NCTR PROTOCOL E0219001

TWO YEAR CHRONIC TOXICOLOGY STUDY OF BISPHENOL A (BPA) [CAS # 80-05-7] ADMINISTERED BY GAVAGE TO SPRAGUE-DAWLEY RATS (NCTR) FROM GESTATIONAL DAY 6 UNTIL BIRTH AND DIRECTLY TO F_1 PUPS FROM POSTNATAL DAY (PND) 1; CONTINUOUS AND STOP DOSE (PND 21) EXPOSURES

STATISTICAL REPORT

STATISTICAL ANALYSIS OF LITTER PARAMETER DATA

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FOR

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Statistical Analysis of Litter Parameter Data

1. Objectives

1.1 Project Objectives

The goal of this two year chronic study is to characterize the long term toxicity of orally administered BPA, including developmental exposure, in the NCTR Sprague-Dawley (CD) rat over a broad dose range.

1.2 Analysis Objectives

The goal of this analysis is to test the treatment effect of exposure to BPA in Sprague-Dawley rats based on litter parameter data.

2. Experimental Design

The study design consisted of first generation female and male rats (F_0) for up to 600 mating pairs randomized to treatment groups in 5 loads. The goal of the F_0 matings was to obtain 352 study litters, 50 per dose group for vehicle controls and five BPA dose groups, 2.5, 25, 250, 2500, and 25000 µg/kg bw/day, and 26 for each of two EE_2 dose groups, 0.05 and 0.5 µg/kg bw/day. Dams were dosed daily from gestation day (GD) 6 until parturition. Dosing was by gavage for F_0 dams and F_1 pups, the second study generation. There were two study dosing arms of F_1 animals, daily continuous dosing to termination, and daily dose stopped at post-natal day (PND) 21. There was a vehicle control group and five BPA groups for each study dosing arm, and EE_2 daily dose groups for the continuous dosing arm only. From the F_1 litters, pups were allocated at weaning, PND 21, to the interim (1 year) and terminal (2 year) sacrifices for the core study. Pups within litter and sex were assigned to different dosing arms and sacrifice times. Additional pups were assigned to other protocols that provided animals and tissues to academic investigators.

Litter Parameter Data

Litter parameter data were collected for dams with pups allocated to either the core study or used for the academic investigator study, which included any litters produced over the core study goals.

3. Statistical Methods

Analyses and adjustments for multiple comparisons were performed separately for the BPA and EE_2 treatments. Sex proportions of pups within litters and pup counts (number alive, males, females, number unsexed, and number born dead), total litter weight and mean pup weight (across and by sex) were analyzed.

Unsexed pups were assigned as male sex for analysis of sex proportions and analyses of female and male counts. Pup counts (number alive, number of males and number of females) were analyzed using Poisson regression. Litter sex proportions were analyzed for treatment effects using logistic regression.

For litter weight data, across and by sex, an analysis was performed using contrasts within a one-way model analysis of variance (ANOVA) to test for treatment effect. For litter mean pup weights, an analysis was performed using contrasts within an analysis of covariance (ANOCOVA) adjusted for litter size to test for treatment effect.

Pairwise comparisons of treatment means to the control group were performed using contrasts with Dunnett's method of adjustment for multiple comparisons. Tests of trend, increasing

treatment effect with increasing dose, were performed for the BPA and vehicle control groups. All tests were performed as two-sided tests.

For litter parameter endpoints, a sensitivity analysis was also performed. For a portion of the gestational period, 85 dams (16 in vehicle control, 50 in BPA 2.5, 25, 250, 2500, and 25000 µg/kg bw/day, and 19 in EE_2 µg/kg bw/day dose groups) were held in the same rooms as a special BPA 250,000 µg/kg bw/day high dose requested by an academic laboratory. In consultation with the Principal Investigator, to address the possibility of inadvertent exposure, a sensitivity analysis excluding litters of these 85 dams was also performed to test the robustness of the results. Additional statistically significant pairwise comparisons from the sensitivity analysis are reported in the text.

4. Results

Tables are included in Appendix A.

4.1 BPA Treatments

Summary statistics for the BPA treatments are presented in Table 1 for litter counts and in Table 2 for litter sex proportions. Summary statistics for the EE_2 treatments are presented in Table 5 for litter counts and in Table 6 for litter sex proportions. The number of pups born dead were not analyzed due to sparse data (of 483 total litters only 7 litters had pups born dead).

Analysis results for BPA are presented in Table 3 for litter counts. Trend was not significant and there was no statistically significant difference for any dosed group compared to control. Analysis results for sex proportions are presented in Table 4. Trend was not significant and there was no statistically significant difference for any dosed group compared to control. In analysis of sex proportions with unsexed pups assigned as female, there were no differences in conclusions.

Summary statistics for BPA treatment average pup and litter weights are presented in Table 5. Results of ANOVA for total litter weights and ANOCOVA for litter mean pup weights with covariate number of pups in the litter are given in Table 6. The ANOVA and ANOCOVA omnibus test results are given for the null hypothesis that all of the control and BPA treatment means for weight are equal. The covariate litter size was a significant effect (all $p<0.001$); there were no other significant effects.

Comparisons of BPA treatments to vehicle groups for total litter weights and pup weights are given in Table 7. There were no significant trends for total litter weights or for pup weights. There were no significant differences between any of the treatment groups and the vehicle control group for total litter weights or for pup weights.

In the sensitivity analyses of litter counts, sex proportions, total litter weights, and pup weights for BPA dose groups, there were no additional statistically significant results.

4.2 EE_2 Treatments

Analysis results for EE_2 are presented in Table 8 for litter counts. There was no statistically significant difference for any dosed group compared to control. Analysis results for sex proportions are presented in Table 9. There was no statistically significant difference for any dosed group compared to control. In analysis of sex proportions with unsexed pups assigned as female, there were no differences in conclusions.

Summary statistics for EE_2 treatment average pup and litter weights are presented in Table 12. Results of ANOVA for total litter weights and ANOCOVA for litter mean pup weights with covariate number of pups in the litter are given in Table 13. The ANOVA and ANOCOVA omnibus test results are given for the null hypothesis that all of the control and EE_2 treatment means for weight are equal. The covariate litter size was a significant effect (all $p<0.001$); there were no other significant effects.

Comparisons of EE_2 treatments to vehicle groups for total litter weights and pup weights are given in Table 14. There were no significant trends for total litter weights or for pup weights. There were no significant differences between the treatment groups and the vehicle control group for total litter weights or for pup weights.

In the sensitivity analyses of litter counts, sex proportions, total litter weights, and pup weights for EE_2 dose groups, there were no additional statistically significant results.

5. Conclusions

In comparisons of BPA dosed groups to the control group, there were no significant differences for litter counts, sex proportions, mean pup or litter weights. In comparisons of EE_2 dosed groups to the vehicle control, there were no significant differences for litter counts, sex proportions, mean pup or litter weights.

Appendices

A. Statistical Tables

a) BPA Treatments Litter Counts and Proportions

Table 1. Summary Statistics for Litter Size by Sex and Number Born Dead for Bisphenol-A											
Dose		***Litter Size***		***# of Males***		***# of Females***		***# of Unsexed***		***# Born Dead***	
(µg/kg$_{BW}$/day)	***N***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***
Control	73	11.8	0.4	5.8	0.2	5.8	0.2	0.2	0.1	0.00	0.00
BPA 2.5	65	12.6	0.3	6.4	0.3	6.0	0.3	0.2	0.1	0.08	0.05
BPA 25	61	11.9	0.5	6.2	0.3	5.6	0.3	0.1	0.0	0.02	0.02
BPA 250	64	11.6	0.5	5.7	0.2	5.7	0.4	0.2	0.1	0.00	0.00
BPA 2500	64	12.3	0.4	6.2	0.3	5.8	0.3	0.3	0.1	0.02	0.02
BPA 25000	64	11.5	0.4	5.5	0.3	5.8	0.3	0.2	0.1	0.02	0.02

Table 2. Summary Statistics for Litter Sex Proportions for Bisphenol-A							
Dose		***Male %***		***Female %***		***Unsexed %***	
(µg/kg$_{BW}$/day)	***N***	***Mean***	***SE***	***Mean***	***SE***	***Mean***	***SE***
Control	73	49.4	1.5	49.5	1.5	1.1	0.4
BPA 2.5	65	51.5	2.3	47.0	2.3	1.6	0.6
BPA 25	61	52.2	2.2	47.0	2.2	0.8	0.3
BPA 250	64	50.6	2.0	47.9	2.1	1.5	0.7
BPA 2500	64	50.9	1.8	46.7	1.7	2.3	0.9
BPA 25000	64	47.3	2.3	51.5	2.3	1.1	0.6

Table 3. Poisson Regression Test of Treatment Effect on Litter Counts for Bisphenol-A [1]

	Dose (µg/kg'BW'/day)																					
	Control			BPA 2.5				BPA 25				BPA 250				BPA 2500				BPA 25000			
Analysis[2]	Mean	SE	P	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P
Alive	11.8	0.4	0.442	12.6	0.4	106.6	0.566	11.9	0.4	101.0	1.000	11.6	0.4	98.2	0.996	12.3	0.4	104.1	0.885	11.5	0.4	97.4	0.981
Females	5.8	0.3	0.859	6.0	0.3	102.6	0.996	5.6	0.3	95.6	0.961	5.7	0.3	98.0	0.999	5.8	0.3	99.4	1.000	5.8	0.3	99.9	1.000
Males	5.8	0.3	0.262	6.4	0.3	109.9	0.519	6.2	0.3	107.5	0.763	5.7	0.3	97.9	0.999	6.2	0.3	106.0	0.881	5.5	0.3	94.7	0.915
Males+	6.0	0.3	0.361	6.6	0.3	110.5	0.450	6.3	0.3	106.2	0.864	5.9	0.3	98.4	1.000	6.5	0.3	108.8	0.617	5.7	0.3	95.0	0.927

[1] All p-values and % are relative to the control group, except for the trend p-value shown below control.
[2] Analysis 'Alive' was based on the sum of counts of unsexed and sexed pups; analysis of 'Males+' included unsexed as well as male pups.

Table 4. Comparison of Sex Proportions for Bisphenol-A (Proportion of Males)

Dose (µg/kg'BW'/day)																							
Control[1]			BPA 2.5				BPA 25				BPA 250				BPA 2500				BPA 25000				
Mean	SE	P	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P
0.505	0.017	0.606	0.524	0.017	103.7	0.914	0.532	0.018	105.2	0.752	0.506	0.018	100.2	1.000	0.528	0.018	104.5	0.833	0.493	0.018	97.5	0.984

[1] All p-values and % are relative to the control group, except for trend shown below control.

b) BPA Treatments Litter Weights

Table 5. Summary Statistics for Litter Weights (g) for Bisphenol-A																		
	Dose (µg/kg$_{BW}$/day)																	
	Control			***BPA 2.5***			***BPA 25***			***BPA 250***			***BPA 2500***			***BPA 25000***		
Analysis	***N***	***Mean***	***SE***	***N***	***Mean***	***SE***	***N***	***Mean***	***SE***	***N***	***Mean***	***SE***	***N***	***Mean***	***SE***	***N***	***Mean***	***SE***
Average Female	73	6.83	0.11	62	6.60	0.10	61	6.80	0.10	64	6.79	0.10	63	6.72	0.13	64	6.90	0.13
Average Male	73	7.06	0.11	62	6.99	0.09	61	7.18	0.14	64	7.11	0.09	63	7.00	0.12	64	7.24	0.12
Average Pup	73	6.96	0.09	62	6.82	0.10	61	7.04	0.11	64	6.93	0.08	63	6.88	0.11	64	7.05	0.12
Females	73	38.49	1.62	62	37.16	2.33	61	36.69	1.97	64	36.44	2.16	63	37.04	1.73	64	37.24	1.74
Males	73	40.10	1.74	62	43.57	2.29	61	43.47	2.15	64	39.14	1.63	63	41.65	1.82	64	38.09	2.04
Total	73	78.59	2.31	62	80.73	2.62	61	80.16	2.81	64	75.58	2.88	63	78.69	2.38	64	75.33	2.59

Table 6. Test of Treatment and Covariate Effects on Litter Weight[1] for Bisphenol-A Dose (µg/kg$_{BW}$/day)

Analysis	*Effect*	*NumDF*	*DenDF*	*Fvalue*	*P value*
Mean Female	Treatment	5	380	0.343	0.886
	Pup Count	1	380	117.263	**<.001**
Mean Male	Treatment	5	380	0.462	0.804
	Pup Count	1	380	86.685	**<.001**
Mean Pup	Treatment	5	380	0.469	0.799
	Pup Count	1	380	147.269	**<.001**
Females	Treatment	5	381	0.147	0.980
Males	Treatment	5	381	1.334	0.248
Total	Treatment	5	381	0.748	0.588

1. Analyses were performed separately for females, males, and totals; ANOVA was performed for litter weights and ANOCOVA was performed for mean pup weights.

Table 7. ANOCOVA of Litter Mean Pup Weight and ANOVA of Litter Weight (g)[1] for Bisphenol-A Dose (µg/kg$_{BW}$/day)

	Control			*BPA 2.5*				*BPA 25*				*BPA 250*				*BPA 2500*				*BPA 25000*			
Analysis	*Mean*	*SE*	*P*	*Mean*	*SE*	*Pct*	*P*	*Mean*	*SE*	*Pct*	*P*	*Mean*	*SE*	*Pct*	*P*	*Mean*	*SE*	*Pct*	*P*	*Mean*	*SE*	*Pct*	*P*
Mean Female	6.816	0.094	0.661	6.685	0.102	98.1	0.824	6.791	0.102	99.6	1.000	6.742	0.100	98.9	0.980	6.760	0.101	99.2	0.994	6.853	0.100	100.5	0.999
Mean Male	7.040	0.096	0.505	7.068	0.105	100.4	1.000	7.177	0.106	101.9	0.814	7.064	0.103	100.3	1.000	7.038	0.104	100.0	1.000	7.194	0.103	102.2	0.726
Mean Pup	6.942	0.081	0.785	6.899	0.088	99.4	0.997	7.037	0.088	101.4	0.900	6.887	0.086	99.2	0.989	6.915	0.087	99.6	1.000	7.001	0.086	100.9	0.985
Females	38.488	1.810	0.667	37.158	1.964	96.5	0.985	36.690	1.980	95.3	0.948	36.436	1.933	94.7	0.909	37.040	1.949	96.2	0.978	37.241	1.933	96.8	0.988
Males	40.104	1.829	0.210	43.574	1.985	108.7	0.584	43.470	2.001	108.4	0.616	39.142	1.953	97.6	0.997	41.649	1.969	103.9	0.972	38.094	1.953	95.0	0.919
Total	78.592	2.439	0.208	80.732	2.647	102.7	0.968	80.161	2.668	102.0	0.992	75.578	2.605	96.2	0.877	78.689	2.626	100.1	1.000	75.334	2.605	95.9	0.840

1. All p-values and % are relative to the control group, except for trend shown below control.

c) EE_2 Treatments Litter Counts and Proportions

Table 8. Summary Statistics for Litter Size by Sex and Number Born Dead for Ethinyl Estradiol

Dose		Litter Size		# of Males		# of Females		# of Unsexed		# Born Dead	
(µg/kg·BW/day)	N	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	73	11.8	0.4	5.8	0.2	5.8	0.2	0.2	0.1	0.00	0.00
EE2 0.5	41	11.8	0.6	6.1	0.4	5.4	0.4	0.3	0.2	0.00	0.00
EE2 5.0	51	12.2	0.4	5.8	0.3	6.1	0.3	0.3	0.1	0.04	0.04

Table 9. Summary Statistics for Litter Sex Proportions for Ethinyl Estradiol

Dose		Male %		Female %		Unsexed %	
(µg/kg·BW/day)	N	Mean	SE	Mean	SE	Mean	SE
Control	73	49.4	1.5	49.5	1.5	1.1	0.4
EE2 0.5	41	53.2	2.4	44.8	2.2	1.9	1.1
EE2 5.0	51	48.0	2.1	49.6	2.1	2.4	1.0

Table 10. Poisson Regression Test of Treatment Effect on Litter Counts for Ethinyl Estradiol Dose (µg/kg·BW/day) [1]

	Control		EE2 0.5				EE2 5.0			
Analysis[2]	Mean	SE	Mean	SE	Pct	P	Mean	SE	Pct	P
Alive	11.8	0.4	11.8	0.5	100.1	1.000	12.2	0.5	103.3	0.769
Females	5.8	0.3	5.4	0.4	93.0	0.598	6.1	0.3	104.6	0.782
Males	5.8	0.3	6.1	0.4	105.4	0.744	5.8	0.3	99.6	0.998
Males+	6.0	0.3	6.4	0.4	107.0	0.607	6.1	0.3	102.1	0.947

[1] All p-values and % are relative to the control group.
[2] Analysis 'Alive' was based on the sum of counts of unsexed and sexed pups.[3] Analysis of 'Males+' included unsexed as well as male pups.

Table 11. Comparison of Sex Proportions for Ethinyl Estradiol (Proportion of Males)

Dose (µg/kg·BW/day)										
Control[1]		EE2 0.5				EE2 5.0			
Mean	SE	Mean	SE	Pct	P	Mean	SE	Pct	P
0.505	0.017	0.540	0.023	106.9	0.371	0.499	0.020	98.8	0.965

[1] All p-values and % are relative to the control group

d) EE_2 Treatments Litter Weights

Table 12. Summary Statistics for Litter Weights (g) for Ethinyl Estradiol

	Dose (µg/kg'BW'/day)								
	Control			EE2 0.5			EE2 5.0		
Analysis	N	Mean	SE	N	Mean	SE	N	Mean	SE
Average Female	73	6.83	0.11	39	6.56	0.14	50	6.87	0.11
Average Male	73	7.06	0.11	39	7.12	0.11	50	7.19	0.14
Average Pup	73	6.96	0.09	39	6.85	0.12	50	7.03	0.12
Females	73	38.49	1.62	39	34.87	2.41	50	39.96	2.31
Males	73	40.10	1.74	39	41.84	2.87	50	38.64	1.99
Total	73	78.59	2.31	39	76.71	3.94	50	78.60	2.93

Table 13. Test of Treatment and Covariate Effects on Litter Weight[1] for Ethinyl Estradiol
Dose (µg/kg'BW'/day)

Analysis	Effect	NumDF	DenDF	Fvalue	P value
Mean Female	Treatment	2	158	2.454	0.089
	Pup Count	1	158	70.962	**<.001**
Mean Male	Treatment	2	158	0.633	0.532
	Pup Count	1	158	39.376	**<.001**
Mean Pup	Treatment	2	158	1.059	0.349
	Pup Count	1	158	67.228	**<.001**
Females	Treatment	2	159	1.325	0.268
Males	Treatment	2	159	0.473	0.624
Total	Treatment	2	159	0.116	0.890

1. Analyses were performed separately for females, males, and totals; ANOVA was performed for litter weights and ANOCOVA was performed for mean pup weights.

Table 14. ANOCOVA of Litter Mean Pup Weight and ANOVA of Litter Weight (g)[1] for Ethinyl Estradiol

	Dose (µg/kg'BW'/day)									
	Control		EE2 0.5				EE2 5.0			
Analysis	Mean	SE	Mean	SE	Pct	P	Mean	SE	Pct	P
Mean Female	6.814	0.085	6.565	0.117	96.3	0.159	6.900	0.103	101.3	0.757
Mean Male	7.040	0.096	7.128	0.131	101.3	0.817	7.208	0.116	102.4	0.443
Mean Pup	6.942	0.079	6.851	0.108	98.7	0.736	7.058	0.096	101.7	0.558
Females	38.488	1.747	34.869	2.390	90.6	0.381	39.958	2.111	103.8	0.823
Males	40.104	1.804	41.838	2.468	104.3	0.804	38.638	2.180	96.3	0.833
Total	78.592	2.492	76.708	3.410	97.6	0.873	78.596	3.011	100.0	1.000

1. All p-values and % are relative to the control group, except for trend shown below control.

B. Data

Litter parameter data were extracted from the Genesis database using SAS Proc SQL, utilizing the Vortex ODBC driver.

Quality Control

1. Data Verification

The extraction of the data into SAS was verified by the reviewer, Paul Felton, by review of the SAS code used to extract and verify the data.

2. Computer Program Verification

SAS programs were used to extract the data, explore the distributional properties of the data, and perform the statistical analysis.

The SAS programs were verified by detailed review of the program code, the program log, and the program output.

3. Statistical Report Review

3.1. Statistical Report Text

The statistical report was reviewed for logic, internal completeness, technical appropriateness, technical accuracy, and grammar. Technical appropriateness was reviewed based on statistical expertise.

Comments and questions were provided from the reviewer to the statistician. The statistician made appropriate changes and returned the report to the reviewer for final verification.

The text of the final statistical report was considered by the reviewer to be logical, internally complete, and technically appropriate and accurate. The statistical results stated in the text accurately presented those in the tables.

3.2. Table Verification

Analysis results were output from SAS to an .rtf file using PROC REPORT, which were then copied into the statistical report.

Statistical report tables were verified by checking the procedure used to create the tables and, additionally, by checking numbers sufficiently to conclude that the tables are correct.

4. Conclusions

The final statistical report has been fully reviewed and is considered by the reviewer to be logical, internally complete, and technically appropriate and accurate.