AFRL-RH-FS-TR-2012-0030

Acute Inhalation Toxicity Study of 1,4-Dioxane in Rats (*Rattus norvegicus*)

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July 2012

Interim Report for March 2008 to September 2011

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The experiments reported were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

AFRL-RH-FS-TR-2012-0030 HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION IN ACCORDANCE WITH ASSIGNED DISTRIBUTION STATEMENT.

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
15-07-2012	Interim	Mar 2008 – Sep 2011

4. TITLE AND SUBTITLE
Acute Inhalation Toxicity Study of 1,4-Dioxane in Rats (*Rattus norvegicus*)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
NA

5c. PROGRAM ELEMENT NUMBER
62202F

6. AUTHOR(S)
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5d. PROJECT NUMBER
OAFW

5e. TASK NUMBER
P0

5f. WORK UNIT NUMBER
OAFWP002

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Materiel Command*
Air Force Research Laboratory
711th Human Performance Wing
Human Effectiveness Directorate
Bioeffects Division
Molecular Bioeffects Branch
Wright-Patterson AFB OH 45433-5707

10. SPONSOR/MONITOR'S ACRONYM(S)
711 HPW/RHDJ

11. SPONSORING/MONITORING AGENCY REPORT NUMBER
AFRL-RH-FS-TR-2012-0030

12. DISTRIBUTION AVAILABILITY STATEMENT
Distribution A: Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
1,4-dioxane has been used as a stability additive in a number of industrial solvents and has found its way into the environment, especially in groundwater. At current and former military installations where solvents were used, there has been growing concern for assessing the health risk involving human exposure to dioxane. Consequently, we conducted an acute inhalation study, in which we exposed male and female Fischer 344 rats for 6 hours to 0, 100, 300, 800, 2200 or 6000 ppm dioxane and assessed them at 2 days post-exposure or 2 weeks following a recovery period. We also conducted a two-week inhalation study, in which we exposed male and female rats 6 hours/day, 5 days/week to 0, 100, 1600 or 3200 ppm dioxane and assessed them at 1 day post-exposure or following the 2-week recovery period. In the acute study, two rats in the highest exposure groups had vacuolar changes in their nasal cavities two days post-exposure but they fully recovered after two weeks. In the two-week study, animals responded with numerous degenerative changes (lesions in nasal cavity, liver and kidney; hepatic single-cell necrosis). Male rats in the mid- and high-concentration groups did not recover fully from hepatic single cell necrosis two weeks after exposure. The lowest observed adverse effect level (LOAEL) for 1,4-dioxane in the 2-week study is 100 ppm.

15. SUBJECT TERMS
1,4-dioxane, 123-91-1, inhalation, toxicity, acute study, two-week study, recovery

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
U					David R. Mattie
a. REPORT	b. ABSTRACT	c. THIS PAGE	SAR	29	19b. TELEPONE NUMBER (*Include area code*)
U	U	U			NA

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI-Std Z39-18

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PREFACE

Funding for this project was provided through the Air Force Environmental Restoration Account to support HQ AFMC and the OSD Chemicals and Materials Risk Management Directorate. This research was conducted under contracts FA8650-05-2-6518 and FA8650-10-2-6062. The program manager for the contracts was David R. Mattie, Ph.D. of 711 HPW/RHPB (now RHDJ).

This study was conducted in the spirit of the United States Environmental Protection Agency (U.S. EPA) Good Laboratory Practice (GLP) Standards (40 CFR Part 792). The study protocols were designed to be in general compliance with the U.S. EPA Office of Prevention, Pesticides and Toxic Substances (OPPTS) Guideline 870. 1300 Acute Inhalation Toxicity (1998) and the Organisation for Economic Co-operation and Development (OECD) Guideline OECD 412 Repeated Dose Inhalation Toxicity: 28-day or 14-day Study (OECD, 1981).

These animal studies were approved by the Wright-Patterson Air Force Base Animal Care and Use Committee (protocol number F-WA-2008-0106-A). The study was conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

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1.0 SUMMARY

1,4-dioxane has been used as a stability additive in a number of industrial solvents to improve the performance and the useful life of the solvents. As a component of a number of solvent-containing processes, dioxane has found its way into the environment, especially in groundwater. At current and former military installations where 1,4-dioxane has been used, there is growing concern for cleaning up contamination and assessing the risk of human exposure to dioxane at those sites. An acute inhalation study was conducted with Fischer 344 rats in order to quantitate and characterize a prospective threshold for nasal and respiratory effects from dioxane. Male and female rats were exposed for 6 hours to 0, 100, 300, 800, 2200 or 6000 ppm 1,4-dioxane and assessed at 2 days post-exposure or 2 weeks following a recovery period. A two-week inhalation study with a two-week recovery group also was conducted to detect early cellular changes and to determine if changes were permanent or transient, in order to assess the mode of action of dioxane. Male and female rats were exposed 6 hours/day, 5 days/week to 0, 100, 1600 or 3200 ppm 1,4-dioxane and assessed at 1 day post-exposure or following the 2-week recovery period.

Few changes were observed in the acute study, even at the highest concentration (6000 ppm). Vacuolar changes were observed in the nasal cavities of two rats in the highest two exposure groups at two days post-exposure. This early epithelial degenerative change was not observed among the rats in the two-week recovery groups. The acute study resulted in no permanent effect. The two-week study animals responded to dioxane exposure with numerous degenerative changes. Necropsy of these animals showed lesions in the nasal cavity, liver and kidney, and a number of hepatic single cell necroses. Male rats in the mid- and high concentration groups did not recover fully from hepatic single cell necrosis two weeks after exposure. Therefore changes in the male rat liver after repeated exposures to high concentrations of dioxane do not appear to resolve completely. Changes in the two-week study were seen to some extent at all levels, with recovery in the lowest exposure group. The lowest observed adverse effect level (LOAEL) for 1,4-dioxane in the 2-week study is 100 ppm.

2.0 INTRODUCTION

1,4-dioxane has been used as a stability additive in a number of industrial solvents to improve the performance and the useful life of the solvents. As a component of a number of solvent-containing processes, dioxane has found its way into the environment, especially in groundwater. It is a volatile organic compound miscible in both water and organic solvents, and its hydrophilicity tends to enhance its presence in and movement with groundwater (ATSDR, 2012). These properties also cause it to be resistant to the treatment and cleanup technologies commonly used to capture and treat most solvents. At current and former military installations where dioxane has been used, there is growing concern for cleaning up contamination and assessing the risk of human exposure to dioxane at those sites.

Male and female rats exposed to various levels of dioxane (10 to 5000 ppm) in chronic (1 to 2 years) drinking water studies develop hepatocellular carcinomas (Argus *et al.*, 1965; 1973; Hoch-Ligeti *et al.*, 1970; Kano *et al.*, 2009; Kociba *et al.*, 1974; NCI, 1978; Yamazaki *et al.*, 1994). Rat hepatic tumor incidence has been shown to occur in a dose-dependent manner (Argus *et al.*, 1973; Kociba *et al.*, 1974; NCI, 1978; Yamazaki *et al.*, 1994). Male and female mice also develop hepatocellular tumors during chronic drinking water studies (500 to 8000 ppm) (Kano *et al.*, 2009; NCI, 1978; Yamazaki *et al.*, 1994).

In addition to hepatocellular carcinomas, nasal tumors were found in several of these chronic drinking water studies in rats (Hoch-Ligeti *et al.*, 1970; Kociba *et al.*, 1974; NCI, 1978; Yamazaki *et al.*, 1994). A few incidences of mouse nasal tumors following exposure to 8000 ppm dioxane in the drinking water were also noted by Yamazaki *et al.* (1994) and Kano *et al.* (2009) but nasal tumors were not found among male or female mice in the National Cancer Institute study at lower exposure concentrations (500 or 1000 ppm) (NCI, 1978).

A two-week drinking water study (1110 to 90000 ppm) resulted in hepatic swelling and vacuole changes in the centrilobular region, enlargement of epithelial cells in the nasal cavity, proximal tubule changes in the kidney and some brain vacuole changes were seen at higher doses in rats. Unlike rats, mice responded with only the hepatocellular changes (JBRC, 1998). The follow-on 13-week subchronic drinking water study (640 to 25000 ppm) resulted in increased plasma levels of liver function markers in both rats and mice. Cellular changes were noted in the nasal passages, liver, kidney and brains of rats, while changes in mice were seen in the nasal passages and liver (Kano *et al.*, 2008). These studies catalog the cellular damage and probable precursor states for non-genotoxic carcinomas seen in the chronic studies.

The specificity of the site of epidermal nasal tumor formation in the rat nose (the anterior portion of the dorsal meatus) led to supposition that dioxane causes these tumors through contact with water when sipped from tubes during drinking water studies (Goldsworthy *et al.*, 1991). Studies by Battelle (2007) and Sweeney *et al.* (2008) independently confirmed direct contact of rat nasal tissues with drinking water from sipper tubes using fluorescent water soluble dyes.

As a volatile organic compound, dioxane exposure may also occur through inhalation. Several inhalation studies have been conducted. In 1974, Torkelson *et al.* reported a chronic inhalation study using male and female Wistar rats. Rats were exposed to 0 or 111 ppm dioxane for 7

hours/day, 5 days/week for 2 years. No treatment-related lesions or carcinomas were found in either the livers or nasal passages of these rats. Another chronic carcinogenicity study was conducted with male Fischer 344 (F344) rats (0, 50, 250 or 1250 ppm, 6 hours/day, 5 days/week, 104 weeks). The high exposure group rats developed nasal squamous cell carcinomas, hepatocellular adenomas and peritoneal mesotheliomas. Incidences of renal cell carcinomas, mammary fibroadenomas and Zymbal gland adenomas increased dose dependently. All exposed groups responded with nasal cavity and hepatic pre-neoplastic lesions, regardless of exposure level (Kasai *et al.*, 2009).

A 13-week inhalation study (100 to 6400 ppm, 6 hours/day, 5 days/week) was conducted with male F344 rats. Nuclear enlargement of the nasal epithelial cells was seen in the 100 ppm group. Plasma liver function markers were elevated and pre-neoplastic lesions were observed in the livers of rats exposed to 1600 ppm dioxane and higher. The lowest observable adverse effect level (LOAEL) was found to be 100 ppm, based on the nasal epithelial changes (Kasai *et al.*, 2008). The responses to dioxane exposure in this study were very similar to the changes seen in the short-term and subchronic drinking water studies discussed above.

Herein described we conducted an acute inhalation study using rats in order to quantitate and characterize a prospective threshold for nasal and respiratory effects from 1,4-dioxane. The two-week study with a two-week recovery group was conducted to detect early cellular changes and to determine if changes were permanent or transient, in order to assess the mode of action of dioxane. In addition, a recovery group had not been included in any repeated exposure studies to date. Both studies help to fill a data gap in the dioxane risk assessment database.

3.0 METHODS

The acute study with sample collection after exposure and following two weeks of recovery was intended to provide the toxicity threshold for dioxane associated with acute inhalation exposure. The study design was based on the U.S. Environmental Protection Agency (U.S. EPA) Office of Prevention, Pesticides and Toxic Substances (OPPTS) 870.1300 Acute Inhalation Toxicity guideline. Every attempt was made to conform to the most recent version of the Good Laboratory Practice (GLP) standard, U.S. EPA Good Laboratory Practice Standards (40 CFR Part 792).

The two-week study design was based on the Organization for Economic Cooperation and Development (OECD) guideline: 412: Repeated Dose Inhalation Toxicity: 28-day or 14-day Study. All portions of this study adhered to the study protocol and standard operating procedures.

3.1 Test Substance

The solvent additive 1,4-dioxane ((formula: $C_4H_8O_2$); CAS # 123-91-1), also known as diethylene dioxide or diethylene ether, has a molecular weight of 88.10 g/mole. The chemical

stock was purchased from Sigma-Aldrich, Inc. (St. Louis MO) with a purity of greater than 99.0 percent.

3.2 Test Animals

Albino inbred Fischer (CDF®) [F344/DuCrl] rats were purchased from Charles River Laboratories (Wilmington MA) for the studies. Males weighed 150-200 g and females weighed 125-175 g at exposure onset.

For the acute study, ten male and ten nulliparous, non-pregnant female rats were randomly selected for each exposure group (Table 1). The number of animals/sex/group (10) was selected to provide sufficient information for determining the threshold as well as setting target exposure concentrations for the two-week inhalation toxicity study.

Table 1. Animals Assigned for Each Target Exposure Group in the Acute Study

Target Exposure Level (ppm)	**Number of Animals**	
	Males	Females
0	10	10
100	10	10
300	10	10
800	10	10
0	10	10
2200	10	10
6000	10	10
Total	70	70

The exposure levels for the two-week study were selected based on results from the acute study. The selected target concentrations for the two-week study were 0, 100, 1600 and 3200 ppm of dioxane (See Table 2). Sixteen animals per group per sex were randomly assigned to exposure groups. The number of animals/sex/group (16) was selected to provide sufficient information for determining the threshold for effects after a repeated series of exposure concentrations.

Table 2. Animals Assigned for Each Target Exposure Group in the Two-Week Study

Target Exposure Level (ppm)	Number of Animals	
	Males	Females
0	16	16
100	16	16
1600	16	16
3200	16	16
Total	64	64

In both studies, rats were individually housed in a polycarbonate shoebox caging system with Cell-Sorb bedding (Fangman Specialties, Inc., Cincinnati OH). Rats were transferred into freshly bedded and sanitized cages at least twice weekly. Prior to and between exposure periods, rats were kept in sanitizable animal holding rooms designed to provide 10 to 15 complete fresh air changes per hour. Room air temperature and humidity were maintained between 64 to 79 °F (21 to 26 °C) and 30 to 70 percent, respectively. Full spectrum fluorescent light was provided on a 12:12 hour light:dark cycle. Rodent chow (Formulab Certified Rodent Diet #5002, PMI Nutrition International, Inc., Brentwood MO) and fresh reverse osmosis treated water were provided *ad libitum* throughout the study, except during exposure periods.

3.3 Exposure Conditions and Administration

Exposures for both studies took place in 690 liter chambers. The chambers were operated at a flow rate of 180 liters per minute total air flow to provide 15 air changes/hour. Animals were individually housed in suspended stainless steel mesh cages in the exposure chambers. Animals were acclimated over five days to chamber conditions prior to exposure. Exposure simulation experiences were limited to 1.5 hours on the first day and incrementally extended to 6.5 hours on the fifth day. Chamber temperature and relative humidity were maintained between 20 to 24 °C and 30 to 70 percent, respectively.

The exposure levels for the acute study were selected based on the literature results of previous oral drinking water studies and a low level inhalation study with dioxane. Five exposure levels were required to identify the threshold exposure level for effects. Because the facility had four exposure chambers available, the study consisted of two sets of exposures, with a control group exposed to clean air for each set. In the first set, target concentrations were 0, 100, 300 and 800 ppm; the second set target concentrations were 0, 2200 and 6000 ppm of dioxane. Acute study rats were exposed for six hours on a single exposure day. A two-week recovery period followed for half the animals.

The two-week study utilized all four exposure chambers for the target concentrations: 0, 100, 1600 and 3200 ppm. Rats were exposed for six hours a day, five days a week for two weeks. A two-week recovery period followed for half the animals.

In both studies, the dioxane was pumped from a glass reservoir using fluid metering injection pumps at a rate corresponding to the desired final concentration. The liquid was introduced into the top of a heated glass column and captured on glass wool. Heated air was passed through the column causing vaporization of the dioxane. Any aerosol developed was captured by a patch of glass wool upstream (Reboulet and Lear, 2010). Fourier transform infrared (FTIR) spectrophotometers were pre-calibrated for each of these concentration ranges. Exposure chamber concentrations were continuously sampled and the concentration determined approximately every 40 seconds by FTIR analysis for each entire 6 hour exposure. Nominal concentrations were determined by measuring the pumping rate in mL/minute prior to the exposure and dividing that by the total flow rate. The levels of test article were generated by dilution with filtered air.

Animals were exposed, whole-body, for six hours to dioxane vapor. Following exposure, the animals remained in the chamber for 20 minutes while fresh air was pumped into the chamber. This post-exposure period was sufficient to remove measurable dioxane levels from the chamber, allowing researchers to handle the rats without exposing themselves.

3.4 Observations and Necropsy

Animals were checked hourly during exposure. Animals were observed directly following removal from the chamber and once daily for overt signs of toxicity. Clinical observations included general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia as well as evaluations of respiration, palpation for tissue masses, circulatory effects, autonomic effects, central nervous system effects, and reactivity to handling or sensory stimuli. Body weights were recorded at randomization, daily prior to the exposure session(s), weekly during the post-exposure period, and at necropsy.

For the acute study, half the rats in each group were euthanized and necropsied two days after the beginning of exposure, per the EPA OPPTS 870.1300 Acute Inhalation Toxicity guideline (U.S. EPA, 1998). The remaining animals were euthanized at two weeks post-exposure, following a recovery period.

For the two-week study, half the rats were euthanized for necropsy one day after the beginning of the final (tenth) exposure. The remaining animals were euthanized at two weeks post-exposure, following a recovery period.

In both studies, necropsy included examination of the external surface and all orifices; the organs and tissues of the cranial, thoracic, abdominal and pelvic cavities and neck; and the remainder of the carcass. Wet weights of the liver and kidneys were obtained after dissection. Histopathology was conducted on liver, kidneys, urinary bladder, lungs, trachea and nasal cavities. Lung and nasal cavity histopathology was performed which included preparation of six levels of the nasal cavities and lung fixation by constant pressure perfusion. Tissues were prepared by formalin fixation and paraffin embedding; 5 μm hematoxylin and eosin stained sections were examined microscopically.

In the two-week study, animals were fasted overnight prior to their respective necropsy days, as diet interferes with clinical chemistry observations. Blood was collected via the inferior vena cava following pentobarbital anesthesia administered intraperitoneally. Blood samples were analyzed for hematology and serum chemistry parameters.

3.5 Data Analysis

Mean body weight values and organ weight values were analyzed by t-test and analysis of variance (ANOVA). Data were tested for sex differences, as well as control versus treated differences. Significance was set at $p < 0.05$.

4.0 RESULTS

4.1 Acute Study

The average observed concentrations of dioxane for each target exposure in the acute study are listed in Table 3.

Table 3. Comparison of Target and Observed Chamber Concentrations during Acute Dioxane Exposure

	Target Concentration (ppm)				
	100	**300**	**800**	**2200**	**6000**
Observed Concentration (ppm)	119.19	281.32	797.54	2197.86	6002.06
Standard Deviation	*108.16	40.44	34.74	19.43	223.24
Nominal ppm	145.0815	361.8721	907.2618	2127.349	6059.01
Percent of nominal	82.15%	77.74%	87.91%	103.31%	99.06%

Note: *A problem in the air handling system of this chamber resulted in a large spike in concentration during the first hour; the issue was resolved.

4.1.1 Nasal Cavity: Vacuolar change was observed in the olfactory and respiratory epithelium of two rats in the 2200 ppm and 6000 ppm exposure groups, respectively, at two days post-exposure. This change was attributed to the test article. Vacuolar changes were not observed among the two-week recovery group.

4.1.2 Incidental Findings: No statistically significant changes in organ or body weight were observed in rats used in the acute study (data not shown).

4.1.2.1 Nasal Cavity: Minimal serous exudate and few acute and chronic leukocyte infiltrates were observed in a small number of rats distributed across all groups, controls and treated, excluding the 300 ppm, in both the 2-day post-exposure and 2-week recovery necropsy groups. The changes are attributed to environment irritants and/or a mild resolving bacterial infection.

4.1.2.2 Lung: One rat in the 100 ppm, 2-week recovery group, had a focal accumulation of alveolar histiocytes.

4.1.2.3 Liver: Minimal chronic infiltrates were found in all groups, control and treated, two-day post-exposure and two-week recovery necropsy groups. The infiltrates are considered an aging change.

4.1.2.4 Kidneys: Renal mineralization, a common finding in rats, especially females, was observed in most females and a few males in all groups. Mineralization is considered an aging change.

4.2 Two-Week Study

The average observed concentrations of dioxane for each target exposure in the two-week study are listed in Table 4.

Table 4. Comparison of Target and Mean Observed Chamber Concentrations during Two-Week Dioxane Exposure

	Target Concentration (ppm)		
	100	**1600**	**3200**
Mean Observed Concentration (ppm)	104.80	1554.24	3245.21
Standard Deviation	4.38	44.46	40.40

4.2.1 Clinical Pathology and Hematology: Both the clinical pathology and hematology analyses showed no statistically significant findings in rats after either two weeks of exposure or two weeks of recovery (data not shown).

4.2.2 Nasal Cavity: Nasal cavity lesions were more severe and prevalent at higher exposure levels. Severity and prevalence increased when given more time post-exposure to develop and progress.

4.2.3 Liver: Single cell necroses occurred proportionally to the exposure level, and was more severe among rats in the one-day post-exposure group as opposed to those in the two-week recovery groups. Hepatic degeneration was observed in the centrilobular area of five females in the 3200 ppm, 1-day post-exposure group. The lesion was minimal to mild in 40 to 60 percent of the affected females, respectively.

4.2.4 Kidneys: Tubular degeneration occurred in all rats tested, except one in the 100 ppm, 2 week recovery group. The severity of tubular degeneration increased with the exposure level. Hyaline droplets were found in all males except those in the 100 ppm, 1-day post-exposure group. Incidence of the droplets in the other one-day post-exposure groups was higher. Chronic interstitial inflammation was an uncommon finding, in males and females, with the exception of a slight increase in severity in five females in the 3200 ppm, 2 week post-exposure group.

4.2.5 Incidental Findings: Multiple incidental findings were noted during necropsy.

4.2.5.1 Trachea: One rat in the 3200 ppm 2-week recovery group had a slight increase in chronic mucosal infiltrates, but the finding was incidental and not clinically significant.

4.2.5.2 Lungs: Two rats, one in the 100 ppm and the other in the 3200 ppm, 1-day post-exposure necropsy groups, had multifocal, mild chronic perivascular infiltrates, but the finding was incidental and not clinically significant.

4.2.5.3 Liver: Chronic hepatic infiltrates were found in all exposure groups, in both males and females. No correlation existed between the incidence of chronic hepatic infiltrates and exposure time, but females were more severely affected, especially in the 1-day post-exposure group (Table 6).

4.2.5.4 Kidneys: All rats in all groups exhibited some degree of degenerative and/or toxic nephropathy. Degenerative nephropathy is not uncommon in rats and is due to the predisposing factors of age, sex, strain, diet, immunity and the endocrine system. Three males in the two-week recovery control group had accumulations of intracytoplasmic protein. This was attributed to early and more acute progression of chronic progressive glomerular nephropathy.

4.2.6 Statistical Analysis: A statistical analysis was conducted to determine the significance of lesions found. Only the presence of lesions was considered, not the number of lesions in a specific organ. Seventy percent of the lesions were rare/minimal, 25 percent mild and 5 percent moderate. None of the lesions were severe. A Fisher's exact test was used to compare the four exposure groups in combination and in pairs. Results are displayed in Table 5.

Table 5. Comparison of Exposure Groups for Lesions in the Nasal Cavity, Liver, and Kidney using Fisher's Exact Test to Compare all Four Exposure Groups Together and Pairwise

Lesion	Time Point	Sex	p-values from Fisher's Exact Test						
			All 4 Exposures	Control vs. 100	Control vs. 1600	Control vs. 3200	100 vs. 1600	100 vs. 3200	1600 vs. 3200
Nasal Cavity									
Nuclear Enlargement: Respiratory Epithelium	1 day	M	1	-	-	1	-	1	1
		F	-	-	-	-	-	-	-
	2 wk	M	0.5871	-	0.4667	1	0.4667	1	1
		F	0.5871	-	0.4667	1	0.4667	1	1
Nuclear Enlargement: Olfactory Epithelium	1 day	M	0.1279	-	0.4667	0.2000	0.4667	0.2000	1
		F	**0.0064**	-	0.4667	0.0256	0.4667	0.0256	0.3147
	2 wk	M	**0.0001**	-	**0.0014**	**0.0070**	**0.0014**	**0.0070**	1
		F	**0.0001**	-	0.0256	**0.0002**	**0.0256**	**0.0002**	0.2000
Erosion: Maxillo-turbinate	1 day	M	0.2258	-	-	0.4667	-	0.4667	0.4667
		F	-	-	-	-	-	-	-
	2 wk	M	**0.0345**	-	1	0.0769	1	0.0769	0.2821
		F	-	-	-	-	-	-	-
Erosion: Vestibule	1 day	M	1	-	-	1	-	1	1
		F	1	-	-	1	-	1	1
	2 wk	M	0.0452	-	0.2000	-	0.2000	-	0.2000
		F	1	-	1	1	1	1	1
Liver									
Necrosis: Single Cell	1 day	M	0.5871	0.4667	1	0.4667	1	1	1
		F	**0.0008**	**0.0406**	**0.0070**	**0.0070**	1	1	1
	2 wk	M	1	1	1	1	1	1	1
		F	0.4443	1	1	0.4667	0.5692	0.2000	1
Inflammation: Chronic	1 day	M	1	1	1	1	1	1	1
		F	0.2258	0.4667	0.4667	0.4667	1	1	1
	2 wk	M	0.2582	1	0.5692	0.1189	1	0.3147	0.6193
		F	0.4232	0.3147	0.3147	1	1	0.6084	0.6084
Degeneration	1 day	M	-	-	-	-	-	-	-
		F	**0.0011**	-	-	**0.0256**	-	**0.0256**	**0.0256**
	2 wk	M	-	-	-	-	-	-	-
		F	-	-	-	-	-	-	-
Kidney									
Nephropathy: Hyaline Droplet	1 day	M	**0.0001**	-	**0.0070**	**0.0002**	**0.0070**	**0.0002**	0.4667
		F	-	-	-	-	-	-	-
	2 wk	M	0.8584	1	1	1	1	0.6084	0.6084
		F	-	-	-	-	-	-	-
Nephropathy: Vacuolar Change	1 day	M	**0.0001**	-	**0.0014**	**0.0002**	**0.0014**	**0.0002**	1
		F	**0.0001**	-	**0.0014**	**0.0002**	**0.0014**	**0.0002**	1
	2 wk	M	-	-	-	-	-	-	-
		F	1	1	-	-	1	1	-
Nephropathy: Tubular Degeneration	1 day	M	0.1279	0.2000	1	0.2000	0.4667	1	0.4667
		F	**0.0081**	0.6084	1	**0.0070**	0.6084	0.0769	**0.0070**
	2 wk	M	0.5871	1	1	1	0.4667	1	0.4667
		F	**0.0001**	0.4667	1	**0.0070**	1	**0.0002**	**0.0014**
Inflammation: Chronic Interstitial	1 day	M	0.2258	0.4667	0.4667	0.4667	-	-	-

Notes: F – female; M = male; wk – weeks; Bolded p values are significant ($p \leq 0.05$)

The presence of chronic hepatic infiltrates was also analyzed statistically. A Fisher's exact test was also used in the same manner described above. Significant lesions were found in the olfactory epithelium, at the 1600 ppm and 3200 ppm exposure levels, with the most significant findings at two weeks post-exposure. Female livers examined one day after exposure showed significant differences from the control of single cell necrosis occurrences at all exposure levels. The nephropathy of males in the 1600 ppm and 3200 ppm, 1-day post-exposure groups showed significant hyaline droplet formation and vacuolar change. In the 1-day post-exposure necropsy, females showed vacuolar change in the 1600 ppm and 3200 ppm exposure groups; in both the 1-day and 2-week post-exposure necropsies, female rats showed degeneration in the 3200 ppm exposure groups (Tables 6 and 7).

Table 6. Comparison of Exposure Groups for Chronic Hepatic Infiltrates at each Location using Fisher's Exact Test to Compare all Four Exposure Groups Together and Pairwise

Time Point	Sex	p-values from Fisher's Exact Test						
		All 4 Exposures	**Control vs. 100**	**Control vs. 1600**	**Control vs. 3200**	**100 vs. 1600**	**100 vs. 3200**	**1600 vs. 3200**
Centrilobular								
1 day	M	1	1	1	1	1	1	1
	F	0.5871	1	0.4667	0.4667	1	1	1
2 wk	M	0.1205	1	0.2000	0.0769	0.5692	0.2821	1
	F	0.5551	0.3147	0.3147	0.6084	1	1	1
Midzonal								
1 day	M	0.1279	1	0.4667	0.4667	0.2000	0.2000	*
	F	**0.0359**	**0.0406**	1	1	**0.0406**	0.1319	1
2 wk	M	0.1279	*	0.4667	0.2000	0.4667	0.2000	1
	F	0.7913	0.5692	1	1	1	0.5692	1
Periportal								
1 day	M	0.2258	*	*	0.4667	*	0.4667	0.4667
	F	0.5871	0.4667	1	*	1	0.4667	1
2 wk	M	1	1	1	1	1	1	*
	F	0.4443	1	1	0.4667	0.5692	0.2000	1

Notes: F – female; M = male; wk – weeks; * indicates no lesions for any exposure group in the test; p-values for paired comparisons are two-tailed; Bolded values are significant ($p \leq 0.05$)

Table 7. Comparisons vs. Control[1] for Lesions and Chronic Hepatic Infiltrates[2]

	1-Day Post-Exposure Group								2-Week Recovery Group							
	Male				Female				Male				Female			
	C	100	1600	3200	C	100	1600	3200	C	100	1600	3200	C	100	1600	3200
Nasal Cavity																
Nuclear Enlargement: Respiratory Epithelium	0	0	0	1	0	0	0	0	0	0	2	1	0	0	2	1
Nuclear Enlargement: Olfactory Epithelium	0	0	2	3	0	0	2	5*	0	0	7**	6**	0	0	5*	8**
Erosion: Maxilloturbinate	0	0	0	2	0	0	0	0	0	0	1	4	0	0	0	0
Erosion: Vestibule	0	0	0	1	0	0	0	1	0	0	3	0	0	0	1	1
Liver																
Necrosis: Single Cell	6	8	7	8	2	7*	8**	8**	8	7	8	8	6	5	7	8
Degeneration	0	0	0	0	0	0	0	5*	0	0	0	0	0	0	0	0
Inflammation: Chronic	3	4	3	4	6	8	8	8	1	2	3	5	3	6	6	4
Centrilobular	3	3	3	3	6	7	8	8	0	1	3	4	2	5	5	4
Midzonal	2	3	0	0	1	6*	1	2	0	0	2	3	1	3	2	1
Periportal	0	0	0	2	0	2	1	0	1	1	0	0	2	3	1	0
Kidney																
Nephropathy: Hyaline Droplet	0	0	6**	8**	0	0	0	0	3	4	4	2	0	0	0	0
Nephropathy: Vacuolar Change	0	0	7**	8**	0	0	7**	8**	0	0	0	0	0	1	0	0
Nephropathy: Tubular Degeneration	5	8	6	8	2	4	2	8**	7	8	6	8	2	0	1	8**
Inflammation: Chronic Interstitial	2	0	0	0	0	0	0	1	2	1	0	0	1	1	0	5

Notes: [1]Fisher's Exact test of Control (C) vs. 100, 1600 and 3200 ppm; [2]Each cell identifies number of rats with lesions where n = 8 rats, except n = 7 for liver/1-day/female/control; * $0.01 < p \leq 0.05$; ** $p \leq 0.01$

4.2.7 Hepatic Single Cell Necrosis (SCN): One female's liver from the one-day post-exposure control group could not be examined. Of the remaining 127 rats, the range of SCN incidence was from 0 to 50 with a median of 3. Sixteen rats exhibited no SCN lesions, with 9 of these coming from the control group and none coming from the 3200 ppm group. An ANOVA was performed for each post-exposure group separately, using SCN as the dependent variable with factors of gender and exposure group. Residual analyses of the skewness of the results did not show serious departures from assumptions of normality or equal variance. There was a significant interaction between gender and exposure group in the one-day post-exposure group {$F(3,55) = 7.16$, $p = 0.0004$} and a near significant interaction in the two-week recovery group {$F(3,56) = 2.74$, $p = 0.0517$}. Based on these results, it was decided to compare exposure groups separately for each combination of post-exposure group and gender (i.e., the exposure group remained the only factor). The F-test showed a significant difference among the exposure groups in the one-day post-exposure group for males ($p = 0.0001$) and females ($p = 0.0001$), and among the two-week recovery group of males ($p = 0.0221$) but not females ($p = 0.1096$). Dunnett's test was used to compare control vs. the 100, 1600 and 3200 ppm exposure groups for each post-exposure group/gender combination. Results are shown in Table 8 and Figure 1.

Table 8. Hepatic Single Cell Necrosis Pairwise Comparisons of Exposure Group for each Post-exposure Group/Gender Combination using Dunnett's Test, Two-Tailed T-Tests with Pooled Error from the ANOVA, Two-Tailed Paired T-Tests with No Pooling from the ANOVA, and Wilcoxon Rank Sum Test

Group	Exposure		Dunnett's Test Means & Difference			Pooled t-test	Non-Pooled t-test		Wilcoxon Rank Sum
	Level 1	Level 2	Level 1	Level 2	Diff	p	DF	p	p
1-Day Post-Exp. Male	Control	100	3.0	1.8	1.3	0.4156	8.5	0.3112	0.7984
	Control	1600	3.0	1.6	1.4	0.3711	9.0	0.2736	0.6454
	Control	3200	3.0	8.9	-5.9**	**0.0006**	14.0	**0.0130**	**0.0177**
	100	1600	1.8	1.6	0.1	0.9347	14.0	0.8257	0.8785
	100	3200	1.8	8.9	-7.1	**0.0001**	7.6	**0.0044**	**0.0005**
	1600	3200	1.6	8.9	-7.3	**0.0001**	7.8	**0.0040**	**0.0005**
1-Day Post-Exp. Female	Control	100	1.4	5.1	-3.7	0.4513	13.0	0.0849	0.0541
	Control	1600	1.4	17.0	-15.6**	**0.0033**	7.7	**0.0092**	**0.0017**
	Control	3200	1.4	26.8	-25.3**	**0.0001**	7.7	**0.0006**	**0.0003**
	100	1600	5.1	17.0	-11.9	**0.0171**	8.9	**0.0325**	**0.0330**
	100	3200	5.1	26.8	-21.6	**0.0001**	8.9	**0.0013**	**0.0002**
	1600	3200	17.0	26.8	-9.8	**0.0465**	14.0	0.1394	0.0939
2-Week Recovery Male	Control	100	3.8	6.3	-2.5	0.2721	9.7	0.2962	0.5054
	Control	1600	3.8	5.4	-1.6	0.4725	14.0	0.2471	0.2786
	Control	3200	3.8	10.9	-7.1**	**0.0035**	14.0	**0.0055**	**0.0030**
	100	1600	6.3	5.4	0.9	0.6980	14.0	0.7080	0.8785
	100	3200	6.3	10.9	-4.6	**0.0475**	14.0	0.1275	0.1176
	1600	3200	5.4	10.9	-5.5	**0.0201**	14.0	**0.0254**	**0.0330**
2-Week Recovery Female	Control	100	1.8	1.6	0.1	0.9092	14.0	0.8999	0.9188
	Control	1600	1.8	4.0	-2.3	**0.0477**	14.0	0.0833	0.0650
	Control	3200	1.8	3.1	-1.4	0.2162	14.0	0.2338	0.1605
	100	1600	1.6	4.0	-2.4	**0.0374**	14.0	**0.0431**	0.0740
	100	3200	1.6	3.1	-1.5	0.1784	14.0	0.1374	0.2147
	1600	3200	4.0	3.1	0.9	0.4275	14.0	0.4731	0.4120

Notes: [1]Dunnett's test (Control vs. 100, 1600 and 3200 ppm): * 0.05, ** 0.01 joint significance level for each post-exposure group/gender combination; Pooled t-test, Non-pooled t-test and Wilcoxon rank sum: Bolded values are significant ($p \leq 0.05$)

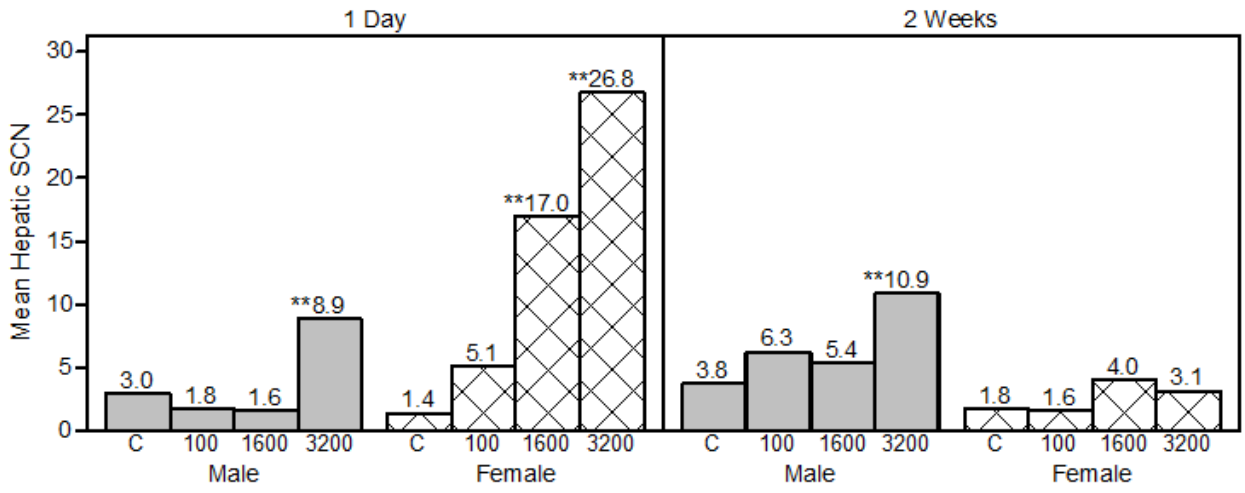

Figure 1. Dunnett's Test (Control vs. 100, 1600 and 3200 ppm) for Hepatic Single Cell Necrosis

Notes: * 0.05 and ** 0.01 joint significance level for each post-exposure group/gender combination; From ANOVA gender*exposure group interaction tests: one-day post-exposure group - p = 0.0004, two-week recovery group - p = 0.0517

5.0 DISCUSSION AND CONCLUSIONS

Both the acute and two-week studies presented herein were designed to detect a prospective threshold and mechanism for nasal and respiratory effects from 1,4-dioxane. Few changes were observed in the acute study, even at the highest concentration (6000 ppm). This exposure level was comparable to the high exposure group in the Kasai *et al.* (2008) study (6400 ppm), which resulted in 100 percent lethality during the first week of a 6 hours/day, 5 days/week, 13-week study.

The only study-attributable finding during necropsy of the acute exposure study was vacuolar change in the nasal cavities of two rats in the highest two exposure groups at two days post-exposure. An early epithelial degenerative change, this observation is consistent with early stages of the degenerative changes seen in the longer term study (Kasai *et al.*, 2008). Since the changes were not observed among the rats in the two-week recovery groups, the changes are considered reversible. The paucity of lesions observed in the acute study is most likely attributed to the brevity of exposure to the test article. Therefore, short-term inhalation exposure to high levels of dioxane would not be expected to result in permanent adverse effects.

Generally, the acute study results help determine the target concentrations for a two-week study. Due to the lack of responses in the acute exposures, the mid and high exposure groups for the two-week study were based on the Kasai *et al.* (2008) 13-week study, where effects were seen beginning at either 1600 or 3200 ppm. Since the two-week study was designed to address the mechanism of action for dioxane, a two-week recovery group was included to determine if changes seen during exposure were permanent or transient. Additionally, the two-week recovery also allowed for potential delayed responses to the test article to manifest.

The two-week study animals responded to dioxane exposure with numerous degenerative changes. Necropsy of these animals showed lesions in the nasal cavity, liver and kidney, and a number of hepatic single cell necroses. These lesions correspond with the more advanced findings in the Kasai *et al.* (2008) 13-week dioxane inhalation study. Male rats in the mid- and high-concentration groups did not recover fully from hepatic single cell necrosis two weeks after exposure. Therefore changes in the liver after repeated exposures to high concentrations of dioxane do not appear to resolve completely. A fairly high incidence of liver and kidney background lesions in the control rats may indicate that this strain is more susceptible to hepatic and renal effects caused by 1,4-dioxane.

Although hyaline droplets were found in all treated male rats except the lowest exposure level at the one-day post-exposure time point, the occurrence of these droplets is a male rat specific response to aromatic hydrocarbon exposure. Hyaline droplets are caused by the formation of α2-microglobulin proteins that are not formed in human kidneys and are therefore not significant to considerations of human health (Alden, 1986; Flamm and Lehman-McKeeman, 1991).

In conclusion, few degenerative changes were seen following an acute six hour exposure of rats to 1,4-dioxane. A two-week recovery period appears to be sufficient to reverse any changes caused by the acute exposure. However, a two-week exposure to levels of dioxane resulted in multiple degenerative changes in agreement with the lesions seen in a longer inhalation study (Kasai *et al.*, 2008). Further, the changes align with the nasal and hepatic neoplasms observed in a chronic two-year inhalation study with dioxane (Kasai *et al.*, 2009). Changes seen in the two-week study were seen to some extent at all exposure levels, with recovery in the lowest exposure group. The LOAEL for 1,4-dioxane in the 2-week study is 100 ppm.

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LIST OF ACRONYMS

ANOVA	analysis of variance
DTIC	Defense Technical Information Center
F344	Fischer 344
FTIR	Fourier transform infrared
GLP	Good Laboratory Practice
LOAEL	lowest observable adverse effect level
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
SCN	single cell necrosis
U.S. EPA	U.S. Environmental Protection Agency