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Toxicology of octamethylcyclotetrasiloxane (D_4)

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ABSTRACT

Octamethylcyclotetrasiloxane (D_4) is a volatile cyclic siloxane used primarily as a monomer or intermediate in the production of some silicon-based polymers widely used in industrial and consumer applications and may be present as a residual impurity in a variety of consumer products. A robust toxicological data set exists for D_4. Treatment-related results from a chronic inhalation study conducted in rats are limited to mild effects on the respiratory tract, increases in liver weight, increases in the incidence of uterine endometrial epithelial hyperplasia, and a dose-related trend in the incidence of endometrial adenomas. The observed increases in liver weight appear to be related to the induction of hepatic metabolizing enzymes, similar to those that are induced in the presence of phenobarbital. D_4 is not mutagenic or genotoxic in standard *in vitro* and *in vivo* tests; therefore, the benign uterine tumors observed likely occur by a non-genotoxic mechanism. Results from mechanistic studies suggest that D_4 has very weak estrogenic and antiestrogenic activity, as well as dopamine agonist-like activity. In rats, D_4 exposure delays ovulation and hypothesized to prolong exposure of the uterine endometrium to endogenous estrogen. Though this mode of action may play a role in the development of benign uterine tumors in the rat, it is considered unlikely to occur in the human due to the marked differences in cycle regulatory mechanisms. Reproductive effects were observed following D_4 exposure in female rats. These effects appear to be related to a delay of the luteinizing hormone (LH) surge, which fails to induce complete ovulation in the rat. However, based on differences in ovulatory control in rats and humans, it appears these effects may be species-specific with no risk or relevance to human health. Results from pharmacokinetic studies indicate that dermal absorption of D_4 is limited, due to its high volatility and, if absorbed via dermal, oral or inhalation exposure, the majority of D_4 is rapidly cleared from the body, indicating bioaccumulation is unlikely.

1. Introduction

Octamethylcyclotetrasiloxane (D_4), CAS RN 556-67-2, is a low-molecular-weight volatile cyclic siloxane used primarily as a monomer or intermediate in the production of silicon-based polymers for industrial and consumer applications and may be present as a residual impurity in a variety of consumer products. The low boiling point of D_4 (175 °C) results in its high volatility, and limited exposure via inhalation is possible in occupational settings. It also displays a rather low water solubility (56 µg/L) and high lipophilicity. As such, there are potential dermal and inhalation routes of exposure to consumers, the general public, workers involved in the manufacture of D_4, and workers involved in the production of polymers and products containing D_4.

This manuscript summarizes the results of the toxicity and mechanistic studies available for D_4, with specific focus on two primary effects seen in animal studies (liver enlargement and reproductive effects). The pharmacokinetics of D_4 are also discussed. Companion manuscripts (Jean and Plotzke, 2017; Jean et al., 2017; Dekant et al., 2017) further review the uterine results reported in rats following chronic exposure (dose-related increased incidences of uterine endometrial epithelial hyperplasia and a dose-related trend in the incidence of endometrial adenomas) and explore the mechanisms by which D_4 exposure may contribute to these reported results.

2. Absorption, distribution, metabolism, elimination of D_4

The pharmacokinetics of D_4 are well characterized. Single exposure inhalation (Utell et al., 1995, 1998) and dermal (Powell et al., 1996; Jovanovic et al., 2008) pharmacokinetic studies have been performed in humans, with single and repeated dose inhalation (Plotzke et al., 2000; Dow Corning Corporation 1995c,d, 1996c) and dermal (Jovanovic et al., 2008; Zareba et al., 2002; Reddy et al., 2007) pharmacokinetic studies performed in experimental animals. Studies to investigate pharmacokinetics following single oral exposures have also been conducted in rats (Dobrev et al., 2008; Sarangapani et al., 2003; Domoradzki et al., 2017). These studies are discussed in more detail in

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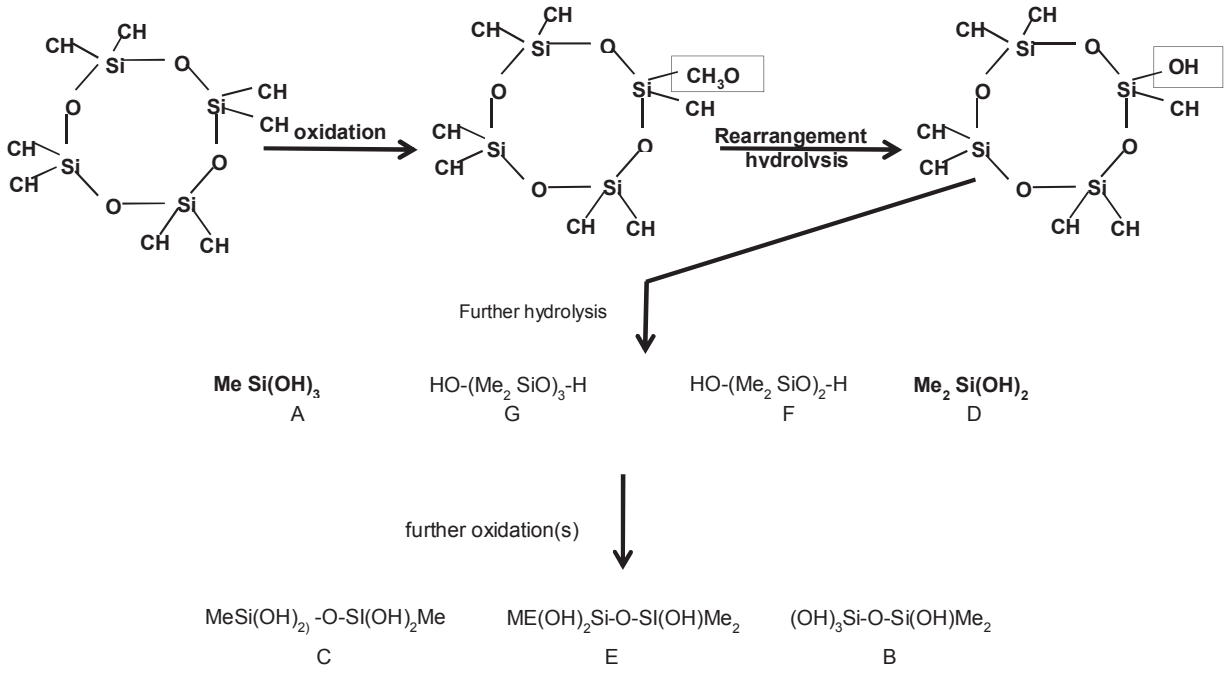

Major metabolites A and D indicated in bold, minor metabolites C, E and B can be produced by multiple pathways (Modified from Varaprath et al. 1999).

Fig. 1. Adapted from Varaprath et al. (1999) – Possible pathways for formation of D_4 metabolites in rat urine.

the following sections.

The metabolism of D_4 was investigated in humans following single inhalation exposure (Utell et al., 1998) and in animals following single or repeated inhalation (Plotzke et al., 2000; Dow Corning Corporation 2000a, 2002), single or repeated intravenous (iv) administration (Varaprath et al., 1999) and single oral exposure (Domoradzki et al., 2017). Based on the metabolite profiles reported in blood, tissues and excreta of rats following exposure to D_4, a metabolic pathway was proposed (Fig. 1) by Varaprath et al. (1999). D_4 is initially oxidized, which leads to ring-opening followed by simple hydrolysis, and eventually two major metabolites (dimethylsilanediol, $Me_2Si(OH)_2$) and methylsilanetriol, $MeSi(OH)_3$ and five minor metabolites are formed, based on analyses of compounds present and identified in urine (Varaprath et al., 1999).

2.1. Inhalation[1]

2.1.1. Humans

Utell et al. (1995, 1998) conducted four double-blind, crossover inhalation studies in humans (single 1-h exposure to 10 ppm D_4) to investigate the pharmacokinetics of inhaled D_4 and one nose-piece exposure system study to compare the deposition of D_4 in the lung during nasal and oral inhalation exposures. The mean D_4 intake, determined from continuously-measured inspiratory and expiratory D_4 concentrations, ranged from 122 to 154 mg (Utell et al., 1995, 1998) and absorption of D_4 was reported to range from 12% to 17% at rest, decreasing with exercise to 10%. Utell et al. (1995) reported that 95% of D_4 absorbed was eliminated in 10 min through post-exposure exhalation. Comparison of nasal breathing (nose-only) to mouth breathing (mouthpiece) exposures indicated that the average total intake of D_4 was 11.5 mg (mouthpiece) and 14.8 mg (nose-only). The estimated uptake of D_4, determined by the product of the mean intake concentration of D_4 and the estimated deposition fraction, was 1.1 mg and 2.0 mg for mouthpiece and nose-only, respectively. D_4 was not found in the urine of exposed volunteers, but three to five minor D_4 metabolites were detected (Utell et al., 1998).

Table 1
Range of Concentrations from studies in ppm and mg/L.

5 ppm	60 mg/L
7 ppm	84.9 mg/L
10 ppm	121.3 mg/L
20 ppm	242.6 mg/L
30 ppm	363.9 mg/L
35 ppm	424.6 mg/L
60 ppm	727.9 mg/L
70 ppm	849.2 mg/L
122 ppm	1480.1 mg/L
150 ppm	1819.7 mg/L
180 ppm	2183.7 mg/L
226 ppm	2741.7 mg/L
300 ppm	3639.5 mg/L
417 ppm	5058.8 mg/L
488 ppm	5920.2 mg/L
500 ppm	6065.8 mg/L
540 ppm	6551 mg/L
700 ppm	8492.1 mg/L
898 ppm	10894.1 mg/L
900 ppm	10918.4 mg/L
1000 ppm	12131.5 mg/L
1076 ppm	13053.5 mg/L
1154 ppm	13999.8 mg/L
2975 ppm	36091.3 mg/L

2.1.2. Rodents – single exposure

Two single-exposure nose only inhalation studies in Fischer 344 (F344) rats (Plotzke et al., 2000) (Dow Corning Corporation, 1995c,d, 1996c); Dow Corning Corporation, 2002) and one in Sprague-Dawley (SD) rats (Dow Corning Corporation, 2002) have been conducted.

[1] Throughout the manuscript, discussion of concentrations in inhalation studies will be reported as ppm and will be the nominal concentrations planned for administration by the study authors. Table 1 provides all concentrations tested as mg/L.

Table 2
Summary of mean total body burden and % D_4 retained in the carcass after single and repeated dose inhalation exposure to concentrations up to 700 ppm of D_4.

Species	Time Points (Hours)	Mean Total Body Burden (μCi)	% Retained in the carcass	Refs.
Single Exposure				
Male F344[a]	0	1.88	4.99	Plotzke et al. (2000)
Male F344[b]	0	2.2	5.47	
Male F344[c]	0	2.18	5.37	
Female F344[a]	0	1.6	5.52	
Female F344[b]	0	1.6	5.19	
Female F344[c]	0	1.63	5.39	
Female F344[a]	0	4.1	8.3	Down Corning Corporation (2000)
Female SD[a]	0	4.2	5.9	
Repeated Dose Exposure				
Male F344[a]	0	2.17	5.95	Plotzke et al. (2000)
Male F344[c]	0	1.84	5.23	
Female F344[a]	0	1.86	1.58	
Female F344[c]	0	6.14	5.75	
Female F344[a]	0	4.5[*]	8.8	Dow Corning Corporation (2002)
Female SD[a]	0	4.7[*]	6.8	

[*]Data was converted from disintegrations per minute (DPM) to μCi (μCi = DPM/2.22 × 10^6).
[a]700 ppm.
[b]70 ppm.
[c]7 ppm.

Animals were exposed to D_4 concentrations of 7, 70 or 700 ppm (F344 rats) (Plotzke et al., 2000) or 700 ppm (F344 and SD rats) (Dow Corning Corporation, 2002), single-exposure nose-only inhalation. In each of the studies, animals were exposed for six hours. Animals were examined for total body burden, tissue distribution and elimination of D_4. Results from the single exposure studies (Table 2) indicate that only a small amount of the administered concentration of ^{14}C D_4 (4.65–8.8%) was retained in the animals (Plotzke et al., 2000; Dow Corning Corporation, 1995c,d, 1996c, 2000a). The reported tissue concentrations (liver, lung, fat, adrenal, kidney, ovaries and testes) of D_4 after a single exposure demonstrated that it rapidly reaches systemic circulation (Table 3).

The metabolism of D_4 in both female F344 and SD rats was compared through the analysis of the percent parent D_4 in the blood, liver, lung, feces, urine and expired volatiles after a single 6 h nose-only exposure to ^{14}C D_4 (700 ppm (Dow Corning Corporation, 2000a)). The results demonstrated differences in the percent of radioactivity (which may be composed of parent D_4 and/or metabolites) in F344 versus SD rats, respectively, in the blood (19 vs. 39%), liver (51 vs. 82%), lung (11 vs. 18%), and expired volatiles (67 vs. 52%). No parent D_4 was present in the urine samples from either strain, but two major metabolites (dimethylsilanediol and methylsilanetriol) were identified that comprised 70–100% of the urinary radioactivity.

2.1.3. Rodents – repeated exposure

To better understand the pharmacokinetics and metabolism of D_4 over a prolonged exposure period, two repeated dose inhalation studies in F344 and one in SD rats have been conducted (Plotzke et al., 2000; Dow Corning Corporation, 1995c,d, 1996c, 2002). Animals were exposed to D_4 concentrations of 7, 70 or 700 ppm (F344 rats) (Plotzke et al., 2000; Dow Corning Corporation, 1995c,d, 1996c) or 700 ppm (F344 and SD rats) (Dow Corning Corporation, 2002), six hours a day for 14 days, and then treated with ^{14}C D_4 on day 15 for six hours. Mean total body burden, concentrations of D_4 in the tissues and blood, metabolism of D_4 and pharmacokinetic parameters (i.e. C_{max} and area under the curve (AUC)) were reported (). Mean total body burden concentrations of D_4 in tissues (Tables 2 and 3) and blood (Table 4), as well as the elimination profile of D_4 (Table 6) were similar to that observed following single exposures (Table 5). Mean total body burden reported suggested a strain-specific difference in the percent of D_4 retained in F344 rats (8.3%) compared to SD rats (5.9%), consistent with the single exposure inhalation studies (Table 2).

The reported tissue concentrations (liver, lung, fat, adrenal, ovaries and testes) in rats of D_4 after repeated exposure demonstrate that D_4 rapidly reaches systemic circulation (Table 3). In addition, the concentrations of ^{14}C D_4 in the tissues over the duration of the study suggest no accumulation of parent D_4 in tissues. Concentrations of D_4 in the blood, liver, lung, feces and expired volatiles suggest strain-specific differences in metabolism between F344 and SD rats (Dow Corning Corporation, 2002). Percent difference in radioactivity and parent were attributed to metabolites during exposure (48.5 versus 66.5%) and post-exposure (21.5 versus 29.1%), as well as in the liver (37.8% versus 71.4%) and lung (13.5% versus 18.5%) for F344 and SD rats, respectively (Dow Corning Corporation, 2002). The F344 rats appeared to metabolize D_4 more readily than SD rats as indicated by the lower percentage of total radioactivity present as parent in the blood and tissues of F344 rats, consistent with the findings after single exposure to D_4. This study also qualitatively identified two major metabolites (dimethylsilanediol and methylsilanetriol) present in the urine of both strains of rats, consistent with that seen in single exposure experiments. The inherent kinetic difference between these two strains suggests that there may be biochemical differences leading to a decreased metabolism of D_4 in female SD rats, compared to female F344 rats (Dow Corning Corporation, 2002). Plotzke et al. (2000), reported that the elimination pathways for repeated exposure were dose-and gender-independent. Distribution and elimination of radioactivity from ^{14}C D_4 was similar to the results reported from single exposure experiments described in Plotzke et al. (2000). Although D_4 was slower to eliminate from fat than from plasma or other tissues, D_4 is not expected to accumulate following repeated exposures due to rapid elimination via exhalation of D_4 in exhaled air and metabolism to polar metabolites (Andersen et al., 2005).

Collectively, the results from these studies demonstrate that D_4 is readily absorbed and reaches systemic circulation rapidly following inhalation exposure. The results also indicate that D_4 is unlikely to accumulate in the fat, even though it is lipophilic, primarily due to three excretion mechanisms: ready clearance as water soluble metabolites from the tissues via the urine; exhalation of parent D_4; and to a lesser extent, excretion of parent D_4 in the feces.

2.2. Dermal

2.2.1. Humans

The bioavailability, or the amount systemically available after

Table 3
Summary of Radioactivity Concentrations measure in tissues of male and Female Rats Following Single and Repeated Dose Inhalation Exposure to 700 ppm of D_4.

Species	Time Points (Hours)	Liver (μg/eq g)	Lung (μg/eq g)	Fat (μg/eq g)	Adrenal (μg/eq g)	Kidney (μg/eq g)	Ovaries (μg/eq g)	Testes (μg/eq g)	Refs.
Single Exposure									
Male F344	0	96.39	458.78	230.59	418.28	115.79	–	–	Plotzke et al. (2000), Down Corning Corporation (1995b)
	24	15.83	170.21	166.92	31.53	11.38	–	–	
	48	11.09	135.54	125.50	11.98	5.75	–	–	
	96	7.34	122.06	98.35	11.07	3.69	–	–	
	168	4.89	80.60	67.11	4.11	2.08	–	–	
Male F344	0	139.87	83.35	132.72	196.5	184.57	–	25.04	Plotzke et al. (2000), Dow Corning Corporation (1995c)
	1	89.69	66.35	189.36	–	137.33	–	–	
	3	57.39	49.49	162.16	–	88.96	–	–	
	6	44.02	41.79	178.24	36.21	64.13	–	23.59	
	12	36.63	32.61	195.56	–	38.95	–	–	
	24	26.22	22.98	184.97	23.76	19.73	–	4.86	
	48	18.97	13.73	152.15	–	9.08	–	–	
	96	11.69	11.41	118.84	–	5.46	–	–	
	120	9.96	9.56	93.25	6.50	4.77	–	1.26	
	168	7.62	8.30	76.20	14.40	4.23	–	2.00	
Male F344	0	127.45	101.73	144.93	196.26	120.67	–	25.13	Plotzke et al. (2000), Dow Corning Corporation (1995d)
	1	72.16	61.99	122.85	112.52	90.90	–	26.40	
	3	48.52	51.79	139.63	39.03	63.90	–	24.90	
	12	26.19	29.22	151.88	35.74	21.74	–	10.08	
	24	20.39	24.23	144.54	28.62	12.24	–	3.97	
	48	14.31	14.31	118.24	11.36	7.28	–	2.07	
	72	11.24	11.24	105.79	15.37	5.21	–	1.66	
	96	8.92	8.92	85.79	6.08	4.84	–	1.47	
	120	8.25	8.25	77.27	4.89	4.43	–	1.36	
	168	5.51	5.51	63.64	3.89	2.69	–	1.40	
Female F344	0	171.24	95.72	247.48	331.97	107.66	151.41	–	Plotzke et al. (2000), Dow Corning Corporation (1995d)
	1	104.71	84.18	241.89	202.13	65.33	114.58	–	
	3	52.32	52.77	284.21	114.17	38.18	64.60	–	
	12	21.20	26.90	221.45	25.33	20.40	14.28	–	
	24	20.93	23.40	280.79	40.79	12.33	14.09	–	
	48	12.45	17.29	234.36	13.53	6.86	9.33	–	
	72	10.56	16.01	154.95	15.58	5.11	8.61	–	
	96	9.27	12.77	145.12	7.75	4.80	4.97	–	
	120	7.43	11.53	126.06	6.42	4.57	5.94	–	
	168	5.86	11.81	94.54	4.53	2.95	5.79	–	
Female F344	0	178.52	164.60	175.77	–	–	–	–	Dow Corning Corporation (2000a)
	2	108.97	102.39	181.67	–	–	–	–	
	12	16.16	44.66	187.32	–	–	–	–	
	72	4.11	25.57	116.94	–	–	–	–	
	120	2.39	24.33	105.97	–	–	–	–	
	168	2.94	29.96	103.30	–	–	–	–	
Female SD	0	166.26	174.27	124.76	–	–	–	–	
	2	94.80	93.33	151.60	–	–	–	–	
	12	13.44	43.98	94.30	–	–	–	–	
	72	3.17	23.61	82.74	–	–	–	–	
	120	2.29	23.49	70.60	–	–	–	–	
	168	1.80	24.06	77.41	–	–	–	–	
Repeated Dose Studies									
Male F344	0	115.17	193.42	138.37	301.90	–	–	26.25	Plotzke et al. (2000), Dow Corning Corporation (1996c)[a]
	1	63.44	127.81	93.52	205.49	–	–	24.94	
	3	48.14	102.71	11.25	104.73	–	–	24.06	
	12	23.20	74.72	112.47	37.81	–	–	7.81	
	24	17.82	60.94	91.07	22.18	–	–	3.73	
	48	12.57	57.59	94.95	14.40	–	–	1.83	
	72	8.42	44.38	56.87	10.18	–	–	1.26	
	96	7.63	42.15	63.17	11.93	–	–	1.53	
	120	6.06	41.74	57.20	6.93	–	–	1.20	
	168	4.27	34.42	45.95	4.47	–	–	0.87	
Female F344	0	129.39	153.45	158.51	502.52	–	125.65	–	
	1	78.64	141.50	179.38	399.03	–	112.05	–	
	3	47.90	104.48	189.48	160.36	–	65.38	–	
	12	21.15	51.17	165.12	57.5	–	23.33	–	
	24	16.21	50.03	167.91	25.35	–	21.81	–	
	48	11.21	45.79	122.71	19.15	–	11.94	–	
	72	9.12	45.37	121.29	12.42	–	13.23	–	
	96	7.35	40.33	90.12	12.09	–	4.52	–	
	120	6.44	34.59	96.94	9.65	–	5.59	–	
	168	4.53	24.84	70.89	7.57	–	7.75	–	
Female F344	0	132.19	117.85	275.78	–	–	–	–	Dow Corning Corporation (2002)
	2	52.89	58.80	246.87	–	–	–	–	
	12	33.67	13.51	317.33	–	–	–	–	

(continued on next page)

Table 3 *(continued)*

Species	Time Points (Hours)	Liver (μg/eq g)	Lung (μg/eq g)	Fat (μg/eq g)	Adrenal (μg/eq g)	Kidney (μg/eq g)	Ovaries (μg/eq g)	Testes (μg/eq g)	Refs.
	72	3.85	22.62	124.47	–	–	–	–	
	120	2.21	18.64	109.38	–	–	–	–	
	168	1.63	19.63	60.35	–	–	–	–	
Female SD	0	151.52	148.58	320.59	–	–	–	–	
	2	78.17	69.18	434.24	–	–	–	–	
	12	34.76	11.98	340.24	–	–	–	–	
	72	3.44	23.13	225.65	–	–	–	–	
	120	1.99	17.89	100.60	–	–	–	–	
	168	1.55	21.20	80.41	–	–	–	–	

[a] Values reported are from Group 2C males and females.

absorption of D_4, was assessed after dermal administration to humans (Jovanovic et al., 2008; Reddy et al., 2007; Powell et al., 1996), rats (Jovanovic et al., 2008), and mice (Zareba et al., 2002). The percutaneous absorption of D_4 was investigated in 13 human volunteers exposed by dermal application of neat D_4 or a 50% solution of D_4 in absolute ethanol for 48 h under occlusive cover. Skin changes, skin blood flow and skin biopsy specimens were examined for D_4 content (Powell et al., 1996). The study results indicate that a single occluded exposure to neat D_4 resulted in a low level (0.1–8.1%) of percutaneous absorption; however, absorption was enhanced when in a solution of ethanol (0.83–18.3%).

Three male and three female human subjects applied 1.4 g (male) or 1.0 g (female) of ^{14}C D_4 to both axillae, uncovered. Blood and exhaled air samples were collected before exposure (baseline) and at 1, 2, 4, 6, and 24 h after application (Reddy et al., 2007). Results indicated ^{14}C D_4 levels were significantly elevated compared to baseline in plasma for up to six hours and in exhaled air at all time points after application for all subjects. Peak D_4 blood levels were observed 1 h after exposure and dropped rapidly with time in both males and females.

In an *in vitro* study (Jovanovic et al., 2008), ^{14}C D_4 was applied to human cadaver skin from six donors using a flow-through diffusion cell technique with provisions to collect material volatilized from the skin by absorption to charcoal traps. The *in vitro* results using human skin indicated that 0.5% of the applied dose of neat ^{14}C D_4 was absorbed, with a similar percentage absorbed (0.49%) following application of D_4 formulated in an antiperspirant.

Due to the differences in the permeability of human and animal skin, a study using the human skin/nude mouse model in female BALB/C nude mice was conducted to determine the percutaneous absorption of neat D_4 (Zareba et al., 2002). Radiolabeled ^{14}C $-D_4$ was applied at a dose of 15.7 mg/cm^2 to human skin grafted onto the skin of BALB/C nude mice under semi-occluded conditions. After 72 h of exposure, 94.6% of the applied dose of ^{14}C D_4 evaporated from the site of application. The average percent of ^{14}C D_4 dermally absorbed by each animal was 1.09%.

2.2.2. Rodents

A dermal absorption study in F344 rats was performed in which three doses of D_4 (2, 4.8, or 10 mg/cm^2) were applied under semi-occlusive cover (Jovanovic et al., 2008). Results suggested an initial rapid elimination of D_4 followed by a slower linear elimination profile. Less than 1% of the applied D_4 dose was absorbed and approximately 60% of the absorbed dose reached systemic compartments. The amount of D_4 absorbed into the skin decreased with time. The authors concluded that the results suggest that D_4 diffuses back to the skin surface and continues to evaporate.

The studies conducted in rodents and using human skin demonstrate very low absorption of neat D_4 (0.49–1.09%) and formulations containing D_4 following *in vitro* and *in vivo* dermal application, with the majority of the D_4 applied to the skin volatilizing from the skin surface.

2.3. Oral

2.3.1. Rodents

Studies have been performed in F344 rats to investigate the pharmacokinetics of D_4 following undiluted (neat) oral administration, or in corn oil, simethicone or a rodent liquid diet (Dow Corning Corporation, 1998, 2013; Domoradzki et al., 2017). A single oral gavage dose (300 mg/kg body weight (bw)/d) of ^{14}C D_4 in corn oil, simethicone, or neat, was administered to groups of female F344 rats (Dow Corning Corporation, 1998). Following oral administration, blood was collected at 15 and 60 min, and at 6, 12, 24, 48, 72, 96, 120, 144 and 168 h post-exposure. Absorption of ^{14}C D_4 was 51.95%, 12.11% and 28.14% in corn oil, simethicone, and neat preparations, respectively. The AUC estimated based on the plasma concentrations of ^{14}C D_4 measured for up to 168 h post–exposure confirmed that D_4 is more readily absorbed in the gastrointestinal tract when administered in corn oil and least available for absorption in simethicone. Regardless of the carrier, the majority of D_4 administered was excreted unchanged in the feces (41–81%). Excretion in urine (27.48%), expired volatiles (15.51%), CO_2 and carcass accounted for approximately 13–53% of the administered dose across the different vehicles, with less than 10% remaining in the carcass.

A single oral gavage dose (30 mg/kg bw/d) of ^{14}C D_4 in liquid diet was administered to female and male F344 rats (Dow Corning Corporation, 2013; Domoradzki et al., 2017). Parent D_4 and total radioactivity were measured in the blood at 15 and 30 min, and at 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 h post-exposure, and in the tissues at 2, 6, 12, 24, 48, 72, 120 and 168 h post-exposure. The mean percentage of the administered dose recovered was 87% and 86% in female and male rats, of which approximately 79% and 75% was absorbed in female and males, respectively. The highest concentration of parent D_4 was found in the fat (52.02 μg/g at 24 h post-exposure in females and 33.48 μg/g in males 12 h post-exposure) and adrenals (127.51 μg/g in females and 75.08 μg/g in males 2 h post-exposure) after exposure to 30 mg/kg bw of D_4. The majority of systemically absorbed ^{14}C D_4 was eliminated in the urine (32.08 and 40.02% for females and males, respectively).

The highest concentrations of D_4 were found in the adrenals (259 μg/g in females 12 h post post-exposure and 200 μg/g in males 6 h post post-exposure) and digestive tract (191 μg/g in females at 2 h post-exposure and 194 μg/g in males 2 h post post-exposure) following exposure to 30 mg/kg bw D_4. Excretion via expired volatiles was reported to be 29.9 and 18.5% for females and males, respectively. Urinary elimination consisted entirely of polar metabolites, while expired volatiles consisted of parent D_4 and metabolites. Both sexes showed comparatively similar disposition patterns at each time point with different concentrations in some organs, as well as presence in sex-specific organs.

A comparison of the low (30 mg/kg bw/d) and high dose (300 mg/kg bw/d) kinetic studies provide evidence of dose dependency with a

Table 4
Summary of D_4 blood and plasma concentrations after single and repeated dose inhalation exposure to 700 ppm D_4.

Species	Time Points (hours)	Blood (μg eq/g)	Plasma (μg eq/g)	Refs.
Single Exposure				
Male F344	0	14.81	15.84	Plotzke et al. (2000), Dow Corning Corporation (1995b)
	24	2.58	3.65	
	48	0.47	1.79	
	96	0.00	0.82	
	168	0.122	0.27	
Male F344	0	16.56	17.86	Plotzke et al. (2000), Dow Corning Corporation (1995c)
	1	17.28	19.11	
	3	16.11	19.94	
	6	14.21	17.58	
	12	8.90	11.79	
	24	3.94	5.56	
	48	2.17	2.84	
	96	0	1.46	
	120	0	1.32	
	168	0	0	
Male F344	0	14.16	16.15	Plotzke et al. (2000), Dow Corning Corporation (1995d)
	1	13.68	16.00	
	3	13.94	17.34	
	12	5.48	7.92	
	24	2.60	3.89	
	48	1.40	2.43	
	72	1.15	1.71	
	96	1.02	1.29	
	120	0.82	1.12	
	168	0.59	0.66	
Female F344	0	11.13	11.14	Plotzke et al. (2000), Corning Corporation (1995d)
	1	10.35	11.27	
	3	8.94	10.78	
	12	5.21	6.75	
	24	3.29	5.19	
	48	1.41	2.31	
	72	0.99	1.57	
	96	0.97	1.30	
	120	0.55	1.002	
	168	0.24	0.74	
Female F344	0	14.77	14.72	Dow Corning Corporation (2000a)
	1	8.98	–	
	2	8.33	–	
	6	8.05	–	
	12	4.40	5.88	
	24	1.98	–	
	48	0.71	–	
	72	0.51	–	
	96	0.68	–	
	120	0.20	–	
	168	0.16	–	
Female SD	0	14.68	12.81	
	1	8.34	–	
	2	7.06	–	
	6	5.41	–	
	12	4.03	5.68	
	24	1.93	–	
	48	0.65	–	
	72	0.44	–	
	96	0.32	–	
	120	0.28	–	
	168	0.21	–	
Repeated Dose Exposure				
Male F344	0	20.40	25.27	Plotzke et al. (2000), Dow Corning Corporation (1996c)
	1	18.25	21.86	
	3	16.85	22.04	
	12	4.62	6.98	
	24	2.57	4.13	
	48	1.41	2.30	
	72	0.82	2.00	
	96	0.67	1.27	
	120	0.00	0.85	
	168	0.00	0.55	
Female F344	0	18.38	20.96	
	1	14.31	18.34	
	3	14.68	18.40	
	12	4.22	5.78	
	24	2.35	3.49	
	48	1.31	1.97	
	72	1.14	1.49	
	96	0.63	1.05	
	120	0.60	0.87	
	168	0.08	0.65	
Female F344	0	17.54	19.33	Dow Corning Corporation (2002)
	1	12.41		
	2	11.84	–	
	6	7.11	–	
	12	3.48	4.30	
	24	0.77	–	
	48	0.27	–	
	72	0.14	–	
	96	0.07	–	
	120	0.08	–	
	168	0.01	–	
Female SD	0	16.50	17.10	
	1	12.13	–	
	2	12.11	–	
	6	7.42	–	
	12	4.63	4.62	
	24	1.66	–	
	48	0.55	–	
	72	0.43	–	
	96	0.26	–	
	120	0.21	–	
	168	0.14	–	

~Values reported are from Group 2A males and females.

greater percentage of the administered dose absorbed following low dose oral gavage administration (77% after 30 mg/kg bw/d of D_4 and 55% after 300 mg/kg bw/d of D_4). In addition, more of the recovered dose was found in expired volatiles and was excreted in urine as metabolites after exposure to 30 mg/kg bw of D_4. No gender differences were noted by the authors, except for the absence of a metabolite (dimethyldixiloxane-1,3,3,3-tetrol) 0–24 h after exposure in females, which was observed in males.

Oral uptake of D_4 was investigated in male F344 rats in an open and closed[2] chamber experiment, in which F344 rats were administered a bolus dose of D_4 (0, 10, 50, 100, 200, or 300 mg/kg bw/d) in corn oil by oral gavage. Concentrations of exhaled D_4 were recorded for up to 10 h post-exposure in both open and closed chamber systems (Dobrev et al., 2008). Peak chamber concentrations of D_4 were reached within 4–6 h of dosing and then steadily declined until the end. An increase in the oral dose from 200 to 300 mg/kg bw/d did not result in a proportional increase in the amount of D_4 exhaled. Attempts to simulate the concentration time courses of exhaled D_4 in air with a PBPK model required smaller absorption (0.5%) after a higher oral dose than lower doses (0.65–1.0%), suggesting a reduced absorption of D_4 at higher doses (Dobrev et al., 2008).

These results suggest that accumulation of D_4 in the body after repeated exposure is unlikely, due to the effective elimination through metabolism and exhalation. In addition, the results suggest a dose-dependency in the kinetics of D_4 that is likely related to a combination of factors, such as saturation of uptake/transport mechanisms and non-linear storage and metabolism.

[2] Closed chamber experiments refer to a closed, recirculating system with an initial concentration of D_4 in the chamber and monitoring of the elimination of the D_4 over time. Open chamber experiments have a constant flow of air containing the experimental concentration of D_4.

Table 5
Disposition Kinetics after single and repeated dose inhalation exposure to 700 ppm of D_4.

Species	Tissue	Time Point (hours)	C_{max} (μg eq ^{14}C D_4/g)	AUC (μg eq ^{14}C D_4 hr/g)	$t_{1/2}$ (hr)	Refs.
Single Exposure						
Male	Blood	0	17.28	371	13	Plotzke et al. (2000), Dow Corning Corporation (1995c)
F3-	Fat	12	195.56	30011	114	
44	Liver	0	139.87	2993	83	
	Lung	0	83.35	2609	158	
	Testes	0	25.04	744	75	
Male	Blood	0	14.16	383.57	97	Plotzke et al. (2000), Dow Corning Corporation (1996c)
F3-	Fat	12	151.88	16965.79	135	
44	Liver	0	127.45	2337.33	91	
	Lung	0	101.73	3081.06	143	
	Testes	1	26.40	573.62	328	
Female	Blood	0	11.13	331.47	75	
F3-	Fat	12	284.21	29062.42	131	
44	Liver	0	171.24	2312.33	111	
	Lung	0	95.72	2972.31	202	
	Ovaries	0	151.41	1908.86	117	
Female	Blood	0	10.89	39	58	Dow Corning Corporation (2000a)
F3-	Fat	12	186.97	21959	392	
44	Liver	0	184.46	900	238	
	Lung	0	81.63	587	125	
	Ovaries	–	–	–	–	
Female	Blood	0	13.54	70	64	
SD	Fat	12	149.4	14543	450	
	Liver	0	181.51	1232	100	
	Lung	0	75.29	934	191	
	Ovaries	–	–	–	–	
Repeated Dose Exposure						
Male	Blood	0	–	–	–	Plotzke et al. (2000)[a]
F3-	Fat	0	138.37	11976.3	144	
44	Liver	0	115.17	1989.31	73	
	Lung	0	193.42	8546.20	183	
	Testes	1	26.25	507.05	128	
Female	Blood	0				
F3-	Fat	3	198.48	19356.66	129	
44	Liver	0	129.39	1964.70	82	
	Lung	0	153.45	7307.04	139	
	Ovaries[b]	0	125.65	2327.81	73	
Female	Blood	0	17.54	145.9	26.6	Dow Corning Corporation (2002)
F3-	Fat	12	317.33	17148	69.3	
44	Liver	0	132.20	1117.4	77.5	
	Lung	0	117.90	2926.6	469.6	
Female	Blood	0	16.50	198.3	59.5	
SD	Fat	2	434.20	22867	69.9	
	Liver	0	151.5	1268.52	83.4	
	Lung	0	148.6	3023.33	762.2	

[a] Values reported are from Group 2C males and females.
[b] The value reported was from Group 3A females due to no information recorded for Group 2A females.

2.4. Intravenous administration

2.4.1. Rodents

The pharmacokinetics of D_4 were studied after single and repeated intravenous (iv) administration of ^{14}C D_4 to SD rats at doses of 7 or 70 mg/kg bw/d (Huntingdon Research Centre, 1995). In the first experiment, animals were administered a single iv injection of 7 mg/kg bw/d or 70 mg/kg bw/d of ^{14}C D_4 emulsion (1:1:7 ethanol, Emulphor and saline) and blood samples were taken at 10, 20, and 40 min, and 1, 2, 4, 6, 12, 24, 30 and 48 h post-exposure. Urine, feces and expired air were collected at 0–6, 6–12 and 24-h intervals for up to 5 days post-exposure. At 120 h post-exposure, animals were sacrificed and the liver, kidneys, lungs, fat, gastrointestinal tract and carcass were measured for radioactivity. Another set of animals were administered ^{14}C D_4 emulsion by iv injection daily for 14 days at a dose of 7 mg/kg bw/d and the concentration of ^{14}C D_4 in the blood was examined 10, 20, 40 min and

Table 6
Percent Excretion of Radioactivity after single and repeated dose exposure to 700 ppm of 14C-D_4.

Species	Time Points (Hours)	Expired Volatiles (% body burden)	Urine (% body burden)	Feces (% body burden)	Refs.
Single Exposure					
Male	1	12.42	–	–	Plotzke et al. (2000), Dow Corning Corporation (1995b)
F344	2	4.35	–	–	
	4	3.35	–	–	
	6	1.83	10.95	0.23	
	12	3.11	10.89	11.13	
	24	3.63	5.37	8.40	
	48	2.79	3.69	5.32	
	72	1.18	1.68	1.84	
	96	0.55	1.19	1.71	
	120	0.29	0.82	0.56	
	144	0.16	0.6	0.36	
	168	0.053	0.54	0.20	
Male	1	16.71	–	–	Plotzke et al. (2000), Dow Corning Corporation (1995c)
F344	2	3.68	–	–	
	3	3.27	–	–	
	6	1.63	5.99	–	
	12	0.60	10.92	2.76	
	24	1.60	12.59	3.98	
	48	0.93	9.43	2.70	
	72	0.74	3.47	1.02	
	96	0.34	1.81	0.60	
	120	0.10	1.35	0.47	
	144	0.0	0.83	0.42	
	168	0.0	0.62	0.37	
Male	1	13.66	–	–	Plotzke et al. (2000), Dow Corning Corporation (1995d)
F344	2	3.20	–	–	
	4	3.20	–	–	
	6	1.03	12.35	0.29	
	12	0.85	11.02	6.45	
	24	1.45	7.11	3.35	
	48	1.22	6.24	1.66	
	72	0.54	2.54	0.65	
	96	0.28	0.95	0.36	
	120	0.25	0.96	0.30	
	144	0.16	0.51	0.22	
	168	0.13	0.64	0.18	
Female	1	12.01	–	–	Plotzke et al. (2000), Dow Corning Corporation (1995d)
F344	2	5.94	–	–	
	4	4.55	–	–	
	6	2.28	3.10	0.13	
	12	0.92	6.63	3.03	
	24	2.43	5.42	3.78	
	48	1.95	9.72	3.67	
	72	1.24	4.45	1.84	
	96	0.43	0.75	0.48	
	120	0.62	1.02	0.43	
	144	0.23	0.52	0.28	
	168	0.24	0.72	0.27	
Female F344	168	3.94	25.54	19.42	Dow Corning Corporation (2000a)
Female SD	168	3.57	32.24	18.16	Dow Corning Corporation (2000a)
Repeated Dose Exposure					
Male	1	30.45	–	–	Plotzke et al. (2000), Dow Corning Corporation (1996c)[a]
F344	2	3.10	–	–	
	4	2.84	–	–	
	6	1.16	19.53	2.97	
	12	0.67	13.81	8.44	
	24	2.00	6.86	2.63	
	48	1.81	4.56	2.21	
	72	0.62	1.70	0.73	
	96	0.40	0.75	0.37	
	120	0.28	0.68	0.28	
	144	0.11	0.43	0.23	
	168	0.10	0.43	0.19	
Female	1	28.24	–	–	Plotzke et al.

(continued on next page)

Table 6 *(continued)*

Species	Time Points (Hours)	Expired Volatiles (% body burden)	Urine (% body burden)	Feces (% body burden)	Refs.
F344	2	3.74	–	–	(2000), Dow Corning Corporation (1996c)[a]
	4	3.29	–	–	
	6	1.91	18.26	1.17	
	12	0.86	12.38	8.19	
	24	1.80	6.32	2.35	
	48	1.33	5.66	2.29	
	72	0.77	2.29	0.76	
	96	0.45	0.80	0.44	
	120	0.33	0.93	0.36	
	144	0.19	0.45	0.26	
	168	0.16	0.60	0.24	
Female F344	168	3.6	37.8	12.8	Dow Corning Corporation (2002)
Female SD	168	3.5	38.3	15.4	Dow Corning Corporation (2002)

[a] Values reported are from Group 2C males and females.

1, 2, 4, 6, 12, 24, 30 and 48 h post-exposure following the last day of administration.

Whole-body autoradiography of animals sacrificed at various times after a single dose of 7 mg/kg bw/d ^{14}C D_4 indicated that radioactivity was well distributed and the main areas of concentration were the fat, liver and kidneys in both male and females. Gender differences were noted in the extent of metabolism of D_4, with the results suggesting that male animals were able to metabolize D_4 more extensively than females. A greater proportion of administered radioactivity was excreted in male urine (48%) and feces (10%) than in female urine (28%) and feces (8%). Expired air from males also contained more $^{14}CO_2$ than females (7% for males and 3% for females) and expired air for females contained more total radioactivity than males (35% for females and 22% for males). Fat had the highest concentration of radioactivity at 120 h post-exposure, compared to other tissues, with a higher concentration of D4 per gram of fat found in female animals compared to males. Plasma radioactivity (^{14}C-D_4) indicated that the relationship between administered dose and AUC was similar in males and females following administration of either 7 or 70 mg/kg bw/d of ^{14}C D_4. A ten-fold dose increase in dose resulted in a proportional increase in the area under the plasma concentration-time curve in both males and females following administration of 7 mg/kg bw/d (66.1 μg.h/mL for males, 48.3 μg.h/mL for females) compared to 70 mg/kg bw/d (546 μg.h/mL for males and 485.4 μg.h/mL).

To investigate the metabolism of D_4, a single iv injection of 70 mg/kg bw/d of ^{14}C D_4 was administered to F344 rats (Varaprath et al., 1999). Two major metabolites (dimethylsilanediol and methylsilanetriol) (Fig. 1), five minor metabolites and no parent D_4 were identified in the urine 12 h following exposure. The major metabolites constituted approximately 75–85% of the total components observed and the five minor metabolites constituted approximately 15–25% of the components observed in the urine (Varaprath et al., 1999). The same major metabolites were reported in rats following a single inhalation exposure to 700 ppm of D4 for 6 h (Plotzke et al., 2000).

A comparison of the results from the single and repeated dose iv studies suggested a proportional increase with dose in the concentration of radioactivity in tissues when comparing results from administration of 7–70 mg/kg bw/d of D_4. The concentration of ^{14}C D_4 in tissues (fat, liver and kidney) of animals administered 7 mg/kg bw/d was 4–5 times higher in all tissues following repeated administration compared to a single administration of the same dose of ^{14}C D_4.

2.5. Oral vs intravenous administration

2.5.1. Rodents

Female F344 rats were administered a single dose (70 mg/kg bw/d) of ^{14}C D_4 via oral gavage or iv injection, and urine, feces, and expired air were analyzed for total radioactivity 72 h post-exposure to determine if the route of exposure impacted the urinary metabolic profile or excretion rate in female rats (Dow Corning Corporation, 1997a). After oral administration, similar percentages of urinary excretion were observed as those following iv administration. At 72 h post-exposure, rats excreted 24% versus 31% of the administered dose of D_4 in the urine after iv and oral administration, respectively. Fecal elimination was a minor route of excretion in rats administered D_4 by iv injection (< 8%). However, in rats administered D_4 orally, 29% of the dose was eliminated in the feces, most likely the result of unabsorbed D_4 and 18% remained in the carcass following administration of D_4 via oral gavage compared to 29% administered intravenously. These differences reflect absorption following oral exposure, which is not a factor following iv administration of D_4.

2.6. Metabolism of D_4 summary

In summary, D_4 is readily metabolized in laboratory animals and humans following inhalation, oral, iv or dermal exposure. After inhalation exposure, a relatively small amount of inhaled D_4 is retained in rats and humans, distributed quickly throughout the body, and readily eliminated through expired volatiles, urine or feces. D_4 is initially oxidized, which leads to ring-opening followed by simple hydrolysis, and eventually, is excreted in the urine as two major metabolites (dimethylsilanediol, $Me_2Si(OH)_2$ and methylsilanetriol, $MeSi(OH)_3$) and five minor metabolites. The results from the available studies indicate that D_4 has similar kinetics after single and repeated inhalation exposure in rats. After oral exposure to D_4, there is evidence of dose dependent related differences in absorption and metabolism at high doses (300 mg/kg bw/d) compared to lower doses (30 mg/kg bw/d) in rats.

2.7. Impact on metabolism following induction or inhibition of CYP450

A study was performed to determine if induction of CYP450 alters the metabolism of D_4 in rats (Dow Corning Corporation, 1997a; Varaprath et al., 1999). Animals were pretreated with an enzyme inducer, phenobarbital (80 mg/kg bw/d, intraperitoneal), an enzyme inhibitor, 3-methylcholanthrene (3-MC) (30 mg/kg bw/d, intraperitoneal), or vehicle (saline), once a day for four days. The day following exposure, rats were administered a single iv dose of 70 mg/kg bw/d of ^{14}C D_4 (Dow Corning Corporation, 1997a). At 72 h post-exposure, urine, feces and expired air were analyzed for radioactivity and metabolite profile. Following iv administration, phenobarbital-pretreated rats excreted 55% of the administered D_4 in the urine, while control and 3-MC pretreated rats excreted 24–27% D_4 in the urine. Rats pretreated with phenobarbital excreted 14% of the D_4 dose as CO_2 compared to 3% in the animals treated with vehicle and D_4 or 3-MC. Only 9% of the D_4 dose was excreted as expired volatiles in phenobarbital pretreated rats, while 29% was excreted in 3-MC pretreated animals and 38% in animals treated with vehicle and D_4 (Dow Corning Corporation, 1997a). These results suggest that pretreatment with selected enzyme inducers (phenobarbital) result in an increase in the amount and rate of urinary excretion of D_4 following a single intravenous dose of ^{14}C D_4 and metabolism of D_4 was not altered following 3-MC pre-treatment of animals.

2.8. Physiologically based pharmacokinetic models

Several physiologically based pharmacokinetic (PBPK) models have

been published over the last decade describing the pharmacokinetic disposition of D_4 in various species following different routes of exposure (Andersen et al., 2001; Reddy et al., 2003, 2007; Sarangapani et al., 2003; McMullin et al., 2016). The most recent model (McMullin et al., 2016) is a harmonized, nested, multi-compound, multi-route and multi-species PBPK model for both D4 and D5 that relies upon aspects of the pharmacokinetics of each siloxane that have been published in seven previous models.

These models used chemical-specific kinetic features of D_4 to determine the parameterization of the common compartmental structures and, where appropriate, to determine the need to include specific tissue compartments relevant to a specific siloxane. The harmonized model consists of six tissue compartments including blood, lung, liver, fat, slowly perfused tissues and rapidly perfused tissues. Chemical-specific metabolite sub-models for D_4 were incorporated into the harmonized model structure. The metabolism of D_4 in this harmonized model produces linear silanols (of variable chain lengths), and the model uses a simple compartmental PK sub-model to simulate distribution and urinary elimination of these linear metabolites.

To evaluate the extent to which highly cleared, lipophilic vapors are expected to accumulate in the blood and tissues, an analysis was conducted using both a generic PBPK model and the more detailed PBPK model for D_4 (Andersen et al., 2008). The results from the Andersen et al. (2008) generic PBPK model analysis indicated that highly metabolized, lipophilic compounds with low blood:air partition coefficients don't accumulate systemically or in the blood after repeated exposure. The results further demonstrated that volatile compounds with partition coefficients in the hundreds and lower hepatic excretion accumulate in the blood after repeated exposure. The authors concluded that in general, poor whole-body clearance, not lipophilicity, results in the accumulation of volatile compounds in the blood and tissues. In addition, the term bioaccumulation should be used to refer to instances where repeated exposures lead to increases in volatile compounds in the blood (Andersen et al., 2008). Therefore, cyclic volatile compounds like D_4 are highly cleared and would not bioaccumulate after repeated exposure, which is consistent with the pharmacokinetic experiment results.

3. Toxicity of D_4

The following sections contain a summary of the available toxicity studies for D_4 by study type. The reliability of the available toxicity studies for evaluating the potential hazards from exposure to D_4 was based on the application of Klimisch scores (Klimisch et al., 1997). This scoring method for assessing the reliability or quality of toxicity studies and their data for use in hazard and risk assessments has been applied for regulatory purposes and is the standard method used in European Union (EU) regulatory schemes. A summary of the scoring method is provided in Table 7. Only studies that received a high reliability score (1 or 2) are discussed in the text. All studies that received a Klimisch score of 3 or 4 are discussed in brief in the Supplementary material (Supplemental Tables S1–S6).

3.1. Acute exposure

Acute oral, dermal and inhalation studies have been conducted for D_4. D_4 has a low potential for toxicity after a single oral administration, with an oral lethal dose in 50% of treated animals (LD50) of > 4800 mg/kg bw estimated in Wistar rats (Bayer, 1979; SCCP, 2005) and 1700 mg/kg in CD-1 mice (Pasquet, 1971). Similarly, after 24 h of dermal exposure to D_4, no overt clinical signs of toxicity were seen in Wistar rats and an acute dermal LD50 of > 2400 mg/kg bw was estimated (IFREB, 1982; Ramm, 1985). After a single four-hour aerosol inhalation exposure to F344 rats, a lethal concentration in 50% of treated animals (LC50) of 2975 ppm was reported (Dow Corning Corporation, 1994). In summary, following acute dermal, oral or inhalation exposure, D_4 exposure has a low potential for acute toxicity.

3.2. Eye and skin irritation and sensitization

Multiple studies have been conducted to evaluate the potential for D_4 exposure to result in eye or skin irritation or sensitization. These studies include one skin irritation study in rabbits (Pasquet, 1971), three eye irritation studies in rabbits (Hazleton, 1985; Dow Corning Corporation, 1997b; Bayer Institute of Toxicology, 1979) and one skin sensitization test in guinea pigs after pretreatment to 1% D_4 in paraffin oil by intracutaneous and closed dermal topical application of neat D_4 for 48 h. Challenge reaction was with undiluted and with 10% test substance in paraffin oil by occluded patch test on day 14 after exposure (Schmidt, 1985). The results indicate that D_4 is not an eye or skin irritant at amounts up to 100 and 500 µL, respectively (Pasquet, 1971; Hazleton, 1985; Dow Corning Corporation, 1997b; Bayer Institute of Toxicology, 1979). D_4 also has been determined not to be a sensitizer in guinea pigs at concentrations up to 10% D_4 in paraffin oil, as well as neat D_4 (Schmidt, 1985). Taken together, these data indicate that, at the doses tested, D_4 has no adverse effects on the skin and is not a sensitizer following contact with skin. SCCS (2010) and REACH (2011) have conducted a critical review and evaluation of the available eye irritation, skin irritation and sensitization studies for D_4 and have concluded that D_4 is not a skin sensitizer, or a skin or eye irritant.

3.3. Repeated dose toxicity

3.3.1. Oral studies

Dow Corning Corporation (1990) completed a subacute (14 day) repeated oral dose, study with doses of 0, 25, 100, 400 or 1600 mg/kg bw/d of D_4 in SD rats. Treatment-related effects were limited to statistically significant increases in relative liver weight in both male and female rats, and a statistically significant increase in absolute liver weight in females in the absence of accompanying liver histopathology following administration of 400 or 1600 mg/kg bw/d, compared to controls. In addition, statistically significant decreases in body weight were observed in male and female rats following administration of 1600 mg/kg bw/d (Dow Corning Corporation, 1990). In summary, D_4 produced significant increases in liver weights, without concomitant changes in liver histopathology in rats following subacute oral exposure

Table 7
Summary of Klimisch et al. (1997) Scoring Method.

Klimish Score	Klimish Scoring Justification
(1) Reliable without restriction	Studies or data from literature or reports that are valid and/or conducted following internationally accepted testing guidelines, or test parameters are based on or closely related to a national testing guideline
(2) Reliable with restriction	Studies or data from literature and reports that are mostly not performed according to Good Laboratory Practices (GLP) but methods are well documented and scientifically acceptable
(3) Not reliable	Studies or data from literature or reports in which the measuring system and test substance have interferences, or organisms or test systems were not relevant to exposure or used a non-acceptable method with insufficient documentation
(4) Not assignable	Studies or data from only short abstracts or secondary literature or do not give sufficient experimental detail

to 400 mg/kg bw/d and greater.

3.3.2. Dermal studies

A repeated dose dermal study with D_4 was performed in male and female New Zealand white rabbits for 21 days under semi-occlusive conditions (Bayer, 1988). No adverse local or systemic effects were noted in either males or females at doses up to 960 mg/kg bw/d. The lack of systemic effects in the dermal toxicity study is consistent with minimal dermal absorption of D_4 reported in humans, rats and mice in the pharmacokinetic studies (Jovanovic et al., 2008; Reddy et al., 2007; Powell et al., 1996; Zareba et al., 2002).

3.3.3. Inhalation studies

Several repeated exposure inhalation studies have been conducted at concentrations up to 700 ppm D_4 (Burns-Naas et al. 2002; Dow Corning Corporation 1989, 1999a; IRDC 1991; Klykken et al. 1999). An important element in evaluating the results from D_4 inhalation exposure studies is that while the saturated concentration of D_4 is approximately 1000 ppm, the maximum vapor concentration that can be consistently maintained over repeated exposures is 700 ppm (the maximum concentration reliably achieved as 100%). Thus, studies using concentrations higher than the maximum vapor concentration of D_4 should be viewed with caution due to the potential formation of aerosols and the potential limitations and complications associated with the dose delivered.

In a 28-day nose-only inhalation study (Dow Corning Corporation, 1995a), groups of 10 male and 10 female F344 rats were exposed to measured concentrations of 0, 226, 417, 700, or 1154 ppm of D_4 for six hours per day, five days per week. Test atmosphere generation was performed with a Hospitak 950 nebulizer designed to produce aerosol principally in the respirable range. Gravimetric and particle size determinations were not possible due to test substance volatility. Relative humidity ranged from 2.9 to 3.4% in exposure groups. During the first five days of exposure, several deaths were noted in the high concentration group, resulting in a decision by the authors to decrease the exposure concentration for this group to 1076 ppm. A statistically significant increase in absolute and relative liver weights were reported following exposure to all concentrations > 417 ppm (Tables 8a and 8b) and a statistically significant increase in absolute and relative adrenal weights in males were reported following exposure to the highest concentration and in females following exposure to the two highest concentrations. Absolute and relative thymus and spleen weights were significantly decreased in males exposed to the highest concentration and in females exposed to the two highest concentrations. Significant changes were noted in several clinical biochemistry parameters including increased glucose concentrations in both sexes (700 ppm), decreased total bilirubin concentration in males (1154 ppm), increased total cholesterol concentrations in both sexes (417 ppm), decreased alkaline phosphatase activity in females (226 ppm) and in males (417 ppm), decreased calcium concentrations in males (700 ppm) and females (1154 ppm), increased sodium in females (700 ppm), increased albumin concentrations in males (226 ppm) and females (700 ppm), increased total protein concentrations in both sexes (417 ppm), increased globulin concentration in males (700 ppm) and females (417 ppm) and decreased albumin-to-globulin ratio in females (417 ppm). Many of these changes did not appear to be treatment-related, and the authors suggested that based on the increases in adrenal weights, it could be assumed that adrenocortical function activity may have an effect on these parameters, especially those associated with carbohydrate, protein and fat metabolism. A significant increase in the incidence of alveolar inflammation in the lungs in all exposed animals was reported from the histopathological examinations. However, this finding was not confirmed following a review of the histopathology from this study by the pathology working group. The pathology

Table 8a
Summary of Male Liver Weights from Inhalation Toxicity Studies.

Species and Period of Treatment	Exposure Period	Control	10 ppm or less	20–50 ppm	60–150 ppm	180–300 ppm	400–600 ppm	700–800 ppm	> 800 ppm	Refs.
F344 Rat	4 wks	5.76 ± 0.39	–	–	–	6.25 ± 0.40	6.72 ± 0.54**	7.01 ± 0.39**	7.27 ± 0.65**	Dow Corning Corporation (1995)
Hartley guinea pigs	4 wks	23.9 ± 4.9	–	–	–	–	–	21.9 ± 1.8	–	Klykken et al. (1999)
LVG Golden Syrian hamsters	4 wks	3.83 ± 0.59	–	–	–	–	–	3.81 ± 0.42	–	
CD-1 Swiss mice	4-wks	1.81 ± 0.14	–	–	–	–	–	2.02 ± 0.21*	–	
New Zealand White rabbits	4-wks	96.4 ± 27.8	–	–	–	–	–	105.3 ± 31.3	–	
F344 Rat	4 wks	5.12 ± 0.15	5.12 ± 0.20	5.31 ± 0.17	5.35 ± 0.15	5.70 ± 0.21	5.95 ± 0.15*	–	–	
Sprague Dawley rats	13 wks	10.8 ± 1.4	–	13.4 ± 2.7**	–	12.7 ± 1.3	–	13.7 ± 1.3**	–	Dow Corning Corporation (1989)
Sprague Dawley rats	13 wks	14.0 ± 3.3	–	–	–	–	–	14.4 ± 2.4	–	
F344 Rat	13 wks	14.49 ± 2.5	13.85 ± 1.5	15.79 ± 3.3	–	14.16 ± 1.2	–	–	–	IRDC (1991)
F344 Rats	3 mo	7.73 ± 2.08	–	7.86 ± 1.61	7.89 ± 1.64	–	8.28 ± 1.07	–	8.41 ± 1.33	Burns-Naas et al. (2002)
F344 Rats	3 mo + 28 d recovery	8.14 ± 0.85	–	–	–	–	–	–	7.64 ± 0.49	
F344 Rats	6 mo	11.23 ± 1.40	12.29 ± 0.66	12.99 ± 0.79**	12.80 ± 0.86*	–	–	14.28 ± 0.44**	–	Jean and Plotzke (2017)
F344 Rats	12 mo	12.96 ± 1.44	14.02 ± 1.48	14.55 ± 1.36	15.08 ± 1.30**	–	–	16.81 ± 1.54**	–	
F344 Rats	12 mo + 12 mo recovery	14.02 ± 1.54	14.42 ± 1.56	15.49 ± 3.17	15.82 ± 3.32	–	–	17.12 ± 4.14	–	
F344 Rats	24 mo	16.07 ± 3.45	15.78 ± 2.53	16.05 ± 2.64	16.24 ± 2.57	–	–	20.48 ± 2.84**	–	

Due to the large number of exposure concentrations across studies, ranges were developed which best captured the study exposures without the need to combine results.
* Significantly different from control at $p < 0.05$.
** Significantly different from control at $p < 0.01$.

Table 8b
Summary of Female Liver Weights from Inhalation Toxicity Studies.

Species and Period of Treatment	Exposure Period	Control	10 ppm or less	20–50 ppm	60–150 ppm	180–300 ppm	400–600 ppm	700–800 ppm	> 800 ppm	Refs.
F344 Rat	4 wks	3.84 ± 0.21	–	–	–	4.25 ± 0.26	4.57 ± 0.31**	5.12 ± 0.29**	5.50 ± 0.49**	Dow Corning Corporation (1995)
Hartley guinea pigs	4 wks	20.4 ± 4.1	–	–	–	–	–	20.6 ± 4.8	–	Klykken et al. (1999)
LVG Golden Syrian hamsters	4 wks	3.56 ± 0.71	–	–	–	–	–	4.05 ± 0.82	–	
CD-1 Swiss mice	4 wks	1.38 ± 0.19	–	–	–	–	–	1.87 ± 0.14**	–	
New Zealand White rabbits	4 wks	102.2 ± 20.3	–	–	–	–	–	99.4 ± 19.3	–	
F344 Rat	4 wk	3.65 ± 0.07	3.61 ± 0.05	3.92 ± 0.07*	3.88 ± 0.09*	3.96 ± 0.08*	4.73 ± 0.12*	–	–	
Sprague Dawley rats	13 wks	8.37 ± 1.82	–	7.95 ± 0.76	–	9.20 ± 1.01	–	10.07 ± 1.60*	–	Dow Corning Corporation (1989)
Sprague Dawley rats	13 wks + 28 d recovery	8.68 ± 1.02	–	–	–	–	–	9.12 ± 1.71	–	
F344 Rat	13 wk	6.69 ± 0.85	7.42 ± 1.1	7.65 ± 1.5	–	8.58 ± 1.1**	–	–	–	IRDC (1991)
F344 Rats	3 mo	4.33 ± 0.68	–	4.47 ± 0.59	4.60 ± 0.47	–	5.18 ± 0.55**	–	5.42 ± 0.32**	Burns-Naas et al. (2002)
F344 Rats	3 mo + 28 d recovery	4.83 ± 0.21	–	–	–	–	–	–	5.01 ± 0.27	
F344 Rats	6 mo	6.79 ± 0.66	7.02 ± 0.48	6.83 ± 0.87	7.44 ± 0.41	–	–	8.16 ± 0.68**	–	Jean and Plotzke (2017)
F344 Rats	12 mo	7.45 ± 0.68	8.13 ± 1.13	7.59 ± 0.41	8.47 ± 0.48**	–	–	9.64 ± 0.66**	–	
F344 Rats	12 mo + 12 mo recovery	10.08 ± 1.02	9.36 ± 1.10	9.72 ± 1.01	9.82 ± 0.93	–	–	9.59 ± 1.34	–	
F344 Rats	24 mo	9.93 ± 1.58	10.47 ± 1.41	10.40 ± 1.54	11.36 ± 1.61**	–	–	12.85 ± 1.86**	–	

Due to the large number of exposure concentrations across studies, ranges were developed which best captured the study exposures without the need to combine results.
* Significantly different from control at $p < 0.05$.
** Significantly different from control at $p < 0.01$.

working group determined that the results support findings of an increased incidence of alveolar inflammation only in males after exposure to the highest concentration of D_4 (Dow Corning Corporation, 2000b). A significant increase in the incidence of goblet cell proliferation in the nasal cavity and of thymic atrophy were reported in the high concentration group in both males and females. However, this finding was not confirmed following a review of the histopathology from this study by the pathology working group, it was determined to not support findings of an increased incidence of goblet cell proliferation (Dow Corning Corporation, 2000b). Utilization of a nebulizer for test atmosphere generation suggests that the exposures may have been primarily to D_4 aerosol with minimal vapor present. The exact proportion of D_4 as a vapor at each level could not be determined and is thus uncertain. When evaluating the results of this study it would be important to take into consideration the potential for the effects to be representative of an aerosol or mixed aerosol/vapor exposure. Several of the original histopathology findings, stated previously, were shown to be not confirmed following the pathology review. However, most of the results of this study were consistent with the pathology working group re-evaluation of the histopathology review and it should be considered that the maximum vapor concentration (700 ppm) was exceeded, which will change the deposition of D_4 in the lung when compared to groups exposed to lower concentrations and, along with the extremely low relative humidity during exposure, may induce stress in the animals.

In a 28-day inhalation study, male and female F344 rats were administered concentrations of 0, 7, 20, 60, 180, or 540 ppm of D_4 via whole-body inhalation for six hours a day, five days per week, and sacrificed following exposure on day 28. One rat from each dose group served as recovery animals and were analyzed 14 days post-exposure (Klykken et al., 1999). Statistically significant increases in liver weights were reported (Tables 8a, 8b), as well as a significant increase in liver-to-body weight ratios, in male rats in the two highest concentration groups and in females administered concentrations > 20 ppm. However, the liver changes had recovered following a 14 day recovery period.

In a separate experiment, the effects of D_4 were investigated on multiple species (SD rats, CD-1 mice, Syrian hamster, New Zealand white rabbits and Hartley guinea pigs) following whole-body inhalation exposure to concentrations of 0, 10 or 700 ppm for six hours per day, five days a week for five weeks (Dow Corning Corporation, 1999a). A 14-day recovery period was included for observation to determine if the effects from D_4 exposure were reversible and to characterize possible species differences in liver responses. Cell replication assays were performed on a group of rats exposed to 700 ppm of D_4 via an osmotic mini pump for six hours per day for three of five days and in a group of rats exposed for 5 days followed by a 14-day recovery period. This study also investigated the effects of D_4 on possible enzyme induction in rats and guinea pigs from each group exposed to 0 or 700 ppm. The authors reported a statistically significant increase in liver weight in male and female hamsters, mice and rats following administration of 700 ppm, but no change in guinea pigs or rabbits; however, these hepatic effects in the hamster, mice and rats were readily reversible, as no changes were noted in any species after the recovery period. Results from the cell replication assays performed in rats showed induced hepatic cell proliferation in females only following administration of 700 ppm, which returned to control levels during the recovery period. Induction of glutathione-S-transferase, epoxide hydrolase and ethoxycumarin deethylase were noted in male rats following administration of 700 ppm and induction in epoxide hydrolase and ethyoxycumarin deethylase were observed in female rats receiving the same concentration, but no enzymes assayed from guinea pigs were induced (Dow Corning Corporation, 1999a).

Three 13-week repeated exposure inhalation toxicity studies were performed (Burns-Naas et al., 2002; Dow Corning Corporation, 1989; IRDC, 1991). In the study conducted by IRDC (1991), SD rats were exposed to concentrations of 0, 5, 10, or 300 ppm of D_4 via whole body

exposure for six hours per day, five days per week, for 13 weeks. Increased liver weights were reported in female SD rats following exposure to 300 ppm that returned to control levels after the exposed animals were allowed to recover for four weeks. The return of liver weights to levels comparable to control animals following a recovery period is similar to the liver weight results reported following a 28-day exposure and recovery period reported by Klykken et al. (1999) (Table 8a, 8b).

In the Dow Corning Corporation (1989) study, SD rats were exposed to D_4 (whole body inhalation exposure) at concentrations of 0, 50, 300 or 700 ppm for six hours per day, five days per week for 13 weeks and an additional 10 animals per sex in the control and high concentration group were observed for an additional four week recovery period. A significant increase in liver weights was observed in all exposed groups of males and in the mid- and high- concentration groups of females. A significant decrease in ovary weights in females in the high concentration group was also reported. Liver weight increases were reversible in males, but not females after the 4-week recovery period.

The effects of nose–only exposure in F344 rats were investigated following administration of measured concentrations of 0, 35, 122, 488 or 898 ppm of D_4, for six hours per day, five days per week for 13 weeks (Burns-Naas et al., 2002). Test atmosphere generation was performed with a Hospitak 950 nebulizer designed to produce aerosol principally in the respirable range. Gravimetric and particle size determinations were not possible due to test substance volatility. Relative humidity was reported between 2.7 and 3.3% for exposure groups. A significant change in the clinical biochemistry indicating hepatocellular alterations was noted following administration of concentrations > 122 ppm in both males and females. Liver and adrenal weights significantly increased in females (> 488 ppm), lung weights in females (898 ppm) and a significant decrease in ovary weight were observed in females exposed to 898 ppm. In females, an increased incidence of ovarian hypoactivity (absence of corpora lutea; normal follicular development present) and increased vaginal mucification was noted in female rats following exposure to 898 ppm but by the end of the recovery period, ovarian hypoactivity was not present in treated females. In male rats a significant increase was seen in heart, kidney and testes weight (488 ppm), and a decrease in brain weight (898 ppm). In the respiratory system, grey-white foci indicative of macrophage accumulation and interstitial inflammation were observed in the lungs of male and female rats in the high concentration group immediately after exposure, as well as after a one-month recovery period, but this observation is generally correlated with the presence of aerosol droplets in the mixed aerosol/vapor that would be present in the high concentration. All histopathological findings were reversible or showed a tendency for reversibility for animals in the recovery group, except for the increase in brain and kidney weights in males. During a re-evaluation of the histopathology from this study it was determined that all results reported were confirmed by the pathology working group with the addition of increased incidence of interstitial inflammation in the lungs was also found in males and females after exposure to 122 and 488 ppm and in females after exposure to 35 ppm (Dow Corning Corporation, 2000b), as well as neutrophil infiltration in the lungs of males and females in the high dose group, but these results were not present after recovery. The pathology working group (PWG) concluded that these effects in the lung appear to be spontaneous, nonspecific background inflammation unrelated to exposure to D_4. Utilization of a nebulizer for test atmosphere generation suggests that the exposures may have been primarily to D_4 aerosol with minimal vapor present. The exact proportion of D_4 as a vapor at each level could not be determined and is thus uncertain. When evaluating the results of this study it would be important to take into consideration the potential for the effects to be representative of an aerosol or mixed aerosol/vapor exposure and may be additionally impacted by the extremely low relative humidity. The results of this study were consistent with the pathology working group re-evaluation of the histopathology review and it should be considered that the maximum vapor concentration (700 ppm) was exceeded, which will change the deposition of D_4 in the lung when compared to groups exposed to lower concentrations and, along with the extremely low relative humidity during exposure, may induce stress in the animals.

In summary, increased liver weights were consistently observed in rats, mice, and hamsters (Table 8a, 8b) following repeated inhalation exposure to D_4. Along with increased liver weights, other histopathological changes reported included concentration-related increases in inflammatory and proliferative changes in the lungs in male and female rats (> 226 ppm) (Dow Corning Corporation, 1995a), as well as increased heart, kidney, spleen, and testes weight in male rats and increased weight of adrenals and decreased weight of thymus in female rats exposed to concentrations of 488 ppm D_4 and greater (Burns-Naas et al., 2002). Observations also included increased lung weights and decreased brain weights in males and female rats, decreased ovary weights and increased incidence of ovarian hypoactivity and vaginal mucification in female rats when the maximum vapor concentration of 700 ppm was exceeded. However, results reported following exposure to concentrations exceeding the maximum vapor concentration should be reviewed with caution as these effects could be considered an artifact of the vapor/aerosol mix and low relative humidity.

3.4. Carcinogenicity/chronic toxicity study

The results from a two-year combined carcinogenicity/chronic toxicity inhalation study conducted in male and female F344 rats exposed to concentrations up to 700 ppm of D_4 have been reported (Jean and Plotzke, 2017). The study was divided into four different subgroups based on duration of exposure (6 months, 12 months, 12 months with 12-month recovery, and 24 months) and all subgroups were administered 0, 10, 30, 150 or 700 ppm D_4. Significant changes were reported in clinical chemistry alterations (decrease in serum enzyme activities, creatinine and increase in total proteins), lymphocytic leukocytosis and respiratory tract irritation in both males and females (Jean and Plotzke, 2017). Significant increases in absolute and relative liver weights were reported in males and females exposed to 700 ppm D_4 (12 and 24 months) and in females exposed to 150 ppm (24 months). Significant increases in testes weight were also reported in males exposed to 700 ppm D_4 (12 and 24 months). Absolute and relative kidney weights were significantly increased in males and females following exposure to 700 ppm (12 and 24 months) and a significant increase in uterine weights was reported in females exposed to 700 ppm (24 months). There was a significant increase in the incidence of endometrial epithelial hyperplasia in females following exposure to 700 ppm (24 months) (Jean and Plotzke, 2017). Uterine benign endometrial adenomas were present in four of sixty animals exposed to 700 ppm D_4 for 24 months, and although not statistically significant compared to controls, the incidence profile across the dose groups had a statistically significant trend. No other toxicologically significant neoplastic or non-neoplastic histopathological findings were reported. An increased incidence/severity of chronic nephropathy was reported in female rats after inhalation exposure to > 30 ppm (24 month) D_4. However, since this is a common age-related spontaneous change in the F344 rat showing variable incidence dependent upon several factors including food intake, its direct relationship to D_4 exposure is unclear (Jean and Plotzke, 2017). The significant increases in liver weights in both sexes that correlated with reported hepatocellular hypertrophy after exposure to 700 ppm in males (12 and 24 month) are considered an adaptive change and would not be considered an adverse effect in the absence of additional hepatic histopathology of clinical chemical changes.

3.5. Reproductive and developmental toxicity

The reproductive and/or developmental toxicity of D_4 was assessed in multiple range finding studies (IRDC, 1993a,b,c; Dow Corning Corporation, 1996a,b), one-generation studies (Dow Corning

Corporation, 1997c,d,e; IRDC, 1993d,e; Siddiqui et al. 2007), phased female studies in rats (Dow Corning Corporation, 1998a, 1999b; Meeks et al. 2007) and in a two-generation reproductive/developmental study in rats (Siddiqui et al., 2007). A brief summary of these studies is provided in the following sections.

3.5.1. Developmental studies

Studies investigating the potential developmental effects of D_4 have been performed in rabbits following oral exposure (IRDC, 1993c) and in rats (IRDC, 1993b,d) and rabbits (IRDC, 1993a,e) following whole body inhalation exposure.

In an oral study conducted in New Zealand white rabbits (dose range-finding study), pregnant New Zealand white rabbits (6 per dose group) were given 0, 50, 100, 500, or 1000 mg/kg bw/d via gavage (in Methocel®) from gestation days 7 through 19. Clinical signs of toxicity in the dams included mucoid stool and tissue and/or red fluid in the cage tray following administration of 500 or 1000 mg/kg bw/d (IRDC, 1993c) and anogenital staining and hair loss following administration of 1000 mg/kg bw/d. A significant increase in abortions (500 and 100 mg/kg bw/d) and in post-implantation loss (1000 mg/kg bw/d), and a significant decrease in the number of live fetuses and gravid uterine weights (1000 mg/kg bw/d) were reported. The significant increases in abortions and post-implantation losses were determined by the authors to be a result of a significant decrease in food consumption.

In the inhalation dose-range finding studies (IRDC, 1993a,b) conducted in Crl:CD rats and New Zealand white rabbits, pregnant females were exposed to whole-body concentrations of 0, 10, 100, 300, or 700 ppm from gestation day 6 through 18 and gestation day 6 through 15 for rabbits and rats, respectively. Maternal effects included significantly decreased body weight and food consumption in rats and rabbits exposed to 700 ppm. In rabbits, maternal effects also included decreased defecation and increased incidence of anogenital staining following exposure to 100, 300 or 700 ppm (IRDC, 1993a). No developmental effects were noted in either rats or rabbits.

Two developmental whole-body inhalation studies were performed in pregnant Crl:CD SD rats and New Zealand white rabbits (IRDC, 1993d,e). Rats were exposed to D_4 concentrations of 0, 100, 300, or 700 ppm from gestation day 6 through 15 and sacrificed on gestation day 20 (IRDC, 1993d). No treatment-related fetal malformations or developmental variations were reported in rats exposed to any concentration. Rabbits were exposed to concentrations of 0, 100, 300 or 500 ppm D_4 from gestation day 6 through 18 and sacrificed on gestation day 29 (IRDC, 1993e). No significant fetal malformations or developmental variations were reported in rabbits exposed to any concentration.

In summary, no developmental effects were observed in either Crl:CD SD rats or New Zealand white rabbits following inhalation exposure to D_4 at the highest concentrations tested (700 ppm in rats and 500 ppm in rabbits). In addition, no developmental effects were observed following oral exposure of New Zealand white rabbits to D_4 at the highest dose level tested (1000 mg/kg bw/d).

3.5.2. Reproductive studies

Two whole-body inhalation dose-range finding studies were performed in SD rats (Dow Corning Corporation 1996a, 1996b). In the first dose-range finding study, SD male and female rats were exposed to concentrations of 0, 70 or 700 ppm D_4 for six hours per day from 28 days prior to mating through mating and up to gestation day 21. (Dow Corning Corporation, 1996a). No effects on mortality, fertility indices, mating indices, days between pairing and coitus, gestation or the process of parturition were reported. Clinical signs of toxicity included dried red material around the nose and dried clear material around both eyes in males and females exposed to 700 ppm, increased number of ejaculatory plugs from day 16 to sacrifice in males, decreased number of implantation sites and decreased mean litter size in females exposed to 700 ppm.

A second dose-range finding study was performed to confirm the findings from the first Dow Corning Corporation (1996a) study. In this second study, Crl:CD SD rats were exposed to concentrations of 0 or 700 ppm for six hours per day from 28 days prior to mating and throughout mating to gestation day 20 (Dow Corning Corporation, 1996b). Clinical signs of toxicity in treated animals included dried red material around the nose in males and females, brown vaginal discharge in females, and increased incidence of ejaculatory plugs in males. Decreased numbers of implantation sites, numbers of corpora lutea, increased pre-implantation loss and reduced mean live litter size were reported in treated females.

Two male (Dow Corning Corporation, 1997c,d) and one female (Dow Corning Corporation, 1997e) 1-generation inhalation studies were conducted, as well as two phased inhalation female studies (Meeks et al., 2007). In the two male reproductive studies (Dow Corning Corporation, 1997c,d), groups of SD rats were exposed to concentrations of 0, 70, 300, 500, or 700 ppm D_4 daily for six hours per day for 70 days prior to mating, during mating and through study day 113. At the end of exposure, rats entered a 4- to 5- week recovery period. No adverse effects on reproduction or adverse effects in the offspring were reported. Dow Corning Corporation (1997d) reported significantly increased liver weights (500 and 700 ppm), and kidney and thyroid weights (700 ppm), which were reversible during the recovery period.

In the female one-generation (Dow Corning Corporation, 1997e), SD rats were exposed to concentrations of 0, 70, 300, 500, or 700 ppm D_4 via whole-body inhalation exposure for six hours a day for 70 consecutive days prior to mating, during mating and gestation, through gestation day 21, and through lactation day 4. Increased liver weights were reported in dams exposed to a concentration of 300 ppm and decreased implantation sites were reported in females exposed to 700 ppm.

The first phased female study (Meeks et al., 2007) consisted of four phases in which SD rats were exposed to different D_4 concentrations (0, 70, 300, 500 or 700 ppm, 6 h a day seven days a week) during selected phases of the reproductive cycle: an overall phase, ovarian phase, fertilization phase, and implantation phase. For the overall phase study, animals were exposed 28 days prior to mating, through mating and until gestation day 19. For the ovarian phase study, animals were exposed 31 days prior to mating until three days prior to the start of mating. For the fertilization phase study, animals were exposed three days prior to mating until gestation day three, and for the implantation phase study, animals were exposed from gestation day two through gestation day five. In the overall phase, a significant decrease was reported in the number of corpora lutea in female rats exposed to > 300 ppm D_4 and in the number of uterine implantation sites and fetuses in rats exposed to 500 or 700 ppm. No significant treatment-related changes were reported in the ovarian and implantation phases. In the fertilization phase, uterine implantation and post-implantation losses were increased in females exposed to 700 ppm, as well as a significant decrease in corpora lutea.

In the second phased female study (Meeks et al., 2007), SD rats were exposed during pre-mating phases and a post-mating phase to a concentration of 0 or 700 ppm D_4 via whole body inhalation for six hours per day. In the pre-mating phases, animals were further subdivided into groups. Group 1 were controls, Groups 2 through 5 were exposed on either the first, second, third, or fourth day prior to mating, respectively; Group 6 animals were exposed from three days prior to mating until one day prior to mating, and Group 7 animals were exposed through a two-day mating phase until gestation day 3. In the post-mating phase, animals were further subdivided into groups again. For this phase, Group 1 were controls, Groups 2–4 were exposed on gestation day 0, 1, or 2, respectively, and Group 5 were exposed on gestation day 0 through gestation day 2. A decreased pregnancy rate in the pre-mating phase (Group 2) was reported. In the pre-mating group 7

(day 3 through gestation day 3), decreased numbers of corpora lutea and implantation sites, increased numbers of small implantation sites, and reduced mean uterine weights were reported. No other significant reproductive changes were noted in any other pre-mating phase group. In the post-mating phase, no evidence of reproductive toxicity was noted. Therefore, the results suggest that exposure to D_4 in the pre-mating phase is critical when considering the reproductive effects of D_4.

In a two-generation study (Siddiqui et al., 2007), the F_0 generation SD males and female rats were exposed to concentrations of D_4 up to 700 ppm. No significant effects on reproductive parameters (sperm number, production rate, motility and morphology) were reported in male rats; however, effects were reported in females exposed to concentrations of 500 ppm or greater. In F_1 females exposed to 700 ppm, the number of ovarian sections containing corpora lutea was significantly decreased and reproductive efficiency was significantly reduced. In the F_1 females exposed to concentrations > 70 ppm, the number of pregnant rats was reduced but didn't reach statistical significance until 500 ppm. A significant increase in estrous cycle length was reported in the F_1 females in the 700 ppm group. A significant decrease was reported in the mean number of live pups per litter in the 500 and 700 ppm groups of the F_0 and F_1 generations (Table 9). A significant decrease in the mean number of total pups per litter was observed in the F_0 (500 and 700 ppm) and F_1 (700 ppm) generations (Table 9). Pup survival was decreased in the F_0 500 ppm PND 4 group, but not the F_0 700 ppm exposure group and in the F_{2b} PND 0 group, but not the F_1, or F_{2a} or F_{2c} 700 ppm groups. The lack of consistent effects across exposure groups or generations calls into question the relevance of this finding.

In summary, in the one-generation and two-generation studies, no significant reproductive effects were noted in male rats exposed to D_4 at any concentration; however, effects were reported in female rats at concentrations of 500 ppm and greater. These effects included decreases in the number of corpora lutea, with an associated decrease in number of uterine implantation sites, total number of pups born and the mean live litter size. Based on the results of the one-generation and two-generation reproductive studies, the reproductive NOAEC for D_4 was determined to be 300 ppm.

3.6. Immunological studies

Immunological studies have been performed in both humans and animals and include an *in vitro* study in human peripheral blood mononuclear cells (Dow Corning Corporation, 2001) and an *in vivo* study in volunteers (Dow Corning Corporation, 1998b; Looney et al., 1998), as well as a series of studies conducted in rats (Dow Corning Corporation, 1997f; Klykken et al. 1999; Nicholson et al. 1996a,b; Naim et al. 1995a,b).

Table 9
Significant reproductive endpoints in a two-generation reproductive study with rats exposed to D_4 (Siddiqui et al., 2007).

Endpoint		Exposure concentration				
		0 ppm	70 ppm	300 ppm	500 ppm	700 ppm
F_1 Generation						
No. Of total pups/ litter	Mean	13.7	13.5	12.2	10.8*	10.0**
	SD	3.1	3.8	3.3	3.7	3.9
	Number	27	24	27	23	23
No. Of live pups/ litter	Mean	13.3	13.4	11.9	10.4*	9.7*
	SD	3.3	3.8	3.1	3.8	3.8
	Number	27	24	27	23	23
F_2 Generation						
Pups born	Mean	13.4	12.5	12.5	11.2	9.0**
	SD	3.2	3.9	3.6	3.3	3.9
	Number	29	26	25	26	17
Live litter size	Mean	13.1	12.0	12.0	10.5*	8.6**
	SD	3.4	3.9	3.7	3.4	3.7
	Number	29	26	25	26	17

* Significantly different from controls at $p \leq 0.05$.
** Significantly different from controls at $p \leq 0.01$.

In the *in vitro* study, cultured human peripheral blood mononuclear cells were exposed to concentrations of 0, 3.5, 10, 35, 105, 350, or 1000 μM D_4 (Dow Corning Corporation, 2001). In cells cultured with D_4 concentrations greater than 10 μM/ml, and in the absence of serum, proliferation induced by phytohemagglutinin was inhibited. This effect was reversed by the addition of small amounts of serum or plasma to the serum-free medium. The authors reported the inhibitory effect occurring in the absence of serum or plasma would likely be irrelevant systemically because high levels of phospholipids in plasma *in vivo* would neutralize such effects.

In a double-blind placebo-controlled crossover study, human volunteers were exposed to either 12 mg/day of D_4 orally in corn oil for 14 days or to D_4 via inhalation at a concentration of 10 ppm for one hour on two occasions, separated by one week (Dow Corning Corporation, 1998b; Looney et al., 1998). Immunotoxicity assays included enumeration of peripheral lymphocyte subset and functional assays using peripheral blood mononuclear cells. No immunotoxic or pro-inflammatory adjuvant effects were reported following repeated oral or inhalation exposure to D_4.

Immunotoxicity of D_4 was assessed in a series of studies in male and female F344 rats following gavage doses of 0, 10, 30, 100, or 300 mg/kg bw/d in corn oil for 28 days (Dow Corning Corporation, 1997f). Immunotoxicity assays performed included splenocyte phenotyping, peripheral blood phenotyping, spleen IgM antibody response to the T-dependent antigen sheep red blood cell (sRBC) serum IgM antibody titers to the T-dependent antigen, sRBC, mixed leukocyte response to Long-Evans and Brown Norway rat spleen cells, peripheral blood leukocytes, clearance of sRBC by the reticulo-endothelial system and natural killer (NK) activity. Overall, the results of these assays indicated that D_4 produced no effects on immune function in male or female F344 rats.

The immunotoxicity of D_4 was also investigated in male and female F344 rats following whole body inhalation exposure to concentrations of 0, 7, 20, 60, 180, or 540 ppm of D_4 for six hours per day, five days per week for four weeks. The ability of D_4 to produce IgM antibody response via a splenic antibody forming cell (AFC) assay and a serum enzyme-linked immunosorbant assay (ELISA) was evaluated. D_4 produced no alterations in immune system functions at any of the exposure levels.

Two studies investigated the immunotoxic potential of D_4 after a single subcutaneous injection (0.2 mL) of neat D_4 to A/J mice (Nicholson et al. 1996a, 1996b). Retro-orbital blood was taken and analyzed for antibody titers to bovine serum adjuvant (BSA) by indirect ELISA. Results indicated that D_4 enhanced antibody production to BSA and induced granulomas at the site of injection.

Finally, in a series of studies performed by Naim et al. (1995a, 1995b), rats were dosed intramuscularly with 1% D_4 in 1000 centistokes silicone oil with bovine collagen II (Naim et al. (1995b)), or a single intramuscular injection of 50 μg D_4 mixed with 50 μg of BSA in phosphate buffered saline (PBS) (Naim et al. (1995a)). The results from Naim et al. (1995b) provided no evidence of arthritis or humoral adjuvant effects in treated animals. Naim et al. (1995a) examined the injection sites histopathologically 98 days post-treatment and reported that anti-BSA antibodies were formed in most rats and these showed a positive delayed-type hypersensitivity reaction.

In summary, while positive immune responses have been noted *in vitro* and *in vivo* in rodents injected subcutaneously or intramuscularly with D_4, no adverse effects have been reported following inhalation or oral exposures in human volunteers. In addition, no immunological effects have been reported in *in vivo* studies conducted in rodents

following exposure to inhalation concentrations up to 540 ppm or oral doses up to 300 mg/kg bw/d.

3.7. Mutagenicity and genotoxicity of D_4

Several studies have been conducted to assess the potential genotoxicity (Isquith et al., 1982; Vergnes et al., 2000) and mutagenicity (Bayer, 1985; Felix et al. 1998; Litton Bionetics Inc, 1978; Vergnes and Morabit, 1993; Vergnes et al., 2000) of D_4, including *in vitro* studies in bacteria, Chinese hamster ovary (CHO) cells, cultured mammalian cells, and *in vivo* studies in rats following exposure via inhalation and oral gavage.

3.7.1. Mutagenicity

Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were incubated with D_4 at concentrations ranging from 1 to 12,500 μg/plate with and without the supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 g for 20 min in a suitable medium and contains cytosol and microsomes (S9) metabolic activation (Bayer, 1985; Litton Bionetics Inc, 1978; Vergnes and Morabit, 1993; Vergnes et al., 2000). No mutagenic activity was observed in any of the five strains tested with or without metabolic activation. In a gene mutation assay with Saccharomyces cerevisiae, D_4 at concentrations of 1–5000 μg/plate, produced no mutations with or without metabolic activation (Litton Bionetics Inc, 1978). In addition, D_4 (50 μM) was negative for mutations in a rat fibroblast cell line (Rat2 λlac1 cells), both with and without activation (Felix et al., 1998). These results indicate that D_4 is not mutagenic. No gene mutations occurred by base pair changes or frameshift in the strains used in these experiments.

3.7.2. Genotoxicity

3.7.2.1. In vitro studies

Chinese hamster ovary (CHO) cells were used to test D_4's potential to induce structural chromosomal aberrations at concentrations of 0, 0.3, 1, 3, 6, or 10 μg/ml in non-activated assays for four hours at concentrations of 0, 3, 6, 10, 20 or 30 μg/ml in the presence of metabolic activation (Vergnes et al., 2000). Results were negative for chromosomal aberrations and sister chromatid exchanges (SCE).

3.7.2.2. In vivo studies

The genotoxic potential of D_4 was assessed *in vivo* in a chromosome aberration assay in the bone marrow cells in Harlan SD rats after inhalation exposure to 700 ppm D_4 for six hours per day for five consecutive days (Vergnes et al., 2000) and a dominant lethal assay in SD rats exposed to D_4 (0, 100, 500, 1000 mg/kg bw/d) via oral gavage five days per week for eight weeks (Isquith et al., 1982). Animals in the chromosome aberration assay were sacrificed six to 24 h after final exposure and bone marrow from the femur was evaluated for chromosome, number, specific chromosome- or chromatid-type of aberration and exchanges. Results from this study showed no statistically significant or exposure-related increases in the incidence of cells with chromosome aberrations (Vergnes et al., 2000). In the dominant lethal assay, males and females were mated on week nine and females were sacrificed 14 days after mating and the numbers of corpora lutea and living and dead implantations were counted for each pregnant female (Isquith et al., 1982). The results provided no evidence of a treatment related effect on measured endpoints. The authors concluded that D_4 did not induce chromosomal damage in germinal tissue.

In summary, the results from the bacteria or mammalian cells *in vitro* chromosomal aberration, SCE assays indicate D_4 is not genotoxic. *In vivo* studies performed (micronucleus and dominant lethal assay) also indicate D_4 is not genotoxic.

4. Results from mechanistic studies with D_4

4.1. Hepatic effects and enzyme induction

As noted in the previous sections, only minimal changes in the liver were reported following subacute or subchronic exposure to D_4 by the oral or inhalation routes. These changes included increased liver weights, centrilobular hepatocyte hypertrophy and enzyme induction (glutathione-S-transferase, epoxide hydrolase and ethoxycumarin deethylase) (McKim et al., 1998). Liver weight increases accompanied with centrilobular hepatocyte hypertrophy have been attributed to "phenobarbital-like" inducers of rat hepatic cytochrome P450 enzymes. A few studies have investigated the mechanistic basis for these effects in rodents exposed to D_4 (Zhang et al., 2000; McKim et al., 2001a), as these hepatic effects have been considered to be adaptive in rodents (Williams and Iatropoulos, 2002).

A study was conducted to define the dose-response curve for liver enlargement and hepatic CYP2B1/2 induction over a wide range of D_4 concentrations (0, 7, 30, 70, 150, 300 or 700 ppm) (McKim et al. (2001a)). In this study, female F344 rats were exposed to whole-body inhalation concentrations of D_4 up to 700 ppm for six hours per day, for five days. A concentration-dependent increase in 7-pentoxyresorufin O-depentylase (PROD) activity and the relative abundance of CYP2B1/2 in animals exposed to 7 ppm and higher was reported, with maximum increases noted in animals exposed to 500 ppm. Expression of CYP2B1/2 in animals exposed to 7 ppm D_4 was confined to the centrilobular hepatic regions, with expansion across the hepatic lobule observed as exposure concentrations increased. These results suggest that D_4 is a "phenobarbital-like" inducer of rat hepatic cytochrome P450 enzymes by the induction of CYP2B enzymes only in the centrilobular region of the liver.

An *in vitro* study was conducted to evaluate the ability of D_4 to inhibit the major P450 enzymes in human liver microsomes at target concentrations of 0, 0.1, 0.3, 1.0 and 3.0 μM (Dow Corning Corporation, 1998c). The results suggested that D_4 has little or no capacity to function as a metabolism-dependent inhibitor of any of the P450 enzymes examined, with the possible exception of rat CYP1A1/2 and human CYP3A4/5, which were weakly inhibited by D_4 in a reversible metabolism-dependent manner. The authors concluded that D_4 appears to be a non-competitive inhibitor of human CYP2B6, CYP2D6, and CYP3A4/5 and a competitive inhibitor of human CYP1A2.

Two studies were conducted to determine the time course for liver enlargement during repeated inhalation exposure to D_4 and to identify the effects of D_4 on liver enlargement during repeated inhalation exposure of rats to whole-body D_4 at concentrations of 0, 70, or 700 ppm for 6 h per day, 5 days per week for 4 weeks (McKim et al., 1998). Results demonstrated a significant increase in PROD activity in male and female treated animals. Results from western blot analysis indicated that an increase in CYP2B1/2 protein was associated with the observed increase in PROD. On day 28, a significant increase in CYP3A was seen in males (700 ppm) and females (70 and 700 ppm) (McKim et al., 1998). No changes in CYP4A activity were observed in males or females in either study.

To further examine the relationship between D_4 exposure and CYP2B1/2 induction, relative to that of phenobarbital, a comparison of the D_4 and phenobarbital induced liver enzyme profile was performed (Zhang et al., 2000). SD male and female rats were exposed to D_4 by oral gavage administration at doses of 0, 1, 5, 20 or 100 mg/kg bw/d for four consecutive days and the resulting liver enzyme profile was compared to a profile induced by a single intraperitoneal injection of phenobarbital (positive control). A significant increase in liver-to-body weight ratios was reported in females administered doses of 20 mg/kg bw/d or greater and an increase in liver-to-body weight ratios was reported in males and females administered the highest dose tested (100 mg/kg bw/d). These results were similar to the liver-to-body weight increases observed in the phenobarbital-induced group.

Induction of liver enzymes were reported in combination with the increasing liver weights, with concentrations of 7-ethoxyresorufin O-deethylase (EROD) significantly increased in males and females administered doses of > 20 mg/kg. Additionally, an increase in PROD activity was observed in males and females administered concentrations of 5 mg/kg bw/d or greater. CYP2B1/2 protein levels increased similarly to PROD, with the one difference being that female rats had a significant increase in CYP2B1/2 levels after treatment with only 1 mg/kg bw/d D_4. There was a slightly significant increase in CYP3A1/2 levels in females administered doses of > 20 mg/kg and in males administered 100 mg/kg bw/d. NADPH cytochrome P450 reductase was also significantly increased in females in the 100 mg/kg bw/d dose group. The authors concluded that D_4 induced CYP2B1/2 in the adult rat liver in a manner similar to phenobarbital and female rats were more sensitive to this induction than were male rats.

A study to investigate the involvement of hyperplasia and hypertrophy in liver enlargement following inhalation exposure to D_4 was performed and results were compared to those obtained with phenobarbital (McKim et al., 2001a). F344 rats were exposed to concentrations of 0, 7, 30, 70, 150, 300 or 700 ppm D_4 by inhalation for six hours per day, five days per week for four weeks or to phenobarbital (500 ppm) in drinking water for four weeks. Liver-to-body weight ratios were increased over control in animals exposed to 700 ppm, similar to the phenobarbital exposed animals. Hepatic hyperplasia increased in an exposure-dependent manner and increased significantly in the groups exposed to > 70 ppm. A significant increase in hypertrophy, measured indirectly by the overall size of hepatocytes evaluated in H & E stained sections of tissue and comparing the total number of cells from treated to the total number or cells from control, was observed on day 6, 13, and 27 after exposure to 700 ppm of D_4. Phenobarbital exposure is typically associated with liver enlargement, hepatic enzyme induction, and transient hyperplasia in liver and thyroid and sustained hypertrophy (McKim et al., 2001a). These observations (transient hyperplasia, hypertrophy, liver enlargement, and enzyme induction) are consistent with phenobarbital-like effects (McKim et al. 1998, 2001a).

Sarangapani et al. (2002) developed a pharmacodynamic extension to a physiologically based pharmacokinetic (PBPK) model to characterize dose-response behaviors of Cytochrome P450 induction following inhalation exposure to D_4. The model simulated tissue D_4 and CYP2B1/2 protein concentrations following exposure to D_4 concentrations of 0, 1, 7, 30, 70, 150, 300, 500, 700 or 900 ppm for 6 h/day, for 5 days. This evaluation showed that at exposures greater than ~300 ppm there was an apparent saturation of liver enzymes with subsequent decreasing liver metabolism.

In summary, the moderate changes in liver weight, hypertrophy and enzyme induction profile in rats exposed to D_4, (Dow Corning Corporation, 1999c; McKim et al., 1998, 2001a; Zhang et al., 2000), are consistent with the classical response observed following phenobarbital treatment. This suggests that D_4 is a weak phenobarbital-like inducer in the rat liver. In the 2-year bioassay liver changes were transient and were not associated with overt hepatotoxicity in male and female rats exposed to concentrations of D_4 up to 700 ppm.

4.2. Reproductive effects/modulation of LH surge

A study was conducted in female SD rats to assess the effect of inhalation exposure to D_4 (0, 700, 900 ppm) on the preovulatory luteinizing hormone (LH) surge, the ability of D_4 to block or delay ovulation and to evaluate the effects of exposure to D_4 on other hormones related to normal reproductive function (Quinn et al., 2007a). General anesthetics and phenobarbital can block or delay the LH surge in rodents and thereby delay ovulation. While hormone concentrations determined on the morning of proestrus were not changed by D_4 inhalation, exposure to 900 ppm D_4 (greater than the maximum vapor concentration of D_4 of 700 ppm) induced a significant reduction of mean proestrus concentrations of LH in female rats with a reduction of peak LH also observed in female rats following exposure to 700 ppm D_4. The D_4-induced delay in the LH surge correlated with blocked or reduced ovulation. On the morning of estrus, concentrations of estradiol were increased and FSH concentrations were decreased in D_4-exposed animals. These results indicate that inhalation exposure to high concentrations of D_4 attenuates the preovulatory LH surge which then would decreases the number of eggs ovulated, and increase the number of anovulatory female rats (Quinn et al., 2007a).

Conclusions from a recent review by Plant (2012) suggest occasional inconsistencies in the literature on whether the neuroendocrine control of preovulatory LH in human females differs from rodents. Therefore, there are some uncertainties regarding the human relevancy of this MOA. In the rodent, the timing of the preovulatory LH surge is determined by the brain initiating a discharge of gonadotrophin releasing hormone (GnRH) by a neural signal that's coupled to the light dark cycle of estradiol in the preoptic area (POA). In rodents, operation of the LH surge may be blocked by barbiturate anesthesia. In contrast, in the higher primates the control system that governs the preovulatory LH surge is located in the mediobasal hypothalamus (MBH)-pituitary unit and is emancipated from control by the POA. It is therefore not subjected to programming by testicular androgens during perinatal development and is resistant to the inhibitory actions of barbiturates on neuronal activity. Because of this, rodent studies intended to examine the impact on the hypothalamic component of neuroendocrine axis regulation of human fertility and ovulation, are inappropriate (Plant (2012)).

The decrease in female rat reproductive capability after inhalation of D_4 is consistent with impaired ovulation due to a shift in preovulatory LH surge. This effect might be due to inhibition of preovulatory prolactin (Quinn et al., 2007a). As reported in Dekant et al. (2017), D_4 might act as a dopamine agonist, perhaps downstream of receptor activation, and might thereby reduce prolactin release. Whereas prolactin is required for normal ovulation in rats, it does not appear to play a role in human ovulation. Indeed, bromocriptine appears to facilitate ovulation even in women with normal plasma prolactin concentrations (Porcile et al., 1990; Yasui et al., 1990). Therefore, the impairment of fertility in female rats exposed by inhalation to D_4 is of questionable relevance for human reproductive risk assessment.

4.3. Uterine effects

In the two-year inhalation exposure study (chronic bioassay) with F344 rats (Jean and Plotzke, 2017), a statistically significant increase in the incidence of uterine endometrial epithelial hyperplasia was reported in female rats exposed to the highest concentration of 700 ppm. Extensive research has been conducted to understand the potential mode of action for these effects and evaluate whether it is relevant to human health (Dekant et al., 2017). Dekant et al. (2017) concludes that the available information suggests that the induction of uterine cystic hyperplasia and adenomas observed in female rats following inhalation of D_4 have no relevance for human health risk characterization. This comment is more fully explored in the next sections.

4.4. Estrogenic, androgenic and progestagenic potential

The estrogenic and androgenic properties of D_4 have been conducted in female F344 and SD rats, female B6C3F1 mice and female estrogen receptor-α knockout (αERKO) mice following oral gavage administration and a receptor binding and luciferase reporter gene assay *in vitro* (He et al., 2003; McKim et al., 2001b; Quinn et al., 2007b).

An *in vitro* receptor-binding experiment and a luciferase reporter gene assay were used to determine if D_4 could activate either the α or β estrogen or progesterone receptors. Receptor binding studies demonstrate that D_4 interacts weakly with the alpha subtype of the estrogen receptor (ERα) but not with beta subtype (ERβ) (Quinn et al., 2007b). D_4 was found not to be a ligand for either progesterone receptor and

was found to have a low binding affinity for the ERα receptor. The *in vivo* rat uterotrophic assay for estrogenic activity was performed in female F344 and SD rats after whole body inhalation over a wide range of D_4 concentrations (0, 7, 30, 70, 150, 300 or 700 ppm) 16 h per day for 3 days, and the Hershberger assay for androgenic activity was performed in male F344 and SD rats, after inhalation exposure to 700 ppm D_4 for 16 h/d for 10 days. A significant increase in both wet and blotted uterine weight and luminal and glandular epithelial cell height was reported in both Female F344 and SD rats. D_4 had no anti-estrogenic activity and was negative in the Hershberger assay, indicating no androgenic activity. D_4 demonstrated a weakly estrogenic response *in vivo*.

Female F344 and SD rats were administered D_4 by oral gavage at doses of 0, 10, 50, 100, 250, 500, or 1000 mg/kg bw/d for four consecutive days (McKim et al., 2001b). Estrogenicity of D_4 was determined by dosing separate groups of female rats in both strains with only ethinyl estradiol (EE) (potent estrogen at concentrations of 1, 3, 10 or 30 μg/kg/day), coumestrol (weak phytoestrogen at concentrations of 10, 35, 75 and 150 μg/kg/day) or diethylstilbestrol diproprionate (non-steroidal estrogen at concentrations of 0.5, 1.5, 5 and 15 μg/kg/day) to place the results from exposure to D_4 into context with the relative potency of known estrogenic compounds. The anti-estrogenic effects of D_4 were also evaluated by co-administering D_4 (500 mg/kg/day) with EE at 1, 3, 10 or 30 μg/kg/day (McKim et al. 2001b). Increased uterine weights were reported in female rats (250–1000 mg/kg bw/d) and mice (250 or 1000 mg/kg bw/d) administered D_4. D_4 significantly inhibited the uterotrophic response of EE, indicating weak anti-estrogenic activity of D_4 when co-administered with a potent estrogen in rats (McKim et al., 2001b). At 50% of the maximal response, D_4 was approximately 1.2–25 million times less potent than known estrogenic compounds (McKim et al., 2001b).

In another study (He et al., 2003), female αERKO -mice were administered 1000 mg/kg bw/d of D_4 orally via gavage for 7 consecutive days to determine E2 and corticosterone levels. In the uterotrophic assay animals were exposed to concentrations of 1000 mg/kg bw/d of D_4 and E2 (10 ug/kg via subcutaneous injection) for 3 consecutive days. Twenty-four hours after exposure to D_4 for 7 days, uterine peroxidase levels (marker for estrogenic activity) were significantly increased in αERKO mice. In another experiment in the same study the αERKO mice pre-treated 30 min prior to D_4 treatment with an estrogenic receptor antagonist (ICI 182,780) (20 mg/kg bw/d) were examined to determine if the estrogenic receptor-alpha (ERα) was important in mediating the estrogenic effects of D_4 in the mouse uterus. The results indicated that pre-treatment with ICI 182,780 blocked D_4-induced increases in uterine weight. In addition, ovariectomized estrogen receptor-knockout mice showed no increase in uterine weights following exposure to D_4. The authors concluded that based on the uterotrophic effects observed in mice, D_4 has a weak estrogenic activity mediated through estrogen receptor binding.

The estrogenicity of D_4 through *in vitro* and *in vivo* assays that employed calcium-binding protein 9K (CaBP-9K) as a biomarker was examined (Lee et al., 2015). For the *in vivo* assay, female rats were examined in an uterotrophic assay for detection of CaBP-9K after subcutaneous injection with D_4. EE (3 μg/kg) or D_4 (0, 500, or 1000 mg/kg bw/d) was administered for 4 days with our without pre-treatment with ICI 183,780 (3 mg/kg bw/d 30 min prior to exposure). In contrast to the He et al. (2003) study cited above, no significant changes in uterine weight were reported following D_4 administration; however, CaBP-9k and progesterone receptor gene expression were induced, compared to controls.

For the *in vitro* assay, GH3 rat pituitary cells were exposed to D_4, 17β-estradiol (E2) or vehicle (corn-oil) with or without ICI 182,780 co-exposure (Lee et al., 2015). Following administration of either D_4 or EE (a steroidal estrogen), CaBP-9K, a validated biomarker for estrogenic activity, was reported to be upregulated along with the progesterone receptor. A decrease in the transcription of the estrogen receptor α was also reported after D_4 exposure. Based on the induction of CaBP-9k, the upregulation of the progesterone receptor and similar results observed following EE exposure, the authors concluded that D_4 has estrogenic potential.

A series of *in vitro* studies have been conducted to evaluate the potential of D_4 to act as a progesterone receptor ligand at concentrations of 0, 0.1, 10, 100, 250, 500 or 1000 μM. The studies assessed the progesterone binding/activation potential of D_4 and whether modulation of progesterone has an impact on estrogenic influences (Dow Corning Corporation, 2005). Two *in vitro* studies were conducted; (A) a progesterone receptor binding and agonism study, which was evaluated utilizing human recombinant progesterone receptor-alpha and −beta and calf uterine preparation ligand binding assays, and (B) a reporter gene assay using transfected human U2-OS osteosarcoma cells. The results from these assays indicated no evidence of progesterone receptor binding and/or activation following D_4 exposure.

In summary, the affinity of D_4 for the estrogen and progesterone receptors is low to non-existent as determined in various *in vivo* studies. Dekant et al. (2017) concluded that “it is unlikely that the very weak activity of D_4 in estrogenic assays is responsible for the increase in the endometrial proliferative lesions seen in the 2-year chronic bioassay”.

4.5. Dopamine agonism

A mode of action based on dopamine agonism for D_4-induced uterine effects has been investigated utilizing a variety of *in vitro* and *in vivo* model systems (Dow Corning Corporation, 2010a,b; Jean et al. 2005, 2017; Dekant et al. 2017).

D_4’s ability to act as a dopamine D2 receptor agonist was assessed by analyzing the ability to lower circulating prolactin levels in aged female F344 rats (Dow Corning Corporation, 2010a; Jean et al. 2005, 2017; Dekant et al. 2017). Animals were exposed to 0 or 700 ppm D_4 by inhalation 6 h/day for five days. At the end of exposure, circulating prolactin levels were not affected by exposure to D_4; however, increased blood prolactin levels were reported at four and eight hours after exposure. Progesterone levels were significantly higher in treated animals, but may not be considered treatment-related due to a noted decrease of progesterone levels in the control animals. No significant effects were found on corticosterone levels or uterine weight after exposure to 700 ppm D_4.

Another *in vivo* study was performed to investigate the ability of D_4 to reduce circulating prolactin levels in reserpine-treated female F344 rats (Dow Corning Corporation, 2010b; Jean et al., 2017; Dekant et al., 2017). After oral reserpine and pergolide treatment, animals were exposed to 700 ppm D_4 for 6 h via inhalation. Prolactin levels were not impacted immediately after exposure to D_4. However, prolactin levels were decreased at 18 h post-exposure, a time when reserpine was losing its effects. Circulating prolactin levels in D_4 treated animals were 87% lower than reserpine treated controls. The authors concluded that the effect of D_4 at 18 h post-exposure may suggest some potential for prolactin secretion modulation (direct or indirect). Further investigation is needed to understand the dopamine agonism potential and the contradictory influence of D_4.

An aged F344 rat model (49–50 weeks at study initiation) assessed the effects of repeated inhalation exposure to 700 ppm D_4 6 h/day, five days a week for 58 weeks. The study assessed vaginal cytology, reproductive senescence and monitored estrus cycle stage by daily vaginal lavage (Jean et al., 2017). A significant increase was noted in both the 1st and 2nd half of the study in percent days in proestrus/estrus after exposure to D_4. Prolonged estrus was significantly higher in the first half of the study in D_4 treated animals. The number of times the animals were in estrus significantly increased in the second half of the study, but not the first half. A significant decrease in estradiol and the ratio of estradiol to progesterone (E2:P4) was noted in the first and second half of the study after exposure to D_4, and a significant increase was noted in estradiol and corticosterone levels. No significant changes were noted in prolactin levels. Histopathological changes noted after

inhalation exposure to D_4 included an increase in the incidence of vaginal epithelial thickness, an increase in vaginal mucification severity but not incidence, and uterine cystic endometrial hyperplasia, and a decrease in the incidence of atretic large ovarian follicles and atretic antral follicles (Jean et al., 2017). It should be noted that these changes were observed in an aging F344 rat and interpretation of the data should take into the account the uncertainties associated from the measured endpoints in the aging animal.

An *in vitro* study was performed to evaluate D_4 interaction and activation of the dopamine D2 receptor in isolated membranes of female F344 rat brain striatum (Baker, 2010; Jean et al., 2017; Dekant et al., 2017). Interaction with the receptor was determined by displacement of specific [125I]iodosulpiride binding, and receptor activation was determined by the stimulation of [35S]GTPγS binding to the receptor coupled G-protein. D_4 was dispersed in aqueous incubation solutions by dissolving in ethanol or by sonication to create a concentration range of 0.1 μM to 1000 μM. Over the concentration ranges using the dispersion method, D_4 had no effect on specific [125I]idosulpiride binding to the D2 receptor. D_4 did not affect the interaction of the selective D2 agonist pergolide, a standard D2 agonist, with the D2 receptor. Over the concentration ranges using either dispersion method, D_4 also did not stimulate [35S]GTPγS binding, but suppressed the basal [35S]GTPγS binding and maximal stimulation produced by pergolide. The authors concluded that a direct interaction with the receptor was not detected and that these effects may be non-receptor related and their cellular or physiological relevance is not obvious.

The MMQ pituitary cell line was used to evaluate the ability of D_4 as a dopamine D2 receptor and/or adenylate cyclase agonist by analyzing its ability to modulate forskolin-induced increases in 3′-5′-cyclic adenosine monophosphate (cAMP) production, cellular viability and cAMP levels (Dow Corning Corporation, 2011; Jean et al., 2017; Dekant et al., 2017). The cAMP-Glo assay was used to analyze forskolin-induced cAMP accumulation following D_4 treatment. MMQ cells, which express functional dopamine receptors and adenylate cylase, were incubated with forskolin (125 nM) to stimulate cAMP production and D_4 at concentrations of 0, 25, 50, or 100 μM. Quinpirole (0, 5.6, 11, 22, 44 μM), a dopamine D2-receptor agonist, was used as a positive control to demonstrate dopamine D2-receptor mediated decreases in forskolin-stimulated cAMP accumulation and raclopride (125 μM) was used to demonstrate antagonism. Due to the lack of antagonism (raclopride) on D_4, an evaluation was conducted to determine whether D_4 activity was dependent on G-protein coupled receptors by pre-treating MMQ cells for 20 h with a G1 inhibitor, pertussis toxin (200 ng ml), and assaying for cAMP production. D_4 caused a decrease in forskolin-stimulated cAMP production in the highest dose group. D_4 did not cause cytotoxicity at any dose and the addition of raclopride or pertussis toxin treatment did not cause an effect on D_4 activity. The authors concluded that D_4 activity does not involve the dopamine D2 receptor but rather directly acts on adenylate cyclase or other mediation of adenylate cyclase.

Short-term *in vitro* and *in vivo* studies (Baker, 2010; Dow Corning Corporation, 2011, 2010b; Jean et al. 2017; Dekant et al. 2017) conducted to assess dopamine receptor interaction have not demonstrated direct interaction of D_4 with aspects of the dopamine receptor as a direct-acting agonist, but have indicated possible interactions with the dopamine pathway through several common characteristics: suppression of basal Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) activity in F344 rat striatal membranes, suppression of cellular cyclic adenosine monophosphate (cAMP) production in the MMQ cell line (a prolactin-secreting clonal cell line responsive to dopamine), increase in prolactin immediately following exposure with subsequent depression in the reserpine rat model, and slight increase in prolactin immediately following exposure in the aged F344 rat model, which may be suggestive of an indirect interaction.

Dopamine agonists have been demonstrated to inhibit prolactin secretion from the pituitary gland in rats, causing luteolysis and reduction in progesterone; which results in an increase in the estrogen:progesterone ratio (Alison et al., 1990). Increases in the estrogen:progesterone ratio lead to persistent endogenous estrogen stimulation of the endometrium, which may lead to endometrial tumors in rats. Most dopamine agonists, such as pergolide, have been reported to produce uterine cystic endometrial hyperplasia and uterine tumors in rats, an effect not seen in any other species (Burek et al., 1988). Clinical studies with dopamine agonists show no effect on follicle stimulating hormone, luteinizing hormone, estrogen levels, progesterone levels or endometrial histopathology in women. Based on the lack of effects observed in the clinical studies, the tumorigenic effect of dopamine agonists in female rats is considered a species-specific effect with no risk to human health (Burek et al., 1988).

In summary, the studies conducted to investigate the direct interaction of D_4 with the dopamine receptor have not provided evidence of a precise mode of action, and the subtlety of the effects observed may prevent further assessment (Dekant et al., 2017). While D_4 was not a direct dopamine agonist, slight alterations in the dopamine activation pathway and modulation of prolactin levels after D_4 exposure are suggestive of some indirect interference with this pathway (Dekant et al., 2017).

5. Discussion and conclusions

No significant toxicological effects were observed following acute dermal, oral or inhalation exposures to D_4. D_4 has a low potential for toxicity after single oral (LD50 > 4800 mg/kg bw/d), inhalation (LC50 = 2975 ppm) or dermal exposure (LD50 > 2400 mg/kg bw/d). D_4 has been reported to not be an eye irritant, a skin irritant or a skin sensitizer.

The changes noted in the lungs of animals following subchronic nose-only inhalation exposure were considered to be a generalized, non-specific adaptive response to a mild irritant, possibly exacerbated by aerosol exposure and extremely low humidity and not to be a specific effect of D_4. In support of this conclusion, no significant adverse changes in the respiratory tract were noted in the 2-year chronic whole-body vapor inhalation study.

The effects on the liver reported following subchronic and chronic inhalation exposure have been attributed to a “phenobarbital-like” induction of rat hepatic cytochrome P450 enzymes. Changes observed in the liver following D_4 exposure were reversible and were not associated with overt hepatotoxicity. The enzyme induction reported was considered an adaptive response to xenobiotics and has no significant impact on determining human risk.

The reproductive effects reported in the female rats in the two-generation reproductive study (Siddiqui et al., 2007) and the additional studies (Quinn et al., 2007a,b; He et al. 2003; Lee et al. 2015) conducted to assess the potential endocrine activity of D_4 have suggested that D_4 has very weak estrogenic and antiestrogenic activity. However, there are observations in the reproductive studies that don’t support the direct effect of D_4 as a weak estrogen and that are inconsistent with this activity (Siddiqui et al., 2007), thus indicating the very weak hormonal potency of D_4. A more relevant explanation for the reproductive toxicity is induction of a delay of the LH surge necessary for optimal timing of ovulation (Quinn et al., 2007a,b). An insufficient or blocked pre-ovulatory LH surge fails to induce complete ovulation in the rat and results in the reduced litter size observed following exposure. However, the current understanding of estrous cyclicity and neural/hormonal regulation of ovulation in humans suggests that the effects of D_4 on fertility as observed in the rat are unlikely to be relevant to humans (Plant, 2012; Dekant et al., 2017).

The remaining treatment-related endpoint identified following inhalation exposure to D_4 was the statistically significant increase in the incidence of endometrial epithelial hyperplasia and a significant positive trend for the incidence of benign endometrial adenomas observed only in female rats exposed to the highest concentration tested

(700 ppm). Dekant et al. (2017) examined the biological relevance and possible modes of action for these effects observed in the F344 rat following chronic inhalation exposure to D_4. They reported that endometrial adenomas are an unusual lesion in rats, have no malignant potential and there is no endometrial lesion in women that is directly analogous to the endometrial adenoma in the rat. They further hypothesize that an alteration in the estrous cycle in the aging F344 rat was the most likely mode of action for the observed uterine effects following chronic inhalation exposure to D_4. It should also be noted that although the mode of action for the induction of uterine adenomas in the female F344 rat has not been specifically defined, the available data suggest that the observed benign tumors are not relevant to human health (Dekant et al., 2017). D_4 has not been shown to be mutagenic or genotoxic in *in vitro* or *in vivo* experimental models, therefore the observed tumors likely occur by a non-genotoxic mechanism. In addition, no tumors were observed in male F344 rats and no proliferative lesions reported in any other hormone dependent tissues other than the uterus of female F344 rats following chronic D_4 exposure.

Declaration of interest statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2017.06.007.

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