APPENDIX XXI

Statistical Analysis of Pregnancy Parameters

Statistical Report

Project #: E02186.01
Project Title: Effect of oxybenzone on fertility and early embryonic development in Sprague-Dawley rats (Segment I)
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Task: Statistical Analysis of Pregnancy Parameters
Statistician: Beth Juliar, Division of Bioinformatics and Biostatistics
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Team Leader – Statistical Support Group Date

Statistical Analysis of Pregnancy Parameters

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 effect of oxybenzone on pregnancy 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 one 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 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.

From the pregnant females on GD 15, the uterus with the right ovary, vagina, and cervix attached was to be removed, weighed and opened to allow evaluation of the number and status of implantation sites. For female animals that bred but appear not to be pregnant, the uterus was to have been stained with ammonium sulfide to detect presence of any implantation sites. Ovaries were to be removed for microscopic counting of corpora lutea.

3. Statistical Methods

Counts and percentages of mated and pregnant dams in each treatment group were calculated. Summary statistics were performed for gravid uterine weight. Mean counts per litter for each treatment group were calculated for corpora lutea, implants, resorptions, and live fetuses. Mean litter percentages were computed for pre-implantation loss and post-implantation loss. Pre-implantation loss was defined as the percentage of corpora lutea that did not result in implantation. Post-implantation loss was defined as the percentage of implantations that were resorbed.

Proportions of mated and pregnant females were analyzed using Fisher's Exact test for comparisons of treated groups to control and using Cochran-Armitage test for trend. Analysis of gravid uterine weight was performed using contrasts within a one-way analysis of variance (ANOVA). Counts of implantation sites were analyzed using Poisson regression with terms for treatment and covariate number of corpora lutea. Counts of resorptions were analyzed using Poisson regression with terms for treatment and covariate number of implantation sites.

Test of trend, increasing treatment effect with increasing dose, was performed for the oxybenzone and control groups. Comparisons of pregnancy proportions of treated groups to control were performed using Holm's method of adjustment for multiple comparisons. For all other analyses, comparisons of treated groups to control were performed with Dunnett's method for adjusted contrasts. Tests were conducted as two-sided at the 0.05 significance level.

4. Results

Tables are presented in appendix A1.

Counts and percentages in each treatment with analysis results using Fisher's Exact test and Cochran-Armitage trend test given in Table 1 for mated females and in Table 2 for pregnant dams. For analysis of mating, pregnant females were assumed to have mated even if the plug was not observed. There were no statistically significant results for proportions of mated females, although there was a difference for pregnant dams in comparison of treatment oyxbenzone 10,000 ppm to the control group (p=0.042, 96.0% versus 64.0%).

Summary statistics for gravid uterine weight are given in Table 3. Thirteen pregnant dams were excluded because GD 6 was undetermined. Vaginal smear sperm evaluation (VSSE) was missed and dosing was not stopped (UIN=5A000002579, 5A000002580, 5A000002600, 5A000002607, 5A000002630, 5A000002636, 5A000002639, 5A000002648, 5A000002652, 5A000002653, 5A000002661, and 5A000002678). One female (UIN=5A000002671) was not monitored for evidence of mating until the 3rd day of pairing, but was plug positive by day 3. Dosing for 4 additional dams was not stopped until GD 8 (UIN=5A000002650, 5A000002658, 5A000002664, 5A000002667), and their gravid uterine weights were excluded. Two dams, sacrificed early in the oxybenzone 10,000 ppm treatment at GD 14 (UIN=5A000002589) and in the EE_2 0.05 ppm treatment

at GD 13 (UIN=5A000002581), are included (analysis excluding data for these two dams did not result in any differences in conclusions).

Results of the ANOVA for gravid uterine weight are given in Table 4. Treatment effect was not statistically significant. Least squares mean comparisons of treated groups to the control group are presented in Table 5. In pairwise comparisons, there was a statistically significant difference for treatment EE_2 0.05 ppm compared to the control group (p=0.030), with the treated group showing mean weight 17.8% less than control.

Summary statistics for counts of corpora lutea, implants, resorptions, resorption loss, and live fetuses, and for pre-implantation loss and post-implantation loss are presented in Table 6. Live fetuses, resorptions, and post-implantation loss % are not reported for dams with undetermined GD 6. There were no late resorptions or dead fetuses.

There was no significant treatment effect in the analysis of implants adjusted for covariate corpora lutea, although there was a significant covariate effect (p=0.008). Results for pairwise comparisons of treatments for counts of implants are presented in Table 7. There was no statistically significant trend and there were no significant differences for treated groups compared to control.

There was no significant treatment or covariate effect in the analysis of resorptions adjusted for implants. Results for trend and pairwise comparisons are presented in Table 7. There was no statistically significant trend, and there were no significant differences in pairwise comparisons.

5. Conclusions

There was a statistically significant difference in pregnancy proportions for treatment oyxbenzone 10,000 ppm compared to the control group in pairwise comparisons, with the treated group showing higher mean pregnancy proportion compared to control. In pairwise comparisons of gravid uterine weight, there was a statistically significant difference for treatment EE_2 0.05 ppm compared to the control group, with the treated group showing lower mean weight relative to control. There were no statistically significant differences for treated groups compared to control for counts of implantation sites or resorptions.

A1. Tables

Table 1. Summary Statistics and Analysis of Mated Females by Treatment[1]				
Treatment (ppm)	***N***	***Mated Count***	***Percent***	***P-value***[2]
CTRL	25	20	80.0	0.422
OXY 3,000	25	22	88.0	1.000
OXY 10,000	25	25	100.0	0.201
OXY 30,000	25	22	88.0	1.000
EE2 0.05	25	22	88.0	1.000

1. P-values are adjusted for multiple comparisons using Holm's method.
2. Fisher's exact test was used for comparisons of treated groups to control; Cochran-Armitage trend test is shown for control.

Table 2. Summary Statistics and Analysis of Pregnancies by Treatment[1]				
Treatment (ppm)	***N***	***Pregnancy Count***	***Percent***	***P-value***[2]
CTRL	25	16	64.0	0.065
OXY 3,000	25	19	76.0	0.588
OXY 10,000	25	24	96.0	0.042
OXY 30,000	25	21	84.0	0.588
EE2 0.05	25	21	84.0	0.588

1. P-values are adjusted for multiple comparisons using Holm's method.
2. Fisher's exact test was used for comparisons of treated groups to control; Cochran-Armitage trend test is shown for control.

Table 3. Summary Statistics for Uterine Weight by Treatment[1]															
CTRL			OXY 3,000			OXY 10,000			OXY 30,000			EE2 0.05		
N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE
12	18.7	0.7	18	16.8	1.2	18	17.8	0.4	17	16.7	0.5	19	15.3	0.9

1. Two females, sacrificed early at GD 13 and 14, are included.

Table 4. ANOVA Results for Uterine Weights[1]				
Effect	NumDF	DenDF	Fvalue	P value
Treatment	4	79	2.169	0.080

1. Two females, sacrificed early at GD 13 and 14, are included.

Table 5. Comparisons of Least Squares Mean Gravid Uterine Weights Across Treatments[1]																		
Control			OXY 3,000				OXY 10,000				OXY 30,000				EE2 0.05			
Mean	SE	P value	Mean	SE	Pct	P value	Mean	SE	Pct	P value	Mean	SE	Pct	P value	Mean	SE	Pct	P value
18.7	1.0	0.254	16.8	0.8	89.9	0.350	17.8	0.8	95.3	0.875	16.7	0.8	89.4	0.318	15.3	0.8	82.2	0.030

1. All p-values and % are relative to the control group, except p-value for trend (excluding the EE2 treatment) shown below control.

Table 6. Summary Statistics of Pregnancy Parameters															
	Treatment (ppm)														
	CTRL			OXY 3,000			OXY 10,000			OXY 30,000			EE2 0.05		
Parameter[1]	N[2]	Mean	SE	N[2]	Mean	SE	N[2]	Mean	SE	N[2]	Mean	SE	N[2]	Mean	SE
Corpora Lutea	16	16.7	0.4	19	17.3	0.6	24	16.7	0.5	21	14.1	0.4	21	13.2	0.3
Implants	16	15.2	0.4	19	13.3	0.9	22	14.2	0.3	20	13.3	0.4	21	11.9	0.4
Resorptions	12	0.6	0.2	18	0.6	0.2	18	0.4	0.2	17	0.5	0.2	19	0.7	0.2
Live	12	14.3	0.5	18	12.7	1.0	18	13.8	0.4	17	12.7	0.4	19	11.2	0.5
Pre-Implantation Loss %	16	8.5	2.5	19	23.2	5.1	22	13.9	2.5	20	5.6	1.5	21	9.9	2.7
Post-Implantation Loss %	12	3.9	1.6	18	4.4	1.7	18	3.2	1.2	17	3.8	1.3	19	6.0	1.6

1. Parameters are counts for corpora lutea, implants, resorptions, and live fetuses.
2. Live or early fetuses, resorptions, and post-implantation loss % are not reported for dams with unknown GD 6.

Table 7. Poisson Regression Test of Treatment Effect on Pregnancy Parameters[1]																			
	Treatment (ppm)																		
	CTRL			OXY 3,000				OXY 10,000				OXY 30,000				EE2 0.05			
Analysis	Mean	SE	Trend	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P	Mean	SE	Pct	P
Implants	14.5	1.0	0.750	12.4	0.9	85.5	0.252	13.5	0.8	93.0	0.816	14.0	0.9	96.2	0.984	13.0	0.9	89.1	0.626
Resorptions	0.6	0.2	0.878	0.6	0.2	109.8	0.999	0.4	0.2	78.1	0.963	0.5	0.2	96.4	1.000	0.7	0.2	131.0	0.945

1. All % and Dunnett adjusted p-values are relative to the control group except the p-value for trend (excluding the EE2 treatment) shown below control.
2. “Implants” analysis was performed with covariate corpora lutea.
3. “Resorptions” analysis was performed with covariate implants.

A2 Data

Pregnancy parameter data were provided in an Excel spreadsheet from the Principle Investigator and data were extracted from the Genesis database using SAS Proc SQL, utilizing the Vortex ODBC driver.

Statistical Analysis of Pregnancy 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 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.