have desirable statistical properties even under cluster sampling (e.g., they are asymptotically normal and unbiased). The key difference from analysis of independent failure time data is that a robust variance estimator (Binder, 1992; Wei et al., 1989; Lee et al., 1992) is used to properly account for the intracluster correlation of responses. This procedure yields valid inferences about treatment effects in the presence of intracluster correlation.

Wald Chi-Square Test statistics are used to evaluate the significance of model parameters relating to treatment effects. For a single degree-of-freedom hypothesis (e.g., high dose vs. control), the Wald Chi-Square Test statistic reduces to a standard normal deviate, or the regression coefficient estimate divided by its standard error. For multiple degree-of-freedom hypotheses (e.g., the overall effect of treatment), the Wald Chi-Square Test is analogous to the ANOVA F-test for linear models with continuous responses.

The robust variance approach for clustered time-to-event outcomes was implemented through the SURVIVAL procedure in SUDAAN[®] 8.0 (RTI, 2001), which uses an estimating equation approach documented by Binder (1992) and Lee et al. (1992).

Learning and Memory: Water-Filled M-Maze. Learning and memory functions were tested on ten randomly chosen F1 pups/sex/group in a water-filled M (Morris)-maze. Each rat was given eight runs/day in the maze for two consecutive study days. Each test was performed for a maximum of 60 seconds, and the inter-trial interval was a maximum of 15 minutes. In each run, the time it took the animal to emerge onto a platform and out of the water (in seconds) was recorded. Animals not reaching the platform at 60 seconds (termination of the observation period) were considered censored at that time.

Analysis of swimming data combines the elements of both developmental landmark analyses and motor activity analyses. That is, these are time-to-event data, with possible

censoring, as well as repeated testing of each animal over time. In this study, each animal was tested at each of 16 runs, and at each run the time to successfully swimming to a platform was recorded. Therefore, the Cox (1972) proportional hazards model was used to test the following effects on swimming time: treatment, trial, study day, and sex. The trial and day effects helped validate learning (the trial effect) and memory (the day effect) in the swimming test. When no interaction was detected between sex and treatment, sex-adjusted treatment effects were reported. Otherwise, treatment effects were evaluated separately for each sex. The treatment-by-study day interaction effect was evaluated in a similar way: if not statistically significant, day-adjusted treatment effects were reported; if statistically significant, the treatment effects were evaluated separately on each study day. The Cox proportional hazards model was used to detect overall heterogeneity in swimming time distributions among the experimental groups, perform individual pairwise comparisons of exposed groups to control, as well as testing for linear trend in dose. Median event times (the amount of time it takes for half the animals to achieve the event in each treatment group) can be computed from the model.

The model requires the user to supply each animal's swimming time (in seconds), an indicator for censoring (yes/no), and a set of covariate values. Animals reaching the platform during the observation period provided complete event times. Animals that did not reach the platform before 60 seconds were censored at that time. All event times, censored and complete, were used in the analysis. The data file consisted of multiple records for each animal, with the number of records from a given animal equal to the number of repeated measurements obtained.

Repeated observations measured on the same animal over time tend to be positively correlated. The tendency for multiple observations from the same animal to be similar is commonly referred to as intra-subject correlation. This implies that responses at multiple time points within the same animal are not statistically independent. Ignoring the longitudinal nature of the design in the statistical analysis can lead to significant bias in the variance of regression coefficient estimates, yielding false-positive tests of treatment effects and other covariates that do not vary over time (time-stationary covariates) and false-negative tests of covariate effects measured at each time point (time-varying covariates, such as the time trend itself). To incorporate the repeated testing over time, a model-fitting method designed specifically for repeated measures with non-normally distributed outcomes was recommended.

Recent advances in analyzing many types of correlated data (Zeger and Liang, 1986) have led to new methods for handling time-to-event outcomes in a longitudinal design. The techniques make no strict distributional assumptions about the endpoint of interest (e.g., normality) or the correlations among clustered observations.

Proportional hazards regression, described above, is used to model the marginal distribution of correlated event times as a function of covariate effects. Estimates of model parameters are obtained using methods equivalent to the univariate failure time setting (i.e., via partial likelihood), while the correlation structure within clusters is treated as a nuisance parameter. The regression parameter estimates are identical to those obtained if the data were independent, and they are known to have desirable statistical properties even for repeated measures designs (e.g., they are asymptotically normal and unbiased). A robust variance estimator is then used to properly account for the intra-animal correlation of responses.

Wald Chi-Square Test statistics are used to evaluate the significance of model parameters relating to treatment, trial, study day, and sex effects. For a single degree-of-freedom hypothesis (e.g., high dose vs. control), the Wald Chi-Square Test statistic reduces to a standard normal deviate, or the regression coefficient estimate divided by its standard error. For multiple degree-of-freedom hypotheses (e.g., the overall effect of treatment), the Wald Chi-Square Test is analogous to the ANOVA F-test for linear models with continuous responses.

The robust variance method for clustered time-to-event outcomes was implemented through the SURVIVAL procedure in the SUDAAN[®] 8.0 software package (Research Triangle Institute, 2001), which uses an estimating equation approach documented by Binder (1992) and Lee et al. (1992).

Personnel

This study was conducted at RTI, Research Triangle Park, NC, under contract to Mentor Corporation. Dr. Maher Michael was the Sponsor's Representative, and Ms. Donna Free was the Study Monitor (both from Mentor Corporation). Dr. R.W. Tyl served as Study Director. Reproductive and Developmental Toxicology personnel included Ms. M.C. Marr (Laboratory Supervisor), Ms. C.B. Myers (Reproductive Toxicity Study Supervisor and Data Analyst), Ms. Robin T. Krebs, Mr. M.D. Crews, Ms. A.J. Parham, Mr. W.P. Ross, Ms. V.I. Wilson, Ms. L.B. Pelletier, Ms. L.L. Macdonald, Ms. J.B. Carnell, Mr. C.G. Leach, Mr. J.E. Gray, Ms. C.R.

Robinson, and Ms. N.M. Kuney. Bulk chemical handling and dose formulations were provided by Mr. M.M. Veselica (Supervisor, RTI Materials Handling Facility), Mr. D.L. Hubbard, Mr. R.A. Price, and Mr. J.E. Larson. Animal care was provided by Dr. D.B. Feldman, DVM, ACLAM, Veterinarian, and Mr. F.N. Ali, Manager of RTI Animal Research Facility. RTI Quality Assurance personnel were Ms. D.A. Drissel (Manager), Ms. C.D. Keller, Ms. M.D. Phillips, Ms. E.D. Shinnald, Ms. D.D. Rowe, Ms. J.E. Jones, Ms. S.C. Wade, Ms. P.D. Hall, Ms. M. Oh, Ms. M. Jones, and Ms. C.A. Ingalls.

The final report was prepared by Dr. R.W. Tyl, with assistance from Ms. C.B. Myers on data compilation, statistical analyses, and generation of figures, and from Mr. T.W. Wiley on data entry. Ms. M.C. Marr was responsible for maintaining and archiving the study records. The individual scientist reports were prepared and signed by the author(s) and are included as appendices to this final report.

The protocol and two amendments detailing the design and conduct of the study are presented in Appendix V of this final report.

Storage of Records

All raw data, correspondence, documentation, records, reports, specimens, blocks, and slides generated as a result of the study will be retained in the RTI Archives under the control of the RTI Archivist in the Health Sciences Unit.

Compliance

The toxicology laboratories at RTI are operated in compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations (U.S. FDA, 2003b). The RTI Animal Research Facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International. Thus, this study was conducted in compliance with FDA Regulations for GLPs (U.S. FDA, 2003b; except for the density determinations on the control CMC gel and the test gel that were performed using a standard method, not validated at RTI) and in compliance with the AAALAC accreditation standards. The Sponsor was responsible for GLP compliance of the chemical characterization of the bulk chemical. The RTI Quality Assurance Unit reviewed the protocol and amendments, inspected critical in-life phases and necropsy, and audited the data and the final report.

RESULTS

Gel Formulations and Analyses

As described in Materials and Methods, the target test gel implant doses (3, 10, and 30 ml/kg) were chosen based on the work by Siddiqui et al. (1994) on a similar implant gel, and the test gels were taken directly from the clinical product. The negative control group (0 ml/kg) received CMC gel by subcutaneous implantation at approximately the same viscosity (3.5% in sterile saline). The actual amount implanted was based on the reasonable assumption (Siddiqui et al., 1994) that the females would all weigh 300 g on gd 0. The doses in ml/kg were calculated for the doses in ml/300 g and, with the density of the test gel at 0.97 g/ml, the target doses were calculated in g/animal. The density of the CMC control gel was 1.01 g/ml to provide the actual amount of control gel administered in grams and ml (Table 1 and Appendix I).

The delivery syringes were modified to allow insertion of the gels into the subcutaneous pockets. The technicians drew up the gel to the correct volume, weighed the full syringes, implanted the gels, and weighed the emptied syringes. This provided the amount of gel implanted in grams. The administered weights (in grams) of these implanted gels were all within 94.6-100.0% of the target values. The administered volumes (in ml) of these implanted gels were all within 94.6-100.0% of the target (Table 1 and Appendix I).

The summary data are presented in Tables 2 through 40 in chronological order of occurrence. Individual animal data for this portion of the study are presented in Appendix VI. Statistical analyses of auditory startle and motor activity data are presented in Appendix II. The pathology report on F0 females and F1 adult males and females is presented in Appendix III. Historical control data from the performing laboratory, comparable to this portion of the study (in the same rat strain), are presented in Appendix IV. The protocol and two amendments are presented in Appendix V.

F0 Females

A total of 120 females (30/group x 4 groups) were implanted with the test/control gel over a three-day surgical period (with one third of the animals in each group implanted each day) prior to mating. The decision to implant 30 females/group was based on the need for 25 dams with litters/group and on the 92-100% pregnancy rate in this species and strain in the performing laboratory. Once all the F0 females had delivered, 25 dams with their litters were randomly

selected per group for retention. The data presented in this report are based on the 25 selected dams and litters/group. All selected 100 F0 dams were present at scheduled sacrifice at the weaning of their F1 litters (Table 2).

F0 Prebreed Exposure Period

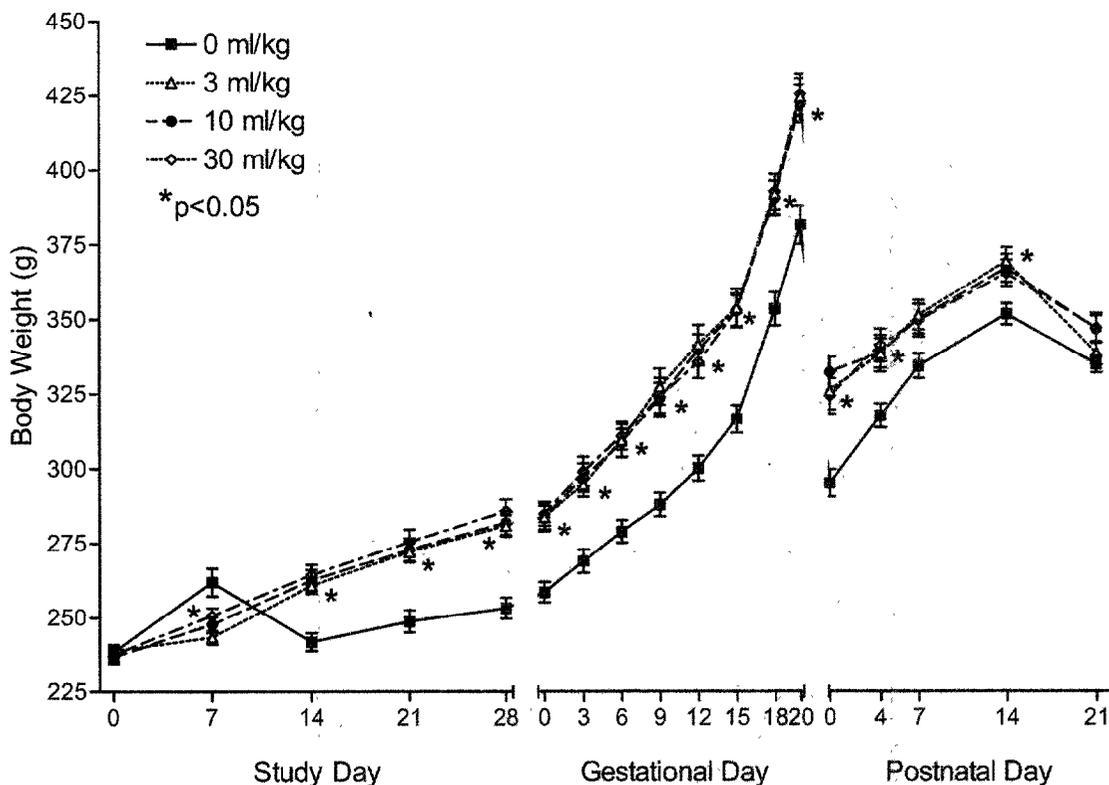
On sd 0 (day of implantation of gels), the mean body weights and standard errors were equivalent across all four groups (Table 3). By sd 7, the CMC-implanted control group females were exhibiting major fluid retention, so that the control mean body weights were significantly greater (by approximately 11-19 g) than the mean body weights of the three groups implanted with the test gel. Prior to sd 14, the study veterinarian aspirated the excess fluid from the control dams (the aspirates were individually weighed and retained frozen) so that the control mean body weights were significantly below the three test gel treatment groups on sd 14. The reduced mean body weights in the CMC-implanted control females continued through sd 21 and 28, the end of the four-week prebreed exposure period (Table 3 and Figure 2). The mean body weights of the three test gel-implanted groups were equivalent for each time point and exhibited the usual and customary 8-18 g weight gain per week. The slopes of the increases in body weight were comparable across all three test gel groups. Maternal body weight changes in the control group reflected the events indicated above; a significant gain of 23.4 g from sd 0-7 (due to fluid retention/exudate accumulation) and a significant loss of 20.1 g from sd 7-14 (due to aspiration of the excess fluid). From that point forward, from sd 14-21 and 21-28, maternal weight gains were equivalent across all groups. Body weight change for the entire four-week prebreed period was equivalent across all three test gel groups and lower in the CMC-implanted control group (Table 3).

Maternal feed consumption (in g/day) exhibited no differences among the test gel treatment groups for the entire prebreed period, with the usual consumption of approximately 20 g/day. The CMC-implanted control group exhibited significant reduction for all intervals (approximately 15-17 g/day; Table 4). When feed consumption was expressed in g/kg body weight/day, the same pattern (no differences among the test gel-implanted groups and reduced consumption in the CMC-implanted control group) was present for sd 0-7, 7-14, and 14-21. By sd 21-28, there were no differences in maternal feed consumption between the CMC-implanted control group value and the mid and high test gel-implanted groups (the value for the low dose

group was still significantly increased over the control value). Feed consumption overall during the entire period (sd 0-28), expressed either in g/day or g/kg/day, exhibited no differences among the test gel treated groups, with significantly lower consumption in the CMC-implanted control group (Table 4).

Figure 2

F0 Female Body Weights



F0 maternal clinical observations during the prebreed, mating, and postmating holding periods (for females not detected sperm or plug positive) are presented in Table 5. There were no dose-related clinical observations. The entire CMC-implanted control group exhibited fluid accumulation in and around the implantation site, with additional swelling on the back and/or side(s) in 18 of the 25 dams. All other observations were related to hardening of the implants, migration of the implants (almost exclusively in the CMC control group and in three dams at 30 ml/kg), staples for occlusion of the implant site in the control group missing (with no leakage), one instance in the control group of blood around the vaginal area on sd 25 after the vaginal

smear (for estrous cyclicity) was taken, and alopecia on multiple areas (predominantly on limbs) in 3, 2, 3, and 5 dams at 0, 3, 10, and 30 ml/kg, respectively (Table 5). From sd 29-52, alopecia was noted in one dam at 0 ml/kg from sd 30-39 and in two dams at 3 ml/kg: in one dam on sd 29-31, and in one dam on sd 32-52. The only other findings during this period were swelling on the left side in one dam at 0 ml/kg on sd 31-33, and staples missing at the implantation site(s) and wound opening for a few females at 3, 10, and 30 ml/kg.

F0 female estrous cyclicity was evaluated during the last two weeks of the four-week prebreed period. There were no differences among all groups for any parameter examined, including number and percent of females cycling/group, number and percentage of females with an abnormal cycle/group, and cycle length in days/group. All groups exhibited the typical and appropriate four-day cycles.

F0 Gestation

F0 maternal body weights during gestation on gd 0, 3, 6, 9, 12, 15, 18, and 20 were equivalent across all test gel-implanted groups. The body weights of the CMC-implanted control group were significantly lower at all time points (Table 7 and Figure 2). Maternal weight changes were equivalent across all groups for all intervals, except for a significant increase at 3 ml/kg for gd 6-9. The body weight change during the entire gestational period (gd 0-20) was equivalent across all test gel-implanted groups and significantly lower in the CMC-implanted control group (Table 7). Feed consumption in g/day was equivalent across all four groups for gd 0-3, 15-18, and 18-20. For gd 3-6, 6-9, 9-12, and 12-15, the values for the test gel-implanted groups were equivalent; the values for the CMC gel-implanted control group were significantly lower. Feed consumption in g/day during the entire gestational period was increased in the 10 and 30 ml/kg groups and equivalent in the 0 and 3 ml/kg groups. Feed consumption in g/kg/day was equivalent across all groups for gd 0-3, 3-6, 6-9, 9-12, 12-15, and 0-20. For gd 15-18 and 18-20, values at 3 and 30 ml/kg were significantly decreased relative to the CMC control group. The value at 10 ml/kg was equivalent to the CMC control group (Table 8).

F0 maternal clinical observations during gestation are presented in Table 9. Alopecia in multiple areas (predominantly on the limbs) was present in 11, 6, 9, and 9 dams at 0, 3, 10, and 30 ml/kg, respectively. Four dams at 30 ml/kg exhibited migration of implant(s) to the left arm, and one dam at 0 mg/kg/day exhibited migration of the implant to the left arm. All

the remaining clinical observations were in the CMC control group and were limited to masses (soft and hard) in multiple areas around the implantation sites, swelling on right side, loose skin, and the wound at aspiration site healing (Table 9).

F0 Lactation

F0 maternal body weights and weight changes are presented in Table 10. Body weights were equivalent across all test gel-implanted groups at all time points (pnd 0, 4, 7, 14, and 21). The CMC gel-implanted group weighed significantly less than the test gel-implanted groups on pnd 0 and 4. All four groups exhibited equivalent body weights on pnd 7 and 21. On pnd 14, the control group value was significantly lower than the values at 3 and 10 ml/kg and equivalent to the value at 30 ml/kg (Table 10 and Figure 2). Maternal lactational weight change was significantly reduced for pnd 0-4 in the 3 and 10 ml/kg groups, relative to the control group, and equivalent at 30 ml/kg. Weight change was equivalent across all groups for pnd 4-7 and 7-14. For pnd 14-21, dams in all groups lost weight (typical for this time in lactation). The body weight loss was greater at 3 ml/kg (-30.7 g) than in the control group (-16.3 g) and equivalent to the control group at 10 (-20.5 g) and 30 (-18.4 g) ml/kg. For the entire lactational period, maternal weight change was significantly higher (39.8 g) in the control group than in the 3 (12.8 g), 10 (14.3 g), and 30 (23.0 g) ml/kg groups.

F0 maternal feed consumption in g/day and g/kg/day was equivalent across all four groups for all lactational intervals (Table 11).

Clinical observations during lactation (Table 12) included alopecia (in various locations) in all groups (12, 8, 8, and 9 at 0, 3, 10 and 30 ml/kg, respectively), chromodacryorrhea at 0 (1), 10 (3), and 30 (1) ml/kg, and migration of implants to the left arm in one dam at 0 ml/kg and 10 dams at 30 ml/kg. Loose skin on the right side was observed in one dam at 0 ml/kg. Piloerection was observed in one dam each at 0, 3, and 10 ml/kg. Sore(s) on the neck was observed in one dam on pnd 8 and 9 at 10 ml/kg.

F0 Reproductive and Lactational Indices

For the 25 dams with litters/groups, precoital interval and gestational length were equivalent across all four groups. The number of uterine implant sites/litter, and therefore total number of pups/litter and number of live pups on pnd 0, were equivalent across all test gel-

implanted groups and reduced in the CMC-control group. Percent postimplantation loss/litter, number of dead pups/litter, stillbirth index, and live birth index were all equivalent across all four groups. The survival indices (pnd 0-4 [precull], pnd 4 [postcull]-7, 7-14, 14-21, and lactational index, pnd 4 [postcull]-21) were all very high and equivalent across all four groups (Table 13).

F1 Litters During Lactation

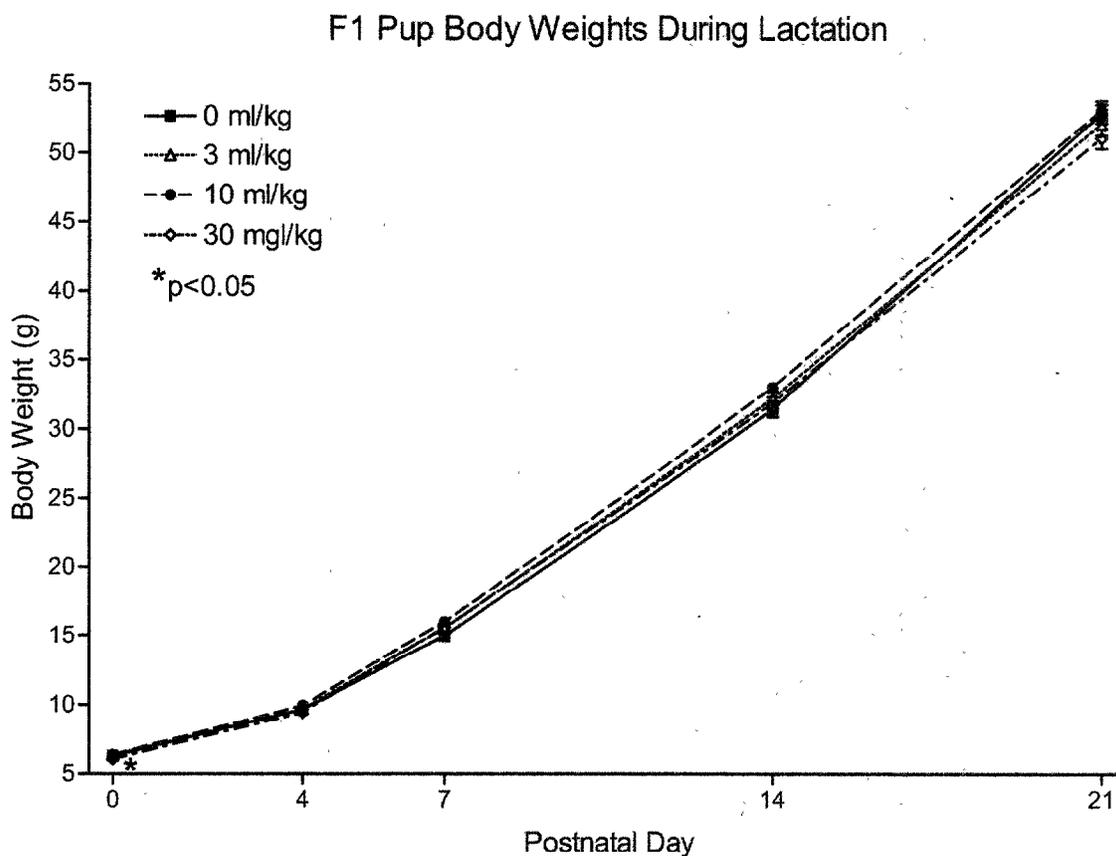
All F1 litters (25/group) survived to weaning on pnd 21. For these litters, the mean numbers of live pups/litter on pnd 0 and 4 (precull) were equivalent across the test gel-implanted groups and were significantly lower in the CMC gel-implanted groups. All litters were standardized to ten pups, with as equal a sex ratio as possible on pnd 4. On pnd 7 (postcull), 14, and 21, the mean numbers of live pups/litter were equivalent across all four groups. Anogenital distance (absolute and adjusted for body weight) on pnd 0 was equivalent across all groups for both males and females (Table 14). Mean pup body weights/litter (separately by sex or for all pups) were equivalent across all groups at all times evaluated (pnd 0, 4, 7, 14, and 21) except for a significant ($p < 0.05$) decrease in combined pup body weights/litter (with no effect in male or female pup body weights/litter) at 30 ml/kg on pnd 0 (Table 14 and Figure 3). Sex ratio (% males) per litter was also equivalent across all four groups at all time points evaluated (Table 14).

F1 pup preweaning acquisition of developmental landmarks is presented in Table 15. There were no effects on any landmark evaluated. These landmarks included average and median ages at pinna detachment, percentage of pups/litter positive for surface righting reflex on pnd 4 (precull), average and median ages at incisor eruption, eye opening, and percentage of pups/litter positive for auditory startle on pnd 13. There were no retained nipples in any F1 male pups in any litter or group. There were no differences among groups in the number of areolae/male or in the percentage of male pups with one or more areolae. This examination was performed on pnd 11-13 (Table 15).

The numbers of F1 pups that died or that were missing and presumed dead were 6, 9, 5, and 6 for pnd 0-4 and 0, 2, 0, and 0 for pnd 5-21 at 0, 3, 10, and 30 ml/kg, respectively. One male pup at 30 ml/kg was euthanized moribund on pnd 0, and one male pup at 10 ml/kg exhibited clubbed hindlimbs on pnd 0. One female pup at 10 ml/kg exhibited lacerations on the

abdomen and right hindlimb on pnd 2, one female pup at 30 ml/kg exhibited no milk band (visualization through the upper abdominal skin of milk in stomach) on pnd 4, one female at 3 ml/kg exhibited a growth on left side of mouth on pnd 8, and one female at 10 ml/kg exhibited head tilt to the right. None of these findings were treatment or dose related (Table 16).

Figure 3



Of the 28 F1 pups that died during lactation, 23 were available for necropsy examination. There were no treatment- or dose-related findings. All findings were typical of dead pups during lactation. Two pups (one unable to sex on pnd 0 and one female on pnd 13) at 3 ml/kg were cannibalized, and four pups exhibited autolysis of abdominal organs (one female on pnd 0 at 30 ml/kg, one male at 3 ml/kg on pnd 0, one male at 0 ml/kg on pnd 3, and one male at 10 ml/kg on pnd 4). Most of the dead pups, especially those dying early in lactation, exhibited patent (open) ductus arteriosus (a fetal state whereby blood is shunted through the ductus from the pulmonary arch to the aortic arch in the absence of oxygenation in the lungs), no air in lungs (on pnd 0) due

to primary atelectasis (failure of expansion of lung alveoli), and no milk in stomach. Conversely, a few dead pups were found with closed ductus, air in lungs, and/or milk in stomach. Of all the pups culled on pnd 4 to standardize litters, one pup in one litter at 0 ml/kg and two pups (one each in two litters) at 30 ml/kg were externally sexed as male and found to be female upon internal examination (Table 17).

F1 Pup Necropsy

F1 Male Pups

The numbers of F1 male pups/group necropsied on pnd 21 were 37, 47, 44, and 50 at 0, 3, 10, and 30 ml/kg, respectively. Anogenital distance (absolute and adjusted for terminal body weight) was equivalent across all four groups. There were no retained nipples or areolae in any weanling males in any group on pnd 21. Sacrifice body weight was significantly reduced at 30 ml/kg and equivalent across all other groups. Absolute organ weights were equivalent across all groups except for absolute paired testis weight that was significantly reduced at 3 and 30 ml/kg when compared to the control value. The values in the test gel-implanted groups were all equivalent. Organ weights relative to terminal body weights were equivalent across all groups except for relative prostate weight that was significantly reduced at 10 ml/kg and equal across all other groups (Table 18).

F1 Female Pups

The numbers of F1 female pups/group necropsied at weaning on pnd 21 were 42, 43, 46, and 48 at 0, 3, 10, and 30 ml/kg, respectively. Anogenital distance (absolute and adjusted for terminal body weight) was significantly shorter in the CMC gel-implanted control group and significantly longer than the control values in the 3 and 10 ml/kg test gel-implanted groups. The anogenital distances in the 30 ml/kg test gel-implanted group were statistically equivalent to the control group values. The anogenital distances in the three test gel-implanted groups were equivalent. Sacrifice body weights were equivalent across all four groups. Absolute organ weights were equivalent across all groups: brain, liver, spleen, paired kidneys, paired ovaries, and uterus plus cervix and vagina. Organ weights relative to terminal body weights were equivalent across all four groups for brain, liver, spleen, and uterus plus cervix and vagina.

Relative paired kidney weight was significantly reduced at 30 ml/kg, and relative paired ovary weight was significantly reduced at 10 and 30 ml/kg (Table 19).

F1 Pup Necropsy Findings on Pnd Day 21

F1 pup gross necropsy findings at weanling sacrifice on pnd 21 are presented in Table 20. There were no treatment- or dose-related clinical findings. In F1 female pups, alopecia (limbs) was observed in one female at 10 ml/kg. Bilateral hydronephrosis was observed in one female at 0 ml/kg and in two females each at 10 and 30 ml/kg. Unilateral hydronephrosis (right or left) was observed in 3, 2, 3, and 3 females at 0, 3, 10, and 30 ml/kg, respectively. One female at 10 ml/kg exhibited pelvis not fused at midline and one female at 3 ml/kg exhibited malocclusion of the lower incisors (Table 20).

For F1 male pups, one or both Cowper's glands (very small in weanlings) could not be located in 2, 3, 1, and 2 males each at 0, 3, 10, and 30 ml/kg, respectively. One male at 30 ml/kg exhibited a diaphragmatic hernia involving the right lateral lobe of the liver. In one male at 30 ml/kg, the caput (head) of the left epididymis was detached from the corpus (body). Unilateral hydronephrosis (left or right) was observed in 4, 1, 2, and 2 males each at 0, 3, 10, and 30 ml/kg. One male at 30 ml/kg exhibited a clear cyst on the liver (1 mm in diameter). In one male at 10 ml/kg, the prostate was reduced in size. In one male at 0 ml/kg, the left testis was undescended (Table 20).

F0 Female Necropsy

All 25 F0 dams were present in all groups at scheduled necropsy. No CMC gel-implanted control F0 females (0%) had implant material present at necropsy. All test gel-implanted F0 females (100%) in all three groups had implant material present at necropsy. Sacrifice body weights were equivalent across all four groups. Absolute organ weights were equivalent across all four groups for the spleen, paired kidneys, paired ovaries, and uterus plus cervix and vagina. Absolute liver weight was significantly reduced at 30 ml/kg and equivalent across the 0, 3, and 10 ml/kg groups. Organ weights, relative to terminal body weights, were equivalent across all groups for the spleen, paired kidneys, paired ovaries, and uterus plus cervix and vagina. Relative liver weight was significantly reduced, relative to the CMC gel-implanted

control group value, in all three test gel-implanted groups, but there were no differences among the test gel groups (Table 21).

F0 female gross necropsy findings (Table 22) exhibited no treatment- or dose-related pattern of incidence or severity. These findings included alopecia (in various areas) in all four groups and red-tinged fluid at or around the implant site in two F0 females in the CMC gel-implanted control group (most likely indicative of vascularization at the site). One F0 female at 0 ml/kg and three F0 females at 3 ml/kg exhibited fluid-filled uteri, most likely due to these animals being in full estrus at demise (Table 22). There were no treatment- or dose related histopathologic findings in the F0 maternal animals, based on 25/group in the high dose and control groups (see Pathology Report in Appendix III).

F1 Retained Offspring

Seventy-five (75) animals/sex/group (three/sex/litter, 25 litters/group and four groups) were retained from weaning to adult necropsy (there were 74 F1 males retained at 10 ml/kg due to an error during weaning). All retained animals survived to scheduled necropsy (Table 23).

F1 Retained Females

In-Life

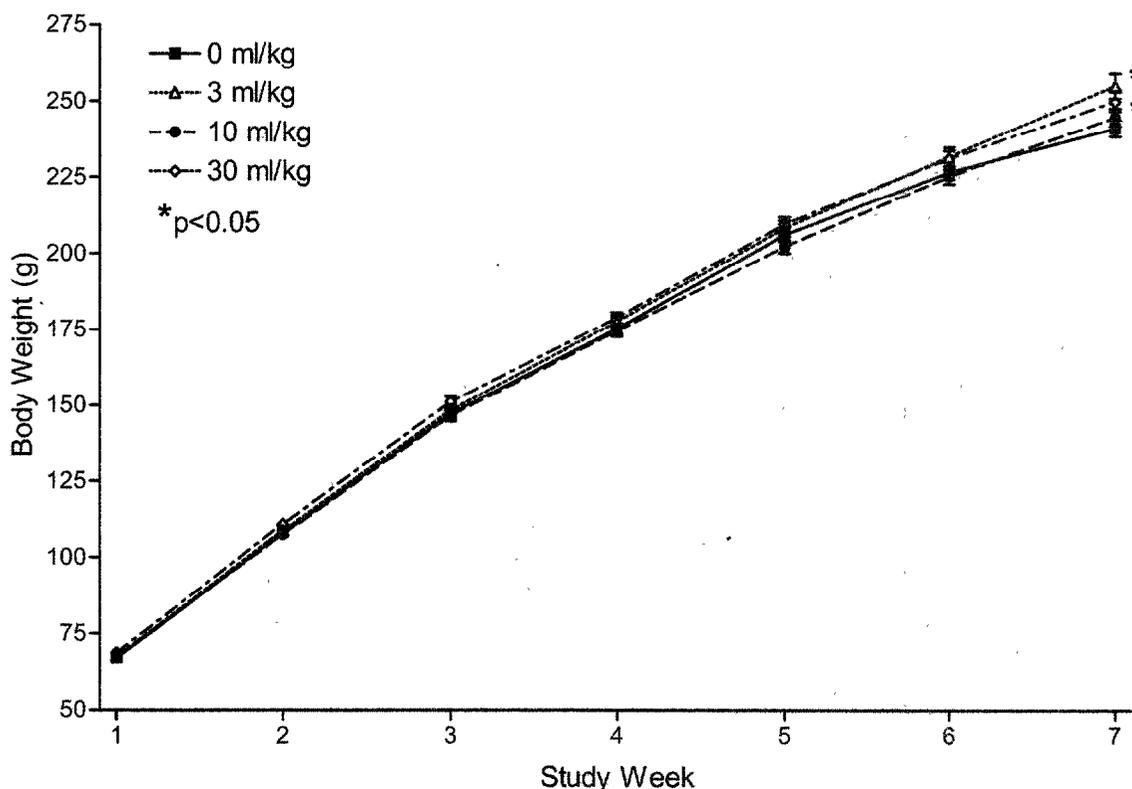
During the seven-week retention period, F1 female body weights were equivalent across all groups for all weekly time points, except for week 7 (day 1) when the mean body weights for F1 females at 3 and 30 (but not 10) ml/kg were significantly increased relative to the control group value (Table 24 and Figure 4). F1 female weekly body weight changes during this period were equivalent across all groups for all intervals, except for weeks 4 to 5 when the 10 (but not the 3 or 30) ml/kg group value was significantly reduced, and for weeks 6 to 7 when the 3 (but not the 10 or 30) ml/kg group value was significantly increased (Table 24).

F1 female feed consumption in g/day was equivalent across all groups for all intervals except for weeks 3-4, 4-5, and 6-7 when the values at 3 (but not 10 or 30) ml/kg were significantly increased, and for weeks 1 through 6 when the values at 3 and 30 ml/kg (but not 10 ml/kg) were also significantly increased (Table 25). F1 female feed consumption in g/kg/day was equivalent across all groups for all intervals (Table 25).

Clinical observations of F1 retained females during the postwean holding period (Table 26) exhibited no treatment- or dose-related changes in incidence or severity. They exhibited alopecia, chromodacryorrhea, dehydration, sore(s) in various locations, and abrasion in and under the left eye in one female at 0 ml/kg, beginning on day 7 of week 3, which slowly resolved after a small, round particle was removed from under the eyelid. The skin was missing from the tail tip or tail tip missing from one female at 10 ml/kg, beginning on week 5, day 6 (Table 26). All of these observations are typical (except for the eye and tail injuries) of rats during this time period.

Figure 4

F1 Female Body Weights



F1 female acquisition of puberty (vaginal patency/opening) was slightly but statistically significantly delayed at 3 (delay of 1.3 days) and 10 ml/kg (delay of 1.4 days) and unaffected at 30 ml/kg. Body weight at acquisition was also significantly increased at 3 (but not 10 or 30)

ml/kg. When age at vaginal patency was adjusted (by analysis of covariance, with body weight at acquisition as the covariate), only the delay at 10 ml/kg (an adjusted delay of 0.9 days) was statistically significant (Table 27).

Evaluation of F1 postweaning females for auditory startle (both force and duration of jump), motor activity (activity during the six intervals and overall), and Morris water maze, to evaluate learning and memory (mean swim time on days 1 and 2 [trial 1] versus days 1 and 2 [trial 8] for learning, and day 1 [trial 1] versus day 2 [trial 1] for memory), were equivalent across all groups for all trials (Table 28).

F1 postweaning female FOB evaluations on pnd 28 and 60 were all equivalent across all groups for both evaluation days by pairwise comparisons. There were overall treatment effects (in the absence of pairwise comparisons) for average hindlimb foot splay on pnd 28 ($p < 0.01$) and for average hindlimb grip strength on pnd 60 ($p < 0.05$) (Table 29).

F1 postweaning female estrous cycling data indicated no effects on the number or percentage of females cycling or on cycle length in days. There were significant increases in the percentage of F1 females with an abnormal cycle at 3 (42.7%), 10 (38.7%), and 30 (48.6%) ml/kg versus the control value (24.3%), with no dose-response pattern (Table 30).

Necropsy

The retained F1 females were necropsied on pnd 70 ± 5 (all 75/group). Terminal body weights were equivalent across all groups. Absolute brain, liver, spleen, paired kidney, and paired ovary weights were equivalent across all groups. Absolute pituitary, paired adrenal gland, and uterus with cervix plus vagina weights were all significantly increased at 30 ml/kg (Table 31).

Relative weights (as percentage of terminal body weight) of the brain, liver, spleen, paired adrenal glands, paired kidneys, and paired ovaries were equivalent across all groups. Relative weights of the pituitary and uterus with cervix plus vagina were both significantly increased at 30 ml/kg (Table 31). F1 female gross findings exhibited no treatment- or dose-related pattern of incidence or severity. They did exhibit alopecia (3, 1, 1, and 2 at 0, 3, 10, and 30 ml/kg, respectively), hydronephrosis (bilateral and/or left or right; 6, 6, 6, and 8 at 0, 3, 10, and 30 ml/kg, respectively), ovarian cysts (one each at 0 and 3 ml/kg and two at 30 ml/kg), cyst in the uterine mesentery (one at 0 ml/kg), enlarged spleen (one at 0 ml/kg), tail tip missing (one

at 10 ml/kg), enlarged thymus (two at 3 ml/kg), and fluid-filled uterus (one each at 0, 3, 10, and 30 ml/kg), the last most likely due to these females being in estrus at demise (Table 32).

There were no treatment- or dose-related histopathologic findings in any F1 female evaluated out of 25/group in the high dose and control groups (Table 32 and Pathology Report, Appendix III).

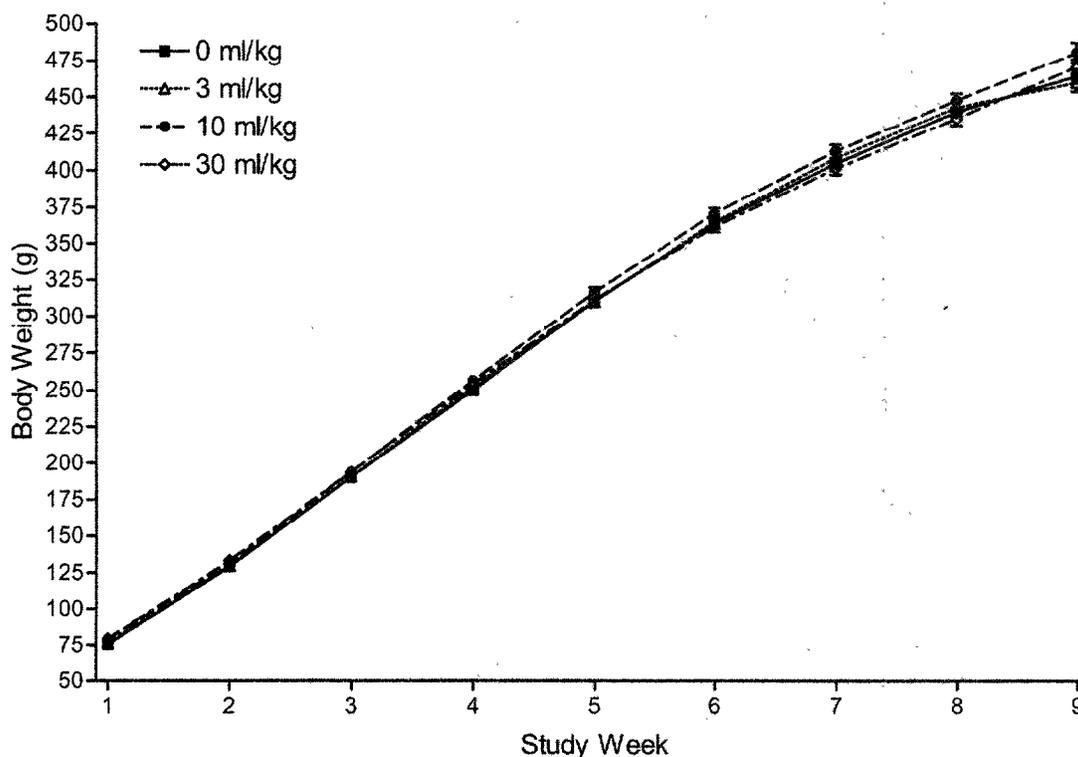
F1 Retained Males

In-Life

During the nine-week retention period, F1 male body weights were equivalent across all groups for all time points (Table 33 and Figure 5). Weekly body weight changes were also equivalent across all groups for all intervals, except for week 6-7 when the mean value at 3 (but not 10 or 30) ml/kg was significantly increased and for week 8-9 when the mean value at 10 (but not 3 or 30) ml/kg was also significantly increased (Table 33).

Figure 5

F1 Male Body Weights



F1 retained male feed consumption in g/day was equivalent across all groups for all nine weeks and for all feed consumption for weeks 1 through 8. F1 retained male feed consumption in g/kg/day was also equivalent across all groups for all nine weeks except for weeks 2-3 when the mean value at 3 (but not 10 or 30) ml/kg was significantly increased. The mean values for overall feed consumption for weeks 1 through 8 were also equivalent across all groups (Table 34).

Clinical observations of F1 retained males during the postwean holding period (Table 35) exhibited no treatment- or dose-related incidence or severity. The signs observed included alopecia (5, 8, 10, and 8 at 0, 3, 10, and 30 ml/kg, respectively), chromodacryorrhea (3, 6, 3, and 6 at 0, 3, 10, and 30 ml/kg, respectively), dehydration and emaciation (one male at 0 ml/kg due to a sipper tube malfunction), lacerations on left side of face (one male at 0 ml/kg), salivation (one male at 0 ml/kg), scab(s) (one male at 10 ml/kg), unilateral (right) micro-ophthalmia (one male at 10 ml/kg) and sore(s) at various locations (4, 6, 2, and 1 at 0, 3, 10, and 30 ml/kg, respectively) (Table 35).

The age at F1 retained male acquisition of puberty (preputial separation) was equivalent across all groups. Body weight at acquisition was slightly but significantly increased only at 10 (but not 3 or 30) ml/kg. When the age at acquisition was adjusted (by analysis of covariance, with body weight at acquisition as the covariate), the values were still equivalent across all groups (Table 36).

Analysis of retained F1 male auditory startle, motor activity, and Morris water maze (Table 37) indicates no differences across groups for auditory startle (in terms of force and duration of jumps), motor activity (in terms of activity during each of six intervals or overall total activity), or Morris water maze in terms of learning (days 1 and 2 [trial 1] versus days 1 and 2 [trial 8]) and memory (day 1 [trial 1] versus day 2 [trial 1]).

F1 retained male FOB was performed on pnd 28 and 60. There were no effects on any parameter (including body weights) across groups on pnd 28. On pnd 60, F1 male body weight was significantly increased at 3 (but not 10 or 30) ml/kg. There were no effects on any other parameter across groups (Table 38).

Necropsy

Retained F1 males were necropsied on pnd 85 ± 5 , with 75/group at 0, 3, and 30 ml/kg, and 74/group at 10 ml/kg. At necropsy, the terminal body weights were equivalent across all groups. Absolute weights of the brain, liver, spleen, paired adrenal glands, paired kidneys, seminal vesicles with coagulating gland, prostate, levator ani bulbocavernosus muscle complex, and paired Cowper's glands were all equivalent across all groups. Absolute pituitary weights were significantly increased at 3 and 30 (but not 10) ml/kg. Absolute paired testes weights were significantly increased at 3, 10, and 30 ml/kg with no dose-response pattern. Absolute paired epididymis weight was significantly increased at 10 (but not 3 or 30) ml/kg (Table 39).

Relative organ weights (as a percentage of the terminal body weights) were unaffected across groups for brain, liver, spleen, paired adrenal glands, paired kidneys, paired epididymides, paired seminal vesicles with coagulating glands, prostate, levator ani bulbocavernosus muscle complex, and Cowper's glands. Relative pituitary weights were significantly increased at 3 and 30 (but not 10) ml/kg. Relative paired testes weights were significantly increased at 3 ($p < 0.05$), 10 ($p < 0.01$), and 30 ($p < 0.05$) ml/kg, with no dose-response pattern (Table 39).

Percent cauda epididymal motile sperm values were equivalent at 0, 3, and 30 ml/kg and significantly increased at 10 ml/kg. Percent cauda epididymal sperm progressive motility was equivalent across all groups (75 males/group). For the remaining parameters (testicular homogenization-resistant spermatid head counts, calculated daily sperm production [DSP; 10^6 /testis], efficiency of DSP [10^6 /gram testis], and percent abnormal cauda epididymal sperm), only the control and high-dose males were evaluated (75 males/group). There were no differences in any values for any of these parameters between 0 and 30 ml/kg (Table 39).

There were no treatment- or dose-related incidences in any gross findings across all groups for the F1 males. Observations included alopecia, reduced size of epididymis, chromodacryorrhea, enlarged heart (one male at 30 ml/kg), right microphthalmia (one male at 10 ml/kg), hydronephrosis (bilateral or right), prostate reduced in size (one male at 3 ml/kg), seminal vesicles reduced in size and/or misshapen, sore(s) at various locations, and testes enlarged (unilateral), flaccid and reduced in size (bilateral), reduced in size (bi- and unilateral) (Table 40). There were no treatment- or dose-related histopathologic findings in F1 males, based on examination of organs from 25/group in the high dose and control groups (Table 40 and Pathology Report in Appendix III).

DISCUSSION

This study was conducted in compliance with FDA Regulations for GLPs (U.S. FDA, 2003b; except for the density determinations on the control CMC gel and the test gel that were performed using a standard method, not validated at RTI) and is similar to a previous one-generation study in rats that evaluated another silicone gel (Schardein, 1991; Siddiqui et al., 1994). This study was performed to determine whether the test substance has reproductive and developmental effects in the implanted F0 dams and in the F1 offspring generation.

The U.S. FDA Center for Devices and Radiological Health issued a document entitled "Guidance for Saline, Silicone Gel, and Alternative Breast Implants; Guidance for Industry and FDA" on February 11, 2003 (U.S. FDA, 2003a). In Chapter 5 (Toxicology Data), Section 5.4 (Special Considerations), the guidance document specifies "Reproductive and teratogenicity studies should measure the rates of conception as well as the number of fetal deaths and malformations. The studies should include at least two generations. You should test individual compounds at the highest possible exposure that does not produce nonreproductive systemic toxicity" (U.S. FDA 2003a, p. 9). In the two-generation reproductive toxicity study design, the initial parental generation, designated the F0 (or P0), begins exposures (via diet, drinking water, oral gavage, etc.) after puberty, so the risks to their reproductive system structures and functions are minimal (and likely transient). The critical generation is the F1 (first filial) generation because they are exposed beginning as gametes in their F0 parents, through gestational and lactational exposure (at least potentially via transplacental and/or translactational processes), and then with direct exposure (if the exposure is via dosed diet, dosed water, gavage, etc.) after weaning to adulthood. In the two-generation reproductive toxicity study, the selected F1 postweanlings (one/sex/litter) are reared to adulthood and then mated within dose groups to produce the F2 generation. The F2 generation is usually only potentially exposed during gestation and lactation since the two-generation study ends at the weaning of the F2 offspring.

The rat reproductive process, per se, is not a sensitive stage for evaluation of adverse effects on the reproductive system (Tyl, 2002). The rat is a robust reproducer, with excess sperm capacity and the ability to successfully breed when the testis is up to 80% destroyed. The sensitive endpoints for assessment of reproduction in the male rat are the reproductive organ weights (absolute and relative to terminal body weight; see list below), histopathology of the reproductive organs such as testes, epididymides, prostate (whole and separately by ventral and

dorsolateral lobes), seminal vesicles, coagulating glands, Cowper's glands, preputial glands (after preputial separation, signaling puberty), and the levator anibulbocavernosus (LABC) complex. Andrological endpoints are also very sensitive and appropriate: cauda epididymal sperm count, sperm morphology and motility, testicular homogenization-resistant spermatid head counts (SHC) to calculate daily sperm production (in millions/testis), and efficiency of daily sperm production (in millions/gram testis). The sensitive endpoints for assessment of reproduction in the female are also absolute and relative organ weights (ovaries, uterus), age and weight at acquisition of puberty (vaginal patency), age of first estrus and first estrous cycle, and estrous cyclicity as adults. These can be assessed in F0 animals with only postpubertal exposure and in F1 animals with exposure beginning as gametes and continuing to adulthood.

Neurobehavioral and other nonreproductive endpoints can also be assessed during the long postwean exposure period in the F1 animals. A full range of preweaning developmental landmarks (e.g., pinna detachment, eye opening, surface righting reflex, incisor eruption, auditory startle, etc.) and postwean neurodevelopmental evaluations (e.g., motor activity, auditory startle, learning and memory, etc.; Bondurant et al., 2000) can and should be performed only on F1 offspring to assess the nonreproductive effects of possible exposure during gestation and lactation (or possible indirect effects on the implanted dam) because of their long exposure duration and the timing of exposure through sensitive pre- and postnatal life stages. Reproductive developmental endpoints can also be assessed on F1 offspring, including anogenital distance (on postnatal day [pnd] 0 at birth, pnd 21 at weaning, and as adults), retention of nipples and areolae in preweaning males (pnd 11-13) at weaning and as adults, and acquisition of puberty.

In females, puberty is signaled by vaginal patency. RTI's historical control database in CD® (SD) rats indicates that the grand mean age of vaginal patency over 15 studies (since 1996) is 31.1 days. In males, puberty is signaled by preputial separation. RTI's historical control database in CD® (SD) rats indicates that the grand mean age in days for preputial separation is 41.9 days.

The two major weaknesses in the current multigeneration study design are: (1) only one F1 animal/sex/litter is retained past weaning for subsequent mating to produce the F2 generation, so only one representative/sex/litter is evaluated for reproduction, necropsy, histopathology, andrology, etc., as an adult and represents the entire litter; and (2) the study design specifies

necropsy of up to three pups/sex/litter at weaning (prior to puberty and adulthood), with the remaining nonretained offspring euthanized and discarded. Retaining additional F1 weanlings/sex to adulthood will not affect the statistical power, per se, to detect effects (if the n = number of litters), because the number of litters will not change. However, retaining additional animals to adulthood will enhance the sensitivity of the statistical analyses in two important ways: (1) evaluating more animals to characterize each litter will better characterize the litter, reducing intra- and inter-litter variability (and if there is an effect, it will better differentiate findings in the control group from those in the treated groups); and (2) evaluating more animals per litter will improve the ability to detect treatment-related effects with low (but biologically significant) incidence. In addition, the use of SUDAAN® statistical software (RTI, 2001) allows use of the individual retained pup as the “ n ” (with intralitter correlations factored in), so retention of more F1 animals per group will increase the statistical power with SUDAAN®. The present study design specifies standardization of F1 litters to ten pups (with as equal a sex ratio as possible) on pnd 4, retention of three pups/sex/litter for postweaning evaluation and adult necropsy (75 pups/sex/group), and necropsy of remaining F1 pups (approximately two/sex/litter) at weaning.

Given the appropriateness of the F1 generation (exposure from gametes to adults) for all of the in-life reproductive and neurobehavioral (and other) evaluations, and for the necropsy and postnecropsy evaluations, and the limitations of the timing and duration of the F2 generation “exposure” with respect to providing useful information, the Sponsor proposed an alternate and more sensitive study design for consideration by the FDA (which FDA accepted in April 2003). This protocol has been called, and includes the best of, an “*in utero*/lactational study,” an “extended one-generation study,” and a modified FDA Segment III study.

The study design is currently being employed in the EPA’s Endocrine Disruptors Screening Program and is being used by other commercial clients for both EPA- and FDA-regulated substances. Several articles have been published on its utility (e.g., Gray, 1998; Gray et al., 1994, 1999a,b, 2000, 2001; McIntyre et al., 2000, 2001a,b; Mylchreest et al., 1998), and it was recommended in the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) report in 1998 (EDSTAC, 1998; Chapter 5) and by other EPA senior staff. As noted above, this study design can be construed as a modified Segment III study design and is also similar to the OECD 415 one-generation study. This approach addresses concerns on the

limitations of the traditional two-generation reproductive toxicity study design and is better than the two-generation study with regard to breast implants, because it provides a focused study design and valuable information on reproductive and nonreproductive effects in adult F0 females “exposed” prior to and during reproduction, in F1 offspring during gestation (in F0 females with gel implants) and lactation (nursed by F0 females with gel implants), and in F1 offspring during the postweaning retention period to adulthood.

The selection of CMC in sterile saline to be implanted in the control group females was the same as that employed in the previous studies (Schardein, 1991; Siddiqui et al., 1994). However, the concentration of CMS used by these authors was 1%, to yield a viscosity of 1000 centipoise. The 1% CMS solution formulated at RTI was thin/too liquid (the consistency of “soup”) and did not provide the appropriate control material to equal the density and viscosity of the test gel at the high dose level and leaked from the wound clip closure of the implantation site. Therefore, in the present study, CMC was used at 3.5% in sterile saline at the same volume as the high test gel dosed group, which provided an extruded CMC gel fully comparable to the extruded test gel.

All the females in the CMC gel implanted group exhibited accumulation of “exudate fluid” around the implant sites within a week after implantation and exhibited a large weight gain for sd 0-7 because of the fluid retention. The exudate was drained from each CMC-control female after sd 7 and before sd 14 (so they exhibited a larger weight loss for sd 7-14), with the fluid weighed and retained frozen. This accumulation of exudate fluid was also observed in Sprague-Dawley rats when CMC (at 2%) was injected into a subcutaneous pouch on the dorsum (Isaji and Naito, 1992). By five days after CMC injection, exudate volume was approximately 12-15 ml/animal, a volume slightly lower than the amount of exudate volume observed in the present study after seven days (after gel implantation). In young Wistar rats exposed to CMC in diet at 0, 2.5, 5, and 10% for three months (Bär et al., 1995), dose-related increases were observed in feed and water intake (with females more affected than males) at 2.5-10% and increases in urine production, urinary sodium, calcium and citrate excretion. Body weights were reduced in females at 10% CMC. Females also exhibited renal pelvic, and corticomedullary nephrocalcinosis and simple epithelial hyperplasia (diffuse and slight) in the urinary bladder at 10% CMC. Female absolute (at 5% CMC) and relative (at 2.5-10% CMC) liver weights were also significantly increased at 2.5-10% CMC for both sexes (Bär et al., 1995).

One additional older study (Bachmann et al., 1978) was found in the open literature that involved repeated oral gavage administration (to ZUR:SIV-Z rats; two times/day, five days/week for four weeks) of CMC-Na (sodium) in distilled water at 0 or 0.2 ml/100 g body weight (2 ml/kg; 10 mg/kg). The animals were sacrificed 16 hours after the last dose at various time intervals (every two to six days). The heart and liver mitochondria and liver endoplasmic reticulum (ER; for mixed function oxidase) were evaluated for effects on oxidative phosphorylation. CMC uncoupled oxidative phosphorylation in both liver and heart mitochondria after the first week of dosing, with maximal inhibition (70% of control) at the start of the third week of dosing. Partial improvement was observed by the end of week 4 in both organs. CMC partially inhibited mixed function oxidase activity in the liver ER, with maximal inhibition (60% of control) in the third week and full recovery by the end of the fourth (last) week. Of the four chemicals tested (gum arabic [20, 40, 200 mg/kg], gum tragacanth [10, 20, 40 mg/kg], methylcellulose [10, 20 mg/kg], and CMC-Na [10 mg/kg]), only methylcellulose at the lowest dose resulted in no effects in either the mitochondrial or ER preparations. Therefore, the effects on F0 maternal animals in the CMC gel-implanted group (accumulation of exudate at the gel implantation sites, reduced body weights and weight gains, and reduced feed consumption) were all resolved by or during lactation. Increased relative liver weight, the slightly reduced number of uterine implants, and total and live F1 pups on pnd 0 and 4, precull) are all most likely due to the physiological effects of the CMC gel implants. These *in vivo* and necropsy effects had no effect on the survival, growth, and development of the F1 offspring through weaning on pnd 21.

There were no effects of test gel implantation on the survival, growth, fertility (precoital interval, gestational length), or fecundity (numbers of uterine implantation sites and total, live, and dead pups/litter) of the F0 maternal females. F0 maternal organ weights at necropsy and estrous cyclicity prior to mating were also unaffected (Table C; presenting the CMC control gel group values, the test gel group values, and the range of historical control mean values for CD® [SD] female rats; taken from Appendix IV). There were also no effects of maternal test gel implantation on the F1 offspring survival, growth, survival indices, sex ratio, anogenital distance (on pnd 0 and 21), nipple and/or areolar retention in preweanling males, acquisition of preweanling developmental landmarks, or on organ weights at pnd 21 necropsy in either sex in any of the test gel-implanted groups (Table D; presenting the CMC control gel group values, the

test gel group values, and the range of historical control values for CD® [SD] offspring rats; taken from Appendix IV).

Overviews of the F1 postweaning offspring endpoints are presented in Table E for F1 females and in Table F for F1 males. There were no treatment- or dose-related findings for F1 females in-life, at necropsy, or for histopathology of selected organs, including no effects on the mammary glands, esophagus, brain, liver, spleen, paired kidneys, or paired ovaries. The absolute and relative weights of the pituitary and the uterus plus cervix plus vagina were increased at 30 ml/kg, with no histopathologic findings (Table E). Comparison of the weights of these organs in the present study, with weights of these organs from females at slightly older ages and higher body weights (Table G), indicates that the absolute organ weight control values in the present study are below the control values, while the treated group absolute organ weights were at or below the previous control values. For F1 males (Table F), there were no treatment- or dose-related effects in-life, at necropsy, or for histopathology of selected organs, including no effects on the brain, liver, spleen, paired adrenal glands, paired kidneys, paired epididymides, seminal vesicles plus coagulating glands (and their fluids), prostate, levator ani bulbocavernosus muscle complex, or paired Cowper's glands. The absolute and relative weights of the pituitary were increased at 3 and 30 (but not 10) ml/kg. The absolute and relative weights of the paired testes were increased at 3, 10, and 30 ml/kg in the absence of any histopathologic findings (Table F). Comparison of the weights of these organs in this study, with the weights of these organs from males at approximately comparable ages and body weights (Table G), indicates that the control values from the present study are either lower than the historical control range or that the control values and test gel-implanted treatment group values are all within the historical control values. This is consistent with the interpretation that the statistical effects in this study are due to the lower control values and do not indicate treatment- or dose-related effects (Table G). In support of this view, testicular toxicity is almost always indicated by decreased absolute and relative paired testes weights associated with histopathologic lesions. The rare treatment-related increased testicular weights are due to testicular edema and calcifications. There were no treatment-related testicular histopathologic lesions observed in F1 adult males in this study.

Table C. Comparison of Control and Treated F0 Maternal Endpoints in This Study Versus Historical Control Mean Data

Parameter	Present Study Means		Historical Control Means (range)
	Control	Test Group (range)	
PREBREED PERIOD			
<u>Body Weights (g)</u>			
sd: 0	238.4	236.7 – 238.8	225.9 – 276.4 ^a
7	261.9	243.3 – 250.6	237.5 – 290.7
14	241.8	260.8 – 264.4	245.9 – 302.3
21	248.7	272.1 – 375.4	247.7 – 309.6
28	253.2	280.9 – 285.7	258.8 – 317.9
<u>Body Weight Change (g)</u>			
sd: 0-7	23.4	4.6 – 13.2	10.7 – 18.7 ^a
7-14	-20.1	13.7 – 17.4	8.4 – 14.7
14-21	6.9	10.3 – 11.3	1.8 – 15.5
21-28	4.6	8.8 – 10.3	3.4 – 11.1
<u>Feed Consumption</u>			
g/day sd: 0-7	15.6	20.9 – 21.4	19.2 – 22.7 ^a
7-14	15.2	21.4 – 21.7	17.0 – 22.9
14-21	16.7	21.2 – 21.4	18.8 – 23.6
21-28	17.6	20.2 – 21.2	17.0 – 22.2
g/kg/day sd: 0-7	62.4	86.8 – 87.5	70.8 – 83.8
7-14	60.5	83.9 – 85.4	69.9 – 84.4
14-21	68.0	78.9 – 80.1	67.1 – 79.7
21-28	69.9	72.9 – 76.5	63.7 – 76.8
<u>Estrous Cyclicity</u>			
% females cycling	92.0	96.0 – 100.0	100.0 – 100.0
% females with abnormal cycles	43.5	12.5 – 20.0	3.3 – 17.24
Cycle length (days)	4.1	3.8 – 4.1	4.11 – 4.69
GESTATION			
<u>Body Weights (g)^b</u>			
gd: 0	258.5	283.6 – 284.8	270.3 – 339.4
20	381.6	422.3 – 425.4	398.4 – 499.6
<u>Body Weight Change (g)^b</u>			
gd: 0-20	123.1	138.6 – 140.7	125.2 – 160.2

(continued)

Table C (continued)

Parameter	Present Study Means		Historical Control Means (range)		
	Control	Test Group (range)			
<u>Feed Consumption (gd 0-20)^b</u>					
g/day	22.4	24.1 – 24.9	21.9 – 28.7		
g/kg/day	73.6	71.0 – 72.7	61.6 – 71.0		
LACTATION					
<u>Body Weights (g)</u>					
pnd: 0	295.2	324.1 – 332.3	309.3 – 385.4		
4	317.6	341.3 – 338.6	319.3 – 392.7		
7	334.4	349.6 – 351.5	328.2 – 399.0		
14	351.8	365.5 – 369.4	341.9 – 401.7		
21	334.9	338.7 – 347.1	324.1 – 380.0		
<u>Body Weight Change (g)^b</u>					
pnd 0-21	39.8	12.8 – 23.0	-7.4 – 17.0		
<u>Feed Consumption</u>					
g/day	pnd:	0-4	34.2	36.3 – 38.8	32.5 – 38.5
		4-7	48.6	49.4 – 51.1	39.8 – 53.0
		7-14	65.4	65.4 – 67.2	36.8 – 69.3
		14-21	77.2	75.7 – 78.5	63.7 – 80.5
		0-21	60.8	61.1 – 63.3	43.8 – 64.3
g/kg/day	pnd:	0-4	110.9	107.4 – 116.2	90.7 – 107.0
		4-7	148.3	143.7 – 148.5	117.3 – 146.5
		7-14	190.0	183.1 – 187.3	95.1 – 173.3
		14-21	225.4	213.0 – 220.1	181.4 – 208.9
		0-21	184.6	178.2 – 181.5	117.3 – 171.8
Precoital interval (days)	3.8	2.1 – 2.8	2.3 ^c		
Gestational length (days)	22.0	22.0 – 22.1	22.0 – 22.6		

(continued)

Table C (continued)

Parameter	Present Study Means		Historical Control Means (range)
	Control	Test Group (range)	
<u>Necropsy</u>			
Body weight (g)	327.88	329.47 – 336.74	292.05 – 361.45
Organ weights:			
Liver	A ^d	18.4544	17.1760 – 17.8861
	R ^d	5.6307	5.1285 – 5.3147
Spleen	A	0.6040	0.5829 – 0.6071
	R	0.1847	0.1745 – 0.1805
Paired kidneys	A	2.7176	2.7782 – 2.8557
	R	0.8300	0.8440 – 0.8493
Paired ovaries	A	0.1307	0.1374 – 0.1431
	R	0.0399	0.0418 – 0.0428
U + C + V ^e	A	0.7400	0.7425 – 0.8086
	R	0.2267	0.2211 – 0.2444

^a For the historical control means (range) for prebreed body weights, weight change, and feed consumption, the values were chosen to best approximate the ages and body weights of the F0 females on this study.

^b In the present study, dams were weighed and feed consumption measured on gd 0, 3, 6, 9, 12, 15, 18, and 20 during gestation. The historical control data set used measurements on gd 0, 7, 14, and 20. Therefore, the only time points and interval common to both the present study and the historical control database are gd 0 and 20. Therefore, data from these two time points (and one interval, gd 0-20) are presented.

^c In the current historical control database (last updated in February 2001; Appendix IV), there were data on the precoital interval only from the most recent study in the database.

^d A = Absolute weight of organ in grams
R = Organ weight relative to terminal body weight

^e U + C + V = Uterus plus cervix plus vagina

^f Uterus weight includes cervix and may or may not contain vaginal weight.

Table D. Comparison of Control and Treated F1 Litter Endpoints During Lactation in This Study Versus Historical Control Mean Data

Parameter	Present Study Means		Historical Control Means (range)
	Control	Test Groups (range)	
No. implantations/litter	13.08	15.52 – 16.32	11.8 – 16.9
% Postimplantation loss/litter	5.79	4.16 – 6.80	0.7 – 20.4
Total no. pups/litter (pnd 0)	12.4	14.5 – 16.2	13.6 – 15.7
No. dead pups/litter (pnd 0)	0.2	0.1 – 0.2	0.0 – 1.4
No. live pups/litter (pnd 0)	12.3	14.4 – 16.0	13.3 – 15.6
Stillbirth index	1.2	0.6 – 1.4	0.2 – 10.2
Live birth index	98.8	98.6 – 99.4	89.8 – 99.8
Survival indices:			
0-4 (precull)	99.5	99.0 – 99.5	95.2 – 99.3
4 (postcull) - 7	100.0	99.6 – 100.0	98.6 – 100.0
7-14	100.0	99.6 – 100.0	98.9 – 100.0
14-21	100.0	100.0 – 100.0	99.3 – 100.0
4 (postcull) - 21	100.0	99.2 – 100.0	98.5 – 100.0
No. live pups/litter:			
pnd: 0	12.3	14.4 – 16.0	13.4 – 15.6
4 (precull)	12.2	14.3 – 15.9	13.2 – 15.3
7 (postcull)	9.7	9.8 – 10.0	7.5 – 9.9
14	9.7	9.8 – 10.0	7.5 – 9.9
21	9.7	9.8 – 10.0	7.5 – 9.9
Pup body weights/litter (g):			
pnd 0: all	6.30	6.07 – 6.39	6.28 – 6.38
male	6.47	6.21 – 6.59	6.46 – 6.56
female	6.13	5.94 – 6.19	6.11 – 6.22
pnd 4: all	9.69	9.46 – 9.94	9.66 – 11.11
male	9.96	9.63 – 10.17	9.91 – 11.35
female	9.44	9.29 – 9.70	9.79 – 10.84
pnd 7: all	14.96	15.52 – 15.97	14.60 – 18.27
male	15.36	15.75 – 16.40	14.83 – 19.01
female	14.58	15.33 – 15.58	14.39 – 17.91
pnd 14: all	31.44	31.89 – 33.00	26.32 – 38.67
male	31.96	32.29 – 33.73	26.63 – 38.92
female	30.93	31.49 – 32.31	26.03 – 38.12
pnd 21: all	52.90	51.10 – 53.14	40.35 – 62.29
male	54.00	52.00 – 54.73	40.67 – 63.20
female	51.75	50.19 – 51.68	40.01 – 61.44

(continued)

Table D (continued)

Parameter	Present Study Means		Historical Control Means (range)
	Control	Test Groups (range)	
Sex ratio (% males/litter):			
pnd: 0	48.7	49.3 – 53.7	44.6 – 53.5
4	48.4	49.3 – 53.0	43.8 – 54.7
7	48.6	48.8 – 51.8	46.8 – 51.7
14	48.6	48.8 – 51.6	46.8 – 51.9
21	48.6	48.8 – 51.6	46.8 – 51.9
No. nipples/male:			
pnd: 11-13	0.0	0.0 – 0.0	0.0 ^a
21	0.0	0.0 – 0.0	-- ^a
No. areolae/male:			
pnd: 11-13	0.28	0.09 – 0.43	0.0 ^a
21	0.0	0.0 – 0.0	-- ^a
No. males with \geq one areola:			
pnd: 11-13	11.86	4.00 – 11.67	0.0 ^a
21	0.0	0.0 – 0.0	-- ^a

^a Based on the most recent study in the historical control database (revised February 2001; Appendix IV); presence of nipples and/or areolae were assessed in F1 males only on pnd 11-13.

Table E. Summary of Retained F1 Female Endpoints During the Postwean Holding Period

Parameter	Mentor Silicone Gel (ml/kg, implant)			
	0	3	10	30
No. F1 females	75	75	75	75
<u>Body Weights (g)</u>				
Weeks 1-6	--	--	--	--
Week 7	--	↑↑	--	↑
<u>Body Weight Change (g)</u>				
Weeks 1-4 and 5-6	--	--	--	--
Weeks 4-5	--	--	↓	--
Weeks 6-7	--	↑↑	--	--
<u>Feed Consumption (g/day)</u>				
Weeks 1-2, 2-3, 5-6	--	--	--	--
Weeks 3-4	--	↑	--	--
Weeks 4-5	--	↑	--	--
Weeks 6-7	--	↑↑	--	↑
Weeks 1-6	--	↑↑	--	↑
<u>Feed Consumption (g/kg/day)</u>				
Weeks 1-7	--	--	--	--
<u>Vaginal Patency</u>				
Age at acquisition (days)	30.3	31.6***	31.7***	30.6
Body weight at acquisition (g)	--	↑↑	--	--
Adjusted age at acquisition	30.6	31.1	31.5***	31.0
<u>Neurobehavioral Assessments</u>				
Auditory startle	--	--	--	--
Motor activity	--	--	--	--
Morris water maze	--	--	--	--
FOB: pnd 28	--	--	--	--
pnd 60	--	--	--	--
Body weight at pnd 60	--	--	--	--
<u>Estrous Cyclicity</u>				
No. (%) females cycling	74 (98.7)	75 (100.0)	75 (100.0)	72 (96.0)
Cycle length in days	4.8	5.4	5.1	5.0
No. (%) females with abnormal cycle(s)	18 (24.3)	32 (42.7)*	29 (38.7)*	35 (48.6)*

(continued)

Table E (continued)

Parameter	Mentor Silicone Gel (ml/kg, implant)			
	0	3	10	30
Necropsy				
Body weight (g)	--	--	--	--
Organ weights:				
Brain	A ^a	--	--	--
	R	--	--	--
Pituitary	A	--	--	↑↑
	R	--	--	↑
Liver	A	--	--	--
	R	--	--	--
Spleen	A	--	--	--
	R	--	--	--
Paired adrenal glands	A	--	--	↑
	R	--	--	--
Paired kidneys	A	--	--	--
	R	--	--	--
Paired ovaries	A	--	--	--
	R	--	--	--
Uterus + cervix + vagina	A	--	--	↑
	R	--	--	↑
Histopathology ^b		--	--	--

^a A = absolute organ weight in grams

R = organ weight relative to terminal body weight

^b Histopathology was performed on organs from 25 females/group for high dose and control groups

-- = not statistically significant different from control group value

↑, ↑↑ = $p < 0.05$, < 0.01 ; statistically significant increase versus control group value

↓ = $p < 0.05$; statistically significant decrease versus control group value

*, *** = $p < 0.05$, $p < 0.001$; statistically significant difference from control group value when means are presented in the text table

Table F. Summary of Retained F1 Male Endpoints During the Postwean Holding Period

Parameter	Mentor Silicone Gel (ml/kg, implant)			
	0	3	10	30
No. F1 males	75	75	74	75
<u>Body Weights (g)</u>				
Weeks 1-9	--	--	--	--
<u>Body Weight Change (g)</u>				
Weeks 1-6, 7-8, 1-8	--	--	--	--
Weeks 6-7	--	↑↑	--	--
Weeks 8-9	--	--	↑	--
<u>Feed Consumption (g/day)</u>				
Weeks 1-9, 1-8	--	--	--	--
<u>Feed Consumption (g/kg/day)</u>				
Weeks 1-2, 3-8, 8-9, 1-8	--	--	--	--
Weeks 2-3	--	↑↑↑	--	--
<u>Preputial Separation</u>				
Age at acquisition (days)	42.4	42.8	43.0	42.3
Body weight at acquisition (g)	--	--	↑	--
Adjusted age at acquisition	42.5	42.7	42.7	42.6
<u>Neurobehavioral Assessments</u>				
Auditory startle	--	--	--	--
Motor activity	--	--	--	--
Morris water maze	--	--	--	--
FOB: pnd 28	--	--	--	--
pnd 60	--	--	--	--
Body weight at pnd 60	--	↑	--	--
<u>Necropsy</u>				
Body weight (g)	--	--	--	--
Organ weights:				
Brain	A ^a	--	--	--
	R	--	--	--
Pituitary	A	↑↑	--	↑↑↑
	R	↑↑	--	↑↑↑

(continued)

Table F (continued)

Parameter	Mentor Silicone Gel (ml/kg, implant)			
	0	3	10	30
Liver	A	--	--	--
	R	--	--	--
Spleen	A	--	--	--
	R	--	--	--
Paired adrenal glands	A	--	--	--
	R	--	--	--
Paired kidneys	A	--	--	--
	R	--	--	--
Paired testes	A	--	↑↑	↑↑↑
	R	--	↑	↑↑
Paired epididymides	A	--	--	↑↑
	R	--	--	--
SV + CG ^b	A	--	--	--
	R	--	--	--
Prostate	A	--	--	--
	R	--	--	--
LABC ^c	A	--	--	--
	R	--	--	--
Paired Cowper's glands	A	--	--	--
	R	--	--	--
<u>Andrology</u>				
Percent motile sperm		--	--	↑↑
Percent progressively motile sperm		--	--	--
Epididymal sperm concentration ^d		--	--	--
Homogenization-resistant spermatid head counts ^d		--	--	--
Daily sperm production (DSP; 10 ⁶ /testis) ^d		--	--	--
Efficiency of DSP ^d		--	--	--
Percent abnormal sperm ^d		--	--	--
<u>Histopathology</u> ^e				
		--	--	--

(continued)

Table F (continued)

^a A = absolute organ weight in grams

R = organ weight relative to terminal body weight

^b SV + CG = seminal vesicles plus coagulating glands

^c LABC = levator ani bulbocavernosus muscle complex

^d Assessed in 75 F1 males/group in the high dose and control groups

^e Organs from 25 F1 males/group in high dose and control groups were evaluated histopathologically

-- = not statistically significantly different from control group value

↑, ↑↑, ↑↑↑ = $p < 0.05$, $p < 0.01$, $p < 0.001$; statistically significant increase versus control group value

Table G. Comparison of Control and Treated F1 Retained Female and Male Selected Necropsy Organ Weights in This Study Versus Previous Control Data

Parameter	Present Study Means		Historical Control Means (range) ^a
	Control	Test Groups (range)	
FEMALES			
Terminal body weights (g)	244.29	241.57 – 252.44	290.4
Organ Weights			
Pituitary	A ^b		
	R		
Uterus + cervix + vagina	A		
	R		
MALES			
Terminal body weights (g)	466.15	462.98 – 476.83	501.31
Organ Weights			
Pituitary	A		
	R		
Paired testes	A		
	R		

^a To match the F1 male and female terminal body weights (and therefore organ weights) in the present study, historical control data from F1 males or females who have been bred and are much older at necropsy cannot be used. Therefore, the F3 male and female control mean data from Tyl et al. (2000; as published in Tyl et al., 2002) were used, since the F3 males and females (unbred) were only slightly older and heavier at demise than the F1 males and females in the present study.

^b A = absolute organ weight in grams

R = organ weight relative to terminal body weight

^c Weight includes uterus plus oviducts plus cervix (not vagina)

CONCLUSIONS

Based on the data from this unaudited draft final report, the conclusions are as follows:

- There were no systemic effects on the test gel-implanted F0 parental females prior to and during reproduction, including mating, gestation, and lactation;
- There were no reproductive effects on the test gel-implanted F0 parental females on mating, gestation, parturition, or lactation;
- There were no organ weight or gross and histopathologic effects in F0 parental females from test gel implantation;
- There were no effects on F1 offspring from possible indirect exposure to test gel leachate during gestation (transplacental) and lactation (from the milk)
- There were no effects on F1 offspring from indirect effects on the test gel-implanted F0 dams during gestation and lactation;
- There were no systemic, developmental, reproductive, or neurologic effects in F1 offspring through weaning from *in utero* and/or lactation exposure to test gel-implanted dams; and
- There were no systemic, developmental, or reproductive effects in F1 retained postweanlings from *in utero* and/or lactational exposure to test-gel implanted dams
 - No effects on acquisition of developmental landmarks
 - No effects on acquisition of puberty
 - No effects on FOB, grip strength, or learning and memory
 - No effects on F1 female estrous cycling
 - No effects on F1 male andrology from *in utero* and lactational exposure to gel-implanted dams
 - No effects on absolute or relative organ weights or on gross or histopathologic findings in adult F1 males and females

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