APPENDIX XXII

Statistical Analysis of Sperm Parameters

Statistical Report

Project #: E02186.01
Project Title: Effect of oxybenzone on fertility and early embryonic development in Sprague-Dawley rats (Segment I)
PI: Amy Inselman
Task: Statistical Analysis of Sperm Parameters
Statistician: Beth Juliar, Division of Bioinformatics and Biostatistics
Reviewer: Paul Felton, Division of Bioinformatics and Biostatistics

Signatures:

[redacted]

Statistician Date

[redacted]

Reviewer Date

[redacted]

Team Leader – Statistical Support Group Date

Statistical Analysis of Sperm Parameters Data

1. Objectives

1.1 Project Objectives

The objective of the study is to examine the reproductive toxicity of oxybenzone in male and female rats and is designed to focus specifically on fertility and early embryonic development to implantation [ICH Guideline S5(R2) 4.1.1]. An additional objective is to compare the results of a typical Segment I, II, III study design with results from a modified one-generation study proposed by the NTP.

1.2 Analysis Objectives

The goal of this analysis is to test the effects of oxybenzone on sperm parameters.

2. Experimental Design

A total of 262 rats were to be requested for this study. Of this number 125 male rats were to be requested along with 125 female rats. Males were to be approximately 5-7 weeks old when delivered to the NCTR, and females were to be approximately 9-11 weeks of age when delivered. All males were to be delivered in one shipment, and all females were to be delivered in a separate shipment. After a two week quarantine period the animals were to be weighed and allocated to the study.

The test article in this study is 2-hydroxy-4-methoxybenzophenone (synonyms: HMB, benzophenone-3, oxybenzone). The animals were to be divided into five treatment groups with 25 male and 25 female rats assigned to each group. The treatment groups were to be four oxybenzone dose levels 0 ppm (control), 3000 ppm, 10,000 ppm, and 30,000 ppm and estrogen ethinyl estradiol (EE_2) 0.05 ppm treatment.

Males were to be dosed for 10 weeks and females for approximately 2 weeks prior to mating. Dosing was to continue until gestational day (GD) 6 for all animals. From GD 6 to GD 15, dams were to receive control chow. All dams were to be sacrificed on GD 15; males were to be sacrificed soon after breeding (approximately dam GD 6).

All animals were to be housed in pairs in cages prior to breeding. For breeding, males and females were to be housed one male: one female for up to 15 days or until animals have mated. Males and females were to be housed individually upon indication of mating (GD 0) until the time of sacrifice.

After sacrifice of the males, the left testis and epididymis were to be used for evaluation of testicular spermatid head counts and epididymal sperm counts, and for morphology and motility evaluations. These parameters will be determined from 10 males selected at random from each treatment group.

3. Statistical Methods

Analysis of sperm morphology data was performed using a generalized linear model with a Poisson distribution and a log link function. Each treatment was compared to the control group, and adjustment for multiple comparisons was performed using Hochberg's method. Percent sperm motility, testes sperm counts, and cauda sperm counts were analyzed using an ANOVA model with Kenward-Roger estimated degrees of freedom (Kenward and Roger, 1997). Each treatment was compared to the control group, and adjustment for multiple comparisons was performed using Dunnett's method. Test of trend, increasing treatment effect with increasing dose, was performed for the oxybenzone and control groups. Tests were conducted as two-sided at the 0.05 significance level.

References

Kenward, M. G. and Roger, J. H. (1997), "Small Sample Inference for Fixed Effects from Restricted Maximum Likelihood," *Biometrics* **53**:983–997.

4. Results

Tables are included in Appendix A1.

Of 50 randomly selected males, 10 per treatment group, 6 animals were excluded from analysis due to undetermined plug dates (UIN=5A000002436, 5A000002445, 5A000002487, 5A000002489, 5A000002528, and 5A000002538). Instead of being removed after mating, these males remained in the breeding cages after dams' GD 0 and continued to be fed dosed chow after dams' GD 6.

Counts and percentages for sperm morphology are given in Table 1. Analysis results for sperm morphology are given in Table 2. There were no sperm head abnormalities. For sperm tail and total abnormalities, the test of trend was not significant and there were no statistically significant differences for treatment groups compared to the control.

Summary statistics for percent sperm motility, cauda sperm counts and testes sperm counts are presented by treatment in Table 3. ANOVA results from analysis of treatment effect for percent motility, cauda counts, and testes counts are presented in Table 4. There were no statistically significant trends or differences for treatment groups compared to the control. Because 2 animals had very low percent motility (5 and 8), a nonparametric analysis was also conducted, but there was no difference in conclusions.

5. Conclusions

There was no statistically significant difference for any treatment group compared to the control for morphology, percent sperm motility, cauda sperm counts, or testes sperm counts.

Appendices

A1 Tables

Table 1. Counts of Sperm Morphology Abnormality by Treatment (mg/kg)				
Treatment	***Type***	***Abnormal Count***	***N (Animals)***	***Percent***
CTRL	Head	0	9	100.0
	Tail	0	7	77.8
		1	2	22.2
	Total	0	7	77.8
		1	2	22.2
OXY 3,000	Head	0	10	100.0
	Tail	0	7	70.0
		1	1	10.0
		2	2	20.0
	Total	0	7	70.0
		1	1	10.0
		2	2	20.0
OXY 10,000	Head	0	8	100.0
	Tail	0	2	25.0
		1	4	50.0
		2	2	25.0
	Total	0	2	25.0
		1	4	50.0
		2	2	25.0
OXY 30,000	Head	0	7	100.0
	Tail	0	4	57.1
		1	3	42.9
	Total	0	4	57.1
		1	3	42.9
EE2 0.05	Head	0	10	100.0
	Tail	0	9	90.0
		1	1	10.0
	Total	0	9	90.0
		1	1	10.0

Table 2. Comparison of Sperm Morphology Abnormality Counts Per Animal Across Groups[1]															
	Treatment (mg/kg)														
	CTRL			OXY 3,000			OXY 10,000			OXY 30,000			EE2 0.05		
Morphology[2]	Mean[3]	SE	Trend	Mean	SE	P value	Mean	SE	P value	Mean	SE	P value	Mean	SE	P value
Abnomality	0.22	0.15	0.685	0.50	0.21	0.483	1.00	0.33	0.163	0.43	0.23	0.483	0.10	0.09	0.483
Tail Abnomality	0.22	0.15	0.685	0.50	0.21	0.483	1.00	0.33	0.163	0.43	0.23	0.483	0.10	0.09	0.483

1. All p-values are relative to control and were adjusted using Hochberg's method, except p-value for trend.
2. Because there were no head abnormalities, analysis was not performed.
3. Mean and standard error were estimated using Poisson analysis.

Table 3. Summary Statistics of Sperm Outcomes by Treatment (mg/kg)															
	CTRL			OXY 3,000			OXY 10,000			OXY 30,000			EE2 0.05		
Outcome	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE
Percent Sperm Motility	9	70.3	8.6	10	74.6	3.7	8	67.1	8.7	7	79.0	2.5	10	77.2	2.7
Cauda Sperm Counts	9	1089.4	73.4	10	1058.2	63.5	8	1105.0	71.7	7	1071.1	63.9	10	1048.9	83.1
Testes Sperm Counts	9	127.6	4.9	10	125.0	5.1	8	120.0	5.4	7	127.8	5.2	10	123.6	7.8

Table 4. ANOVA Comparison of Least Squares Mean Sperm Outcomes Across Treatments[1]															
	Treatment (mg/kg)														
	CTRL			OXY 3,000			OXY 10,000			OXY 30,000			EE2 0.05		
Outcome	Mean	SE	Trend	Mean	SE	P value	Mean	SE	P value	Mean	SE	P value	Mean	SE	P value
Percent Sperm Motility	70.3	5.8	0.359	74.6	5.5	0.955	67.1	6.1	0.987	79.0	6.5	0.721	77.2	5.5	0.805
Cauda Sperm Counts	1089.4	72.2	0.947	1058.2	68.5	0.994	1105.0	76.6	1.000	1071.1	81.9	0.999	1048.9	68.5	0.983
Testes Sperm Counts	127.6	5.9	0.839	125.0	5.6	0.994	120.0	6.3	0.796	127.8	6.7	1.000	123.6	5.6	0.968

1. All p-values are relative to the control group and were adjusted using Dunnett's method, except p-value for trend shown below control.

A2 Data

Sperm parameters data were provided in an Excel spreadsheet from the Principle Investigator.

Statistical Analysis of Sperm 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, and by independent verification of the results.

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 independent verification of the numerical results.

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.