APPENDIX XVI

Statistical Analysis of Litter Parameters

Statistical Report

Project #: E02187.01
Project Title: Effect of oxybenzone on fertility and early embryonic development in Sprague-Dawley rats (Segment II)
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Task: Statistical Analysis of Litter Parameters
Statistician: Beth Juliar, Division of Bioinformatics and Biostatistics
Reviewer: Paul Felton, Division of Bioinformatics and Biostatistics

Signatures:

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Reviewer Date

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Team Leader – Statistical Support Group Date

Statistical Analysis of Litter Parameters

1. Objectives

1.1 Project Objectives

This experiment is a study of embryo/fetal development [ICH Guideline S5(R2) 4.1.3] to determine the potential developmental toxicity of oxybenzone.

1.2 Analysis Objectives

The goal of this analysis is to assess potential developmental toxicity of oxybenzone effects on litter parameters.

2. Experimental Design

Oxybenzone is used in sunscreens and many commercial products to absorb UV radiation and prevent UV-induced photodecomposition in plastics and cosmetics. There has been recent interest in the biological activity of oxybenzone due to its high volume of use and its detection in the urine of a large percentage of the population. This study is designed to address concerns expressed by CDER that oxybenzone may have endocrine disruptor activity.

The test article in this study is 2-hydroxy-4-methoxybenzophenone (synonyms: HMB, benzophenone-3, oxybenzone). Dose levels were to be 0 ppm (control), 3,000 ppm, 10,000 ppm, and 30,000 ppm with approximately 25 animals per treatment group.

Date-mated females (approximately 11- 13 weeks old) were to be delivered in 5 loads to the NCTR on GD 3 or 4 (day of vaginal plug detection= GD 0). They were to be placed on control chow initially, and randomized to treatment groups. All animals were to be placed on dosed chow on GD 6 continuing to GD 15; all animals were to be fed control chow from GD 15 until sacrifice at GD 21. Feed and water were to be provided *ad libitum*. All animals were to be individually housed.

At sacrifice on GD 21, the uterus was to be removed and the fetuses were to be separated from the placenta, individually identified, weighed, sexed, examined, and sacrificed by decapitation. Each fetus was to be given a complete fetal evaluation.

3. Statistical Methods

Data were collected for individual fetuses, except for two fetuses that were not sexed.

Summary statistics are presented for number of female, male, and unsexed live fetuses, for litter sex percent, for fetal weight, and for litter weight. Counts of live fetuses, by sex and overall, were analyzed using Poisson regression. Unsexed fetuses were assigned as male for analysis of litter counts.

Sex proportions within litters were analyzed for treatment effects using logistic regression. Unsexed fetuses were assigned as male sex for analysis of sex proportions.

Analysis of weight by sex was performed using data of weighed female and male fetuses. For litter weight analysis, weight was combined across sex including unsexed fetuses. Mean weights were calculated as the weights divided by the number weighed. For litter weight data by sex, analysis was performed using contrasts within a one-way analysis of variance (ANOVA) model to test for treatment effect. For litter weight, analysis was performed using contrasts within an analysis of covariance (ANOCOVA) adjusted for litter size (number of live fetuses) to test for treatment effect.

Comparisons were performed with Dunnett's method for adjusted contrasts for treatment groups versus the control group. Tests were conducted as two-sided at the 0.05 significance level

4. Results

Tables are presented in appendix A1.

4.1 Analyses of Litter Counts

Summary statistics are given by treatment group in Table 1 for litter size and number of males, females, and unsexed fetuses. Treatments 3,000 mg/kg and 30,000 mg/kg oxybenzone each had one fetus that was not sexed. There were no dead fetuses in any treatment group.

Comparisons of treatments to the control group are presented for litter size and number of female and male fetuses in Table 5. There were no significant treatment effects for the counts of live, female, or male fetuses. There were no statistically significant treatment effects for female or male counts whether counting unsexed fetuses as females or males.

4.2 Analysis of Sex Proportions in Litters

Summary statistics for sex percentage are given in Table 2. Comparisons of treatments to the control are presented in Table 6. The proportion of males was analyzed as the outcome for sex proportion in litters.

There were no statistically significant treatment effects for litter sex proportion whether counting unsexed fetuses as females or males.

4.3 Analyses of Litter Weights

Summary statistics are given in Table 3 for fetal weights by sex and in Table 4 for litter weight. For analysis of fetal weight by sex, data of weighed female and male fetuses were used. For analysis of litter weight, the combined weight of fetuses was used (female, male, and unsexed).

Results of ANOVA for litter weight by sex, and ANOCOVA for litter and mean fetal weights with covariate litter size are given in Table 7. Pairwise comparison results from the ANOVA and ANOCOVA are presented in Table 8. In the analyses of litter weight by sex, there were no significant effects of treatment group for either females or males. In

the analysis of mean litter weight, there was no treatment effect, but there was a significant effect of covariate litter size ($p<0.001$). There were no significant trends or pairwise comparisons of fetal weights by sex or litter weights.

5. Conclusions

Compared to the control group, there were no significant differences for treatment groups in counts of live female, live male, or total live fetuses. There were no significant differences between the treatment and control groups in sex proportions of females and males. In analyses of mean fetal weight by sex and litter weight, there were no significant differences between the treatment and control groups.

A1. Tables

Table 1. Summary Statistics for Litter Fetal Counts									
		Litter Size		# of Males		# of Females		# of Unsexed	
Treatments	N	Mean	SE	Mean	SE	Mean	SE	Mean	SE
CTRL	19	12.53	0.50	6.37	0.38	6.16	0.56	0.00	0.00
OXY 3,000	21	13.00	0.47	6.71	0.45	6.24	0.51	0.05	0.05
OXY 10,000	22	12.27	0.64	6.05	0.47	6.23	0.39	0.00	0.00
OXY 30,000	19	13.37	0.56	6.74	0.58	6.58	0.53	0.05	0.05

Table 2. Summary Statistics for Litter Sex Percentages							
		Male %		Female %		Unsexed %	
Treatments	N	Mean	SE	Mean	SE	Mean	SE
CTRL	19	51.85	3.40	48.15	3.40	0.00	0.00
OXY 3,000	21	51.87	3.08	47.74	3.25	0.40	0.40
OXY 10,000	22	48.44	2.69	51.56	2.69	0.00	0.00
OXY 30,000	19	49.72	3.58	49.90	3.65	0.38	0.38

Table 3. Summary Statistics for Fetal Weights (g) by Sex						
Treatment	*Sex*	*N*[1]	*Fetal Mean*	*Fetal SE*	*Total Mean*	*Total SE*
CTRL	F	19	5.02	0.07	30.76	2.79
	M	19	5.25	0.06	33.46	2.08
OXY 3,000	F	21	5.04	0.08	31.16	2.47
	M	21	5.28	0.06	35.18	2.28
	U	1	5.45	-	5.45	-
OXY 10,000	F	22	4.93	0.08	30.85	2.03
	M	22	5.26	0.05	31.70	2.40
OXY 30,000	F	19	5.09	0.11	33.27	2.59
	M	19	5.31	0.10	35.12	2.89
	U	1	5.13	-	5.13	-

1. Treatments 3,000 and 30,000 ppm each had 1 litter with an unsexed fetus.

Table 4. Summary Statistics for Litter Weights (g)					
Treatment	*N*	*Fetal Mean*	*Fetal SE*	*Litter Mean*	*Litter SE*
CTRL	19	5.15	0.07	64.22	2.31
OXY 3,000	21	5.16	0.07	66.60	2.01
OXY 10,000	22	5.07	0.07	62.55	3.38
OXY 30,000	19	5.19	0.10	68.65	2.39

Table 5. Poisson Regression Test of Treatment Effect on Litter Counts															
	Treatment														
	CTRL			OXY 3,000				OXY 10,000				OXY 30,000			
Analysis[2]	Mean	SE	Trend[1]	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P
Females	6.16	0.57	0.581	6.24	0.55	101.3	0.999	6.23	0.53	101.1	0.999	6.58	0.59	106.8	0.916
Males	6.37	0.58	0.696	6.76	0.57	106.2	0.928	6.05	0.52	94.9	0.953	6.79	0.60	106.6	0.920
Live	12.53	0.81	0.507	13.00	0.79	103.8	0.952	12.27	0.75	98.0	0.991	13.37	0.84	106.7	0.807

1. All p-values and % are relative to the control group except the p-value for trend.
2. Analysis "Live" is based on the sum of counts of unsexed and sexed pups; unsexed pups were classified as male for analyses by sex.

Table 6. Comparison of Litter Sex Proportions Across Treatments[1]				
	Mean	SE	Pct	P value[2]
CTRL	0.508	0.032	-	0.914
OXY 3,000	0.520	0.032	102.3	0.987
OXY 10,000	0.493	0.023	96.9	0.959
OXY 30,000	0.508	0.035	99.9	1.000

1. In the analysis of sex proportions, unsexed pups were classified as males.
2. All p-values and % are relative to the control group except p-value for trend.

Table 7. ANOVA and ANOCOVA Tests of Treatment on Litter Weight[1]					
Sex	**Effect**	**NumDF**	**DenDF**	**Fvalue**	**P value**
Females	Treatment	3	77	0.637	0.593
Males	Treatment	3	77	0.030	0.992
Litter	Treatment	3	76	0.367	0.777
	Count	1	76	814.817	<.001

1. Weight analyses were performed separately using ANOVA by sex and ANOCOVA across sex.

Table 8. ANOVA of Fetal Weight and ANOCOVA of Litter Weight (g)															
	Treatment														
	CTRL			OXY 3,000				OXY 10,000				OXY 30,000			
Analysis	**Mean**	**SE**	**Trend[1]**	**Mean**	**SE**	**Pct**	**P**	**Mean**	**SE**	**Pct**	**P**	**Mean**	**SE**	**Pct**	**P**
Mean Female	5.02	0.08	0.517	5.03	0.08	100.3	0.998	4.93	0.08	98.3	0.798	5.09	0.08	101.4	0.884
Mean Male	5.25	0.07	0.800	5.27	0.07	100.3	0.998	5.26	0.07	100.1	1.000	5.28	0.07	100.5	0.985
Litter	65.36	0.80	0.581	65.59	0.76	100.4	0.993	64.85	0.74	99.2	0.934	65.97	0.80	100.9	0.907

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

A2 Data

Litter parameter data were provided in an Excel spreadsheet from the Principle Investigator.

Statistical Analysis of Litter Parameter Data– QC

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 presented 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 conducting a number of “spot-checks”.

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.