Effect of Oxybenzone on Embryo/Fetal Development in Sprague-Dawley Rats (Segment II)

NCTR Experiment E02187.01

Personnel[a]:

Study Director   Amy L. Inselman, Ph.D., Division of Systems Biology (DSB), NCTR

Co-Investigators   Deborah K. Hansen, Ph.D., DSB, NCTR
Noriko Nakamura, Ph.D., DSB, NCTR
Wafa Harrouk, Ph.D., Center for Drug Evaluation and Research
Paul Foster, Ph.D., National Toxicology Program
Barry McIntyre, Ph.D., National Toxicology Program

Consultant   James B. LaBorde, NCTR (Retired)

<u>Support Staff</u>

Dose Preparation   Jim Carson, B.S., Priority One Services, NCTR
Andy Matson, B.S., Priority One Services, NCTR

Dose Analysis   Matthew Bryant, Ph.D., Team Leader, Chemistry Support Group, Division of Biochemical Toxicology (DBT), NCTR
Raul A. Trbojevich, Ph.D., Chemistry Support Group, DBT, NCTR

Surveillance Diagnostic Program Certification
      Sung Guk Kim, Ph.D., Office of Scientific Coordination (OSC), NCTR

Animal Care   Mark Moore, Animal Care Diet Prep, Priority One Services, NCTR
Jeff Carraway, D.V.M., M.S., D.A.C.L.A.M., OSC, NCTR
Neera Gopee, D.V.M, Ph.D., DABT, OSC, NCTR

Computer Support   Debbie Lester, Project Manager, ICF International
Kathy Carroll, Experimental Liaison, ICF International
Sherry Smith, Experimental Liaison, ICF International

Pathology   William Witt, D.V.M., Ph.D., General Manager, TPA, Inc.
John R. Latendresse, D.V.M., Ph.D., TPA, Inc.
Kelly Davis, D.V.M., Ph.D., TPA, Inc.
Greg R. Olson, D.V.M., Ph.D., TPA, Inc.

Clinical Tests	Ralph Patton, Clinical Services Manager, TPA, Inc. Kristie Voris, TPA, Inc.
Fetal Evaluations	Gene White, Manager, TPA, Inc.
Statistical Support	Paul Felton, M.S., Biometry Statistical Support Group, Division of Bioinformatics and Biometry (DBB), NCTR Beth Juliar, M.S., Statistical Support Group, DBB, NCTR
Quality Assurance	Joe M. Fowler, B.S., RQAP-GLP, Regulatory Compliance and Risk Management, Quality Assurance Unit, NCTR Peggy Webb, Regulatory Compliance and Risk Management, Quality Assurance Unit, NCTR

[a]Note: Between the end of the in-life portion of this study and completion of the final report there were changes in the organizational structure at NCTR so that affiliations of some of the listed personnel changed. Namely, the computer support changed from ICF International to the Office of Scientific Coordination, NCTR. Dr. Jeff Carraway (OSC/Animal Care) retired and Mr. Gene White (TPA, Inc.) passed away before completion of the final report. James B. LaBorde, a retired NCTR employee from the former Division of Reproductive and Developmental Toxicology, served as a consultant and verified all findings in the teratological examination of the fetuses.

Study Laboratory: National Center for Toxicological Research (NCTR)
3900 NCTR Road
Jefferson, AR 72079

Study Initiation: April 6, 2012
Study Completion: September 21, 2015

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EXECUTIVE SUMMARY

Background

Oxybenzone (OXY; CAS No. 131-57-7) is an organic ultra-violet absorbing compound used in a variety of personal care products, including sunscreens as well as plastic products, because of its ability to block UVA and UVB radiation and prevent UV-induced photodecomposition. There has been recent interest in the biological activity of oxybenzone due to its high volume of use, its detection in the urine of a large percentage of the U.S. population and its reported estrogenic activity. This study is designed to address concerns expressed by the Center for Drug Evaluation and Research (CDER) that oxybenzone may have endocrine disruptor activity.

Methods

The study examined the effects of oxybenzone on embryo/fetal development. Time-mated female Sprague-Dawley rats (Harlan) were given dosed feed that contained 3,000, 10,000 or 30,000 parts per million (ppm) of oxybenzone which resulted in estimated ingested doses of 242, 725 and 3,689 milligrams (mg) of oxybenzone per kilogram (kg) of body weight per day, respectively. Dams were dosed from the time of implantation (gestational day (GD) 6) to the closure of the hard palate (GD 15); all animals were then fed control chow until sacrifice on GD 21. At sacrifice, the number and status of each implantation site was recorded (live, dead, early resorption or late resorption). Each live fetus was removed, weighed, sexed, and examined for gross abnormalities (including cleft palate). Fetuses were subjected to a fresh dissection and examined for developmental abnormalities in internal organs. Skeletons were also evaluated for potential abnormalities in the bone and cartilage resulting from oxybenzone administration. Abnormalities were classified as either variations or malformations.

Results

Female rats given 10,000 and 30,000 ppm of oxybenzone in their feed had lower overall body weights compared to unexposed animals; significant decreases were observed at GD 10, GD 14 and GD 17. An overall significant increase in food consumption was observed in the OXY 30,000 ppm treatment group relative to controls; the observed increase, however, may likely be the result of increased spillage due to palatability issues. Hematological evaluation of the dams indicated

lower hemoglobin concentration (HGB), packed cell volume (PCV) and red blood cell (RBC) numbers in the OXY 30,000 ppm treatment group. Platelet (PLT) levels were significantly higher in the OXY 30,000 ppm treatment group relative to controls. Hematocrit (HCT) measurements were also significantly affected by oxybenzone treatment; lower levels were observed in all treatment groups relative to controls. Clinical chemistry analysis of the dams also revealed differences in total bile acids (TBA), cholesterol (CHOL) and blood urea nitrogen levels (BUN) between the control and OXY 30,000 ppm treatment dams. TBA and CHOL levels were higher in oxybenzone treated animals while BUN levels were lower. However, oxybenzone treatment had no apparent effects on embryo/fetal development as the proportion of pregnant dams, number of implantations, number of resorptions, litter size, litter weights, fetal weights, or the number of visceral variations/malformations did not vary between treatment and control groups. The only statistically significant effect observed on embryo/fetal development was an increase in total skeletal variations between the control and OXY 3,000 ppm treatment group; however, this finding was not considered treatment related as a dose response was not observed with increasing oxybenzone concentration.

Conclusions

We conclude that exposure to oxybenzone from implantation (GD 6) to the closure of the hard palate (GD 15) did not affect embryo/fetal development at dose levels that induced minimal effects on maternal toxicity. Exposure to oxybenzone did, however, lead to decreases in maternal body weight at 10,000 and 30,000 ppm and affected hematological and clinical chemistry parameters in the dams primarily at the 30,000 ppm dose.

ABSTRACT
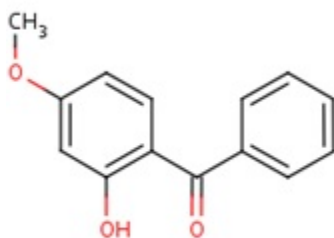
OXYBENZONE

CAS No. 131-57-7

Chemical Formula: $C_{14}H_{12}O_3$ Molecular Weight: 228.24

Synonyms: 2-hydroxy-4-methoxybenzophenone, HMB, benzophenone-3, (2-hydroxy-4-methoxyphenyl)-phenylmethanone

Oxybenzone (OXY) is an ultra-violet absorbing compound (UV-filter). Due to its ability to absorb both UVA and UVB, it is synthesized for incorporation into a wide variety of commercial personal care products including sunscreens, creams and lotions. It is also commonly found in nail polishes, perfumes, lipsticks, hair sprays and conditioners as a photostabilizer and/or a fragrance enhancer (Environmental Working Group (EWG); http://www.ewg.org/skindeep). Oxybenzone is listed as an allowable sunscreen compound in the U.S. Food and Drug Administration's (FDA) sunscreen monograph 21 CFR 352.10 with a permissible concentration not to exceed 6% (CFR, 2013b). In addition to personal care products, oxybenzone is also commonly found in plastics due to its ability to act as an ultraviolet light absorber and stabilizer (Hazardous Substance Data Bank (HSDB); http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB). It has also been approved by the FDA as an indirect food additive used in food contact substances (21 CFR 177.1010) (CFR, 2013a).

Concerns have been raised regarding the biological activity of oxybenzone due to the high volume of use and subsequent detection in the urine of a large percentage of the U.S. population (Calafat et al., 2008). In particular, a number of studies have examined the endocrine disrupting potential of the compound. These studies have indicated that oxybenzone or its primary metabolite, 2,4-dihydroxybenzophenone (DHB), may have estrogenic activity.   Due to these concerns,

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oxybenzone was selected as a compound to be examined in an International Conference on Harmonisation (ICH) guideline S5(R2) 4.1.3 (Segment II) study to examine its effects on embryo/fetal development (ICH, 1993). An additional objective was to compare the results obtained from typical ICH Segment I, II and III studies with results obtained from a modified one-generation study conducted in parallel and concurrently with the National Toxicology Program (NTP).

Data from a preliminary reproductive and developmental dose-finding feed study (National Center for Toxicological Research (NCTR) Protocol No: E02178.01; non-GLP) that utilized exposure concentrations of up to 50,000 ppm in conjunction with the results from an NTP dose-finding feed study were used to select dietary exposure concentrations of 0, 3,000, 10,000 and 30,000 ppm for the current study. These dietary doses resulted in ingested concentrations of approximately 0, 242, 725, and 3,689 mg oxybenzone per kg body weight per day during the time the animals were directly consuming dosed feed (GD 6 –GD 15).

The current study focused specifically on embryo/fetal development in response to oxybenzone exposure. For this study, 100 date-mated female Sprague-Dawley rats were obtained from Harlan Industries (Indianapolis, IN). Animals arrived in five separate shipments with 20 animals per shipment. Animals arrived at the NCTR laboratories on GD 3 (day of vaginal plug detection = GD 0) and were assigned to treatment groups by a weight-ranked randomization procedure upon arrival. They were fed a soy- and alfalfa-free Purina 5K96 diet to reduce potential endocrine related changes in the tested parameters. Twenty-five animals were assigned to each exposure group and were dosed from the time of implantation (GD 6) to the closure of the hard palate (GD 15). All animals were given control chow prior to GD 6 and from GD 15 to the time of sacrifice on GD 21.

Endpoints of reproduction and pregnancy were not adversely affected by oxybenzone treatment under the conditions of this experiment. There was no difference in the pregnancy rates of control and oxybenzone treatment groups nor was there any difference in the number of implantation sites or in the number of resportions observed. Gravid uterine weights were not affected by oxybenzone treatment.

Litter parameters were not influenced by exposure to oxybenzone from GD 6 to GD 15. There were no differences in the total numbers of live fetuses on GD 21 or in the number of male and female fetuses per litter. Mean total litter weights and the mean male and female fetal weights were not affected. The portion of male to female fetuses also did not change in response to oxybenzone exposure.

No observations were noted during the external examinations of the pups. Seventy eight observations were noted across control and oxybenzone treatment groups as a result of visceral examinations. Observations included, but were not limited to, dilated ureter, cleft palate and malpositioned testes. Dilated ureter was the most common finding and present in 1.23 fetuses/litter in the OXY 30,000 ppm treatment group compared to 0.53 fetuses/litter in the controls. There were significant trends for total numbers of variations/malformations and for the total number of pups with dilated ureters. However, pairwise comparison using Dunnett's method indicated no significant differences between the control and oxybenzone treatment groups. Analysis of the fetal skeletons revealed a statistically significant increase in the total number of skeletal variations, which included those of the ribs, sternebrae and centra, observed between control and OXY 3,000 ppm treatment groups in pairwise comparisons. However, the increase was not considered to be treatment related. A significant trend for rib variations, which included observed 14th thoracic ribs, left, right and bilateral rudimentary ribs, was also noted; however, the trend did not appear to be related to oxybenzone exposure due to the lack of a dose dependent increase in rib variations.

The only significant toxicological findings following oxybenzone exposure from GD 6 – GD 15 appeared in the treated dams when compared to controls. Oxybenzone treatment significantly reduced the body weights of dams. Maternal weights were significantly lower in the OXY 10,000 and OXY 30,000 ppm treatment groups at GD 10, GD 14 and GD 17; reductions ranged from 2.5-3.8% in the OXY 10,000 ppm group to 4.0-7.3% in the OXY 30,000 ppm group. Mean overall body weights were also lower for the same two treatment groups (2.7% and 3.8%, respectively). The decrease in maternal body weights was, however, not accompanied by a decrease in food consumption as pregnant dams in the OXY 30,000 ppm treatment group consumed overall

significantly more chow per day than controls in pairwise comparisons. However, palatability issues with the highest oxybenzone dose may also have attributed to increased spillage. Hematological evaluation of the dams indicated lower HGB, PCV and RBC numbers in the OXY 30,000 ppm treatment group. PLT levels were significantly higher in the OXY 30,000 ppm treatment group. HCT measurements were also significantly affected by oxybenzone treatment; lower levels were observed in all treatment groups relative to controls. Clinical chemistry analysis of the dams also revealed differences in TBA, CHOL and BUN levels between the control and OXY 30,000 ppm treatment dams. TBA and CHOL levels were higher in oxybenzone treated animals while BUN levels were lower.

Summary

Dietary exposure to oxybenzone did not significantly impact embryo/fetal development in Sprague-Dawley rats when exposed from implantation (GD 6) through closure of the hard palate (GD 15) at doses (10,000 and 30,000 ppm) which produced some maternal toxicity. No significant changes were observed in pregnancy rates, gravid uterine weights, number of implantations, number of resorptions, litter size, litter weights, or male and female fetal weights. While a significant trend was noted for the total number of visceral variations/malformations and for the number of dilated ureters observed, these differences were not significant in pairwise comparisons. Oxybenzone treatment, however, did negatively impact body weight of the dams at 10,000 and 30,000 ppm. The decrease in body weight of the dams was accompanied by a significant increase in food consumption between the OXY 30,000 treatment group and controls in pairwise comparisons. Oxybenzone treatment also affected a number of hematological and clinical chemistry parameters in the dams, primarily between the control and highest treatment group.

Table S1: Summary of the Embryo/Fetal Development Feed Study of Oxybenzone[a,b]

Endpoint	
Maternal Body Weight	
GD 6	-
GD 10	↓ (10,000; 30,000; Trend)
GD 14	↓ (10,000; 30,000; Trend)
GD 17	↓ (10,000; 30,000; Trend)
GD 21	↓ (Trend)
GD 6 - GD 21	↓ (10,000; 30,000; Trend)
Feed Consumption	
GD 6-10	↑ (30,000; Trend)
GD 10-14	↑ (30,000; Trend)
GD 14-17	↑ (30,000; Trend)
GD 17-21	↑ (30,000; Trend)
GD 6 - GD 21	↑ (30,000; Trend)
Hematology	
WBC	-
NEU (%)	-
LYM (%)	-
MON (%)	-
EOS (%)	-
BAS (%)	-
NEU	-
LYM	-
MON	-
EOS	-
BAS	-
RBC	↓ (30,000; Trend)
HGB	↓ (3,000; 30,000)
HCT	↓ (3,000; 10,000; 30,000)
MCV	-
MCH	-
MCHC	-
PLT	↑ (30,000; Trend)
PCV	↓ (30,000)
Clinical Chemistry	
SDH	-
TBA	↑ (30,000; Trend)
ALB	-
ALT	-
ALP	-
AST	-
TRIG	-
CHOL	↑ (30,000; Trend)
TP	-
CK	-
CREAT	-
BUN	↓ (30,000; Trend)
GLU	-

Table S1: Summary of the Embryo/Fetal Development Feed Study of Oxybenzone[a,b] (cont.)

Endpoint	
Maternal Organ Weights -Relative	
Liver	↑ (Trend)
Kidney (paired)	-
Ovary (paired)	-
% Pregnant	-
Gravid Uterine Weight	-
Number of Implants	-
Number of Resorptions	-
Litter Size	-
Number Male	-
Number Female	-
Sex Proportions	-
Litter Weights	-
Male	-
Female	-
Skeletal Variations	
Total	↑ (3,000)
Ribs	↓ (Trend)
Sternebrae	-
Centra	-
Visceral Variations/Malformations	
Total	↑ (Trend)
Dilated Ureter	↑ (Trend)

[a] GD = gestational day; WBC = white blood cell; NEU = neutrophils; LYM = lymphocytes; MON = monocytes; EOS = eosinophils; BAS = basophils; RBC = red blood cells; HGB = hemoglobin concentration; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLT = platelets; PCV = packed cell volume; SDH = sorbitol dehydrogenase; TBA = total bile acids; ALB = albumin; ALT = alanine aminotransferase; ALP = alkaline phosphatase; AST = aspartate aminotransferase; TRIG = triglycerides; CHOL = cholesterol; TP = total protein; CK = creatine kinase; CREAT = creatinine; BUN = blood urea nitrogen; GLU = glucose.

[b] ↑ or ↓, significant increase or decrease relative to controls at the exposure indicated in parentheses, or, where indicated, significant overall exposure concentration trend; "-", no exposed group significantly different from the control group in pairwise comparisons. Only females that were pregnant were included in the analyses. Details of the specific statistical analysis used for each endpoint are described in the body of the report. For organ weights, results are shown for organ weights after adjustment for body weight.

INTRODUCTION AND STUDY RATIONALE
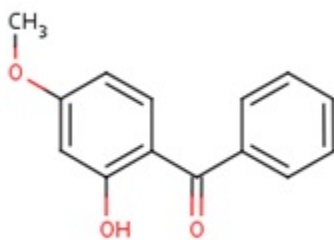
OXYBENZONE

CAS No. 131-57-7

Chemical Formula: $C_{14}H_{12}O_3$ Molecular Weight: 228.24

Synonyms: 2-hydroxy-4-methoxybenzophenone, HMB, benzophenone-3, (2-hydroxy-4-methoxyphenyl)-phenylmethanone

Physical Properties, Production, and Uses

Oxybenzone (OXY) [IUPAC name: (2-hydroxy-4-methoxyphenyl)-phenylmethanone] is a monomethoxylated derivative of 2-hydroxybenzophenone (Cosmetic Ingredient Review, 1983). It occurs naturally in flower pigments (Stecher, 1958) and is a white to light yellow crystalline powder that is insoluble in water, has a melting point of 66°C, low volatility and is stable at room temperature (O'Neil et al., 2012).

Oxybenzone is synthesized for commercial use by the Friedel-Crafts reaction of benzoyl chloride with 3-hydroxyanisole. The resulting product is then re-crystallized from water/methanol and dried (Cosmetic Ingredient Review, 1983). Oxybenzone is included on the U.S. Environmental Protection Agency's (EPA) High Production Volume (HPV) list (http://www.epa.gov/hpv/index.htm); the HPV list is based on the 1990 Inventory Update Rule (IUR) (40 CFR part 710 subpart B; 51FR21438). Chemicals listed as high production volume were either produced or imported into the U.S. in >1 million pounds in 1990 and/or 1994. As of April 4, 2011 thirteen companies were listed as manufacturers of oxybenzone according to the HSDB (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

Commercially, oxybenzone is widely used in sunscreens and sunscreen containing products because of its ability to act as a UV filter, blocking both UVA and UVB rays (Klein, 1992). In addition to sunscreens, it is found in a wide range of personal care products including nail polishes, lipsticks, perfumes, hair sprays and shampoos where it is included as a photostabilizer and/or fragrance enhancer (EWG; http://www.ewg.org/skindeep). The incorporation of oxybenzone in personal care products has risen over the years. In 1976 oxybenzone was found in 47 products, in 62 products in 1979 (Cosmetics Ingredient Review, 1983) and in 451 products in 2002 (Cosmetics Ingredient Review, 2005). The largest increase in product incorporation during this period of time was in lipsticks, moisturizers and suntan gels, creams and liquids (Cosmetics Ingredient Review, 2005). A more recent search using the EWG's Skin Deep Cosmetics Database found oxybenzone listed as a component of 1,283 products (as of December 20, 2013). Sunscreens continue to be the most common oxybenzone containing personal care product followed by moisturizers, nail polish and fragrances (EWG; http://www.ewg.org/skindeep).

Concentrations of oxybenzone in products vary and have been reported to range from <0.1-1% in 1976 to 0.005-7% in 2002 (Cosmetics Ingredient Review, 2005). Oxybenzone is listed as an allowable sunscreen compound in the U.S. FDA's sunscreen monograph 21 CFR 352.10 with a permissible concentration not to exceed 6% (CFR, 2013b). In addition to personal care products, oxybenzone is also commonly found in plastics because of its ability to absorb ultraviolet light and act as a stabilizer (HSDB; http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB). It has also been approved by the FDA as an indirect food additive used in food contact substances (21 CFR 177.1010) (CFR, 2013a).

Exposures

Exposure to oxybenzone may occur through occupational sources or through contact with consumer products. The National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health (NIOSH) in 1981-1983 estimated that 27,518 workers were potentially exposed to oxybenzone on the job-site with 8,664 being female (http://www.cdc.gov/noes//noes2/occssyna.html). More recent analyses, however, have estimated the prevalence of oxybenzone exposure to be much higher due to its widespread incorporation in personal care products (Calafat et al., 2008). The levels of oxybenzone or its metabolites were

measured in urine samples collected from 2,517 individuals from 2003-2004 as part of the National Health and Nutrition Examination Survey (NHANES). Upon analysis over 96.8% of the samples had measurable levels of oxybenzone with a geometric mean value of 22.9 µg/L (22.2 µg/g creatinine) and a 95th percentile concentration of 1,040 µg/L (1,070 µg/g creatinine). The values for non-Hispanic whites were significantly higher than those for other racial groups. Additionally, females had significantly higher levels than did males, and non-Hispanic white women were 6.8 times and Mexican American women were 4 times more likely to have levels above the 95th percentile than were non-Hispanic black women (Calafat et al., 2008).

Measurable levels of oxybenzone have also been reported in 96% of urine samples collected and analyzed from 90 6- to 9-year old girls with levels ranging from <0.2 – 26,700 µg/L (geometric mean of 30.8 µg/g creatinine) (Wolff et al., 2007). In addition, oxybenzone has been detected in the urine of 40 out of 42 premature infants with a geometric mean of 3.4 µg/L (range of LOD – 176 µg/L) (Calafat et al., 2009).

In the environment, oxybenzone has been detected in wastewater samples (31-168 ng/L; 11-286 ng/L) (Rodil et al., 2008; Pedrouzo et al., 2009), in wastewater treatment plants (<LOD-16 ng/L) (Rodil et al., 2008; Balmer et al., 2005) as well as in the surface waters of lakes (<2-125 ng/L) (Poiger et al., 2004) and in the coastal waters of beaches (46.6 ng/L) (Rodríguez et al. 2015). Concentrations were found to be seasonal and tended to be higher in the warmer months, perhaps as a result of increased sunscreen usage (Poiger et al., 2004; Rodil et al., 2008). Oxybenzone has also been found in sediments (1.6 ng/g) collected in Southern California (Schlenk et al., 2005), but was below the limit of quantification (LOQ, 8.2 ng/g) in hornyhead turbot livers (Kwon et al., 2009).

Absorption, Metabolism and Distribution

Oxybenzone is metabolized to 2,4-dihydroxybenzophenone (DHB), 2,2'-dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxybenzophenone (THB). DHB and DHMB are the major detectable metabolites while THB is present at much lower levels (Jeon et al., 2008). Oxybenzone and its metabolites can be conjugated at the hydroxyl groups as glucuronides or as sulfates (El Dareer et al., 1986; Jeon et al., 2008).

Oxybenzone was found to be quickly absorbed from the gastrointestinal tract after a single dose (100 mg/kg body weight) when administered orally to male Sprague-Dawley rats with the maximum plasma concentration being reached within 3 hours of administration and declining rapidly thereafter (Jeon et al., 2008). This is in contrast to the predominant metabolite, DHB, which decreases more slowly with time (Jeon et al., 2008). The main route of elimination was via the urine (over 75% urinary excretion occurred in the first 6-12 hours), followed by the feces (90% of fecal excretion in the first 24 hours) (Jeon et al., 2008). Previous studies which looked at tissue distribution determined the liver contained the highest amount of compound followed by the kidney (El Dareer et al., 1986; Kadry et al., 1995). These studies are in general agreement with a previously sponsored NTP study that looked at oral, intravenous (IV) or topical administration of the compound (El Dareer et al., 1986). In this study they also found the compound to be rapidly absorbed, metabolized and excreted.

Endocrine Disruptor Potential

A number of studies have examined the endocrine disruption potential of oxybenzone. These studies have included both *in vitro* (Schlumpf et al., 2001; Nakagawa and Suzuki., 2002; Wada et al., 2004; Suzuki et al., 2005; Kunz et al., 2006) and *in vivo* (French, 1992; Datson et al., 1993; Schlumpf et al., 2001; Schlecht et al., 2004; Suzuki et al., 2005; Kunz et al., 2006; Coronado et al., 2008) analyses. Overall, the studies demonstrate that oxybenzone and/or the primary metabolite, DHB, have weak estrogenic activity. These compounds are approximately 10^5 to 10^6 less potent than estrogens such as estradiol, but have similar estrogenic potency as bisphenol A.

Dose Selection for the Embryo/Fetal Developmental Feed Study of Oxybenzone

Results from a developmental dose range-finding feed study of oxybenzone are presented in an NCTR report for Protocol No: E02178.01 (non-GLP) that utilized exposure concentrations of up to 50,000 ppm. The doses used in this study, in conjunction with the results of an NTP dose-finding feed study, were used to select dietary exposure concentrations of 0, 3,000, 10,000 and 30,000 ppm for the current study.

Dietary exposures of 0, 1,000, 3,000, 10,000, 25,000 and 50,000 ppm were evaluated in the developmental dose range-finding study. Date-mated female Sprague-Dawley rats (Harlan) were administered oxybenzone via Purina 5K96 chow beginning on GD 6. Dosing continued until the time of sacrifice on GD 10, GD 15, GD 20 or postnatal day (PND) 23. A decrease in maternal body weight was observed as early as GD 10 for the highest oxybenzone dose group and continued through GD 20. Although oxybenzone at the two highest treatment concentrations produced maternal toxicity evidenced by weight loss, no differences were observed in the number of implantations, live fetuses or fetal weights at GD20. At PND 23, there were no body weight differences between the treated groups of maternal animals, however, administration of 10,000 ppm and greater was associated with higher relative liver weights and increases in clinical chemistry parameters suggestive of liver toxicity. Oxybenzone did affect postnatal pup weight at 50,000 ppm. Decreased postnatal pup weight was observed at PND 4 for females and at PND 7 for males. Overall, the results from the developmental dose range-finding feed study of oxybenzone indicated a lowest adverse effect level (LOAEL) to be 10,000 ppm for maternal toxicity and 25,000 ppm for pups.

Study Objective

This study was designed to determine the potential toxicity of oxybenzone on embryo/fetal development in Sprague-Dawley rats. 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 U.S. population. This study was conducted to address concerns raised by CDER that oxybenzone may have endocrine disruptor activity and influence embryo/fetal development.

The study was designed and conducted according to the recommendations outlined in the ICH Guideline S5(R2) 4.1.3 (ICH, 1993); a modified one-generation study was also conducted on oxybenzone by the NTP to compare results from the two study designs.

MATERIALS AND METHODS

Materials and methods and experimental design are outlined in Table 1. Additional information is provided below and in the referenced Appendices.

Procurement and Characterization of Oxybenzone

Oxybenzone (CAS No. 131-57-7) was obtained in a single lot (1F100604) from Ivy Fine Chemicals (Cherry Hill, NJ). Identity and purity analyses were conducted by the Chemistry Support Group located in the Division of Biochemical Toxicology at the NCTR (Jefferson, AR). Reference standards were purchased from Sigma-Aldrich (St. Louis, MO; Lot S42088).

Bulk chemical was stored in the original shipping container(s) protected from light at room temperature. Purity and identity was assessed prior to study initiation using the methods described in brief below; detailed descriptions on analyses performed can be found in Appendix IV. Proton nuclear magnetic resonance (NMR) spectroscopy of the test article indicated that the test article was consistent with the structure of oxybenzone and matched the reference standard. Other than the resonances of the residual non-deuterated solvent and water, no other significant traces of contaminants were detected, indicating the test article was of high purity. The purity of the test article was also assessed by reverse phase high-performance liquid chromatography (HPLC) with photodiode array (PDA) and mass spectrometry (MS) detection. HPLC-MS analysis revealed a single UV peak that was identical to that of the reference standard. HPLC-PDA analysis also revealed one major peak with an identical UV spectrum to that of the oxybenzone standard; purity was determined to be greater than 99%.

Background Isoflavone Content of Purina 5K96 Chow

In an attempt to minimize background exposure to phytoestrogens, animals were maintained on a soy- and alfalfa-free rodent chow, designated 5K96 [verified casein diet 10 IF, irradiated], obtained through Purina Mills, Inc. (St. Louis, MO). Isoflavone levels, defined as the total amounts of genistein and daidzein, in each lot of feed were determined by the Chemistry Support Group, Division of Biochemical Toxicology, NCTR (Jefferson, AR) prior to utilization. The composition of the diet and results of isoflavone/contaminant analyses are presented in Appendices V and IV, respectively, for feed used throughout the study. Based on historical data on lots of 5K96

previously used at NCTR the tolerance level was set at 2 ppm for total isoflavone levels. Analyses indicated that the total levels of daidzein and genistein did not exceed the specified limit of 2 ppm and averaged 0.07 ± 0.07 ppm for daidzein and 0.15 ± 0.14 ppm for genistein (mean total for daidzein and geinstein: 0.23 ± 0.20 ppm).

Preparation and Analysis of Dose Formulations
The Diet Preparation Group, Priority One Services at NCTR prepared all dose formulations for the current study. Dose formulations were prepared as needed by mixing oxybenzone with 5K96 chow. Mixing was performed in a Patterson-Kelly blender using an intensifier bar. Formulations were stored in stainless steel cans at $4°C \pm 2°$. Details on the dose formulation procedure can be found in Appendix V.

Homogeneity and stability studies using lot 1F100604 of oxybenzone were previously conducted as part of the dose range-finding study (E0217801 Dose-Finding Oxybenzone-NTP; non-GLP). Homogeneity (1,000 and 50,000 ppm dose levels) was verified and stability of the 1,000 ppm dose formulation was confirmed through seven weeks[1] at refrigerated temperature (see Appendix IV for detailed descriptions).

Dose certifications and homogeneity analyses were conducted by the Chemistry Support Group for each batch of dosed chow that was mixed. Samples were prepared for analysis by HPLC-PDA as outlined in Appendix IV. Oxybenzone was prepared at three dose concentrations (3,000; 10,000; 30,000 ppm). One sample from each concentration was supplied as aliquots labeled top, middle and bottom for analysis. In each instance, the dose formulations of oxybenzone were within 10% of the target concentrations with a CV of 10% or less (Appendix IV). A sample of the 0 ppm diet was also supplied.

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[1]Stability at the time the protocol was approved was six weeks. Refer to the Analytical Chemistry Report (Appendix IV) for data supporting stability of seven weeks.

Source and Specification of Animals

The study design was modeled on the recommendations presented in ICH Guideline S5(R2) 4.1.3 for assessing effects on embryo-fetal development (ICH, 1993). A total of 100 time-mated female Sprague-Dawley rats were obtained from Harlan Industries (Indianapolis, IN) and arrived at the study laboratory on GD 3. The date of vaginal plug detection or sperm positive lavage was designated as the day of conception or GD 0. Twenty-five animals were assigned per treatment group and fed diets containing 0, 3,000, 10,000 or 30,000 ppm oxybenzone from GD 6 (implantation) through GD 15 (closure of the hard palate). Approximately one day prior to parturition on GD 21 the dams were sacrificed and fetuses evaluated as described below.

Animal Maintenance and Health

Animals were allocated to treatment groups by a weight-ranked randomization procedure upon arrival such that the mean weight of each treatment group was approximately the same (Appendix VIII). Animals were identified by tail tattoo with their cage number and were singly housed throughout the study in solid-bottom polysulfone cages with hardwood chip bedding.

Animals were maintained on a soy- and alfalfa-free Purina 5K96 chow. All animals received control chow from their arrival on GD 3 until dosing began on the morning of GD 6. Exposed animals received dosed chow until the morning of GD 15; all animals received control chow until sacrifice on GD 21. Purina 5K96 chow and Millipore®-filtered tap water were available *ad libitum*. The 5K96 diet underwent routine concentration analysis as well as analysis for isoflavone concentrations as described above. Feed consumption was measured at least twice weekly and at the start and stop of dosing; water consumption was not measured. Cages were changed weekly. Information on feed consumption and contaminants can be found in Table 4 and Appendix IV, respectively.

Traditional quarantine guidelines could not be followed due to the type of animals (time-mated females) and dosing scheduled required. Therefore, "quarantine procedures" were implemented and included entrance only via the clean side and exit via the return side of the animal room; personal protective equipment was donned upon entry and discarded upon exit; no entry into other animal rooms was permitted after exit unless showers were taken and clothing changed. The health

of the animals was monitored according to the protocols of the study laboratory's Microbiology Surveillance and Diagnostic Program (Appendix VI). Fourteen additional non-mated animals were ordered; four animals were used as sentinels and arrived with load one animals. Ten animals (two per shipment/five shipments) were ordered for microbiology surveillance. NTP protocol E02188.01 ran concurrently with this study and as a result animals for microbiology surveillance were shared between protocols as the animals for study arrived in the same shipment.

In-Life Examinations and Pathology
Data collected during the in-life phase of the study and at necropsy are detailed in Table 1. The Multigeneration Support System (MGSS), which was developed by the former R.O.W. Sciences at the NCTR, was used to collect in-life animal data. Twice daily morbidity and mortality checks were performed; clinical observations were recorded once daily. Body weights of animals at allocation and at GD 6, GD 10, GD 14, GD 17 and GD 21 were also recorded.

Dams were euthanized by over-exposure to carbon dioxide approximately one day prior to expected parturition on the morning of GD 21. Blood was removed by cardiac puncture from all dams at sacrifice. Ten pregnant dams from each treatment group, stratified by treatment and load, were randomly selected for analysis of clinical chemistry and hematology parameters. Females not initially selected for clinical chemistry and hematological analysis were ranked for use as backups in case the initial animal selected was not pregnant (see Notes to Study File – Randomization for Clinical Chemistry, Appendix IX). The abdominal and thoracic cavities of the dams were examined for abnormalities, gross lesions and the collection of liver and kidney weights. For animals that did not appear pregnant, the uterus was stained with ammonium sulfide to detect any implantation sites.

Fetal examinations were performed as outlined in TPA's SOP No. 337.00. The gravid uterus with the right ovary attached was removed from the dams and weighed. The number and status of each implantation site (live, dead, early resorption or late resorption) was recorded. Each live fetus was removed, weighed, sexed, and examined for gross abnormalities (including cleft palate) and sacrificed by decapitation or by an intraperitoneal injection of Euthasol followed by bilateral thoracotomy. The heads of the fetuses that were decapitated were fixed in Bouin's fixative and

evaluated using Wilson's free-hand razor dissection technique (Wilson, 1965) as outlined in TPA's SOP No. 338.01. A fresh tissue dissection method was used to examine the internal organs of all pups in each litter for malformations (Sutckhardt and Poppe, 1984). The skeletons were macerated and stained with Alcian Blue and Alizarin Red S; the cartilage and ossified portions of the skeleton were evaluated (LaBorde et al., 1995). The bones of the skull were also stained and examined in the fetuses not selected for free-hand razor dissection. The skeletons were examined for any potential effects on bone or cartilage formation as outlined in TPA's SOP No. 339.01. Sections from the individual heads and skeletons were stored in plastic vials at room temperature in a 1:1 solution of glycerol/ethanol (95%). The samples were sent to the NCTR Archives for storage.

Ovaries, after weighing, were retained for corpora lutea counts as described in TPA's SOP No. 331.00. After counts were obtained the ovaries were fixed in 10% neutral buffered formalin and embedded in Tissue Prep II. Blocks were sent to the NCTR Archives for storage.

Records Storage

The signed and dated copy of the final report and supporting study information will be stored in the NCTR archives. Raw data, including handwritten data, collected during the course of the study will also be placed into the NCTR archives.

STATISTICAL METHODS

The following paragraphs outline the statistical methods used for analysis of the study data. Detailed statistical reports for all endpoints evaluated are included as Appendices XI- XVII. For *post hoc* analysis, comparisons of treatment groups to control were performed with Dunnett's method for adjusted contrasts (Dunnett, 1955), unless noted. Tests were conducted as two-sided at the 0.05 significance level.

Maternal Body Weights (Appendix XI)

To assess the effects of oxybenzone on maternal body weight at GD 6, 10, 14, 17 and 21 the maternal body weight at GD 3, prior to dosing, was used as the baseline weight. Pairwise comparisons of means were performed using contrasts within a two-way repeated measures, mixed model analysis of covariance (ANOCOVA) with terms for treatment group, GD, interaction, and baseline weight. Within-group correlations were modeled using a heterogeneous first-order autoregressive (ARH(1)) correlation structure, which allowed for correlated differences in variability across time points.

Maternal Food Consumption (Appendix XII, Addendum I)

Food consumption was measured at least twice weekly during the study. For analysis, food consumption data was analyzed at the following gestational day intervals: GD 4-6, GD 6-10, GD 10-14, GD 14-17 and GD 17-21. Pairwise comparisons of means were performed using contrasts within a two-way repeated measures, mixed model analysis of variance (ANOVA) with terms for treatment group, GD, and interaction. Within-group correlations were modeled using a heterogeneous first-order autoregressive (ARH(1)) correlation structure.

Maternal Organ Weights (Appendix XIII)

Analyses of maternal organ weights were conducted on the combined weights of the paired organs, if applicable, at sacrifice. Pairwise comparisons of dosed groups to the control were performed using contrasts within an ANOCOVA with terms for treatment group and weight at sacrifice.

Maternal Hematology and Clinical Chemistry (Appendix XIV)

For each hematological or clinical chemistry endpoint, an ANOVA was performed to determine the effect of treatment using a nonparametric method with midranks (using the average of left and right ranks for ties) and an unstructured covariance (Brunner *et al.*, 2002).

Pregnancy Parameters (Appendix XV)

The percentage of pregnant dams in each treatment group was calculated and the proportions were analyzed using Fisher's Exact Test for comparisons of treatments to control and the Cochran-Armitage test for trend. Summary statistics were performed for gravid uterine weights; analysis was done using contrasts within a one-way ANOVA. The mean counts of corpora lutea, number of implants, number of resorptions, and number of live fetuses per litter were calculated for each treatment group. Mean percentages for pre-implantation loss and post-implantation loss were also determined. For analysis, 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. 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.

Litter Parameters (Appendix XVI)

Data were collected for individual fetuses, except for two fetuses that were not sexed. Counts of live fetuses, across and by sex, 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 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 ANOVA model to test for treatment effect. For litter weight, analysis was performed using contrasts within an ANOCOVA adjusted for litter size (number of live fetuses) to test for treatment effect.

Fetal Variations/Malformations (Appendix XVII)

Skeletal and visceral observations were analyzed separately. Due to sparse data, counts were combined across sex. Fetal counts were calculated within litter for fetuses with skeletal and visceral observations. Each fetus was counted only once for a specific variation/malformation. Skeletal observation count data within litter are presented for rib, sternebrae, centra, and others. The total count of skeletal observations in each litter was calculated as the sum of rib, sternebrae, centra, and other observations. Counts for any variation/malformation were calculated as the number of fetuses with one or more observation. Counts for each skeletal observation, total observations, and number of fetuses with an observation in each litter were analyzed using Poisson regression with terms for treatment and covariate number of live fetuses. Visceral observation count data within litter were determined for cleft palate, dilated ureter, malpositioned testes, and others. The total count of visceral variations/malformations in each litter was calculated as the sum of cleft palate, dilated ureter, malpositioned testes, and other observations. Counts for dilated ureter and total observations in each litter were analyzed using Poisson regression with terms for treatment and covariate number of live fetuses. Due to sparse data for cleft palate, malpositioned testes, and other observational counts, statistical analysis was not performed.

RESULTS

In-Life Observations and Clinical Signs

No in-life observations or clinical signs were noted that were considered related to oxybenzone exposure.

Maternal Body Weights

Significant differences were observed in body weights of pregnant rats exposed to dietary oxybenzone from GD 6 to GD 15. Maternal weights were significantly lower in the OXY 10,000 ppm and OXY 30,000 ppm treatment groups when compared to controls at GD 10, 14 and 17 (Figure 1). Reductions in weight ranged from 2.5-3.8% for the 10,000 ppm treatment group and from 4.0-7.3% for the 30,000 ppm treatment group. The overall mean maternal body weight (defined as the mean body weight at GD 6 through GD 21) for the OXY 10,000 and OXY 30,000 treatment groups were also significantly different from control (Figure 2). A similar trend was observed in the body weight gain data (defined as weight at GD 6 through GD 21 minus weight at GD 3) (Figure 3) and in the analysis of body weight gain by GD intervals (GD 3-6, 6-10, 10-14, 14-17 and 17-21) (Table 3). However, due to the similarities of the maternal body weight and maternal body weight gain data sets a statistical analysis was not performed on the weight gain data (total or GD interval). Least square means for maternal body weights are presented in Table 2 and Appendix XI; summary statistics for maternal weight gain and for maternal weight gain by GD intervals are presented in Figure 3 and Table 3, respectively as well as in Appendix XI and Appendix XI, Addendum I. Non-pregnant animals, confirmed by ammonium sulfide staining of the uterus, were excluded from analysis of body weight and body weight gain.

Maternal Food Consumption

Pregnant dams were fed control chow from their arrival on GD 3 until the morning of GD 6 when they were placed on dosed chow; dosing continued until the morning of GD 15 at which time the pregnant dams were returned to control chow until their sacrifice on GD 21. Food consumption data was collected for the entire study period (18 days) and analyzed at the following intervals:

GD 6-10, GD 10-14, GD 14-17 and GD 17-21 (Appendix XII, Addendum I).   An overall analysis of food consumption was also performed and covered GD 6-21.

Pairwise comparisons indicated significant differences between the OXY 30,000 ppm treatment group and controls during all GD intervals examined (p = <0.001 for GD 6-10, GD 10-14 and GD 6-21; p = 0.004 for GD 14-17; p = 0.020 for GD 17-21) with the OXY 30,000 ppm treatment group consistently having higher mean values than controls (Table 4). A 66.7% increase in daily food consumption was observed for the OXY 30,000 ppm treated animals during dosing at GD 6-10; a 70.0% increase between the same treatment groups was observed for the dosing period GD 10-14. Overall, a 41.8% increase in daily food consumption, covering GD 6-21, was observed for the OXY 30,000 ppm group relative to control dams. There was no difference in food consumption levels in any treatment group prior to dosing on GD 6 (data not shown). Estimated amounts of ingested concentrations of oxybenzone are shown in Table 5.

Terminal Sacrifice and Necropsy Evaluations
At sacrifice on GD 21 the uterus of any animal that did not appear pregnant was stained with ammonium sulfide to detect early implantation sites. Six females from the CTRL treatment group, four from the OXY 3,000 ppm treatment group, three from the OXY 10,000 ppm treatment group and six from the OXY 30,000 ppm treatment group were confirmed as non-pregnant. These animals were excluded from all analyses.   One non-pregnant female in the OXY 30,000 ppm treatment group was noted as having hydrometra and a fluid distended vagina at sacrifice.

Maternal Organ Weights
At sacrifice the liver, kidneys and ovaries were removed from each dam and weighed. Individual weights of the left and right kidneys and left and right ovaries were recorded (see Table 7 for summary statistics). Relative organ weights (mg) to body weight (g) ratios of dams at sacrifice were also calculated and the summary statistics are presented in Table 8.

For statistical analyses the paired weights of the kidneys and ovaries were used (Table 6). ANOCOVA analysis indicates oxybenzone had no significant treatment effects on the liver, kidney

(paired), or ovarian (paired) weights when adjusted for body weight at sacrifice. Pairwise comparisons performed using Dunnett's method for adjusted contrasts also indicated no significant differences between control and oxybenzone treatment groups on liver, kidney (paired), or ovarian (paired) weights, although a significant trend was observed for the liver (p = 0.025).

Maternal Hematology/Clinical Chemistry

A total of ten pregnant dams from each treatment group were randomly selected for hematological and clinical chemistry analysis. Each dam selected for analysis was sacrificed by 10:30 AM on GD 21. An ANOVA was performed to determine whether oxybenzone treatment had any effect on the hematological parameters listed in Table 9 and the clinical chemistry parameters in Table 10. ANOVA results indicated a significant treatment effect on the hematological measurements of RBCs, PLTs, and on the proportion, by volume, of blood that consists of RBCs defined as the HCT. Clinical chemistry measurements of TBA and CHOL also indicated significant treatment effects.

For hematological parameters pairwise comparisons of oxybenzone treatment groups to control indicated significantly lower HGB concentrations and HCT measurements (p=0.039 and p=0.026, respectively) between the OXY 3,000 ppm and control group. Comparison of control to the OXY 10,000 ppm treatment group also identified a significant difference for HCT (p=0.042) with lower values again observed in the treatment group relative to control. The greatest number of significant differences was, however, observed between the control and OXY 30,000 ppm treatment group. Measurements of RBCs, PLTs, HGB, HCT and PCV were all statistically significant (p =0.012, p=0.009, p=0.036, p=0.014, and p=0.024, respectively). Except for PLT numbers, which were higher relative to control, RBC, HGB, HCT and PCV values were lower. Analysis of trends also indicated significance for RBCs and PLTs (both p <0.05), although as indicated, only the high oxybenzone dose group differed from the control in pairwise comparisons (Table 9).

Pairwise comparisons of clinical chemistry parameters revealed significant differences in BUN, TBA, and CHOL between the control and OXY 30,000 ppm treatment groups (p=0.039, p=0.001 and p=0.017, respectively). Levels of BUN were lower in the high dose group relative to control

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while levels of TBA and CHOL were higher. Significant trends for TBA, CHOL, and BUN (all p <0.05) were also observed (Table 10).

Pregnancy Parameters

The results of exposure to oxybenzone on reproductive/pregnancy related parameters are reported in Table 11. Treatment with oxybenzone from GD 6 to GD 15 had no effect on the overall number of pregnant females per treatment group. Pregnancy rates ranged from 76.0% for control and OXY 30,000 ppm treatment groups to 88.0% for the OXY 10,000 ppm treatment group. All gravid uterine weights, which included the weight of the right ovary, were not statistically significant when analyzed using contrasts within a one-way ANOVA. Additionally, no statistically significant trend or statistically significant differences in pairwise comparisons were observed. Counts of implantation sites, adjusted for the number of corpora lutea, and the number of resorption sites, adjusted for the number of implantations, were not statistically significant between treatment and control groups. Of the resorptions detected, all were classified as early resorptions. No late resorptions or dead fetuses were observed on study. The summary statistics for the number of corpora lutea, the number of live fetuses, the percentage of pre-implantation loss and the percentage of post-implantation loss are also reported in Table 11 and Appendix XV. Overall, there were no significant differences between the control group and the treatment groups upon exposure to oxybenzone in the reported reproductive/pregnancy parameters. The analysis on the number of live fetuses per litter is discussed in detail in the Litter Parameters section below.

Litter Parameters

Inadvertently, the sex of two fetuses was not recorded during the visceral exam; one fetus was in the OXY 3,000 and the other in the OXY 30,000 ppm treatment groups. For the data presented in this report, unsexed fetuses were assigned as male for analysis of the number of live male and live female fetuses per litter and for the determination of sex ratios. However, there were no differences in the statistical results when the unsexed fetuses were designated as either male or female (data not shown). Unsexed fetuses were not included in the analysis of fetal weights; for analysis of litter weights, weight was combined across sex including those of the unsexed fetuses.

There were no significant differences observed between the control and oxybenzone treatment groups in the total number of live fetuses per litter or in the number of live male and female fetuses per litter after exposure to oxybenzone from GD 6 to GD 15 (Table 12). There were no dead fetuses in any treatment group. There was also no observed difference in total litter weights or in the mean male and female fetal weights upon oxybenzone treatment (Table 12). Additionally, oxybenzone exposure did not influence the proportion of male to female fetuses (Table 12).

Fetal Evaluations – Visceral

Each live fetus was subjected to a visceral exam upon sacrifice. A total of 78 observations were noted across control and treatment groups and included, but was not limited to, dilated ureter, cleft palate and malpositioned testes (a complete list of observations and summary statistics are reported in Table 14 and Appendix XVII). Of the observations noted only cleft palate was classified as a malformation. All observations of dilated ureters were either mild to moderate and therefore, were not considered malformations, but variations.

Dilated ureter accounted for 84.6% of the total observations noted. The mean number of variations/malformations per litter for each treatment group is presented in Table 13. There were significant trends for total numbers of variations/malformations (p=0.020) and for the total number of pups with dilated ureters (p=0.010). Pairwise comparison using Dunnett's method for adjusted contrasts, however, indicated no significant pairwise differences between control and treatment groups.

Fetal Evaluations – Skeletal

The skeletal data for one litter of fetuses in the control group (dam 2; UIN 5A000001143) was excluded from analysis due to a discrepancy between the teratological examination sheet, the skeletal evaluation sheets and the number of specimens. Upon analysis of the skeletons, it was discovered that an additional fetus was included within the litter for dam 2. The investigators were unable to determine what treatment group/litter the extra fetus came from or identify which skeleton belonged to the extra fetus. Therefore, the whole litter was excluded from the analysis.

Variations in skeletal structures were grouped into four main categories (ribs, sternebrae, centra and other) for analysis. A statistical analysis was performed on each category as well as on the total number of skeletal variations. A complete listing of the observed skeletal variations is included in Table 16.

A significant trend for rib variations was observed (Table 15) which included the presence of a 14th thoracic rib or left, right and bilateral rudimentary ribs; however, the trend did not appear to be related to oxybenzone exposure as no dose dependent increase in rib variations was observed. There were also no significant trends or pairwise differences from controls observed for sternebrae and centra variations. Pairwise analysis indicated a significant difference in the total number of variations for the OXY 3,000 ppm treatment group compared to controls; however, this was not considered treatment related as no dose response was observed upon increasing oxybenzone concentration.

DISCUSSION

Oxybenzone is an organic ultra-violet absorbing compound that is widely used in commercial personal care products, in particular sunscreens, because of its ability to block UVA and UVB radiation (Klein, 1992). Oxybenzone is listed as an allowable sunscreen compound in the FDA's sunscreen monograph 21 CFR 352.10 with a permissible concentration not to exceed 6% (CFR, 2013b). It is also a common component of plastics and has been approved by the FDA as an indirect food additive (21 CFR 177.1010) (CFR, 2013a). There has been heightened interest in the biological activity of oxybenzone due to its high volume of use, detection in the urine of a large percentage of the U.S. population (Calafat et al., 2008) and numerous *in vitro* (Schlumpf et al., 2001; Nakagawa and Suzuki., 2002; Wada et al., 2004; Suzuki et al., 2005; Kunz et al., 2006) and *in vivo* (French, 1992; Datson et al., 1993; Schlumpf et al., 2001; Schlecht et al., 2004; Suzuki et al., 2005; Kunz et al., 2006; Coronado et al., 2008) studies indicating endocrine disruptor activity. The present study was designed to examine the effects of oxybenzone on embryo/fetal development and was conducted using the recommendations in ICH guideline S5(R2) 4.1.3 (ICH, 1993). Data from a preliminary reproductive and developmental dose range finding experiment with oxybenzone (NCTR Protocol No: E02178.01; non-GLP) in conjunction with the results from an NTP dose-finding study were used to select dietary exposure concentrations of 0, 3,000, 10,000 and 30,000 ppm for the current study. These dietary doses resulted in ingested oxybenzone doses of approximately 0, 242, 725 and 3,689 mg oxybenzone per kg body weight per day during the time the animals were directly consuming dosed feed (GD 6 – GD 15) (Table 5).

Prior to the conduct of this study there was no published information available on the impact of oxybenzone exposure to the developing embryo and fetus. Limited and conflicting information was available regarding its effects on reproduction and fertility (NTP, 1990; French, 1992; Datson et al., 1993). The results of the present study indicate that exposure to oxybenzone from implantation (GD 6) through closure of the hard palate (GD 15) does not adversely affect embryonic and fetal development when fed to rats at concentrations up to 30,000 ppm. Analysis of reproductive and pregnancy parameters revealed no significant differences in the number of pregnant dams, in the number of corpora lutea, or in the percentage of pre-implantation loss between the oxybenzone treatment and control groups. However, as dosing did not begin until

after mating these results were not unexpected. Reproductive and pregnancy parameters including the number of implantation sites, the number of resorptions, the number of live fetuses and the percentage of post-implantation loss were more likely to be affected by gestational exposure to oxybenzone. However, analysis of each parameter indicates no significant treatment effects. Furthermore, there were no late resorptions or dead fetuses observed in any of the treatment groups.

Assessment of litter parameters also indicated no adverse effects of oxybenzone exposure on the fetus. The overall numbers of live male and female fetuses and the proportion of male to female fetuses were not influenced by gestational exposure to oxybenzone. Additionally, total litter weights and the average weights for male and female fetuses did not differ from the controls at sacrifice.

Visceral exams of the fetuses did, however, reveal over a two-fold increase in the total number of variations/malformations observed per litter upon treatment with the highest concentration of oxybenzone. Of all the observations noted, dilated ureter was the most common averaging 0.53 fetuses per litter for the control group and 1.23 fetuses per litter for the OXY 30,000 ppm group. While the trends for total observations ($p = 0.020$) and the number of dilated ureters ($p = 0.010$) were statistically significant, pairwise comparisons indicated no differences between control and treatment groups. Examination of the fetal skeletons did reveal a statistically significant increase in the total number of skeletal variations between the control and OXY 3,000 ppm treatment group. However, this result appeared sporadic and not related to treatment. There was also a significant trend observed for rib variations ($p = 0.012$), but this was not attributed to oxybenzone exposure as it was not related to dose.

While it may be possible to link exposure to oxybenzone to the increase in the incidence of dilated ureters or potentially even the other observed variations/malformations, we cannot rule out the possibility that the increased incidence of variations/malformations might simply be a common occurrence in the Harlan Sprague-Dawley strain of rat. It is known that underlying genetic factors are important determinants in the type, frequency and even severity of defects observed in both toxicological and teratological studies. Unfortunately, at present there is no historical database for the Harlan Sprague-Dawley strain of rat in order to compare the observed rate of

malformations/variations in the current study to. Without historical control data for comparison, we cannot conclude if the over two-fold increase in the incidence of dilated ureters is a direct consequence of oxybenzone exposure or if the data falls within the typical background range for this strain of rats.

The only significant toxicological findings related to oxybenzone exposure from GD 6 to GD 15 were observed in the dams. Oxybenzone exposure resulted in decreased maternal body weights. Significant declines were reported at GD 10 for the OXY 10,000 and OXY 30,000 ppm treatment groups and were observed at GD 14 and GD 17 as well. Overall, a 2.7% decrease in body weight was observed in the OXY 10,000 ppm group and a 3.8% reduction in the OXY 30,000 ppm group relative to controls. Decreases in body weight in response to oxybenzone treatment were previously reported for F344/N rats and B6C3F$_1$ mice in 13-week dosed feed studies sponsored by the NTP (French, 1992). However, unlike those studies in the rat which saw a general decrease in food consumption with increasing oxybenzone concentration, animals on the current study appeared to consume significantly more chow than control animals. An approximate 41.8% increase in food consumption was observed for the OXY 30,000 ppm group relative to controls from GD 6 to GD 21. A similar increase was observed in the 2-week and 13-week dosed feed studies with the B6C3F$_1$ mice which appeared to consume more chow with increasing oxybenzone concentration (French, 1992). However, it should be noted that while food consumption did increase we cannot rule out possible palatability issues with the highest dose level that may have resulted in increased spillage as well.

The liver and kidneys have previously been shown to be target organs for oxybenzone toxicity (French, 1992). Consistent findings from both the dosed-feed and dermal application studies of oxybenzone sponsored by the NTP were increases in liver and kidney weights (French, 1992). However, in the current study no effects on maternal liver and kidney weights were observed (paired or relative). While a significant trend (p = 0.025) was noted for liver weights (increasing with oxybenzone concentration), there were no significant differences in pairwise comparisons. It is important to note, however, that histopathological analyses were not conducted in the current study. Therefore, we cannot rule out possible histological changes in these target organs. No gross observations of the kidney or liver were noted at sacrifice. Additionally, exposure length could

also be a contributing factor to the differences observed between studies. In the NTP sponsored studies some animals were fed dosed chow for 13 weeks, compared to the current study where dosing only occurred from GD 6 to GD 15.

Treatment with oxybenzone also significantly impacted a number of hematological and clinical chemistry parameters in the dams in the current study. The OXY 30,000 ppm treatment group was the dose group primarily impacted. This group saw a significant decrease in the number of RBCs and in the PCV; a significant increase was also observed in PLT counts. Additionally, HGB concentration levels were significantly lower in the OXY 3,000 and OXY 30,000 ppm treatment groups while HCT values were lower in all treatment groups. Significant changes in clinical chemistry parameters included higher TBA and CHOL levels and lower BUN levels between the control and highest oxybenzone treatment groups.

The effects of oxybenzone on various hematological and clinical chemistry parameters have previously been reported for male and female F344/N rats in 13-week feed and dermal application studies conducted by the NTP (French, 1992). In the feed studies the hematologic change that was considered relevant was a mild to moderate increase in PLT counts in dosed male rats at various time points and in dosed females at 12 weeks. A possible explanation given for the observed increase in PLT counts, or thrombocytosis, was the observed renal lesions which included tubule dilatations with regeneration of the tubular epithelial cells, interstitial inflammation and papillary necrosis (French, 1992). However, a clear association was not established as other mechanisms such as myeloproliferative disorders, infectious and inflammatory disorders, anemia due to iron deficiency as well as a variety of other miscellaneous causes outside of injury to the kidney could also have accounted for the increased number of PLTs (French, 1992; Wintrobe, 1981; Schafer, 2004). As the pathology of the kidney was not investigated in the current study we cannot speculate as to whether renal injury is a contributing factor to the increased PLT numbers, however, there were no observed differences in kidney weights or gross observations in the current study. In the previous study by French (1992) absolute and relative kidney weights were increased in the 50,000 ppm treatment group for male rats and in the 25,000 and 50,000 ppm treatment group for female rats in the 13-week feed study. In the 2-week feed study, significant changes in relative

kidney weights were only observed in the males; therefore, length of treatment, sex, strain or combination of factors might account for the differences in kidney weights between the studies.

It is of interest to note the hematological profiles of the dosed dams in the current study are suggestive of anemia (lower RBC, HGB concentration and HCT levels) which might also be a contributing factor to the observed increase in PLT counts. In a study conducted by Lewerenz and colleagues (1972) they reported lowered HGB levels and leukocytosis with increases in lymphocytes and a decrease in neutrophiils after 6 weeks of feeding female rats oxybenzone at 0.5% and 1% of their diets. After 12 weeks, anemia and lymphocytosis with a reduction in granulocytes was observed; degenerative nephrosis of the kidneys was also noted for both sexes at 1% oxybenzone concentration (Lewerenz et al., 1972). A subchronic study in rats also fed oxybenzone up 0.5% and 1.0% of their diets noted leukocytosis, anemia and degenerative nephrosis as well (Cosmetic Ingredient Review, 1983).

However, dermal application studies have reported no hematological changes or changes that were not considered biologically important in response to oxybenzone treatment. Dermal application resulted in no relevant hematologic changes in males while sporadic changes in HGB concentration and in RBC, PLT, leukocyte and lymphocytes in females were not considered biologically important when F344/N rats were dosed up to 200 mg/kg body weight (French, 1992). Another topical administration study found no hematological or clinical chemistry parameter changes when oxybenzone was administered for 4 weeks to male Sprague-Dawley rats at a 100 mg/kg body weight twice daily (Okereke et al., 1995). A possible explanation for the lack or sporadic hematological changes observed in the dermal application studies might be the overall lower systemic exposure levels.

Summary

Dietary exposure to oxybenzone did not significantly impact embryo/fetal development in Sprague-Dawley (Harlan) rats when exposed from implantation (GD 6) through closure of the hard palate (GD 15) at doses which produced some maternal toxicity (10,000 and 30,000 ppm). No significant changes were observed in pregnancy rates, gravid uterine weights, number of implantations, number of resorptions, litter size, litter weights, or male and female fetal weights.

While a significant trend was noted for the total number of visceral variations/malformations and for the number of dilated ureters observed, these differences were not significant in pairwise comparisons to controls and may be due to strain sensitivity. Oxybenzone treatment, however, did negatively impact body weight of the dams at 10,000 and 30,000 ppm. The decrease in body weight of the dams was accompanied by a significant increase in food consumption between the OXY 30,000 ppm treatment group and controls in pairwise comparisons; however, this increase might be due to palatability issues. Oxybenzone treatment also affected a number of hematological and clinical chemistry parameters, primarily between the control and highest oxybenzone treatment group.

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NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH
QUALITY ASSURANCE STATEMENT

Title of study: E02187
Effect of oxybenzone on embryo/fetal development in Sprague-Dawley rats- Segment II

Study Director: Amy L. Inselman, Ph.D.

This study was audited and inspected by the National Center for Toxicological Research (NCTR) Quality Assurance Unit (QAU) located in Jefferson, Arkansas. The NCTR Study Director and designated Management were informed of the results of each inspection.

Phase	Date(s) of Inspection	Date Reported to the Study Director and Test Facility Management
Protocol	3/09-21/2012	3/21/2012
Amendment #1	6/22-25/2012	6/25/2012
Amendment #2	7/27/2012	7/27/2012
Dose Formulation	8/23/2012	8/24/2012
Dose Certification	8/24/2012	8/24/2012
Necropsy	8/31/2012	8/31/2012
Clinical Pathology Data	12/04/2012	12/04/2012
Technical Report	5/30/2015 - 6/30/2015	6/30/2015

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National Center for Toxicological Research
Quality Assurance Unit

9/18/2015
Date