REPORT D

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1993	Journal article

4. TITLE AND SUBTITLE
Developmental toxicity of OTTO fuel II in the rat and rabbit

5. FUNDING NUMBERS
PE - 63706
PR - M0096
TA - 004
WU - 0006

6. AUTHOR(S)
Cooper JR, Lee LH, Macys DA

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

8. PERFORMING ORGANIZATION REPORT NUMBER
NMRI 93-109

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Medical Research and Development Command
National Naval Medical Center
Building 1, Tower 12
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5606

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
DN377025

11. SUPPLEMENTARY NOTES
Reprinted from: Journal of Applied Toxicology 1993, Vol.13 No.4 pp.235-239

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS
OTTO Fuel II; teratology; rat; rabbit; propylene glycol dinitrate (PGNN)

15. NUMBER OF PAGES
5

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

JOURNAL OF APPLIED TOXICOLOGY, VOL. 13(4), 235–239 (1993)

Developmental Toxicity of OTTO Fuel II in the Rat and Rabbit

James R. Cooper†
Comparative Medicine Branch/Armstrong Laboratory, Wright-Patterson Air Force Base, OH 45433-6573, USA.

Lanfong H. Lee and David A. Macys
Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base, OH 45433-6573, USA

Key words: OTTO Fuel II; teratology; rat; rabbit; propylene glycol dinitrate (PGDN).

OTTO Fuel II (OFII) is a propellent used by the United States Navy in its Mk 46 and Mk 48 torpedoes. Owing to the possibility of human exposure during fueling and defueling operations, studies were initiated to determine if OFII was a developmental toxin. Phase I of the investigation involved dosing four groups of time-mated Fischer-344 rats with OFII. The fuel was administered dermally at the rate of 0, 400, 2000 and 4000 mg kg^{-1} day^{-1}. A significant reduction in body weight was seen at necropsy in dams receiving 2000 and 4000 mg kg^{-1} day^{-1} of OFII. Fetuses from these dams also weighed significantly less than control fetuses.

Phase II of the investigation involved dosing of artificially inseminated rabbits with OFII. The fuel was administered to the skin of the animal's back at the rate of 0, 100, 316 and 1000 mg kg^{-1} day^{-1}. Maternal toxicity was evidenced by a significant reduction in body weight of the dams in the 1000 mg kg^{-1} day^{-1} dose group on days 20 and 25 of gestation. There were no significant differences observed in maternal weight or fetal weight at necropsy. Morphological examination of both rat and rabbit fetuses failed to reveal significant evidence of fetal malformations.

INTRODUCTION

OTTO Fuel II (OFII) is composed of 76% propylene glycol dinitrate, 22.5% di-*n*-butyl sebacate and 2% 2-nitrodiphenylamine. The use of OFII by the Navy as a propellant for Mk 46 and Mk 48 torpedoes is based on its ability to support combustion in the absence of oxygen. The fuel's potential toxicity is derived from its propylene glycol dinitrate (PGDN) component. An excellent review of PGDN toxicology and epidemiology was published by Foreman in 1988.[1]

PGDN is primarily eliminated by metabolism in the blood, *in vitro* studies indicate that this metabolism occurs within the erythrocyte.[2,3] The principal metabolite of PGDN is nitrate, which is eliminated in the urine. *In vivo* experiments conducted in dogs revealed an elimination rate constant of 0.895 min^{-1} for PGDN in the blood of this species.[4] The primary systemic effect associated with acute PGDN exposure is vasodilatation and subsequent hypotension. The vasodilatory effect of PGDN on the vasculature of the brain is the probable cause of headaches reported in a survey of workers who contacted this compound in the workplace.[5] Prolonged exposure of animals to high concentrations of PGDN has been shown to induce formation of methemoglobin, methemoglobinemia being the most probable cause of death.[6] Susceptibility to methemoglobinemia subsequent to PGDN exposure has been found to vary considerably among species (dog > guinea pig > rat > man).[7]

The LD_{50} dose of PGDN following subcutaneous injection in the female rat is 463 mg kg^{-1}.[2] Additional studies by Clark indicated that ca. 10% of topically applied PGDN is absorbed through the skin of this species.[3] Studies by Jones[6] involving the dermal application of PGDN to rabbits for a period of 20 days revealed no systemic effects at a dose of 1 g kg^{-1} day^{-1}, while 2 g kg^{-1} day^{-1} resulted in cyanosis and rapid breathing. A 4 g kg^{-1} day^{-1} dermal dose of PGDN resulted in methemoglobin levels of 34.5% and death within 5 days in the rabbit.[6]

Dibutyl sebacate is added to OFII as a desensitizing agent. Its role as a potential reproductive toxicant was evaluated by Smith in 1953.[8] In this study, 20 male and female rats were exposed to a diet containing 6.25% dibutyl sterate for a period of 10 weeks prior to breeding. Evaluation of the resulting offspring revealed no effect on fertility, litter size or pup survival. Pups from exposed dams did, however, display a decrease in body weight as compared to pups from control animals.[8] 2-Nitrodiphenylamine is incorporated into OFII as a stabilizer. No references were found relevant to the effects of this compound on reproduction.

Carcinogenicity studies conducted on OFII have yielded negative results. Dogs exposed via inhalation to OFII at the rate of 1.4 mg m^{-3}, 6 h each day for 14 months, failed to demonstrate an increased incidence of tumors. The dogs did display a reduction in red blood cell (RBC) counts, hematocrit and hemoglobin levels, and an increase in methemoglobin levels during the exposure period.[9] In the same study, rats and mice were exposed via inhalation to OFII at the rate of 240 mg m^{-3}; examination of the above-cited hematologic parameters in these animals did not demonstrate

† Author to whom correspondence should be addressed.

0260–437X/93/040235–05$07.50

Received 5 February 1992
Accepted (revised) 16 October 1992

significant differences from controls. The mice and rats exposed to OFII in this study also failed to demonstrate an increased incidence of tumors.

The mutagenic activity of OFII is documented in a report authored by Jagannath in 1982.[10] Salmonella and Saccharomyces assays performed both in the absence and in the presence of liver microsomal enzyme preparations did not reveal mutagenic effects. Mutations were found in the thymidine kinase locus of the mouse lymphoma cells when OFII was employed in toxic concentrations. Tests designed to evaluate the ability of OFII to induce sister chromatic exchanges were negative. Similarly, OFII failed to induce clastogenic changes in mouse bone marrow cells following either acute or subchronic administration.

MATERIALS AND METHODS

Test substance

OTTO Fuel II (OFII) is a red-orange, free-flowing liquid with a density of 1.232 g ml^{-1} and a vapor pressure of 0.877 mmHg. The OFII used in this study was supplied by the Naval Ordnance Station, Indian Head, MD.

Rat teratology

Crl:CD BR rats (Charles River Laboratories, Kingston, MA) were used in the rodent segment of the study. Female rats weighing an average of 170 g were mated with proven breeder males. Pregnancy was assessed on a daily basis by vaginal wash. The day on which the females were found to be sperm-positive was considered day 0 of pregnancy. On day 5 of pregnancy, the lumbar area of the rat's back was shaved with a number 40 blade Oster clipper. Animals were then randomly divided into a control and three treatment groups. The dosing procedures involved applying OFII, in neat form, to the shaved lumbar area of each rat's back at the rate of 0, 400, 2000 or 4000 mg kg^{-1} day^{-1}. The fuel was applied with an Eppendorf pipette in five divided doses administered at 2-h intervals. The surface area of skin in contact with the fuel varied in accordance with the dose applied. The control animals were dosed with 1 ml kg^{-1} day^{-1} of sterile water in a similar manner.

On day 20 of pregnancy the dams were euthanized and weighed following removal of the uterus. The total number of fetuses, corpora lutea, implantation sites and resorption sites were recorded. The fetuses were then removed from the uterus, classified as living or dead, sexed, weighed and examined for grossly visible defects.

The uteri of animals that appeared to be non-pregnant were re-examined following emersion in a solution of 10% sodium sulfide. Half of the fetuses were further examined for soft tissue abnormalities using techniques as outlined by Wilson.[11] The remainder were processed and examined for skeletal deformities in accordance with techniques described by Staples.[12]

Rabbit teratology

Female New Zealand white rabbits, 5 months of age (Hazelton, Inc.), were used in Phase II of the study. Artificial insemination was accomplished with ejaculates collected from adult male rabbits of the same strain. Following collection, semen was examined microscopically for concentration and motility. Acceptable samples, i.e. 60% motile and a minimum of 12 million sperm ml^{-1}, were used for insemination. Approximately 3 million live sperm were infused into each female using techniques described by Gibson.[13] Immediately following insemination, the female rabbit received an intramuscular injection of 100 units of human chorionic gonadotropin (Pregnyl). The day of insemination was designated day 0 of pregnancy.

Inseminated female rabbits were randomly divided into four dose groups of sixteen animals each. On the day prior to the initiation of dosing, hair was shaved from a 4 × 6 in. area of each animal's back using a number 40 blade Oster clipper. Dose groups were exposed to either 1 ml kg^{-1} day^{-1} water or neat OFII at the rate of 100, 316 or 1000 mg kg^{-1} day^{-1}. The calculated dose of OFII was applied to the back of each rabbit on days 6–18 of pregnancy. The fuel was delivered to the rabbit's skin using an Eppendorf pipette in five divided doses administered at 2-h intervals. The surface area of skin exposed to the OFII varied in accordance with the dose applied. On day 28 of pregnancy, the rabbits were euthanized with an overdose of barbiturate (Secumb) administered via the marginal ear vein.

Uterine and fetal tissues were harvested and inspected using procedures as outlined for the rat in Phase I of the study. Each fetus was then dissected and examined for visceral abnormalities of the head and trunk.[14,15] Following visceral examination, the fetuses were skinned and prepared for detailed skeletal examination in accordance with techniques described by Dawson.[16]

Data analysis

Statistical analysis of the data was conducted using the litter as the basic experimental unit. Bartlett's test for homogeneity of variance was performed on all data to be examined by analysis of variance (ANOVA) procedures. Data related to maternal body weight and fetal body weight was analyzed using ANOVA. When ANOVA analysis revealed a significant difference between group means, pairwise comparisons were performed using Tukeys' procedure. Nominal data, including fetal malformations and variations, were first analyzed using a chi-squared test. If significant group differences were noted, Fisher's exact probability test was used to perform pairwise comparisons. In all cases, $P < 0.05$ was accepted as the level of significance.

RESULTS

Rat study

Dams in both the 2000 and 4000 mg kg^{-1} day^{-1} OFII dose groups exhibited a significant reduction in body

Table 1. OFII dermal teratology: maternal and fetal reproductive parameters in the rat

	OFII (mg kg 1 day $^{-1}$)			
	0.0	400	2000	4000
Animals mated	28	28	28	47
Animals that died	0	0	0	25
Animals that delivered	1	1	2	0
Animals C-sectioned	27	27	26	22
Non-gravid	7	6	3	3
Gravid	20	21	23	19
Resorptions per dam	0.85 ± 0.49[a]	0.47 ± 0.16	0.61 ± 0.16	6.7 ± 0.9[b]
Dams with live young	20	21	23	5
Fetuses per dam	9.05	10.42	8.56	2.15[b]
Viable fetuses	181	219	197	41[b]
Corpora lutea per dam	12.45 ± 0.3	12.7 ± 0.3	12.8 ± 0.3	9.7 ± 0.8
Implantations per dam	9.9	10.9	9.1	9
Necropsy weight (g)	198 ± 2.3	191 ± 2.3	190 ± 2.1[b]	180 ± 2[b]
Male fetuses	82	109	104	18
Female fetuses	99	110	93	23
Fetal weight (g)	3.05 ± 0.05	3.18 ± 0.04	2.88 ± 0.03[b]	2.57 ± 0.08[b]

[a] Mean ± SE.
[b] Significantly different from controls at $P < 0.05$

weight at necropsy (Table 1). Animals receiving the 4000 mg kg^{-1} day^{-1} dose also displayed a moderate erythemia of the skin in the area of the back exposed to the fuel, together with a 53% mortality rate. Analysis of a blood sample from one of the moribund animals revealed a methemoglobin level of 56.7%.

Examination of rat fetuses harvested at necropsy demonstrated that those pups from dams exposed to 2000 and 4000 mg kg^{-1} day^{-1} of OFII weighed significantly less than pups from control dams. Further evidence of fetal toxicity in animals receiving 4000 mg kg^{-1} day^{-1} was indicated by a significant increase in the number of fetal resorptions in this group, together with a decrease in the number of viable fetuses (Table 1). Inspection of fetuses for both visceral and skeletal abnormalities revealed no evidence of terata in any of the animals examined. Several fetuses did display anatomical variations and delays in development. These changes included the lack of a fully developed renal papillae, slight distention of the lateral ventricle and the incomplete ossification of several skeletal structures. The incidence with which these events occured was not significantly higher in pups from treated versus those from control dams.

Rabbit teratology

The rabbits dosed with 1000 mg kg^{-1} day^{-1} of OFII weighed significantly less than those animals in the control, 100 and 316 mg kg^{-1} day^{-1} dose groups on days 20 and 25 of pregnancy. These differences were not present at sacrifice on day 28 of pregnancy or following removal of the pregnant uterus (Fig. 1). Marked erythemia was evident in the area of skin exposed to OFII in the 1000 mg kg^{-1} day^{-1} dose group. Maternal and fetal data collected at necropsy did not reveal significant differences between OFII and control animals (Table 2). Likewise, the overall incidence of malformations and variations noted in the fetal rabbits did not differ significantly between treated versus non-treated animals (Table 3).

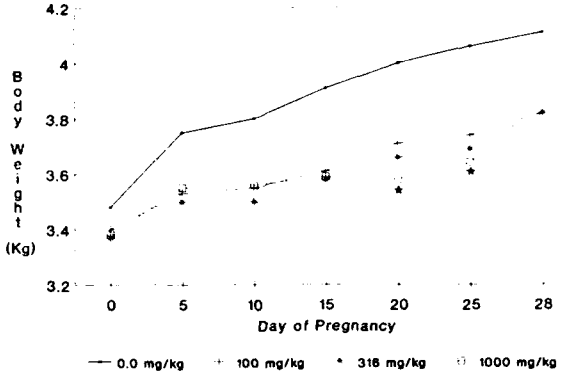

Figure 1. Body weight of female rabbits following dermal exposure to OTTO Fuel II during pregnancy. *Significant difference from controls $P < 0.05$.

DISCUSSION

OFII was administered to the animals' skin in order to emulate the most likely root of human exposure. Likewise, the calculated dose of OFII was administered in small increments over an 8-h period to stimulate exposure to the compound occurring continuously during the workday.

No attempt was made to cover or otherwise restrict the area of skin exposed to OFII. The animals were not observed to lick the area of skin to which the OFII had been applied; however, there is no way to assure that this did not occur to a limited extent. The fact that a small amount of the applied dose may have subsequently been ingested must therefore be considered when evaluating the data.

Pregnant rats exposed to OFII in this study displayed significant maternal toxicity in both the medium and high dose groups. All evidence of fetal toxicity was confined to the offspring of these animals. Analysis of data from the rabbit portion of the study failed to

Table 2. OFII dermal teratology: maternal and fetal reproductive parameters in the rabbit

	OFII (mg kg $^{-1}$ day $^{-1}$)			
	0.0	100	316	1000
Animals inseminated	19	19	19	18
Animals that died	0	0	0	0
Animals C-sectioned	19	19	19	18
Non-gravid	7	8	3	6
Gravid	12	11	16	12
Resorptions per dam	7	7	9	5
Fetuses per dam	5.1 ± 0.75*	6.3 ± 0.92	5.8 ± 0.63	4.7 ± 0.75
Viable fetuses	71	70	94	52
Corpora lutea per dam	10.3 ± 3.8	10.8 ± 4.0	12.4 ± 3.9	12.9 ± 5.2
Implantations per litter	5.57 ± 2.34	6.08 ± 3.0	6.38 ± 2.73	5.58 ± 2.94
Necropsy weight (kg)	3.77 ± 0.07	3.53 ± 0.1	3.51 ± 0.09	3.59 ± 0.14
Male fetuses	33	32	54	28
Female fetuses	38	38	40	24
Fetal weight (g)	40.19 ± 0.57	37.56 ± 0.74	37.95 ± 0.74	38.01 ± 0.83

* Mean ± SE.

Table 3. Summary of the incidence of fetal malformations and developmental variations of rabbits exposed to OTTO Fuel II (mg kg^{-1} day^{-1})

	Control (0)		Low (100)		Medium (316)		High (1000)	
	No. of fetuses	No. of litters	No. of fetuses	No. of litters	No. of fetuses	No. of litters	No. of fetuses	No. of litters
Fetuses examined for soft tissue defects	62	12	71	12	94	16	52	12
Retrocaval ureter	1	1	0	0	0	0	0	0
Cataracts in both eyes	0	0	1	1	0	0	0	0
Common truncus arteriousus	0	0	0	0	1	1	0	0
Postcaval lung agenesis	0	0	0	0	1	1	0	0
Thin areas in muscle of right ventricles	0	0	0	0	0	0	1	1
Short tail	0	0	0	0	0	0	1	1
Fetuses examined for hard tissue defects	62	12	71	12	94	16	52	12
Sternebrae 5 and 6 unossified	3	3	7	7	6	6	4	3
13th rib rudimentary	2	2	3	3	2	2	4	4
Flexure of left wrist	0	0	1	1	0	0	0	0
Delayed ossification of astrangalus bone	0	0	0	0	0	0	1	1

indicate significant fetal toxicity in any of the dose groups. These data suggest that the developmental effects observed following OFII exposure are the result of maternal toxicity.

Acknowledgements

The authors wish to thank Tim Bausman, Brenda Schimmel and Susan Young for their technical assistance, and Roxanne Baer for administrative assistance in preparing the manuscript. This work was supported by the Naval Medical Research and Development Command, Research Task No. 63706NM0096.004.0006. The opinions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23, Revised 1985, and the Animal Welfare Act of 1966, as amended.

REFERENCES

1. S. Foreman, A review of propylene glycol dinitrate toxicology and epidemiology. *Toxicol. Lett.* **43**, 51–65 (1988).
2. D. Clark and M. Litchfield, Toxicity, metabolism, and pharmacologic properties of propylene glycol 1,2-dinitrate. *Toxicol. Appl. Pharmacol.* **15**, 175–184 (1969).
3. D. Clark and M. Litchfield, Metabolism of ethylene glycol dinitrate and its influence on the blood pressure of the rat. *Br. J. Ind. Med.* **24**, 320–325 (1967).
4. S. Erk, P. Newton, J. MacEwen and E. Vernot, Evaluation of the toxicokinetic study of 1,2-propanediol dinitrate (PGDN) in the dog. *Technical Report AFAMRL-TR-82-27.* National Technical Information Service, Springfield, VA (1982).
5. E. Horvath, R. Ilka and J. Boyd, Evaluation of the neurophysiologic effects of 1,2-propylene glycol dinitrate by quantitative ataxia and oculomotor function tests. *Am. J. Ind. Med.* **2**, 365–378 (1981).
6. R. Jones, J. Strickland and J. Siegal, Toxicity of propylene glycol 1,2-dinitrate in experimental animals. *Toxicol. Appl. Pharmacol.* **22**, 128–137 (1972).
7. J. Wyman, B. Gray, L. Lee, J. Coleman, C. Flemming and D. Uddin, Interspecies variability in propylene glycol dinitrate-induced methemoglobin formation. *Toxicol. Appl. Pharmacol.* **81**, 203–212 (1985).
8. C. Smith, Toxicity of butyl stearate, dibutyl sebacate, dibutyl phthalate, and methoxyethyl oleate. *AMA Arch. Ind. Hyg. Occup. Med.* **7**, 310–318 (1953).
9. C. Goworski, H. Leahy, W. Bashe, J. Macewen and E. Vernot, One-year inhalation toxicity study of otto fuel II. *Technical Report AAMRL-TR-85-071, NMRI-85-56.* National Technical Information Service, Springfield, VA (1985).
10. D. Jagannath, Mutagenicity evaluation of otto fuel number 2 in the ames salmonella/microsome plate test. In *Segment Report: NTIS/AD-A112 227/4,* Government Reports Announcement & Index, Issue 15. National Technical Information Service, Springfield, VA (1982).
11. J. Wilson, Embrylogical considerations in teratology. In *Teratology: Principles and Techniques,* ed. by J. Wilson and J. Warkany, pp. 262–277. University of Chicago Press, Chicago (1965).
12. R. Staples and V. Schnell, Refinements in rapid learning techniques in the KOH-alzarin red S method for fetal bone. *Stain Technol.* **39**, 61–61 (1964).
13. J. Gibson, R. Staples and J. Newberne, Use of the rabbit in tertatogenicity studies. *Toxicol. Appl. Pharmacol.* **9**, 398–408 (1966).
14. J. Stuckhardt and S. Poppe, Fresh visceral examination of rat and rabbit fetuses used in teratogenicity testing. *Teratogen. Carcinogen. Mutagen.* **4**, 181–188 (1984).
15. E. Van Julsingha and C. Bennett, Dissecting procedure for the detection of anomalies in the rabbit fetal head. In *Methods in Prenatal Toxicology,* pp. 126–144 PSG Publishing, MA (1977).
16. A. Dawson, A note on the staining of the skelton of cleared specimens with alzarin red S. *Stain Technol.* **1**, 123–124 (1926).