

## Research Paper

# Neither antibody to a group B streptococcal conjugate vaccine nor the vaccine itself is teratogenic in rabbits

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Group B *Streptococcus* (GBS) is a leading cause of human neonatal bacterial disease, resulting in pneumonia, sepsis, meningitis and sometimes, death. Supportive preclinical studies of GBS capsular polysaccharide (CPS)-protein conjugate vaccines have led to several phase 1 and phase 2 trials in healthy, non-pregnant adults, which demonstrated that the vaccines, produced at the Channing Laboratory, were safe and immunogenic. However, evaluation of the safety and immunogenicity of a GBS conjugate vaccine administered to pregnant women demanded that it be manufactured under current good manufacturing practices (cGMP) and that it undergo developmental toxicity evaluation. In this report, we describe a GBS type III CPS-tetanus toxoid (III-TT) vaccine lot 3-1-96 manufactured and vialled under cGMP and our evaluation of the effect of this vaccine and of GBS type III CPS-specific antibody on conception and early- and late-stage fetal development in rabbits. III-TT lot 3-1-96 was compositionally similar to prototype III-TT lot 91-1, produced under non-GMP, and was potent in a mouse maternal vaccination-neonatal pup challenge model of GBS disease. Four groups of 30 female rabbits each were randomized to receive III-TT lot 3-1-96 vaccine, saline-alum, or combinations of these treatments before and after insemination. The dose of conjugated CPS on a weight basis was 1 µg/kg, mimicking the anticipated actual human dose. Based on the weight of the rabbits, this was 20- to 100-fold greater than the expected human dose. Does were pre-assigned to deliver litters naturally or have their kits delivered by Caesarean-section at gestation day 29, to assess late fetal development. Sera from does and kits were collected, and the presence of type III CPS-specific IgG was confirmed by quantitative ELISA. Based on all assessments, GBS type III-TT lot 3-1-96, nor antibody to it did not affect embryo-fetal viability, sex ratio, growth or cause malformations (i.e., it was non-teratogenic). In addition, that III-TT lot 3-1-96 was found to be safe and immunogenic in two clinical studies involving healthy

non-pregnant adults supports a clinical evaluation of this vaccine in pregnant women.

## Introduction

*Streptococcus agalactiae* or Lancefield's group B *Streptococcus* (GBS) is a leading cause of morbidity and mortality among neonates in the United States.<sup>1</sup> Of the many serotypes that colonize humans, Ia, Ib, II, III and V cause the majority of invasive GBS disease, with serotype III most commonly associated with meningitis in infants.<sup>2</sup> Because newborns acquire the infection from a mother who is vaginally and/or rectally colonized with GBS, vaccine strategies have been directed at the development of a maternal vaccine. A correlation between neonatal GBS disease and low maternal levels of antibody to the capsular polysaccharide (CPS) of GBS<sup>3</sup> led to several clinical trials with purified CPS as vaccines,<sup>4</sup> culminating in immunization of pregnant women with purified type III CPS.<sup>5</sup> This landmark trial showed that vaccination was safe and well-tolerated, that a direct relationship between maternal- and cord-blood specific IgG resulted, but that maternal type III CPS-specific IgG levels were not optimal and were dependent upon the pre-vaccination antibody level. Subsequent efforts focused on improving the immunogenicity of all GBS CPSs by covalently linking them to immunogenic proteins,<sup>4</sup> an approach that had been highly successful with *Haemophilus influenzae* type b and *S. pneumoniae* polysaccharides.<sup>6,7</sup> The ultimate goals of the academic GBS vaccine development program were to establish a solid record of safe and immunogenic conjugate vaccines against all of the prevalent GBS serotypes and to provide the rationale necessary to produce under current good manufacturing practices (cGMP) a vaccine that could be evaluated in pregnant women to determine whether protective levels of CPS-specific IgG could be achieved safely.

In 1993, an opportunity arose for Channing scientists to direct the production of a GBS type III-tetanus toxoid (III-TT) conjugate vaccine under cGMP. Up to that time, all non-GMP lots of GBS conjugate vaccines tested clinically were manufactured at the Channing Laboratory, Brigham and Women's Hospital, Boston, Massachusetts. Evaluation in pregnant women was the principal goal of preparing a cGMP lot of III-TT vaccine. GBS III-TT lot 3-1-96 was manufactured under cGMP and controlled with use of batch records for each step in production at The Salk Institute, Swiftwater, Pennsylvania.

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**Table 1 Comparison of group B streptococcus conjugate vaccine III-TT lot 91-1 and lot 3-1-96**

Parameter	Lot 91-1	Lot 3-1-96
Type III polysaccharide		
manufacture site	Channing Laboratory (non-cGMP)	Salk Institute (cGMP)
size fractionated	Yes	No
size (peak Mr, range)	220,000 (50,000 - 1 x 10 <sup>6</sup> )	185,700 (40,700 - 1 x 10 <sup>6</sup> )
(peak Kav, range)	0.39 (0.56–0.18)	0.33 (0.51–0.10)
composition	Pass	Pass
Degree of sialic acid oxidation prior to coupling	26%	30%
Tetanus toxoid		
manufacture site	SSI	MDPHL
purified to monomer	Yes	Yes
size (Mr)	150,000	150,000
Conjugation method	RA	RA
Compositional analysis		
amt. CPS per 0.5 ml dose	58 µg	50 µg
amt. TT per 0.5 ml dose	42 µg	64 µg
Final container	Aqueous, PBS + thimerosal	Lyophilized, sucrose excipient

cGMP, current good manufacturing practices; SSI, Statens Serum Institut; MDPHL, Massachusetts Department of Public Health Laboratory; RA, reductive amination; PBS, phosphate-buffered saline.

Herein we compare the characteristics of the prototype III-TT lot 91-1 prepared at the Channing Laboratory with lot 3-1-96 prepared under cGMP, describe the developmental toxicity evaluation of lot 3-1-96 in rabbits, and present a retrospective analysis of the supporting clinical studies performed to date with this vaccine prior to its evaluation in pregnant women.

## Results

**Comparison of III-TT lot 91-1 and lot 3-1-96.** The prototype GBS CPS conjugate vaccine was III-TT lot 91-1, manufactured at the Channing Laboratory in 1991; a phase 1/2 study demonstrated the safety and immunogenicity of this vaccine in healthy, nonpregnant adult women.<sup>8</sup> The peak geometric mean concentration (GMC) of type III CPS-specific IgG elicited by lot 91-1 in healthy adults was 4.9 µg/ml attained two weeks after administration of a single 58 µg-dose a level superior to that attained with a 50-µg dose uncoupled type III CPS.<sup>8</sup>

GBS III-TT lot 3-1-96 is only the second lot of III-TT vaccine prepared for clinical use, and it is the first lot prepared under cGMP that could be compared to the prototype lot 91-1 with respect to composition and potency in animals.

**Composition.** CPSs used in the generation of the two III-TT vaccines were extracted from type III strain M781 by the same purification procedures and passed all chemical analyses (component sugar analysis, purity and composition). Because the batch of CPS used to prepare lot 91-1 had a broad size range, it was fractionated to reduce the polymer distribution. The batch of CPS used to prepare lot 3-1-96 was not size-fractionated, but the peak  $M_r$ ,  $K_{av}$  and ranges were similar to that of the sized CPS used to prepare lot 91-1 (Table 1). The degree of sialic acid oxidation of the CPS prior to coupling was also similar for the two vaccines, and the conjugation method for both vaccines was reductive amination. The Statens Serum Institut was the source of TT used in lot 91-1, and the Massachusetts Department of Public Health Laboratory was the source used in lot

3-1-96; monomeric TT was purified from polymeric TT for both lots using the same chromatographic methods. Lot 91-1 was vialled as single-dose aqueous preparations, and a 58-µg dose contained 42 µg of TT; whereas lot 3-1-96 was vialled as multidose lyophilized preparations with sucrose excipient and a 50-µg CPS dose contained 64 µg TT (Table 1).

**Potency in animals.** Vialled GBS vaccines were tested periodically for potency, using the mouse maternal vaccination-neonatal kit challenge model of GBS disease.<sup>9</sup> The efficacy of the aqueous III-TT vaccine lot 91-1 was 100% (all of 25 pups survived challenge) two months after it was vialled but decreased to 43% (25 of 51 pups survived challenge) 35 months later, and thus was decertified for use in human clinical trials.<sup>10</sup> However, the lyophilized III-TT vaccine lot 3-1-96 was 98% (52 of 53 pups survived challenge) efficacious one month after it was vialled and remained at this high level (range 91–100% survival) of efficacy 4.5 years later.<sup>10</sup> Control groups of pups born to dams vaccinated with saline, uncoupled type III CPS, or TT were not completely protected against challenge (34% or less) in these studies.

**Summary of the rabbit developmental toxicity study.** Administration of III-TT vaccine lot 3-1-96 to rabbits at a weight-equivalent human dose did not affect maternal body weights, body weight changes or absolute or relative feed consumption values throughout the study period (Table 2). The litter averages for corpora lutea, implantations, litter sizes, live fetuses, early- and late-resorptions, percent resorbed conceptuses, fetal body weights, live fetal weights and percent male fetuses were comparable among the four groups and did not differ significantly (Table 3). All placentae appeared normal.

No gross external, soft tissue, or skeletal malformations or variations in the fetuses were considered effects of III-TT vaccine. All other pregnant does assigned to natural delivery had a litter with one or more live-born kits, and no natural delivery or litter parameters were affected by III-TT vaccine. Averages for duration of gestation,

**Table 2 Rabbit body weights and food consumption**

	Group (prime/boost)			
	IA (saline/saline)	IB (saline/vaccine)	IIA (vaccine/saline)	IIB (vaccine/vaccine)
<b>Body weight (kg)</b>				
All Rabbits (n)	30	30	30	30
Day 1 (Preinsemination)	3.42 ± 0.20 <sup>a</sup>	3.42 ± 0.20	3.42 ± 0.23	3.41 ± 0.21
Day 28 (Preinsemination)	3.82 ± 0.20	3.85 ± 0.21	3.82 ± 0.26	3.81 ± 0.24
Δ Days 1–28 <sup>b</sup>	+0.41 ± 0.11	+0.43 ± 0.10	+0.39 ± 0.13	+0.40 ± 0.10
Pregnant Rabbits (n)	27	25 <sup>b</sup>	27	23 <sup>c</sup>
Day 0 (Gestation)	3.82 ± 0.20	3.86 ± 0.22	3.82 ± 0.24	3.78 ± 0.23
Day 29 (Gestation)	4.25 ± 0.31	4.40 ± 0.33	4.34 ± 0.34	4.22 ± 0.35
Δ Day 0–29	+0.44 ± 0.20	+0.54 ± 0.17	+0.52 ± 0.17	+0.44 ± 0.25
Natural Delivery Does (n)	12	12	12	12
Delivered Litters (n)	9 <sup>b</sup>	10	11	8 <sup>b</sup>
Day 1 (Lactation)	3.74 ± 0.34	3.93 ± 0.51	3.84 ± 0.39	3.77 ± 0.45
Day 21 (Lactation)	3.95 ± 0.31	4.19 ± 0.44	4.05 ± 0.26	3.94 ± 0.28
Δ Days 1–21	+0.21 ± 0.16	+0.26 ± 0.16	+0.21 ± 0.21	+0.17 ± 0.21
<b>Feed consumption (g/kg/day)</b>				
All Rabbits (n)	30	30	30	30
Days 1–28 (Preinsemination)	39.3 ± 2.1	39.7 ± 2.0 (n = 18) <sup>d</sup>	39.2 ± 3.0 (n = 18) <sup>e</sup>	39.9 ± 2.5
Days 0–29 (Gestation)	38.9 ± 4.3 (n = 26) <sup>b</sup>	39.3 ± 3.2	39.3 ± 3.3	39.1 ± 4.7 (n = 21) <sup>b</sup>
Days 1–14 <sup>f</sup> (Lactation)	59.1 ± 5.0	58.3 ± 4.0	58.6 ± 5.1	59.7 ± 2.8

<sup>a</sup>Mean ± standard deviation; <sup>b</sup>Excludes values for does that died or aborted; <sup>c</sup>Excludes value for doe that had a litter that consisted of one early resorption; <sup>d</sup>Number excludes unrecorded value; <sup>e</sup>Number includes rabbits injected on the same day; <sup>f</sup>It is presumed that kits consume maternal feed after day 14 of lactation.

implantation sites per delivered litter, number of does with stillborn kits, the fertility and gestation indices, kit deaths, liveborn litter sizes and viability indices were comparable among the four groups (Table 3). Live litter sizes over the lactation period, kit survival, litter sex ratios, viability index and kit body weights did not differ significantly among the four groups. Necropsy of kits that were stillborn, found dead, moribund sacrificed, or sacrificed on lactation day 21 did not reveal dosage-dependent or significant differences among the groups.

**Adverse events in the rabbit developmental toxicity study.** One doe that was given two doses of saline (group IA) and one doe that was given the III-TT vaccine died (group IIB). The cause of death of the former doe is unknown. The latter doe had a gastric trichobezoar, which was believed to have contributed to the death. One doe given two preinsemination doses of saline (group IB) and three does of vaccine group IIB aborted or had total litter loss. The doe in the saline group and two of the three does in the vaccine group had litters consisting of only 1 to 3 early resorptions, events that are relatively common in artificially inseminated does<sup>11,12</sup> and considered unrelated to the vaccine. The third doe was assigned to natural delivery and had a litter in which all kits died or were missing on day 1 postpartum, an event considered unrelated to the vaccine because it was a single incident. No clinical or necropsy observations were deemed to be related to the III-TT vaccine.

As previously noted, all gross external, soft tissue and skeletal malformations and variations in the fetuses were considered unrelated to the vaccine. The only statistically significant ( $p \leq 0.01$ ) increase in alterations was an increase in the fetal incidence of irregular

ossification of the skull in group IIB (Table 3). Litter averages for the percent fetuses with any alterations was 5.8%, 1.5%, 7.9% and 15.8% for groups IA, IB, IIA and IIB, respectively (Table 3). This observation was considered unrelated to the vaccine because (1) the litter value for this common variation,<sup>13</sup> the more relevant parameter,<sup>14</sup> was not significant; (2) the subcategory incidences for these irregular suture lines and/or inter- or intra-sutural bones (specifically, inter- and intra-frontal irregular suture or irregular frontal ossification; irregular nasal suture, midline nasal suture displacement; extra ossification of the anterior fontanelle) did not differ among the four groups. Moreover, all rabbit skull ossification values were within the historical ranges of the testing facility. For example, of the 1,068 litters and 8,860 fetuses examined between January 2004 and January 2006, 375 (35.1%) and 580 (6.5%), respectively, exhibited irregular ossification of the skull (M.S. Christian and A.M. Hoberman, unpublished data).

**GBS type III CPS-specific IgG in rabbits.** Four groups of rabbit does were vaccinated with two priming doses of vaccine or saline before insemination, followed by three booster doses after conception (Fig. 1). During the two-week period (i.e., during insemination and implantation stages) after the rabbits were given a second priming dose of III-TT vaccine (groups IIA and IIB), the geometric mean concentration (GMC) of type III CPS-specific IgG rose 20- to 29-fold from 0.51–0.76 µg/ml at -7 day of conception to ~15.00 µg/ml at 7 to 14 days of conception (Table 4). Subsequent booster doses of vaccine did not increase the level of antibody in these groups. As expected, type III CPS-specific antibody was absent from does in group IA. Does that were given vaccine during pregnancy

**Table 3 Rabbit pregnancy and delivery events**

	Group (prime/boost)			
	IA (saline/saline)	IB (saline/vaccine)	IIA (vaccine/saline)	IIB (vaccine/vaccine)
<b>Pregnant and Caesarean-sectioned rabbits on gestation day 29; (n)</b>	17	16 <sup>a</sup>	16	14 <sup>b</sup>
Corpora Lutea (n)	11.0 ± 2.5 <sup>c</sup>	9.0 ± 2.3	11.3 ± 3.0	10.5 ± 2.7
Implantations (n)	8.2 ± 2.7 <sup>c</sup>	7.6 ± 3.3	7.2 ± 2.8	7.8 ± 3.0
Litter Sizes (n)	7.9 ± 2.6 <sup>c</sup>	7.5 ± 3.4	6.6 ± 2.5	7.7 ± 3.1
Resorptions (n)	0.3 ± 0.6 <sup>c</sup>	0.1 ± 0.2	0.6 ± 1.0	0.2 ± 0.4
Does with any resorptions; n (%)	4 (23.5)	1 (6.7)	6 (37.5)	2 (15.4)
Litters with fetuses with irregular skull ossification; n (%)	3 (17.6)	1 (6.7)	2 (6.2)	5 (38.5) <sup>d</sup>
Litters with fetuses with any observed alteration; n (%)	8 (47.0)	2 (13.3)	6 (37.5)	6 (46.2)
<b>Fetuses (n)</b>	134	113	105 <sup>e</sup>	100
Live male fetuses/litter (%)	55.7 ± 21.2 <sup>c</sup>	44.6 ± 14.6	41.7 ± 22.9	48.2 ± 20.0
Live fetal body weights (g/litter)	43.74 ± 5.67 <sup>c</sup>	44.15 ± 5.22	44.97 ± 6.14	43.16 ± 5.40
Fetuses with any observed alteration; n (%)	9 (6.7)	2 (1.8)	9 (8.6)	14 (14.0) <sup>d</sup>
Fetuses with irregular skull ossification; n (%)	3 (2.2)	1 (0.9)	2 (1.9)	7 (7.0)
Fetuses with any alteration/litter (%)	5.8 ± 6.6 <sup>c</sup>	1.5 ± 3.9	7.9 ± 11.8	15.8 ± 20.4
<b>Natural delivery rabbits (n)</b>	12	12	12	12
Pregnant (n)	10	10	11	9
Fertility index (%) <sup>f</sup>	83.3 <sup>c</sup>	83.3	91.7	75.0
Gestation (days)	32.3 ± 0.7 <sup>c</sup>	32.8 ± 1.0	32.4 ± 0.5	32.2 ± 0.7
Delivered litters with >1 kit	9	10	11	8
Total kits delivered (n)	72	64	82	55 <sup>g</sup>
	8.0 ± 1.3 <sup>c</sup>	6.4 ± 2.2	7.4 ± 2.4	7.8 ± 2.8
Viability index n alive/n total (% alive)	63/70 (90.0)	58/60 (96.7)	74/81 (91.4)	49/54 (90.7)
Lactation index <sup>h</sup>	57/63 (90.5)	54/58 (93.1)	59/74 (79.7)	47/49 (95.9)
Male kits (%) of those sexed on day 21	35.2 ± 13.4 <sup>c</sup>	48.6 ± 30.9	53.9 ± 21.3	61.0 ± 27.0
Kit weight/litter (g)				
Day 4	73.5 ± 12.5 <sup>c</sup>	92.2 ± 33.4	80.1 ± 27.8	79.6 ± 23.1
Day 21	287.0 ± 44.0 <sup>c</sup>	339.5 ± 137.3	315.5 ± 78.7	275.7 ± 59.0

<sup>a</sup>N = 15 included in analysis due to a doe that had a litter that consisted of three early resorptions; <sup>b</sup>N = 13 included in analysis due to a doe that had a litter that consisted of one early resorption; <sup>c</sup>Mean ± standard deviation; <sup>d</sup>p ≤ 0.01 compared to group IA due to fetal incidence of irregular skull ossification; <sup>e</sup>One dead fetus excluded from analyses; <sup>f</sup>No. of matings/no. of pregnancies; <sup>g</sup>Excludes litter that had no live kits on day 1 postpartum; <sup>h</sup>No. of live kits on day 21 postpartum/no. of liveborn kits on day 4 postpartum.

(group IB) achieved a GMC of type III CPS-specific IgG of 5.57 µg/ml five days after receiving the third dose (Table 4).

A direct positive correlation ( $r = 0.97$ ) was found between the GMC of type III-specific IgG measured in maternal and fetal kit sera at gestation day 29 (Table 5). Antibody persisted in rabbits that were given the III-TT vaccine to lactation day 21; antibody half-life in rabbits was approximately 14 days (Table 5).

As expected, kits born to does that were given the III-TT vaccine either before or after insemination had type III CPS-specific IgG (Table 6). The GMC of specific IgG varied between litters within each group, with the highest levels measured in kits born to groups IIA and IIB does that were given the vaccine as the first dose, with up to 10-fold less IgG in kits from group IB does that were given the vaccine as a second dose (Table 6). Low to no type III CPS-specific IgG was measured in litters born to group IA does that were given saline. These data allow for an evaluation of the developmental toxicity not only of III-TT vaccine itself but also of vaccine-induced antibody levels.

**Conclusions of the rabbit developmental toxicity study.** The maternal and developmental no-observable-adverse-effect-levels

(NOAELs) for III-TT lot 3-1-96 is the highest dose tested, 1.0 µg/kg, when administered twice before gestation and three times during the gestation period. No effects were observed in any of the III-TT groups. The developmental NOAEL is also 1.0 µg/kg, when administered twice before gestation and three times during the gestation period. There were no adverse effects on the embryo-fetal or kit development as evaluated in this study. In conclusion, based on these data, GBS III-TT lot 3-1-96 conjugate vaccine should not be considered teratogenic. These data supported clinical evaluation of the vaccine in pregnancy.

**Retrospective analysis of phase 1 clinical trials of III-TT lot 3-1-96.** The safety and immunogenicity of III-TT lot 3-1-96 was confirmed in two separate clinical trials involving non-pregnant healthy adults. Fifty healthy adults between the ages of 18 and 45 received a 12.5-µg CPS dose of III-TT lot 3-1-96 alone or in combination with a GBS type II-TT conjugate vaccine.<sup>15</sup> In a second trial, 36 of these participants received a second 12.5-µg CPS dose of III-TT lot 3-1-96 vaccine 21 months later to examine the effect of a second dose of vaccine on the immune response.<sup>16</sup> The III-TT vaccine was well-tolerated (pain level was mild to

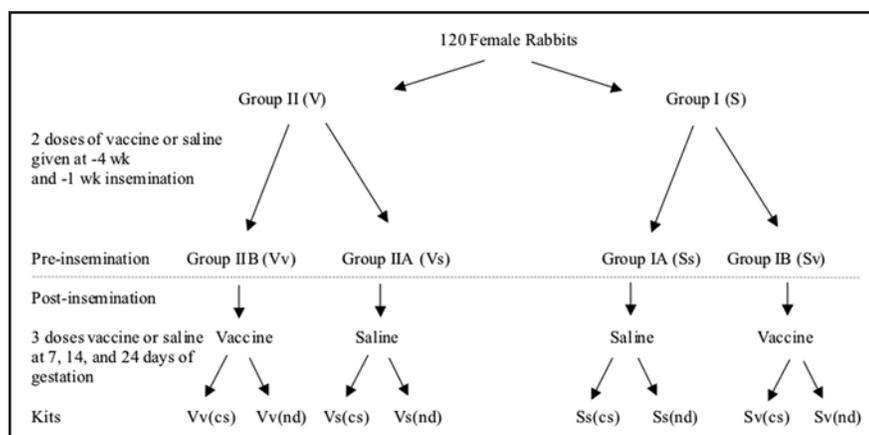


Figure 1. Schematic of the teratogenicity evaluation of GBS III-TT in New Zealand White rabbits. V, dams ( $n = 30$ ) received III-TT vaccine prior to insemination; S, dams ( $n = 30$ ) received saline prior to insemination; v, dams ( $n = 30$ ) received III-TT vaccine during pregnancy; s, dams ( $n = 30$ ) received saline during pregnancy; cs, kits ( $n = 18$  does) delivered by Caesarean-section and sacrificed one day prior to parturition; nd, kits ( $n = 12$  does) delivered naturally and weaned. The vehicle for the III-TT vaccine was 0.45% sodium chloride with 0.01% thimerosal. Alhydrogel (1.3% aluminum hydroxide gel) was incorporated into the 0.45% saline vehicle for this study.

moderate for most recipients) with low redness or swelling scores, with the exception of two males who developed a low-grade fever (100.4°F and 100.6°F) with chills, malaise and headache within 17 h after vaccination, which resolved completely with use of a nonsteroidal anti-inflammatory agent 22 h after onset. The type III CPS-specific IgG responses in these individuals rose sharply from 0.4  $\mu\text{g/ml}$  before to approximately 16  $\mu\text{g/ml}$  one month after vaccination,<sup>15</sup> with the magnitude of the response influenced strongly by the pre-existing level of type-specific antibody.<sup>16</sup>

## Discussion

The academic vaccinologist assumes the challenge of marshalling a candidate antigen from discovery through proof-of-principle and preclinical testing in a suitable animal model in order to evaluate its potential as a vaccine. Positive findings in preclinical immunogenicity, and in some cases, safety, tests could lead to evaluation of the candidate vaccine in phase 1 and phase 2 clinical trials to determine if it is safe and immunogenic in healthy humans.<sup>17</sup> Armed with calls from advocacy groups for a viable vaccine and a clear mandate from the National Academy of Sciences, Institute of Medicine ranking an effective GBS vaccine a Level I (most favorable) priority,<sup>18</sup> we sought to further the progress of a GBS vaccine beyond clinical trials in healthy non-pregnant adults to healthy pregnant women, the population targeted to be immunized in order to protect newborns against GBS disease. The path toward this goal led from the generation of a conjugate vaccine prepared under cGMP with transfer of technology from the Channing Laboratory, a research laboratory, to the Salk Institute, a vaccine production facility. Although tolerance ranges for each vaccine parameter were not established, there was remarkable similarity between the prototype lot 91-1 and lot 3-1-96, especially with respect to the size of type III CPS and the degree of sialic acid oxidation, parameters that impact cross-linking of the CPS with the tetanus toxoid carrier protein.<sup>19</sup> Experience with lyophilized CPS antigens and conjugate vaccines at the Channing Laboratory

suggested that a lyophilized preparation might offer longer stability and potency than if the vaccine was vialled as an aqueous preparation, a hypothesis that was later proven correct by extended potency testing of GBS vaccines in mice<sup>10</sup> and chemical analyses of lyophilized *Haemophilus influenzae* type b conjugate vaccines.<sup>20</sup> Although most licensed conjugate vaccines are now vialled as aqueous preparations, GBS III-TT lot 3-1-96 was vialled as a lyophilized preparation with sucrose excipient with no thimerosal or other preservatives.

III-TT lot 3-1-96 was the first GBS conjugate vaccine to be examined as a reproductive and developmental teratogen. The vaccine itself, as well as antibody to it, were evaluated for potential harmful effects on developmental events occurring from pre-conception and implantation to early- and late-fetal development, including organogenesis and newborn health and survival rates. Tested at 100 times the maximum anticipated dose in humans on a weight basis, there were no deleterious effects attributed to the III-TT vaccine in comparison with the placebo group given saline and alum adjuvant.

Differences in pregnancy and abortion rates among the four groups were within the variation observed at the testing facility. For example, in 78 studies performed between January 2000 and January 2002 involving 1,187 rabbits, the pregnancy rate was 90.9%, 0.8% died, 1.9% spontaneously aborted, and 0.3% delivered prematurely (Christian MS and Hoberman AM, unpublished data). The sole statistical difference among the four groups in our study was the overall incidence in the variation of fetal skull ossification. We consider this variation to be of minor importance because there was no differences in the subcategory analyses and because the observations are within historical values of the testing facility.

Serum type III CPS-specific IgG confirmed that the III-TT lot 3-1-96 vaccine was active in rabbits, with the highest levels detected in sera from the two groups that were given the vaccine prior to insemination (IIA and IIB). At gestation day 29, transfer of type III CPS-specific IgG from does to kits in the three vaccine groups was apparent, with persistence of specific antibody in sera of 21-day-old kits. Transfer of functional specific maternal IgG following vaccination with a GBS conjugate vaccine has been demonstrated in several animal models including mice, rabbits and baboons.<sup>21-23</sup>

GMP lot 3-1-96 was compositionally similar to the prototype III-TT vaccine lot 91-1, which allowed for direct comparison of safety and immunogenicity. Lot 3-1-96 was evaluated in two phase 1/2 clinical trials in healthy non-pregnant adults.<sup>15,16</sup> These trials showed that III-TT lot 3-1-96 was safe and immunogenic and that the vaccine-induced, CPS-specific antibody was functionally active in vitro, findings sufficient to forward this lot of vaccine for phase 1 evaluation as a maternal vaccine in healthy pregnant women. A preliminary report from a double-blinded, placebo-controlled trial involving pregnant women (18 to 45 years of age) between 30 and 32 weeks of gestation described the III-TT lot 3-1-96 vaccine as well-tolerated and immunogenic and that it elicited high levels of maternal antibody that were functionally active in vitro.<sup>24</sup> Importantly, there was a strong, positive correlation of type III CPS-specific IgG in maternal and delivery-cord blood and infant sera:  $>0.5 \mu\text{g/ml}$  of

Table 4 Immunogenicity of GBS III-TT vaccine in rabbit does

Group (Prime/Boost)	Geometric mean GBS type III CPS IgG concentration ( $\mu\text{g/ml}$ ), n, [95% CI] (range) at preinsemination/gestation day:					
	-28 <sup>a</sup>	-7 <sup>a</sup>	7 <sup>b</sup>	14 <sup>b</sup>	24 <sup>b</sup>	29 <sup>c</sup>
IA (Saline/Saline)	0.00, n = 20 <sup>d</sup> [-0.13-0.07] (0.00-0.39)	0.01, n = 20 [-0.00-0.08] (0.00-0.39)	0.00, n = 20 [0.00] (0.00)	0.00, n = 20 [0.00] (0.00)	0.00, n = 19 [0.00] (0.00)	0.11, n = 12 [0.39-1.28] (0.00-1.61)
IB (Saline/Vaccine)	0.00, n = 22 [-0.02-0.07] (0.00-0.47)	0.01, n = 22 [0.00-0.08] (0.00-0.44)	0.00, n = 20 [0.00] (0.00)	0.00, n = 20 [-0.7-0.21] (0.00-1.34)	1.60, n = 20 [-0.71-15.4] (0.00-78.31)	5.57, n = 12 [2.06-17.89] (0.45-47.09)
IIA (Vaccine/Saline)	0.00, n = 21 [-0.11-0.40] (0.00-2.53)	0.76, n = 20 [-0.13-7.19] (0.02-34.88)	15.00, n = 21 [16.03-29.74] (0.38-60.95)	10.03, n = 20 [15.93-30.74] (0.00-58.01)	10.59, n = 20 [10.37-17.63] (1.15-28.29)	7.59, n = 12 [6.41-13.60] (2.04-18.38)
IIB (Vaccine/Vaccine)	0.00, n = 19 [-0.01-0.34] (0.00-0.19)	0.51, n = 18 [0.34-1.09] (0.14-3.26)	11.76, n = 19 [10.94-21.48] (1.31-43.62)	14.84, n = 20 [12.27-25.89] (2.91-70.49)	10.02, n = 19 <sup>e</sup> [8.26-15.39] (3.39-33.68)	10.83, n = 12 [8.24-16.30] (4.74-25.62)

Dose administered <sup>a</sup>before and <sup>b</sup>after insemination. <sup>c</sup>Caesarean-sectioned rabbits only. <sup>d</sup>Number of samples measured.

Table 5 Immunogenicity of GBS III-TT vaccine in rabbit does and fetuses

Group (Prime/Boost)	Geometric mean type III IgG concentration ( $\mu\text{g/ml}$ ) [95 % CI] (range)			
	Does at gestation day 29	Fetal kits at gestation day 29	Does at lactation day 7	Does at lactation day 21
IA (Saline/Saline)	0.11, n = 12 <sup>a</sup> [0.39-1.28] (0.00-1.61)	0.00, n = 10 <sup>b</sup> [0.00] (0.00)	0.00, n = 6 <sup>c</sup> [0.00] (0.00)	0.00, n = 6 <sup>c</sup> [0.00] (0.00)
IB (Saline/Vaccine)	5.57, n = 12 <sup>a</sup> [2.06-17.89] (0.45-47.09)	8.51, n = 11 <sup>d</sup> [-0.42-36.30] (1.10-97.34)	3.95, n = 6 <sup>e</sup> [0.08-9.57] (1.14-12.61)	2.86, n = 6 <sup>e</sup> [1.51-5.64] (1.57-5.38)
IIA (Vaccine/Saline)	7.59, n = 12 <sup>a</sup> [6.41-13.60] (2.04-18.38)	14.23, n = 9 <sup>f</sup> [10.09-25.24] (3.26-34.92)	8.52, n = 8 [5.45-16.08] (1.43-20.79)	3.43, n = 8 [2.02-6.71] (0.58-8.67)
IIB (Vaccine/Vaccine)	10.83, n = 12 <sup>a</sup> [8.24-16.30] (4.74-25.62)	15.01, n = 7 <sup>g</sup> [11.08-20.15] (10.25-24.81)	8.20, n = 5 <sup>h</sup> [1.81-18.03] (2.83-20.19)	3.63, n = 5 <sup>h</sup> [0.90-8.09] (1.37-7.72)

<sup>a</sup>Caesarean-sectioned rabbits only; <sup>b</sup>Two serum samples inadvertently destroyed; <sup>c</sup>One rabbit not pregnant, one rabbit died; <sup>d</sup>One rabbit not pregnant; <sup>e</sup>Two rabbits not pregnant; <sup>f</sup>One rabbit not pregnant, two serum samples inadvertently destroyed; <sup>g</sup>Two rabbits not pregnant, two serum samples inadvertently destroyed, one fetus resorbed; <sup>h</sup>Two rabbits not pregnant, one rabbit aborted.

specific IgG promoted  $>1 \log_{10}$  reduction of GBS type III colony forming units by human polymorphonuclear leukocytes, suggesting that functional immune protection in the infant was passively acquired from the mother.

III-TT lot 3-1-96 was one of three lots prepared at the Salk Institute under cGMP; the other two lots, prepared with CPS purified from the same GBS culture, were denoted lots 1-1-95 and 2-1-96. All three lots were potent as determined with the mouse maternal vaccination-neonatal pup challenge model of GBS disease<sup>10</sup> and could be used in clinical trials. Experiences gained from producing cGMP lots of GBS conjugate vaccine, the fact that lot 3-1-96 was not teratogenic, and the preliminary report of the safety and immunogenicity of III-T T lot 3-1-96,<sup>24</sup> advanced our understanding and expanded our knowledge of GBS vaccines, as the task of providing a safe and efficacious GBS vaccine is now transferred from academia to industry.

## Materials and Methods

**Preparation of GBS type III capsular polysaccharide.** Master and production seed stocks of *Streptococcus agalactiae* strain M781,

obtained from the Channing Laboratory, were established and stored at  $-70^{\circ}\text{C}$  at the Salk Institute. A 200-L fermentor containing 100 L of Columbia broth supplemented with 7.2% dextrose was seeded with 10-L culture of GBS type III strain M781. The culture was incubated for 16 h with pH maintained at 7.0 by the addition of NaOH, and the dissolved oxygen level maintained at 50%. The culture yield was  $8 \times 10^9$  CFU/ml.

CPS, removed from cells by mutanolysin extraction and from culture supernatant fluids by ethanol precipitation, was purified, using procedures described in detail previously.<sup>25</sup> Purified type III CPS was free of protein ( $<0.5\%$ ) and passed all chemical tests for identity including component sugar analysis, NMR analysis and immunoreactivity with type III CPS-specific antibody.

**Manufacture of III-TT.** Bulk tetanus toxoid (TT, lot LP951P, obtained from the Massachusetts Public Health Biologic Laboratory, Boston, MA) was concentrated by ultrafiltration, and the protein monomer ( $150,000 M_r$ ) was isolated by gel filtration chromatography. Purified type III CPS was oxidized with sodium periodate<sup>25</sup> to yield oxidation of 30% of the sialic acid residues. Oxidized type III CPS was combined with monomeric TT and the reductive

**Table 6 Group B streptococcal type III capsular polysaccharide-specific IgG in naturally delivered rabbit kits at 21 days of age**

Group (Prime/Boost)	Doe number	No. of kits	Geometric mean type III CPS-specific IgG concentration ( $\mu\text{g/ml}$ ) [95 % CI] (range)
IA (Saline/Saline) <sup>a</sup>	4107	7	0.00 [0.00] (0.00)
	4110	5	0.03 [0.03–0.04] (0.03–0.04)
	4111	6	0.00 [0.00] (0.00)
	4117	6	0.16 [0.14–0.18] (0.13–0.19)
	4119	4	0.00 [0.00] (0.00)
	4120	8	0.02 [0.01–0.03] (0.01–0.04)
IB (Saline/Vaccine) <sup>b</sup>	4133	6	0.19 [0.15–0.24] (0.16–0.28)
	4134	7	0.08 [0.07–0.08] (0.07–0.09)
	4137	8	0.19 [0.16–0.22] (0.13–0.25)
	4138	4	0.12 [0.11–0.14] (0.11–0.13)
	4139	7	0.25 [0.22–0.28] (0.19–0.28)
	4140	6	1.11 [0.94–1.31] (0.89–1.33)
IIA (Vaccine/Saline)	4164	6	3.81 [2.93–4.83] (3.20–5.65)
	4166	3	1.90 [1.71–2.09] (1.81–1.95)
	4167	5	3.86 [2.85–5.01] (3.18–5.25)
	4169	4	0.31 [0.25–0.36] (0.28–0.36)
	4172	6	3.42 [2.87–4.04] (3.03–4.48)
	4174	6	3.55 [2.99–4.18] (2.94–4.48)
	4175c	7	6.05 [5.16–7.08] (5.06–8.17)
	4176	5	1.74 [1.09–2.54] (1.09–2.59)
IIB (Vaccine/Vaccine) <sup>d</sup>	4197	7	1.36 [1.15–1.60] (1.01–1.74)
	4201	5	2.32 [1.81–2.88] (1.94–3.04)
	4205	4	0.73 [0.47–1.02] (0.55–0.90)
	4208	6	7.11 [6.42–7.86] (5.83–7.80)
	4209	7	1.11 [0.98–1.27] (0.94–1.39)

<sup>a</sup>One rabbit died, one rabbit not pregnant; <sup>b</sup>Two rabbits not pregnant; <sup>c</sup>One kit sacrificed (moribund); <sup>d</sup>Two rabbits not pregnant, one rabbit aborted.

amination conjugation reaction initiated by addition of sodium cyanoborohydride. The conjugate was purified by gel filtration chromatography, unreacted aldehydes on sialic acid residues were reduced by the addition of sodium borohydride, and the conjugate dialyzed against water prior to lyophilization. The composition of the vaccine was determined by using protein and CPS assays as described previously.<sup>25</sup> GBS type III-TT vaccine lot 3-1-96 was mixed with sucrose in multidose vials and lyophilized. Final containers of III-TT lot 3-1-96 passed tests for sterility, general safety and pyrogenicity as required by 21CFR610.<sup>11-13</sup>

**Potency testing of GBS III-TT vaccine.** The mouse maternal immunization-neonatal mouse challenge model of GBS disease<sup>10</sup> was used to measure the efficacy of GBS conjugate vaccines.

**Rabbit developmental toxicity study.** The purpose of this study was to detect adverse effects of GBS III-TT lot 3-1-96 given to female rabbits from implantation through lactation on gestation, parturition, lactation and maternal behavior and on the development of the offspring of the vaccinated rabbits. The New Zealand White [Hra:(NZW)SPF] rabbit was selected as the test system because: (1) it is a non-rodent mammalian species accepted and widely used throughout the industry for preclinical studies of developmental toxicology (embryo-fetal toxicity/teratogenicity); (2) this strain of rabbit has been demonstrated to be sensitive to developmental toxins;

(3) it possesses a hemichorial placenta; (4) historical data and experience exists at the testing facility;<sup>11,12</sup> and (5) the test article (III-TT vaccine) is pharmacologically active in this species and strain.<sup>25</sup> The rabbits were approximately 5 months of age at the onset of the study and weighed between 2.90 and 3.92 kg. This study evaluated ICH Harmonised Tripartite Guideline stages C through E. FDA standards (21 CFR Part 58) were used as the basis for compliance with good laboratory practices.

The two items evaluated in the rabbit developmental toxicity study were GBS III-TT lot 3-1-96 and the vehicle (saline; 0.45% sodium chloride containing 0.01% thimerosal). Alhydrogel (1.3% aluminum hydroxide gel) was incorporated into the saline vehicle to enhance immunogenicity in the rabbit.

The reproductive toxicology study design is shown in Figure 1. The estimated human dose of vaccine may range from 5  $\mu\text{g}$  to 50  $\mu\text{g}$  of conjugated type III CPS. The human dose on a weight basis administered to rabbits was 1.0  $\mu\text{g}/\text{kg}$ , a value based on the highest anticipated dose of 50  $\mu\text{g}$  of conjugated CPS and the weight of an average female of 50 kg.

Rabbits in groups IA and IB were given a single intramuscular injection of saline ~4 weeks and 1 week prior to insemination, whereas rabbits in groups IIA and IIB were given III-TT vaccine ~4 weeks and 1 week prior to insemination. Rabbits in groups IA

and IIA then were given single intramuscular injections of saline on gestational days 7, 14 and 24, while rabbits in groups IB and IIB were given single intramuscular injections of III-TT on these same gestational days. This gestational period encompassed post-implantation through organogenesis in rabbits. A dosage volume of 0.04 ml/kg was used, adjusted based on body weights recorded before injection.

Within each of the four treatment groups of 30 rabbits each, 12 were assigned to natural delivery and weaning of their kits, and the rest were assigned to Caesarean section delivery. The female rabbits were observed for viability at least twice daily and for general appearance weekly during the acclimation and pre-insemination periods and on gestational day 0 (insemination = gestation day 0). Additional examinations for clinical observations of the effect of III-TT vaccine on abortions, premature deliveries and deaths were made daily beginning on gestational day 6. Post-vaccination observations were recorded ~1 h post-injection. Clinical observations were also recorded on lactation days 1, 4, 7, 14 and 21 (birth = lactation day 1) for the rabbits that delivered litters. Body weights were recorded once during acclimation, weekly before insemination, on the day of injection, on the day of insemination, and daily after gestational day 6. For the rabbits that delivered litters, body weights were also recorded on lactation days 1, 4, 7, 14 and 21. Feed consumption values were recorded daily.

The rabbits assigned to natural delivery were evaluated for duration of gestation, litter sizes, and kit viability. Maternal behavior was evaluated daily when the kits were examined during the 21-day postpartum period and recorded on lactation days 1, 4, 7, 14 and 21. Any variations from expected maternal behavior were recorded on all other days of the postpartum period.

Kits that either appeared stillborn or that died before initial examination of the litters for viability were examined for vital status at birth, unless precluded by autolysis or cannibalization by the doe. Lungs were removed and immersed in water, and kits with lungs that sank were considered stillborn; kits with lungs that floated were considered liveborn and to have died shortly after birth. Gross lesions were retained in buffered 10% formalin.

Kits in each litter were counted and physical signs recorded on lactation day 4 and daily after lactation day 7. Kit body weights and nursing behavior were recorded on days 4, 7, 14 and 21 after birth.

Blood samples were collected from the first 12 rabbits in each group assigned to Caesarean section and from the first eight rabbits in each group assigned to natural delivery on the days of both pre-insemination injections and on gestational days 7, 14 and 24 before injection. In addition, rabbits assigned to Caesarean section had blood drawn on the day sacrificed (day 29). Rabbits assigned to natural delivery had blood samples collected on lactation day 7 and at sacrifice. On the day of Caesarean section, blood samples were collected from the umbilical cords of all fetuses from the first 12 litters per group. On lactation day 21, blood samples were collected from all kits from the first eight does assigned to natural delivery.

Rabbits assigned to Caesarean section were sacrificed on day 29, and a gross necropsy of the thoracic, pelvic and abdominal viscera was performed. The number of corpora lutea in each ovary was recorded; and the uterus of each rabbit was excised and examined for pregnancy, number and distribution of implantations, live and dead fetuses, and early- and late-resorptions. Each fetus was

removed from the uterus, weighed and examined for sex and gross external alterations. All fetuses were examined for skeletal alterations. Fetal alterations were defined as: (1) malformations (irreversible changes that occur at low incidences in this species and strain); (2) variations (common findings in this species/strain, and reversible delays or accelerations in development).

All rabbits assigned for natural delivery and their kits were sacrificed on postpartum/lactation day 21, and a gross necropsy of the thoracic, pelvic and abdominal viscera was performed. The number and distribution of implantation sites were recorded for the does, and all kits were examined internally for sex and gross lesions.

**Measurement of rabbit antibody to GBS type III capsular polysaccharide (CPS).** Serum was collected from ~20 (two-thirds) randomly chosen rabbits from each of the four groups, from fetal kits at gestation week 29, and from kits at 21 days of age, and GBS type III CPS-specific IgG was quantified with use of an ELISA.<sup>26</sup> Briefly, flat-bottom, 96-well microtiter plates (Maxisorp, NUNC, Denmark) were coated with 250 ng/well of GBS type III CPS-human serum albumin (III-HSA) conjugate as the target antigen. A standard curve was prepared by coating the first two rows of wells with goat F(ab')<sub>2</sub> anti-rabbit IgG (ICN, Costa Mesa, CA) as the capture antibody at a concentration of 250 ng/well. Checkerboard titrations with the target antigen and the capture antibody were performed to determine optimal coating concentrations. Coated plates were statically incubated at 30°C for 6 h. Plates were then washed five times with 10 mM Tris containing 154 mM NaCl, 0.1% Brij 35, and 0.05% NaN<sub>3</sub>, pH 7.4 (wash buffer) before addition of the test sera. Standard rabbit reference serum to GBS type III-TT vaccine (SRRS III) was used as the positive control and normal rabbit serum as the negative control. Sera samples from adult rabbits were tested at serial two-fold dilutions beginning at a dilution of 1:5,000; samples from fetal and newborn kits were tested beginning at 1:100. SRRS III was raised in rabbits to an oligosaccharide III-TT conjugate vaccine<sup>27</sup> and used at an initial 1:5,000 dilution. The negative control serum was used at a 1:200 dilution. Serum samples (100 µl per well) were added to each coated plate, and the plate was incubated 16 h at 4°C. All sera were diluted in incubation buffer (10 mM PBS containing 0.05% Brij 35, 5% newborn calf sera and 0.05% NaN<sub>3</sub>). Plates were then washed five times with wash buffer before the addition to each well of 100 µl of alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) at a dilution of 1:2,000. Plates were incubated for 2 h at 30°C, washed five times with wash buffer, 100 µl of *p*-nitrophenyl phosphate (1 mg/ml phosphatase 104, Sigma Chemical Company, St. Louis, MO, pH 9.8) with 0.3 mM MgCl<sub>2</sub> was added, and the plates were allowed to develop for 1 h at 37°C with orbital shaking. The absorbance at 405 nm ( $A_{405\text{ nm}}$ ) was determined with use of a kinetic reader (Bio-Tek Instruments, Winooski, VT).

The amount of GBS type III CPS-specific IgG in each serum sample was determined by comparison of the  $A_{405\text{ nm}}$  of the test sample with the linear portion of a standard curve generated on a separate ELISA using the goat F(ab')<sub>2</sub> anti-rabbit IgG and known concentrations of rabbit IgG (Southern Biotechnology Associates).<sup>26</sup> Dilutions of test sera that resulted in an  $A_{405\text{ nm}}$  closest to 1 were used in antibody calculations. The assay was deemed valid if the following criteria were met: (1) the concentration of type III CPS-specific IgG in SRRS III was within the range of 14.1 to

17.3 µg/ml (i.e., ± 10% of the mean of 15.7 µg/ml that resulted from six individual determinations); (2) the negative control sera had an  $A_{405\text{ nm}}$  value between 0.1 to 0.25; and (3) the test serum samples  $A_{405\text{ nm}}$  values used for comparison to the standard curve were between 0.5 and 1.1. If the sample exceeded an  $A_{405\text{ nm}}$  value of 1.1, it was retested at higher dilutions.

**Statistical analyses.** Clinical observation and other proportional data were analyzed by using the Variance Test for Homogeneity of the Binomial Distribution.<sup>28</sup> Continuous data (e.g., maternal body weights, body weight changes, feed consumption values, and litter averages for percent male fetuses and kits, percent resorbed conceptuses, fetal and kit body weights, fetal anomaly data and fetal ossification site data) were analyzed by using Bartlett's Test of Homogeneity of Variances<sup>29</sup> and Analysis of Variance<sup>30</sup> when appropriate (i.e., Bartlett's Test was not significant at  $p > 0.05$ ). If the Analysis of Variance was significant (i.e.,  $p \leq 0.05$ ), the Dunnett's Test<sup>31</sup> was used to identify the statistical significance of the individual groups as compared with the control group. If the Analysis of Variance was not appropriate (i.e., Bartlett's Test was significant at  $p \leq 0.05$ ), the Kruskal-Wallis Test<sup>32</sup> was used, when ties  $\leq 75\%$  were present. When this test was statistically significant at  $p \leq 0.05$ , Dunn's Method of Multiple Comparisons<sup>33</sup> was used to identify the statistical significance of the individual groups. If there were  $>75\%$  ties, then Fisher's Exact Test<sup>34</sup> was used to analyze the data. Count data obtained at Caesarean section and all other natural delivery and discrete data (e.g., number of fetuses or kits, length of gestation) were evaluated by using the procedures described above for the Kruskal-Wallis Test. For all assessments,  $p < 0.05$  was considered significant.

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