Kwok and Silva, Cell Dev Biol 2013, 2:3
http://dx.doi.org/10.4172/2168-9296.1000123

Cell & Developmental Biology

Research Article Open Access

Re-evaluation of Developmental and Reproductive Toxicity of *Ortho*-Phenylphenol (OPP) and Sodium *Ortho*-Phenylphenate (SOPP)

Eric S.C. Kwok* and Marilyn Silva

Medical Toxicology Branch, Department of Pesticide Regulation (DPR), California Environmental Protection Agency (CalEPA), Sacramento, California, USA

Abstract

Background: Due to widespread usage, the general public including pregnant women is routinely exposed to the fungicides, *Ortho*-Phenylphenol (OPP) and Sodium *Ortho*-Phenylphenate (SOPP), from many sources. Previous data reviews concluded no effects on development or reproduction in animal studies but upon re-analysis we realize that alternative interpretation may exist.

Methods: Developmental and reproductive effects were assessed from studies performed in rats, mice, and rabbits. We identified the most sensitive endpoint(s) for OPP or SOPP as they related to fetal developmental or reproductive toxicity. For quantifying the potential health risk associated with the exposure to OPP or SOPP, we used the lowest dose that caused no developmental or reproductive toxicity or benchmark dose analysis.

Results: Developmental effects in OPP-treated rats and mice were decreased fetal body weight, increased incidences in delayed skeletal ossification and post-implantation loss. In addition, fetal mice exposed to SOPP exhibited malformation. Similar to rats and mice, post-implantation loss was the developmental effect noted in OPP-treated rabbits. Except for the rats, maternal toxicity appeared to be minimal (mice) or not observed (rabbits) at the lowest dose where developmental effects occurred (mice: cleft plate; rabbits: resorptions). We did not find evidence of OPP affecting reproductive functions but significant deviations from FIFRA Guidelines in these studies may prevent adequate assessment of the reproductive toxicity especially the effects on fertility and mating.

Conclusions: The revised data analysis suggests that OPP and SOPP induce fetal toxicity in the absence of maternal effects. Our re-evaluation would be useful in the formulation of a current or updated regulatory strategy for the developmental toxicity of OPP and SOPP.

Keywords: OPP; SOPP; Data analysis; FIFRA deviations; Post-implantation loss; Resorption

Introduction

Ortho-Phenylphenol (OPP) and its sodium salt, Sodium Ortho-Phenylphenate (SOPP) fungicides are broadly applied in California for postharvest treatment of citrus fruits and as disinfectants and preservatives [1]. Formulations containing these active ingredients (a.i) are used in agricultural, residential, and public access areas; as disinfectants in food handling, commercial/institutional/ industrial and medical settings, as well as a material preservative for a variety of products, including wood [2]. SOPP is also formulated as an inert ingredient for approximately 123 registered products in the United States [2]. Due to widespread usage, the general publics including pregnant women are exposed to OPP and SOPP from many sources.

The Department of Pesticide Regulation (DPR) within the California Environmental Protection Agency (CalEPA) is charged with assessing potential associations between pesticide use and human health risk including the exposure *in utero*. Registrant-submitted studies performed in conformance with the Federal Fungicide, Insecticide and Rodenticide (FIFRA) Guidelines are required for registering pesticides in California according to the Birth Defects Prevention Act (1984) and the Food Safety Act (1989). To this end, the risk assessment for OPP and SOPP involved a review of all available developmental and reproductive toxicity reports submitted by the registrant and in the open literature.

Since all developmental and reproductive toxicity studies for OPP and SOPP were performed via diet or gavage treatment, the oral route is the focus for this article. After passing through the sites of absorption (e.g., gastrointestinal tract), SOPP (pKa=9.55) dissociates in water and regenerates the parent OPP and hydroxyl ion (OH^-). OPP is further metabolized into biologically active compounds including phenylhydroquinone (PHQ) and phenylbenzoquinone (PBQ) [3]. Toxicology database of OPP and SOPP is extensive, but the main focus is their carcinogenicity and the associated mode-of-action (MOA). To illustrate, in the past two decades, over 20 studies in the open literature investigated their carcinogenesis in the urinary bladder (rats) and liver (mice) [1,2] and more than 70 studies aimed to characterize the carcinogenic MOA [4]. By contrast, there are seven reports on their developmental or reproductive effects.

The United State Environmental Protection Agency (USEPA) and others have evaluated these developmental and reproductive studies of OPP and SOPP. All concluded that "there is no increased concern for developmental toxicity of ortho-phenylphenol when comparing effects in adult animals with those in offspring" [1,2]. However in the course of reviewing the studies for risk assessment, we realized that alterative interpretations of the data may be possible. Upon the re-evaluation, it appeared that fetal toxicity may occur at doses with no clear indication of toxicity in the OPP- or SOPP-treated dams. This paper presents the re-evaluation of the database, along with the methods of analysis which led us to offer alternative conclusions and developmental endpoints to those presented in other risk assessments [1,2].

***Corresponding author:** Eric SC Kwok, Medical Toxicology Branch, Department of Pesticide Regulation (DPR), California Environmental Protection Agency (CalEPA), Sacramento, California, USA, Tel: 916-324-7842; Fax: 916-324-3508; E-mail: ekwok@cdpr.ca.gov

Received August 27, 2013; **Accepted** October 01, 2013; **Published** October 03, 2013

Citation: Kwok ESC, Silva M (2013) Re-evaluation of Developmental and Reproductive Toxicity of *Ortho*-Phenylphenol (OPP) and Sodium *Ortho*-Phenylphenate (SOPP). Cell Dev Biol 2: 123. doi:10.4172/2168-9296.1000123

Methods

The DPR database contained five registrant-submitted developmental toxicity studies performed with OPP: two in rabbits [5,6]; one in mice (OPP and SOPP) [7]; two in rats [8,9] and two rat reproduction studies with OPP [10,11]. These studies are available as open literature or unpublished report, and the letter can be obtained for review via the procedures as described on the DPR website: http://www.cdpr.ca.gov/public_r.htm. In general, for the purpose of toxicity-endpoint identification for risk assessment, we evaluated studies based on adherence to the FIFRA Guidelines [12-14]. Those designated as "acceptable" were considered fulfill the intent of the Guideline requirement and deemed suitable for the toxicity-endpoint identification. Others were identified as "unacceptable" (e.g., missing information as required by the Guidelines) or "supplemental" (e.g., a research publication from academia lacking individual animal data). From the latter two categories, we determined whether their deviations from FIFRA Guidelines would affect data interpretation and hence, usefulness for endpoint selection in risk assessment. After analyses we identified the most sensitive endpoint(s) for characterizing the effects of OPP or SOPP as they related to fetal developmental or reproductive toxicity.

For quantifying the potential health risk associated with the exposure to OPP or SOPP, we determined the lowest dose employed that caused no developmental or reproductive toxicity (i.e., No-Observed-Effect-Level: NOEL). Studies with the toxicity exhibited at the lowest dose tested (i.e., lowest-Observed-Effect-level: LOEL), we performed either LOEL-to-NOEL extrapolation using a 10x Uncertainty Factor (UF) or a benchmark dose analysis (BMD) [15] to establish a point of departure (POD).

Results

Developmental toxicity studies

Rat: Kaneda et al. [9]: In this open literature publication, pregnant Wistar rats (18-20 dams/dose; 11 dams at the highest dose tested [HDT]) were treated with OPP by gavage at 0 (aqueous gum arabic), 150, 300, 600, or 1200 mg/kg/day on gestation days (GD) 6 through 15. The animals were sacrificed on GD 20. Data at 1200 mg/kg/day were not included for evaluating the maternal and fetal effects because 10/11 dams died after 3-9 days of treatment. The high mortality noted may have been due to the toxic effects of OPP since the median acute Lethal Dose (LD_{50}) of OPP in rats has been reported as ~2500 mg/kg [16].

At 600 mg/kg/day, 2 of the 20 dams died. At ≥ 300 mg/kg/day, there was a dose-related increase in ataxia (no data presented). Body-weight gains were reported for GD 6, 9, 12, 15, and 20. Although the intervals were not specified, we have assumed that the values were relative to maternal body weights as measured from GD 0. At ≥ 300 mg/kg/day, dams had decreased body-weight gain from GD 9 (Table 1). Effects to fetuses from OPP exposure *in utero* at 600 mg/kg/day group appeared as an increased ($p<0.01$) incidence of resorptions and reduced fetal body weights (both sexes) (Table 1). Nevertheless, the fetus (not the litter) was the experimental unit for the statistical analysis of resorptions and therefore, the increased resorption in OPP-treated dams may be equivocal. Also included in this article was a dominant-lethal study to assess the effects of OPP on sperm in C_3H mice. OPP was administered by gavage to male mice (15/dose) at 0 (aqueous gum arabic), 100 or 500 mg/kg/day for 5 days. Ethyl Methyl Sulfonate (EMS) served as the positive control. Mating was initiated immediately after the final treatment and continued for 6 weeks. Males showed slight decreases in body weight at 500 mg/kg/day, in addition to a "temporary depression"

Parameters	OPP, mg/kg/day[a]			
	10	150	300	600
Maternal Effects				
Number Pregnant	20	20	20	18
Mean Maternal Body-Weight Gain (g)[b]				
GD 6	21	23 (110%)	22 (105%)	19 (90%)
GD 9	30	31 (103%)	25 (83%)*	12 (40%)***
GD 12	45	42 (93%)	37 (82%)**	22 (49%)***
GD 15	61	56 (92%)	44 (72%)***	23 (38%)***
GD 20	121	111 (92%)	97 (80%)***	65 (54%)***
Fetal Effects				
Number Examined				
Litters	20	20	20	18
Fetuses	230	230	237	188
Resorptions[c]	13.9%	13.9%	15.4%	25.7%**
Mean Fetal Body Weight (gram)[d]				
Males	4.11	4.12 (100%)	4.04 (98%)	3.87 (94%)**
Females	3.87	3.78 (98%)	3.71 (96%)	3.55 (92%)**

[a] Data for the 1200 mg/kg/day group do not appear in the table because the reported fetal and maternal values were derived from the one animal that survived till the schedule time.
[b] The value as a percent of the controls is given in parentheses. The intervals covered by the gain data were not specifically stated by the authors. However, the values for the body weight gains for the negative controls suggest that the intervals begin with GD 0; i.e., it is expected that pregnant Wistar would gain a total of 121 grams between GD 0 and GD 20, with 50 of the gain occurring between GD 15 and GD 20.
[c] This value is the number of resorbed fetuses divided by the total number of implants and is expressed as a percentage, as reported by the investigators.
[d] Fetal body weight measured on GD 20. The value as a percent of the controls is given in parentheses.
*, **, *** Significantly different from the controls at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, as reported by the investigators.

Table 1: Maternal and fetal effects observed in a developmental-toxicity study of OPP using Wistar rats [9].

(term not defined). Females were killed on GD 12-13 and inspected for numbers of corpora lutea, implants, living embryos, and early or late embryonic deaths. There were no dominant lethal or fertility effects; but the study authors did not describe their methods for measuring "fertility." The positive control, EMS, functioned as expected.

Kaneda et al. [9] demonstrated that OPP had fetal effects but only at maternally toxic doses (maternal NOEL=150 mg/kg/day based on decreased body weight gain and the appearance of ataxia at ≥ 300 mg/kg/day). The developmental NOEL was placed at 300 mg/kg/day based on reduced fetal body weights and, possibly, an increased incidence of resorptions at 600 mg/kg/day. These studies were not designed in conformance with FIFRA Guidelines and were considered as supplemental data (summary Table 8).

John et al. [8,17]: This study was performed according to the 1984 FIFRA Guidelines [8] and was evaluated along with the published article of the same study [17]. In this FIFRA study, pregnant Sprague-Dawley (SD) rats (24-26 dams/dose; controls=36 animals) were treated with OPP by gavage at 0 (cottonseed oil), 100, 300, or 700 mg/kg/day OPP on GD 6 through 15 (sacrificed GD 21). The dose levels were based on a range-finding study where, sperm-positive dams (5-6 dams/group) were gavaged at 0, 250, 400, 800, 1200 or 2000 mg/kg during gestation (dosing days not specified) and sacrificed on GD 16. Deaths occurred only at the HDT. Dams exposed to 800 or 1200 mg/kg/day exhibited gastric irritation, decreased maternal body weight and food consumption, and increased water consumption. On this basis, the investigators selected 700 mg/kg/day as the high dose for the main study.

In the main study, results were not recorded for two control dams and four dams at 700 mg/kg/day because they were given the wrong dose, were not pregnant, or delivered early. One dam died at 700 mg/kg/day due to dosing error but there were no treatment-related deaths. Maternal toxicity occurred primarily at the HDT (Table 2). Compared to the controls, the high-dose dams exhibited reductions ($p<0.05$) in body-weight gain on GD 6-9 (Table 2) and in food consumption on GD 9 11 (by 9%) and increased ($p<0.05$) water intake on GD 12-14 and 15-17 (by 26% and 16%, respectively). Increased ($p<0.05$) water intake also occurred on GD 12-14 in the 300 mg/kg/day group (by 17%). Absolute maternal liver weight was reduced by 8% ($p<0.05$) at 700 mg/kg/day; but when viewed relative to body weight, the reduction was not statistically significant.

There were no effects on fetal developmental parameters such as body weights or crown-rump lengths. No external or visceral effects were observed, however only 1/3 of the fetuses in each treatment group were examined, as opposed to ≥ 50% recommended in the current FIFRA guidelines. Skeletal examinations were performed on all fetuses and three skeletal anomalies were statistically significantly increased (~13-15%) at 700 mg/kg/day (delayed ossification of sternebrae, pinpoint holes in the occipital or interparietal plates in the skull, and skull bone island). Delayed ossification in the sternebrae was observed in 3% of fetuses and 30% of litters at 700 mg/kg/day and was outside the historical controls (5% fetuses and 28% litters). Pinpoint holes in the occipital or interparietal plates in the skull increased at ≥ 300 mg/kg/day and bone-island was increased at all doses. Historical controls for these effects in the skull was 0/2,320 litters [18].

Uteri from animals that did not appear to be pregnant were stained with 10% solution of sodium sulfide [19]. This procedure was performed only to test for implantation sites, and a different procedure (not explained) was used to determine fetal resorptions. John et al. [8,17] calculated pre-implantation loss by a proportion of the numbers of corpora lutea not associated with implantation (Table 2). Their report did not subsequently address this effect, although our analysis of their data (Table 2) indicated a statistically significant ($p<0.05$) increase in pre-implantation loss at 700 mg/kg/day. The analysis was

Parameters	OPP, mg/kg/day			
	0	100	300	700
Females mated	35	27	27	25
Deaths	0	0	0	1
Number pregnant	34	25	26	23
Body weight gain				
GD 6-9	14 ± 9	15 ± 11	13 ± 8	5 ± 12*
GD 10-15	44 ± 14	44 ± 11	39 ± 12	40 ± 13
Mean fetal body weight (g)	5.64 ± 0.29	5.70 ± 0.36	5.74 ± 0.38	5.59 ± 0.23
Total number of litters with live pups	34[a]	25	26	20
Pre-implantation Loss[b]				
Litter incidence	16/34 (47%)[c]	15/25 (60%)	17/26 (65%)	15/20 (75%)
Percent pre-implantation loss[d]	11.3 ± 21.7	13.4 ± 20.3	17.4 ± 22.8	13.4 ± 11.0#
Implantation Data				
Total number of implants	444	322	305	259
Total number of live fetuses	416	305	277	252
Litter incidence (post-implantation loss)	14/34 (41%)[c]	8/25 (32%)	8/26 (31%)	7/20 (35%)
Percent post-implantation loss[d,e,f]	7.2 ± 15.7	5.7 ± 10.5	9.6 ± 23.0	2.6 ± 3.6

[a] Number of litter examined was 34 instead of 36 due to two dams died from erroneous dosing.
[b] Pre-implantation loss, reported as "pregnancy wastage," in a litter was the proportion of corpora lutea that was not associated with an implantation in a litter, as reported in John et al. [8].
[c] The value as a percent of the controls is given in parentheses.
[d] Percent pre- or post-implantation loss is the sum of pre- or post-implantation loss (%) per litter divided by total number of litters.
[e] Resorptions only; the investigators reported no data on dead fetuses.
[f] Resorptions which were detected only by sodium sulfide staining were not included for counting total number of resorptions by the investigators and therefore, also excluded in our calculation of percent post-implantation loss.
* Significantly different from the controls at $p<0.05$, as reported by the investigators.
# Non-parametric multiple comparison test with the percent pre-implantation loss per litter as an experimental unit [20,21,29], significant at $p<0.05$.

Table 2: Maternal and fetal effects observed in a developmental toxicity study of OPP using Sprague-Dawley rats [8].

performed using the percent pre-implantation loss per litter as an experimental unit and nonparametric (i.e., distribution free) tests for multiple comparison [20,21]. The occurrence of pre-implantation loss is an unexpected finding because treatments started after implantation had occurred. Because resorptions detected only by sodium sulfide staining were not counted toward total resorptions, it is possible that some of the instances of pre-implantation loss at 700 mg/kg/day might be instances of early resorption (i.e., post-implantation loss). Unfortunately, historical control data from the conducting laboratory are unavailable for further evaluating the biological significance of this finding.

The study authors considered the NOEL to be 300 mg/kg/day in dams based on systemic effects at the high dose, and the fetal NOEL was 700 mg/kg/day. However, if resorptions were underestimated and contributed to the increased pre-implantation loss at 700 mg/kg/day, the developmental NOEL would have been 300 mg/kg/day. The USEPA established a developmental NOEL of 700 mg/kg/day; however, the maternal NOEL was 100 mg/kg/day based on the decreased body weight gains, and food consumption at 300 mg/kg/day [2] (summary in Table 8).

Mouse: Ogata et al. [7]: Developmental effects in mice were reported in a registrant-submitted study translated from Japanese to English. The report consisted of two studies; one with OPP and the second with SOPP. In the first study, four groups of Jcl:ICR mice bearing vaginal plugs (21 animals/dose) were treated by gavage at 0 (olive oil), 1450, 1740, and 2100 mg/kg/day OPP on GD 7 through 15 and sacrificed on GD 18. Dose selection was based on LD_{50} data for OPP in rat (but not mice). Maternal body weight gain was presented as a graph (no summarized or individual data presented) but it was evident that at the mid- and high dose there was a decrease from the first day of treatment (no statistical analysis provided). A dose-related increase in maternal deaths was observed at all levels with 16/20 dying at the HDT (Table 3). Two females that died on study at the HDT (2100 mg/kg/day) had bleeding from the vaginal orifice prior to death. Although maternal deaths occurred at each dose level, inhibition of maternal body-weight gain occurred only at 1740 and 2100 mg/kg/day. Therefore, the evidence for maternal toxicity at 1450 mg/kg/day (low dose) was 4/21 maternal deaths.

Statistical analyses by the investigators indicated that OPP reduced ($p<0.01$) fetal body weight and increased ($p<0.01$) skeletal

Outcomes	OPP, mg/kg/day			
	0	1450	1740	2100
Maternal				
Mated females at start of dosing	21	21	21	21
Unscheduled deaths	0	4	7	16
Impregnated based on laparohysterectomy	20[a]	14[a]	14	5
Mean corpora lutea/Dam	13.5 ± 3.1	14.5 ± 3.0	13.1 ± 2.1	13.2 ± 1.8
Litters with resorptions only	0	0	0	0
Litters with live fetuses	20	14	14	5
Fetal				
Mean implantation scars/Dam[b]	12.4 ± 2.9	12.6 ± 2.2	11.0 ± 1.2	12.8 ± 1.9
Mean litter size (Live fetuses)[b]	10.9 ± 3.2	11.8 ± 2.5	10.6 ± 1.6	11.2 ± 1.1
Late resorptions/litter	2.3 ± 4.2	1.6 ± 4.1	2.2 ± 4.4	5.0 ± 11.2
Mean fetal body weight (g)[c]				
Male	1.4	1.3 (96%)**	1.3 (95%)***	1.1 (80%)***
Female	1.3	1.2 (92%)***	1.2 (96%)***	1.0 (80%)***
Skeletal variations				
Frequency of fetuses with cervical ribs[d]	0	6.6 ± 10.1**	8.9 ± 12.2**	17.0 ± 28.2***
Ossified phalanges forelegs (left/right)	2.43 ± 0.51 2.47 ± 0.51	2.14 ± 0.53 2.18 ± 0.53	2.06 ± 0.44* 2.10 ± 0.43*	1.50 ± 0.87** 1.52 ± 0.38**
Ossified phalanges hindlegs (left/right)	2.85 ± 0.42 2.95 ± 0.44	2.37 ± 0.43** 2.50 ± 0.39***	2.63 ± 0.41 2.69 ± 0.44	1.89 ± 1.09* 1.96 ± 1.14***
Mean ossified posterior lumbar vertebrae	12.94 ± 1.24	12.07 ± 1.89	12.10 ± 1.24	10.22 ± 2.15**
External malformations[e]				
Cleft palate	1 [1]	1 [1]	4 [4]	1[1]
	(5%)	(7%)	(29%)	(20%)
Open eyelids	1 [1]	4 [7]	6 [6]	1 [1]
	(5%)	(29%)	(43%)	(20%)
Exencephalia	0	3 [6]	0	0
		(21%)		
Frequency of fetuses with externally visible malformations (All types combined)[d]	0.67 ± 2.05	6.21 ± 8.03*	6.14 ± 5.96*	3.64 ± 4.98

[a] The investigators explained that the difference between the number of mated females surviving to laparohysterectomy and the number found pregnant at laparohysterectomy represented those mated females that did not become pregnant (no implantation sites).
[b] Means ± one standard deviation, as reported by the investigators.
[c] The value as a percent of the value for the negative controls is given in parentheses; body weight measured on GD 18.
[d] Mean proportion of fetuses affected per litter ± one standard deviation for groups of 5-20 litters, as reported by the investigators. The investigators stated that a fetus with more than one malformation was counted only once.
[e] Number of affected litters, with number of affected fetuses in brackets and the percent of litters affected in parentheses, as reported by the investigators.
*,**,*** Significantly different from the negative-controls at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, as reported by the investigators.

Table 3: Maternal and fetal effects observed in a developmental-toxicity study of OPP using Jcl:ICR mice [36].

developmental delays (cervical ribs) in each of the OPP treated groups, with both changes showing dose dependency (Table 3). The average number of ossified phalanges in hind legs (>1740 mg/kg/day), in the foreleg (2100 mg/kg/day), and in ossified posterior lumbar vertebrae (2100 mg/kg/day) were decreased statistically significantly, indicating additional developmental delays.

Increased ($p<0.05$) overall incidence of severe external malformations (cleft palate, open eye, and exencephalia) occurred at the low and mid doses (Table 3). At the high dose, despite having only five litters for examination at laparohysterectomy, the overall incidence of malformations was increased; and when maternal uterine contents were examined, there was a 2.2-fold increased incidence in late fetal resorptions. No maternal and developmental NOELs could be determined from this study because both maternal and fetal effects occurred at the lowest dose tested (i.e., LOEL).

In the study with SOPP, four groups of Jcl:ICR mice bearing vaginal plugs (20 animals/dose) were dosed by gavage at 0 (water), 100, 200, or 400 mg/kg/day SOPP on GD 7 through 15 and sacrificed on GD 18. Maternal deaths occurred during GD 11-18 at 200 and 400 mg/kg/day (4 and 16 deaths, respectively). The investigators indicated that each of the SOPP-treated groups had inhibition of the maternal body weight gain; the onset times were GD 12-13, GD 11, and GD 8 for the 100, 200, and 400 mg/kg/day groups, respectively. Vaginal bleeding was the only clinical sign noted, and it occurred in all animals that died. The investigators attributed the vaginal bleeding to "abortions." There was no discussion on the detection times for the blood or the condition of the uterine contents.

Fetuses had decreased body weights ($p<0.001$) at all doses, although the magnitude of the reductions did not increase with dose (Table 4). Decreases ($p<0.05$) in the number of implantation sites per litter and live fetuses occurred at 200 mg/kg/day. Comparable decreases (not statistically significant) also occurred at 400 mg/kg/day, albeit only

Outcomes	SOPP, mg/kg/day			
	0	100	200	400
Maternal				
Mated females at start of dosing	20	20	20	20
Unscheduled deaths	0	0	4	16
Impregnated based on laparohysterectomy	17[a]	19[a]	15[a]	4
Mean corpora lutea/Dam	13.5 ± 6.4	13.1 ± 2.2	14.5 ± 2.0	14.5 ± 2.1
Litters with resorptions only	0	0	2	0
Early resorptions	3.7 ± 6.8	5.4 ± 13.0	4.9 ± 9.7	10.0 ± 15.9
Litters with live fetuses	17	19	13	4
Fetal				
Mean implantation scars/Dam[b]	13.2 ± 1.6	12.9 ± 2.1	11.0 ± 3.9*	11.3 ± 5.9
Mean litter size (Live fetuses)[b]	12.6 ± 1.8	11.9 ± 2.9	10.0 ± 4.0*	10.5 ± 6.0
Mean fetal body weight (g)[c]				
Male	1.4	1.2 (85%)***	1.3 (92%)***	1.2 (85%)***
Female	1.3	1.1 (88%)***	1.2 (92%)**	1.1 (85%)***
Skeletal variations				
Frequency of fetuses with cervical ribs[d]	1.2 ± 2.7	2.9 ± 9.0	4.0 ± 6.5	4.2 ± 4.9
Ossified phalanges forelegs (left/right)	3.20 ± 0.38 3.23 ± 0.40	2.35 ± 0.91** 2.42 ± 0.90**	2.36 ± 0.95** 2.29 ± 0.56**	1.32 ± 1.10** 1.59 ± 1.09**
Ossified phalanges hindlegs (left/right)	2.80 ± 0.50 2.82 ± 0.53	1.88 ± 0.69*** 1.94 ± 0.71**	2.07 ± 1.02* 1.97 ± 1.08*	2.05 ± 2.14 2.15 ± 2.05
Mean ossified posterior lumbar vertebrae	13.22 ± 1.3	10.79 ± 2.19	11.37 ± 2.47	10.11 ± 1.62**
External malformations[e]				
Cleft palate	1 [1]	6 [28][f]	1 [1]	1 [3]
	(6%)	(32%)[#]	(8%)	(25%)
Open eyelids	5 [6]	1 [3]	1 [1]	0
	(29%)	(5%)	(8%)	
Exencephalia	0	1 [1]	0	0
		(5%)		
Frequency of fetuses with externally visible malformations (All types combined)[d]	3.3 ± 5.9	12.5 ± 23.6	3.2 ± 8.0	5.8 ± 11.5

[a] The investigators explained that the difference between the number of mated females surviving to laparohysterectomy and the number found pregnant at laparohysterectomy represented those mated females that did not become pregnant (no implantation sites).
[b] Means ± one standard deviation, as reported by the investigators.
[c] The value as a percent of the value for the negative controls is given in parentheses; body weight measured on GD 18.
[d] Mean proportion of fetuses affected per litter ± one standard deviation for groups of 4-19 litters, as reported by the investigators. The investigators stated that a fetus with more than one malformation was counted only once.
[e] Number of affected litters, with number of affected fetuses in brackets and the percent of litters affected in parentheses, as reported by the investigators.
[f] In one of the 6 affected litters, 15 of the16 fetuses exhibited cleft palate.
*,** Statistically significant at $p<0.05$ and $p<0.001$, respectively, as reported by the investigators.
[#] Fisher exact test, $p=0.06$ (our calculation, based on 1/17 vs. 6/19; see text for further discussion of significance).

Table 4: Maternal and fetal effects observed in a developmental-toxicity study of SOPP using Jcl:ICR mice [36].

four litters were available for examination at laparohysterectomy. The numbers of corpora lutea per dam were comparable among the four groups; however the decreases in the numbers of implantation sites per dam at 200 and 400 mg/kg/day were consistent with pre-implantation loss. As with John et al. [8,17], treatments commenced on GD 7, which was after the interval that implantations occur in the mouse (GD 4.5- 5) [22]. The apparent pre-implantation loss might reflect early post-implantation loss that went unrecognized in the study (staining methods not described). While increased incidences of cervical ribs occurred dose-dependently in the SOPP treated groups, they were not statistically significant (Table 4). External malformations at 100 mg/kg/day showed a large increase in the overall incidence (12.5 ± 23.6%) at 100 mg/kg/day. Cleft palate was high (6 litters with 28 cleft palate total), and one litter had 15 of the 28 total cleft palate. There were no individual data provided for fetal parameters in the SOPP (and also the OPP) study.

The study investigators concluded that SOPP (and OPP) was not teratogenic since there was no dose response at the higher doses in either study, the compounds induced no unique malformation (i.e., cleft palate occurred in the concurrent controls), and most affected fetuses treated with SOPP at 100 mg/kg/day originated from a single dam. However, we considered these objections as being not sufficient for dismissing the possible teratogenic effect based on the following considerations.

Although Ogata et al. [7] effectively exposed the animals to OPP in both studies; these studies need to be considered separately given some inconsistencies in their findings when viewed collectively. For example, SOPP at 400 mg/kg/day (equivalent to 258 mg/kg/day of OPP) caused 80% of the dams to die (Table 4). Based on this, one would have expected >80% mortality in the OPP testing at 1450 mg/kg/day but the mortality rate was only 19% (4/21) (Table 3). A similar inconsistency is seen in comparing the fetal body-weight data from the two studies. At 400 mg/kg/day of SOPP, the mean fetal body weights for both sexes were reduced by 15% relative to the controls (Table 4) while OPP at 1450 mg/kg/day showed a reduction of only 4-8% (Table 3). One possibility is that the use of olive oil with OPP may have altered the uptake (or metabolism) of OPP in relation to SOPP, which was delivered as aqueous solution. There is no reason to expect that if OPP and (or) SOPP truly were developmental toxicants, they necessarily would induce a type of malformation that does not occur "spontaneously" in fetuses from control animals.

Severe fetal malformations including cleft palate were observed from SOPP treatment. Although the elevated incidence of cleft palate did not occur at 200 and 400 mg/kg/day, both groups showed evidence of embryo-fetal death and embryo-fetal death is known to reduce the number of fetuses at risk for malformation [23]. Another study by Ogata et al. [24] showed a low spontaneous cleft palate incidence in Jcl:ICR mice (4/412 fetuses from 34 controls; max=12% or 4/34 if one per litter). In contrast, SOPP at 100 mg/kg/day had 28 fetuses with cleft palate, involving 6/19 litters (32% litter incidence). It should be noted that the olive oil control group in the OPP testing had a single fetus with cleft palate (5% litter incidence) as did SOPP control group (6% incidence) (Table 3 and 4).

Based on reduced fetal body weight (both sexes) and an increased incidence of cleft palate, the developmental LOEL was set at 100 mg/kg/day. Using a 10x UF for LOEL-to-NOEL extrapolation, an estimated NOEL was 10 mg/kg/day. Reduced body weight gain could be the basis for the maternal LOEL; however, there are insufficient data in the report for the reduced maternal body weight gain to be distinguished from the 15% fetal body weight reduction that also occurred at this dose (Table 4). Otherwise, there were no deaths or clinical signs in the dams at 100 mg/kg/day. In conclusion, this study documented that SOPP affected the fetuses and that the increased toxicity to the fetuses occurred at the same or lower doses as those causing maternal toxicity.

Rabbit: Zablotny et al. [5]: Prior to the definitive developmental study, a range-finding study was performed. New Zealand white rabbits (NZW; 7 inseminated/dose) were gavaged with 0 (corn oil), 250, 500, or 750 mg/kg/day on GD 7-19 and sacrificed GD 20. Deaths at 250, 500 and 750 mg/kg/day were one, two (2 dosing errors), and six (1 dosing error), respectively. One at 750 mg/kg/day survived to scheduled sacrifice but exhibited clinical signs of "blood in the pan" (presumptive abortion) on GD 17-18; the uterus contained two resorptions. At 500 mg/kg/day, one surviving rabbit aborted two fetuses on GD 20 before sacrifice. Four of 7 dams at 500 mg/kg/day survived until scheduled sacrifice. At 250 mg/kg/day, 1/7 dams passed blood-stained feces on GD 19 and died on GD 20. The report did not describe the uterine contents, except to indicate that the animal was pregnant.

Reduced maternal body weight and body-weight gain occurred at ≥ 500 mg/kg/day. Renal tubular degeneration in dams occurred at each dose level. The incidence was 33% (2/6) at 250 mg/kg/day; all were slight-grade. At 500 mg/kg/day, the incidence was 80% (4/5); the lesions were slight grade, except for a single case that was moderate grade. At 750 mg/kg/day, the one animal to survive to scheduled sacrifice (GD 20) exhibited moderate-grade renal tubular degeneration.

There were increased incidences of litters having resorptions: 43% (3/7), 83% (5/6) and 60% (3/5) at 0, 250, and 500 mg/kg/day, respectively. The report did not provide data for fetal examinations. Based on these results, the investigators selected 250 mg/kg/day as the high dose for the full study.

Zablotny et al. [6]: The definitive study had two phases. In the first phase, artificially inseminated New Zealand White rabbits (16/dose) were gavaged at 0 (corn oil), 25, 100, or 250 mg/kg OPP on GD 7 through 19 and sacrificed on GD 28. After the first phase, only 10 litters with live fetuses remained at 250 mg/kg/day (see Table 5). However, FIFRA Guidelines recommend 20 rabbit litters with implants per dose group. To compensate, the investigators conducted a second phase. Two and eight inseminated females received OPP at 0 and 250 mg/kg/day, respectively. The insemination of second-phase animals occurred five days after the last laparohysterectomy in the first phase; consequently, the second phase was completed one month after the first phase but the report summarized both together. Hence, unless stated otherwise, the following discussion was based on the combined data.

As in the probe study [5], OPP had no effect on maternal body weight or body-weight gain in animals dosed up to 250 mg/kg/day; there was no effect on maternal absolute and relative liver or kidney weights. The evidence of maternal toxicity at 250 mg/kg/day included renal tubular degeneration and inflammation. Histological examination showed no renal lesions occurred at 0, 25, or 100 mg/kg/day but at 250 mg/kg/day there was renal tubular degeneration (33% [8/24 litters] incidence); five were slight-grade lesions and three were moderate-grade lesions (identified by footnotes b, g, and j in Table 5).

Cageside observations reported the occurrence of blood. Although hematuria and perigenital blood staining accompanied the OPP induced urinary-tract toxicity in rats [25], the blood findings may have been associated with toxicity other than urinary-tract effects (Table 5). That is, one of the sources may relate to the finding in three litters that consisted only of early resorptions (identified by footnotes k, l, and m

Event/Outcome	OPP, mg/kg/day					
	0		25	100	250	
	1st Phase	2nd Phase	1st Phase	1st Phase	1st Phase	2nd Phase
Inseminated females on GD7	16	2	16	16	16	8
Not pregnant,[a] discovered at:						
laparohysterectomy (GD28)	1	0	0	2 (one animal, BIP: 19)	1 (BIP: 23)	0
moribund sacrifice	0	0	0	0	1 (16)[b]	0
Pregnant when found dead	1 (16)[c]	0	1 (23)[d]	1 (14)[e]	2 (15 [BIF: 14])[f], (16 [RU,BIP: 13-15])[g]	1 (15 [BIP:11; BIF: 12-14])[h]
Aborted before GD28	1 (24)	0	1 (23)[i]	0	1 (21)[j]	0
Litters at laparohysterectomy	13	2	14	13	11	7
Litters with resorptions only	1 (RU: 18-19)[k]	0	1 (BIP: 24)[l]	0	1 (BIP: 20)[m]	0
Litters with live fetuses	12	2	13	13	10	7

Note: The gestation day when an animal was found dead, was sacrificed, or aborted fetuses is noted in parentheses. For animals with these fates as well as those whose litters contained only resorptions, the types and times of cageside observations involving blood also are noted (blood in pan, BIP; blood in association with feces, BIF; and reddish urine, RU). Other blood observations made during the study are discussed in the text. Footnotes are for abnormal necropsy findings and related comments:
[a] Non-pregnancy confirmed by sodium sulfide staining. Not necropsied, unless noted.
[b] No movement of hind legs (GD16) suggests a broken back. Stomach: hairball, hemolyzed blood. Colon: dark mucosa. Kidneys (histology): tubular degeneration, moderate grade.
[c] Umbilical hernia with volulus.
[d] Stomach: large hairball, mucosal lesions. Left kidney: hypertrophy, dilated pelvis, pale areas in cortex.
[e] Gavage error (lungs).
[f] Colon: hemolyzed blood, mucosal hemorrhage.
[g] Stomach: hairball, hemorrhage. Kidney (histology): tubular degeneration, moderate grade.
[h] Perineal blood staining. Stomach: small hairball. Intestines: blood.
[i] Perineal blood staining. Stomach: lumen occluded by hairball, mucosal lesions.
[j] Kidneys: pale cortices. Kidneys (histology): tubular degeneration, moderate grade. Stomach: mucosal lesions.
[k] 8 early resorptions.
[l] This animal delivered one resorbed fetus on GD28 prior to laparohysterectomy. Uterus was classified as having one implantation site, an early resorption. However, an expelled fetus would not leave an early resorption. Therefore, there is uncertainty over the number or types of implantations for this animal.
[m] 1 early resorption.

Table 5: Maternal outcomes in the first and second phases of a developmental-toxicity study of OPP using New Zealand White rabbits [6].

in Table 5). Further review of the cageside observation data indicates that other animals also had blood in the collecting pan (two 100 mg/kg/day animals) or blood in association with the feces, along with perineal blood staining (one 250 mg/kg/day animal). In each of these three cases, the observation of blood occurred on GD 25 and upon sacrifice on GD 28, the animals exhibited one or two late resorptions. This suggests that the resorptions were related to the blood detected in the pan, the feces, or urine during cageside observations. By corollary, the observation of blood in the pan with two of the three animals that were supposedly not pregnant in Table 5, one 100 mg/kg/day animal and one 250 mg/kg/day animal (1st phase), raises the possibility that these animals were pregnant but had suffered resorptions.

OPP exerted no significant effect on fetal body weight or litter size nor did it induce external, soft tissue, or skeletal anomalies or malformations (data not shown). The only developmental effect of OPP in rabbits was increased incidence of litters with resorptions. However, in both the original report [6,26] and the subsequent re-evaluation [27], the investigators dismissed the possible effect of resorptions. First, these investigators found no statistically significant increase in resorptions [6]; the statistical method employed was censored Wilcoxon test for pairwise comparison [28] with a Bonferroni correction for controlling Type I error and the number of affected fetuses per litter as an experimental unit. Second, in the re-evaluation [27], it was argued that the number of resorptions per litter and percent implantations resorbed at the high dose were slightly higher than the concurrent control but within or marginally above the historical controls (Table 6). Adding support to these arguments was the lower "number of resorptions per litter with resorptions" at 250 mg/kg/day compared to the control and the non-statistically significant increase in the "percent post-implantation loss" at the mid and high doses (method not explained) (Table 6). Third, a "weight-of-evidence" (WOE) analysis [27] indicated that the probe study which preceded the main study showed no increase in resorptions at higher doses [5]. The studies with rats showed no effect on resorption rate [17] or if there was an increase, it only occurred in the presence of significant maternal toxicity [9].

Since this was a registrant-submitted study we were able to examine individual animal data. Our analyses indicate that their dismissal of the possible toxicological significance of the reported resorptions may not be appropriate. For evaluating discrete-response variables like resorptions in a developmental toxicity study, Haseman and Piegorsch [29] recommended that the statistical analysis should be based on proportion of affected fetuses instead of the number affected fetuses; the latter metric gives no consideration to the potential effect of the test chemical on litter size. Also, in an article by Haseman et al. [30], concern was raised regarding the application of Bonferroni correction to the p-values when making pairwise comparison due to a relatively high false-negative rate. These authors suggested that Bonferroni correction would be unnecessary if multiple comparison procedures were used. By following these recommendations, we analyzed the resorptions in OPP-treated rabbits using the percent resorptions per litter as an experimental unit and nonparametric (i.e., distribution free) tests for dose response [31,32] and multiple comparison [20,21]; we found that resorptions exhibited a significant ($p<0.05$) dose-related trend and were significantly ($p<0.05$) increased at 100 and 250 mg/kg/day using the first phase data alone (Table 7). Likewise, our analysis of the combined data from both phases (following the approach by Zablotny et al. [6]) indicates a statistically significant increase in effects at 100 and 250 mg/kg/day (Table 7). Historical control data for percent litters with resorptions in the conducting laboratory were submitted by the investigators [26] (Table 6) and applied to our calculations. From Table 7, the percent litters with resorptions (i.e., incidence of resorptions) in

Parameters[a]	OPP, mg/kg/day				Historical Control[b]	
	0	25	100	250	Range	Mean
Number of resorptions per litter[c]	0.9 (14/15)	0.9 (12/14)	1.4 (18/13)	1.1 (20/18)	0.1-1.1	0.55
Percent implantations resorbed[d]	12.8 (14/109)	11.7 (12/103)	20.2 (18/89)	14.6 (20/137)	1.8-13.9	7.0
Percent litter with resorptions[e]	33.3 (5/15)	57.1 (8/14)	76.9 (10/13)	72.2 (13/18)	11.1-66.7	36.2
Number resorption per litter with resorption(s)[f]	2.8 (14/5)	1.5 (12/8)	1.8 (18/10)	1.5 (20/13)	1.0-2.8	1.5
Percent post-implantation loss[g]	12.2	16.7	19.2	18.3	NA	NA
Percent live litter	87.8	83.3	80.9	81.7	NA	NA

Abbreviation: NA: not available, as indicated by the investigators.
[a] Parameters calculated using combined data from Phase I and Phase II, as reported by the investigators. Definition of each of these parameters was not provided by these investigators but is given in the corresponding footnote in this work.
[b] Historical controls values from the conducting laboratory, as reported by the investigators.
[c] Number of resorptions per litter is defined as total number of resorptions divided by total number of litters.
[d] Percent implants resorbed is defined as the total number of resorptions divided by total number of implants.
[e] Percent litter with resorptions is the litter incidence of resorptions.
[f] Number resorption per litter with resorption is equal to total number of resorptions divided by total number of litters with resorptions.
[g] Percent post-implantation loss is the sum of percent resorptions or dead fetuses per litter divided by total number of litters. Because there were no dead fetuses, the percent post-implantation loss is equivalent to the sum percent resorptions per litter divided by total number of litters (see also Table 7). Using the statistical analysis method "as per current practice" (not explained), these investigators reported a p-value of 0.17 for the mid-dose group and 0.2 for the high-dose group.

Table 6: Resorption rate and related parameters of OPP gavage teratology study in New Zealand White rabbits as complied by Carney and Zablonty [27].

Litters[a]	mg/kg/day					
	0		25	100	250	
	1st Phase	2nd Phase	1st Phase	1st Phase	1st Phase	2nd Phase
1	100[b]		100	60.0	100	
2	33.3		36.4	50.0		33.3
3		22.2	33.3	25.0		33.3
4	14.3		20.0	22.2	28.6	
5	12.5		14.3	20.0	25	
6	0[c]		11.1	20.0		20
7	0		9.1	16.7	16.7	
8	0		9.1	12.5	16.7	
9	0		0	12.5	14.3	
10	0		0	10	12.5	
11	0		0	0		11.1
12	0		0	0	9.1	
13	0		0	0	9.1	
14	0		0		0	
15		0			0	
16						0
17						0
18						0
First Phase Data Only						
Litter incidence	4/13 (31%)		8/14 (57%)	10/13 (77%)	9/11 (82%)	
Percent post-implantation loss[d]	12.3 ± 28.1[+]		16.7 ± 26.9	19.2 ± 18.1*	21.1 ± 27.6*	
Combined Data						
Litter incidence	5/15 (33%)		8/14 (57%)	10/13 (77%)	13/18 (72%)	
Percent post-implantation loss[d]	12.2 ± 26.4[+]		16.7 ± 26.9	19.2 ± 18.1*	18.3 ± 23.3*[#]	

Abbreviations: NS: not significant. Shading identifies data from the second phase of testing.
[a] In columns 2-6, litters are presented in an ordered fashion. The first column only provides a visual aid for showing the number of litters per group.
[b] Percent implantations that were resorptions in a litter; e.g., 100% means that all of the implantations were resorptions.
[c] Litter with no resorptions.
[d] Percent post-implantation loss is the sum of percent resorptions per litter divided by the total number of litters.
[+] Nonparametric (i.e., distribution free) ranked-based trend test for ordered alternatives [24, 30] with the percent affected per litter as an experimental unit [20], significant at p≤0.05.
* Non-parametric multiple-comparison test [55, 56] with the percent affected per litter as an experimental unit [20], significant at p≤0.05.
[#] Calculated t-value (1.68) was comparable to the table value of 1.72 at α=0.05 [55].

Table 7: Occurrence of litters with resorptions in a developmental-toxicity study of OPP using New Zealand White rabbits [6].

the first phase for the 0, 25, 100, and 250 mg/kg/day groups were 31%, 57%, 77%, and 82%, respectively. The resorptions at 100 and 250 mg/kg/day were double that were observed in the concurrent controls and clearly exceeded the historical control range (i.e., 66.7%).

Carney and Zablonty [27] acknowledged that the percent post-implantation loss was slightly (but not statistically significantly) higher than the controls. However, there were no details on how the statistical analysis was performed (Table 6). In addition, "litter" was not the experimental unit used for calculating parameters such as number of resorptions per litter, percent implantations resorbed, and number resorption per litter with resorptions (Table 6); hence, their use in characterizing resorptions in OPP-treated rabbits may not be appropriate. Also, these investigators did not address whether combining data from two rabbit studies could have contributed to a slight downturn of the dose responses of percent litter with resorptions and percent post-implantation loss.

With respect to the weight-of-evidence argument put forth by the study authors, we provide an alternative interpretation as follows. In the two rabbit studies, we observed a resorption incidence at 250 mg/kg/day for the first phase (82%) in the main study to be comparable to the incidence in probe study at this dose (83%). In evaluating evidence of the interspecies effects of OPP and SOPP, we believe that some difficulties exist when applying a "weight-of-evidence" type of analysis. For example, the developmental toxicity database of OPP and SOPP has only 1-2 studies per species, in contrast to the more extensive toxicity database of OPP and SOPP (4 or more studies per species) for carcinogenic/non-carcinogenic endpoint identification. In addition, we have shown that a significant deviation from the FIFRA Guidelines may contribute to the ostensible negative results in some studies. The perception of a negative response could limit interest in further research on potential developmental effects of OPP and SOPP. In general, for identifying developmental hazards, the FIFRA Guidelines require two species (including one non-rodent) to be tested; however, the USEPA Guidelines for developmental toxicity risk assessment [33] stated that an adverse developmental effect in a single, appropriate, well-conducted study in a single experimental animal species is sufficient to judge the existence of a potential hazard. This regulatory difference stems from known species specific effects of certain developmental toxicants (e.g., thalidomide induced phocomelia in humans was observed in NZW rabbits but not in rats and mice) [34-36]. Hence, the evidence of developmental toxicity of OPP in rabbits does not necessarily need to be validated by similar adverse effects in rodents including rats.

Based on the increased litter incidence of resorptions at 100 mg/kg/day, the developmental NOEL was set at 25 mg/kg/day. We subsequently performed a BMD [15] at the lower 95% confidence limit of the effective dose required to cause a benchmark response of 10%. Results showed an increased incidence of resorptions at 2.5 mg/kg/day (Log-Logistic model as the best fit based on Chi-square goodness-of-fit [$p>0.05$], Akaike's Information Criterion [lowest value], and scaled residual [minimum]). This BMD_{10}, albeit a factor of 10 lower than the experimental NOEL, is consistent with the observations that resorptions were distributed across in the treated groups in a dose-dependent manner (Table 7) and that the litter incidence of resorptions at 25 mg/kg/day was nearly double that of the controls. The maternal NOEL was set at 100 mg/kg/day based on an increased incidence of renal tubular degeneration at 250 mg/kg/day (summary Table 8). The USEPA conducted no statistical tests on the incidence of resorptions and determined the developmental NOEL ≥ 250 mg/kg/day [2].

Reproductive Toxicity Studies

Two 2-generation rat reproduction studies were submitted by the registrant (two litters per generation). The first report was produced in two versions: initially as Eigenberg [37] and subsequently revised as Eigenberg [10]. The studies were not acceptable according to previous, or current FIFRA Guidelines, therefore, a second study was conducted, Eigenberg and Lake [11].

Eigenberg [10]: Four groups of Sprague Dawley (SD) rats (35 animals/sex/dose) received diets containing OPP at nominal doses of 0, 40, 140, or 490 mg/kg/day for two generations. The exposure to OPP in F0 rats occurred for 15 and ~31 weeks before their 1st and 2nd matings, respectively, and for a total of ~43 weeks before sacrifice. The exposure to OPP in F1 rats (F1b offspring of F0 parents) occurred for 10 and ~22 weeks before their 1st and 2nd matings, respectively; they were 34-40 weeks old when sacrificed. The number of parental animals dead or sacrificed due to their moribund state was nineteen (12 F0 animals [6/sex] and 7 F1 animals [5 males, 2 females]).

There were deviations from the Guideline protocol that may have affected mating results (e.g., 56 instances, dams were cohoused with a male for only 1-2 days per mating week). Given that the estrus cycle in young rats is typically 4-5 days and that the cycle shifts to even longer durations with increasing age, the reason for cohousing for less than 4 days (i.e., less than one cycle) was not known. Dams that were classified as having not mated in the study almost categorically had not been cohoused with a male for the 21- day minimum given in earlier FIFRA Guidelines or the 16- day minimum (4×4) indicated in the conducting laboratory's Standard Operating Procedures (SOP). In the case of 9 F0 dams, their total number of cohousing days was only 11-13. In 12 instances, dams were noted as having a sperm plug in their bedding or in one case in the dam's vagina (F1b dam) but these dams were not classified as having mated based on finding these plugs. It should be noted that the current and former FIFRA Guidelines specify that a plug is taken to be evidence of mating and that the day of its finding is used to define day 0 of the pregnancy. It was noted that dams possibly had sperm in their vaginal wash but were not designated as having mated and this may have affected the male fertility index. We considered that the assessments on fertility in this study were inconclusive.

Eigenberg and Lake [11]: Four groups of SD rats (30 animals/sex/dose) received OPP in diet at 0, 20, 100, or 500 mg/kg/day. The exposures to OPP in F0 rats occurred for 10 and 21 weeks before the 1st and 2nd matings, respectively, and continued to terminal sacrifice at study weeks 43-44. The exposure to OPP in F1 rats (F1b offspring of F0 parents) occurred for 12 and ~22 weeks before their 1st and 2nd matings, respectively and continued to terminal sacrifice at 34-40 weeks of age. There were 12 parental animals found dead or sacrificed moribund (F0: 2 males, 6 females; F1: 4 males).

Both sexes of F0 animals dosed at 500 mg/kg/day showed reduced ($p<0.05$) body weight, starting after three weeks of treatment in the females and 10 weeks in the males. At 500 mg/kg/day, F1 animals exhibited reduced body weight as weanlings and in the premating period. In the F1 males, the body weight stayed reduced by 10-11% ($p<0.05$) throughout the F1 portion of the study, including at the F1 terminal sacrifice. In the F1 females, by the end of the first premating period, the reduction in body weight was 9% ($p<0.05$); however, by the F1 terminal sacrifice, the reduction in body weight was only 4% (statistically not significant). The only treatment-related clinical observation in adults was an increase of urine stain in the 500 mg/kg/day male groups (F0 and F1). Urine staining tended to start at study week 18 and to last until termination.

Species/Exposure	Effects at LOEL	NOEL mg/kg/day	Reference
	OPP Developmental Toxicity (Gavage)		
Wistar Rat: GD 6-15 vehicle=gum arabic	Dam : ↓bodyweight gain, ataxia Fetus: ↓body weight; ↑ resorptions	Dam=150 Fetal=300	[9]
Sprague-Dawley Rat: GD 6-15 vehicle=cottonseed oil	Dam : ↓bodyweight gain; ↓food consumption; ↑ water consumption Fetus:↑ percent pre-/post-implantation loss	Dam=100-300 Fetal=300-700	[8,17]
New Zealand White rabbit vehicle=corn oil	Dam : Renal tubular degeneration & inflammation Fetus: ↑ resorptions	Dam=100 Fetus (BMD_{10})[a]=2.5	[6]
	SOPP Developmental Toxicity (Gavage)		
Jcl:ICR Mouse: GD 7-15 vehicle=water	Dam : ↓bodyweight gain Fetus: ↓bodyweight; ↑cleft palate	Dam ≥ 10[b] Fetus=10[b]	[7]
	OPP 2 Generation Reproductive Toxicity (Diet)		
Sprague-Dawley rat: 2 matings per generation	Parental: ↑ urine stain; ↓bodyweight; ↑urinary bladder, ureter & kidney pathology; Pup: ↓bodyweight	Parental=100 Pup =100	[11]

Abbreviations: GD: Gestation Day; LOEL: Lowest Observed Effect Level; NOEL: No Observed Effect Level.

[a] Dose Analysis: A point of departure (POD), based on increased resorptions was estimated by a Benchmark Dose Analysis at the lower 95% confidence limit of the effective dose required to cause a benchmark response of 10% (BMD_{10}; Log-Logistic model as the best fit based on Chi-square goodness-of-fit [p>0.05], Akaike's Information Criterion [lowest value], and scaled residual [minimum]).

[b] Estimated NOEL using a 10x UF for LOEL-to-NOEL extrapolation (see text).

Table 8: Summary of the developmental and reproductive effects of OPP and SOPP.

Parental males exhibited treatment-related effects in the urinary bladder at 500 mg/kg/day (percent F0 and F1 incidences in parentheses): increased ($p<0.01$) incidences of chronic inflammation (53% and 63%) and simple hyperplasia (73% and 90%). At this same dose, OPP also appeared to affect the kidneys and ureters in the F0 and F1 males. The effects identified were dilatation and hyperplasia of the ureters, chronic active inflammation in the kidneys, and debris in the renal pelvis. Although the incidence of each of these effects by itself was not statistically significant, when viewed collectively, it would suggest a treatment-related effect on the kidneys and ureters. No other F0 and F1 males in this study, including the controls, exhibited lesions in the kidneys or ureters.

OPP had no effect on the F0 and F1 dam mating or delivery parameters (e.g., mating data, survival, litter data, mean number of live births); estrous-cycle length and periodicity, gestation duration, and sex ratio were also unaffected by treatment (data not shown in the current review). The control- and low-dose fertility (number pregnant/number mated) and fecundity indices were low compared with those at the mid and high dose for the F1 (F1a mating) as were the fecundity indices (number of live deliveries/number mated) for the F1 (F2b mating). The fecundity indices at 500 mg/kg/day for F1 (F1a and F2a matings) were statistically significantly increased over controls. It is a concern that the least ability to procreate was seen in the controls of the F2a and F2b mating trials: fecundity indices for the controls were 0.5 (15/30) and 0.6 (18/30), respectively. A similar situation also occurred in the first reproduction study [10] with the F1b control group: the dam fecundity index was only 0.23 (7/31). Adding to the concern is that the ability to procreate (as indicated by the fertility index) increased with increasing dose in two consecutive mating trials (F2a and F2b). When evaluating both the fecundity and fertility indices, it appeared that the control group did not function as would be expected. When this occurs, the potential for identification of true effects induced by treatments is limited.

OPP showed pup effects in the F1 and F2 litters. As a group, pups from F0 and F1 dams at 500 mg/kg/day had decreased body weights (10-12%) in each of the four mating trials that remained to lactation day 21 ($p<0.01$). Reductions of 3-7%, were also present on lactation day 14 (statistically significant ($p<0.05$)) in the F2a and F2b litters.

The parental NOEL was 100 mg/kg/day based on decreased body weights (F0 and F1 dams and F1 males), increased histopathology in the urinary bladder, ureter, and kidneys in F0 and F1 males at 500 mg/kg/day. The pup NOEL also was 100 mg/kg/day, based on decreased body weights in the F1 and F2 pups (see summary Table 8). However, these NOELs may be subjected to revision because of the aforementioned problems with the study. USEPA established 100 mg/kg/day as both the parental and reproductive NOELs based on the noted effects [2].

Discussion

Developmental effects were observed in rat, mice, and rabbits treated with OPP or SOPP. Among the experimental animals tested, post-implantation loss was a common developmental effect. Published and unpublished reviews have concluded that OPP and SOPP induce developmental effects only at doses where maternal effects are observed [1,2,38]. However, our analyses indicate that fetal effects may also occur at doses where maternal toxicity is either minimal or not present (summary Table 8). Studies performed in Wistar rats [9] and Sprague-Dawley rats [8,17] showed that maternal toxicity (decreased gestational body weight gain and ataxia) was significant at a dose that caused developmental effects: decreased fetal body weight, delayed ossification, and possibly, increased incidence of resorptions. Ogata et al. [7] showed that in SOPP treated Jcl:ICR mice there was an increased fetal incidence of cleft palate along with reduced maternal body weight gain at the same dose. However, in OPP-treated New Zealand White rabbits [6], developmental effects in fetuses (resorptions) occurred at a dose lower than the systemic effects in dam (e.g., renal tubular degeneration: summary Table 8). The rabbit study also provided the lowest point of departure (POD) (based on BMD_{10}=2.5 mg/kg/day) for assessing the developmental effects of OPP and SOPP.

Substituted phenols including those with hydroxy-substituent (OH) group have been shown to reduce live litter size at birth and (or) to increase perinatal offspring loss in rats after a single dosing at mid gestation [39]. Effects included a dose-dependent decrease in implantation viability (i.e., post-implantation loss) in Sprague-Dawley rats treated via gavage at 100-1000 mg/kg catechol or hydroquinone on GD 11 (the potencies of hydroquinone and catechol were ~20 times higher than phenol). In this same study, the potency of post-implantation loss was correlated statistically (i.e., regression analysis) with three properties of the substituent group: lipophilicity (as measured by octanol/water partition coefficient [Kow]), electron

withdrawing ability (Hammett constant [σ]), and bulkiness (molar refractivity [MR]). These physicochemical properties were considered as important parameters for controlling across membrane transport and may determine the extent to which the substituted phenol interacts with its macromolecular targets [39]. OPP and its major metabolite, PHQ, are the 2-phenyl derivative of phenol and hydroquinone, respectively. Of the three physicochemical properties, two support a stronger regression correlation of OPP than phenol for the induction of post implantation loss: 2-phenyl group of OPP is electron donating (σ constant of 0.01 *vs.* 0 of H-atom) and more bulky (MR of 25.36 *vs.* 1.03 of H atom) than hydrogen-atom of phenol [40]. For PHQ, an added electron-donating OH group on the OPP nucleus is expected to further enhance the biological activity by reducing the molecule's lipophilicity and increasing its bulkiness. This expectation appears to be consistent with the lower LOEL of OPP than phenol for inducing post-implantation loss in rats [8,39].

Mode-of-action that might account for the developmental effects noted is not known. However, OPP is an established carcinogen in rats [41] and mice [42], and the carcinogenesis may involve, at least in the rats, a genotoxic MOA. Because carcinogenesis and teratogenesis could share a common MOA [43], the genotoxic and cytotoxic effects of OPP and its metabolites, PHQ and PBQ, may have contributed to the reported developmental effects.

Another potential MOA for the developmental effects noted may be associated with endocrine disruption. OPP was positive in several studies for endocrine disrupting potential *in vitro* [44-48]. The assay systems used were estrogen-receptor binding (non-competitive), estrogen-induced cell proliferation (e.g., MCF-7 human breast cancer cells), and estrogen-receptor transcription activity in cells (e.g., MVLN cell line). In addition, Freyberger and Degen [49] discovered that in ovine seminal vesicles, OPP as well as its metabolite PHQ were inhibitors of prostaglandin synthase; the lowest 50% inhibition concentrations (IC50) obtained were 13 μM and 17 μM, respectively, depending on the concentration of arachidonic acid used in the assay. Habicht and Brune [50] determined an IC_{50} value of 2.5 μM for OPP inhibition of the release of prostaglandin E2 using phorbol ester stimulated mouse peritoneal macrophages in testing *in vitro*. Therefore, OPP and PHQ may be acting *in vivo* as inhibitors of prostaglandin metabolism. It should be noted that some inhibitors of prostaglandin (e.g., Nonsteroidal Anti-inflammatory Drugs [NSAID]) have been reported to increase resorptions in rats [51,52] and rabbits [53] and to induce cleft palate in mice [54].

Currently, FIFRA Guideline studies are considered to be the "gold standard" on which regulatory decisions for pesticide registration are based, for example, by the USEPA. At times variations in Guideline protocols may be unavoidable, but significant deviations can render the resulting data difficult to interpret. This uncertainty may become an issue when the information is used in risk assessment for human health protection. The 2-generation reproductive toxicity study in rats [11] is an example of such a concern. While information in the 2-generation reproductive toxicity study could have been useful, the uncertainties introduced by the controls cause a definitive conclusion difficult to reach.

Conclusion

Methods for toxicological data interpretation and analysis have undergone significant advancement since the studies described above were performed. In light of an increased understanding of the potential for long term effects of toxic compounds after *in utero* or post-natal exposure (e.g. Food Quality Protection Act, 1996) [55], it would be useful to re-analyze relevant older studies using the latest data analytical methods to investigate the veracity of previously accepted conclusions. As we have demonstrated, the re-evaluation would minimize the uncertainties introduced by experimental mishap, maximize the usefulness of all currently available data for protecting public health, and ensure that future health hazard decisions are not based on outdated data reviews. This is especially relevant for using *in vivo* animal studies to validate new methodologies as prescribed by the new risk assessment paradigm (e.g., Toxicity Testing in the 21st Century [56-58]).

In summary, we determined a pattern for developmental effects associated with OPP and SOPP treatment across all species examined. Although further studies are needed to elucidate the developmental toxicity of OPP and SOPP, our re-evaluations indicated that fetal effects (e.g., resorption) occurred in the absence of maternal toxicity. For the purpose of public health protection, the effects that we have identified would be useful in the formulation of a current or updated regulatory strategy for OPP and SOPP.

Acknowledgments

We would like to thank Dr. Stephen Rinkus for his excellent assistance in preforming this work and Dr. Svetlana Koshlukova for her editorial input. The views expressed in this work are those of the author(s) and do not necessarily reflect the view or policy of the California Department of Pesticide Regulation.

References

1. FAO (2000) 2-Phenylphenol and its Sodium Salt. In Pesticide residues in food - 1999. Toxicology Evaluations. World Health Organization, WHO/PCS/004, 2000, nos 957-968: 57.
2. USEPA (2006) Reregistration Eligibility Decision for 2-phenylphenol and Salts (Orthophenylphenol or OPP). Office of Prevention, Pesticides, Toxic Substances, United States Environmental Protection Agency: 156.
3. Kwok ES, Eastmond DA (1997) Effects of pH on nonenzymatic oxidation of phenylhydroquinone: potential role in urinary bladder carcinogenesis induced by o-phenylphenol in Fischer 344 rats. Chemical Research in Toxicology 10: 742-749.
4. Brusick D (2005) Analysis of genotoxicity and the carcinogenic mode of action for ortho-phenylphenol. Environmental and Molecular Mutagenesis 45: 460-481.
5. Zablotny CL, Breslin WJ, Kociba RJ (1991) Ortho-phenylphenol (OPP): Gavage teratology probe study in New Zealand White rabbits. Dow Chemical Company.
6. Zablotny CL, Breslin WJ, Kociba RJ (1991) Developmental toxicity of ortho-phenylphenol (OPP) in New Zealand White rabbits. Dow Chemical Company.
7. Ogata A, Ando H, Kubo Y, Hiraga K (1978) Teratological tests of o-phenylphenol (OPP) and sodium o-phenylphenol (OPP-Na) in mice. Tokyo Metropolitan Research Laboratory of Public Health 29: 99-103.
8. John JA, Murray FJ, Crawford AA, Murray JB, Pilny MK, et al. (1978) The effects of orally administered ortho-phenylphenol on rat embryonal and fetal development. Toxicology Research Laboratory, Health and Environmental Research, Dow Chemical, Midland, Michigan USA.
9. Kaneda M, Teramoto S, Shingu A, Shirasu Y (1978) Teratogenicity and dominant-lethal studies with o-phenylphenol. Journal of Pesticide Science 3: 365-370.
10. Eigenberg DA (1989) Two generation dietary reproductive study in rats using orthophenylphenol-Revised Report. Mobay Corporation.
11. Eigenberg DA, Lake SG (1995) A two generation dietary reproductive study in Sprague-Dawley rats using technical grade ortho-phenylphenol. Bayer Corporation.
12. USEPA (1984) Pesticide assessment guidelines, subdivision F: hazard evaluation: human and domestic animals (Revised edition). In: United States. Environmental Protection Agency. Office of Pesticide Programs., W, D.C., United States. Environmental Protection Agency. Office of Pesticides and Toxic Substances., S, VA (eds.) U.S. Environmental Protection Agency 760.

13. USEPA (1998) OPPTS 870.3700 Prenatal Developmental Toxicity Study [EPA 712–C–98–207]. Health Effects Test Guidelines.

14. USEPA (1998) OPPTS 870.3800 Reproduction and Fertility Effects [EPA 712–C–98–208]. Health Effects Test Guidelines.

15. USEPA (2011) Benchmark Dose Software (BMDS) Version 2.2 User Manual. The U.S. Environmental Protection Agency.

16. Kwok ESC (2007) Ortho-Phenylphenol (OPP) and Sodium Ortho-Phenylphenate (SOPP) Risk Characterization Document: Dietary Exposure. Medical Toxicology Branch, California Department of Pesticide Regulation 227.

17. John JA, Murray FJ, Rao KS, Schwetz BA (1981) Teratological evaluation of orthophenylphenol in rats. Fundamental and Applied Toxicology 1: 282-285.

18. MARTA (1996) Historical Control Data (1992 — 1994) for Developmental and Reproductive Toxicity Studies using the Crl:CD®(SD)BR Rat.

19. Kopf R, Lorenz D, Salewski E (1964) Procedure for staining implantation sites of fresh rat uteri. Naunyn-Schmiedebergs (Arch Exp Pathol Pharmacol Res) 247: 121-135.

20. Williams DA (1972) The comparison of several dose levels with a zero dose control. Biometrics 28: 519-531.

21. Williams DA (1986) A note on Shirley's nonparametric test for comparing several dose levels with a zero-dose control. Biometrics 42: 183-186.

22. Brinster RL (1975) Teratogen testing using preimplantation mammalian embryos. In: Miller, JR, Marois, M, Shepard, TH (eds.) Methods for detection of environmental agents that produce congenital defects : proceedings of the Guadeloupe Conference Sponsored by 1'Institut de la Vie,. North-Holland Pub. Co.; American Elsevier Pub. Co., Amsterdam, New York 113-124.

23. Beck F, Lloyd JB (1963) An Investigation of the Relationship between Foetal Death and Foetal Malformation. Journal of Anatomy 97: 555-564.

24. Ogata A, Ando H, Kubo Y, Hiraga K (1984) Teratogenicity of thiabendazole in ICR mice. Food and Chemical Toxicology 22: 509-520.

25. Wahle BS, Christenson WR (1996) Technical grade ortho-phenylphenol: A combined chronic toxicity/oncogenicity testing study in the rat. Bayer Corporation, Germany.

26. Breslin WJ, Kociba RJ, Landenberger BD (1992) Response to CDPR MT Record Number 097303: ortho-Phenylphenol (OPP) Gavage Teratology Study in New Zealand White Rabbits (Additional data to record number 97303 in Volume 129-0148). The Dow Chemical Company.

27. Carney E, Zablotny C (2006) Developmental toxicity endpoint. Response to Department of Pesticide Regulation Ortho-Phenylphenol (OPP) and Sodium Ortho-Phenylphenate (SOPP) Risk Characterization Document (RCD): Dietary Exposure Draft. Lanxess Corporation and The Dow Chemical Company 27-30.

28. Haseman JK, Hoel DG (1974) Tables of gehan's generalized Wilcoxon test with fixed point censoring. Journal of Statistical Computation and Simulation 3: 117 - 135.

29. Haseman JK, Piegorsch WW (1994) Statistical Analysis of Developmental Toxicity Data. In: Kimmel, CA, Buelke-Sam, J (eds.) Developmental toxicology, 2nd ed edn. Raven Press, New York 349-362.

30. Haseman JK, Bailer AJ, Kodell RL, Morris R, Portier K (2001) Statistical issues in the analysis of low-dose endocrine disruptor data. Toxicolological Sciences 61: 201-210.

31. Jonckheere AR (1954) A distribution-free k-sample test against ordered alternatives Biometrika 41: 133-145.

32. Lehmann EL, D'Abrera HJM (1975) Nonparametrics : statistical methods based on ranks, San Francisco Holden-Day.

33. USEPA (1991) Guidelines for Developmental Toxicity Risk Assessment. U.S. Environmental Protection Agency, Risk Assessment Forum, Washington, DC, EPA/600/FR-91/001, 1991. Federal Register 56: 63798-63826.

34. Somers GF (1962) Letter to the editor: Thalidomide and congenital abnormalities. Lancet: 912-913.

35. Palmer AK (1981) Regulatory requirements for reproductive toxicology: Theory and practice. In: Kimmel, CA, Buelke-Sam, J (eds.) Target organ toxicology series: Developmental toxicology. Raven Press, New York 340.

36. Greek R, Shanks N, Rice MJ (2011) The History and Implications of Testing Thalidomide on Animals. The Journal of Philosophy, Science & Law 11.

37. Eigenberg DA (1989) Two generation dietary reproductive study in rats using orthophenylphenol. Mobay Corporation.

38. Bomhard EM, Brendler-Schwaab SY, Freyberger A, Herbold BA, Leser KH, Richter M (2002) O-phenylphenol and its sodium and potassium salts: a toxicological assessment. Critical Review Toxicology 32: 551-625.

39. Kavlock RJ (1990) Structure-activity relationships in the developmental toxicity of substituted phenols: in vivo effects. Teratology 41: 43-59.

40. Hansch C, Leo A (1979) Substituent constants for correlation analysis in chemistry and biology. Wiley, New York.

41. Hiraga K, Fujii T (1984) Induction of tumours of the urinary bladder in F344 rats by dietary administration of o-phenylphenol. Food and Chemical Toxicology 22: 865-870.

42. Quast JF, McGuirk RJ, Kociba RJ (1997) Results of a two-year dietary toxicity/oncogenicity study of ortho-phenylphenol(OPP) in B6C3F1 mice. Toxicologist 36: 341.

43. Vainio H (1989) Carcinogenesis and teratogenesis may have common mechanisms. Scandinavian Journal of Work, Environment & Health 15: 13-17.

44. Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicological Sciences 54: 138-153.

45. Miller D, Wheals BB, Beresford N, Sumpter JP (2001) Estrogenic activity of phenolic additives determined by an in vitro yeast bioassay. Environmental Health Perspective 109: 133-138.

46. Rehmann K, Schramm KW, Kettrup AA (1999) Applicability of a yeast oestrogen screen for the detection of oestrogen-like activities in environmental samples. Chemosphere 38: 3303-3312.

47. Routledge EJ, Sumpter JP (1997) Structural features of alkylphenolic chemicals associated with estrogenic activity. Journal of Biological Chemistry 272: 3280-3288.

48. Soto AM, Fernandez MF, Luizzi MF, Oles Karasko AS, Sonnenschein C (1997) Developing a marker of exposure to xenoestrogen mixtures in human serum. Environmental Health Perspective 105 Suppl 3: 647-654.

49. Freyberger A, Degen GH (1998) Inhibition of prostaglandin-H-synthase by o-phenylphenol and its metabolites. Archives of Toxicology 72: 637-644.

50. Habicht J, Brune K (1983) Inhibition of prostaglandin E2 release by salicylates, benzoates and phenols: a quantitative structure-activity study. Journal of Pharmacy and Pharmacology 35: 718-723.

51. Hallesy DW, Shott LD, Hill R (1973) Comparative toxicology of naproxen. Scandinavian Journal of Rheumatology: Suppl 2:20-28.

52. McClain RM, Hoar RM (1980) Reproduction studies with carprofen, a nonsteroidal anti-inflammatory agent in rats. Toxicology and Applied Pharmacology 56: 376-382.

53. O'Grady JP, Caldwell BV, Auletta FJ, Speroff L (1972) The effects of an inhibitor of prostaglandin synthesis (indomethacin) on ovulation, pregnancy, and pseudopregnancy in the rabbit. Prostaglandins 1: 97-106.

54. Montenegro MA, Palomino H (1990) Induction of cleft palate in mice by inhibitors of prostaglandin synthesis. Journal of Craniafacial Genetics and Developmental Biology 10: 83-94.

55. USEPA (1997) 1996 Food Quality Protection Act, Amendments to the Laws Governing the Regulation of Pesticides; EPA's Implementation Plan. Federal Register 62: 12829-12830.

56. Bhattacharya S, Zhang Q, Carmichael PL, Boekelheide K, Andersen ME (2011) Toxicity testing in the 21 century: defining new risk assessment approaches based on perturbation of intracellular toxicity pathways. PloS one 6: e20887.

57. Martin MT, Knudsen TB, Reif DM, Houck KA, Judson RS, Kavlock RJ, Dix DJ (2011) Predictive model of rat reproductive toxicity from ToxCast high throughput screening. Biology of Reproduction 85: 327-339.

58. Rotroff DM, Dix DJ, Houck KA, Knudsen TB, Martin MT, et al. (2013) Using in vitro high throughput screening assays to identify potential endocrine-disrupting chemicals. Environmental Health Perspective 121: 7-14.