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Oral repeated-dose systemic and reproductive toxicity of 6:2 fluorotelomer alcohol in mice

Pushkor Mukerji[a,*], Jessica Caverly Rae[a], Robert C. Buck[b], John C. O'Connor[a]

[a] E I. duPont de Nemours and Company, Inc., Haskell Global Centers for Health & Environmental Sciences, P.O. Box 30, Newark, DE 19714, United States

[b] E I. duPont de Nemours and Company, Inc., Chemicals and Fluoroproducts, Wilmington, DE 19805, United States

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ABSTRACT

6:2 fluorotelomer alcohol (6:2 FTOH) was evaluated for potential systemic repeated-dose and reproductive toxicity in mice. 6:2 FTOH was administered by oral gavage to CD-1 mice as a suspension in 0.5% aqueous methylcellulose with 0.1% Tween-80 at dosages of 1, 5, 25, or 100 mg/kg/day. The no-observed-adverse-effect level (NOAEL) for systemic toxicity was 25 mg/kg/day (males) and 5 mg/kg/day (females), based on effects at higher doses on mortality, clinical observations, body weight, nutritional parameters, hematology (red and white blood cell), clinical chemistry (liver-related), liver weights, and histopathology (liver, teeth, reproductive tract, and mammary gland). However, 6:2 FTOH was not a selective reproductive toxicant. The NOAEL for reproductive toxicity was >100 mg/kg/day; no effects on reproductive outcome were observed at any dosage. The NOAEL for viability and growth of the offspring was 25 mg/kg/day, based on clinical signs of delayed maturation in pups, and reductions in pup survival and pup body weight during lactation at 100 mg/kg/day. While the severity of the effects was generally greater in mice than previously reported in CD rats, the overall NOAELs were identical in both species, 5 mg/kg/day for systemic toxicity and 25 mg/kg/day for offspring viability/growth. 6:2 FTOH was not a selective reproductive toxicant in either species; no effects on reproductive outcome occurred at any dose level, and any effects observed in offspring occurred at dose levels that induced mortality and severe toxicity in maternal animals.

1. Introduction

Fluorotelomer alcohols [FTOHs; $F(CF_2CF_2)_nCH_2CH_2OH$, where $n \geq 3$] are industrial raw materials used to manufacture fluorotelomer-based products. The performance of these products is largely derived from the fluorotelomer functionality, which is covalently bound as a side-chain to a polymer backbone, or to additional hydrocarbon groups to create fluorosurfactants [1–5]. Polymeric products provide oil and water repellency and stain release protection that cannot be achieved with non-fluorinated alternatives. Fluorosurfactants provide unparalleled surface tension lowering, wetting and leveling properties. Recently, major global fluorotelomer manufacturers have committed to replace historic long-chain products that are potential precursors to long-chain perfluorocarboxylic acids (PFCAs, $C_nF_{2n+1}COOH$, where $n \geq 7$), such as perfluorooctanoate (PFOA) [6–8]. Alternatives, which cannot break down to long-chain PFCAs, such as products based on short-chain 6:2 FTOH ($C_6F_{13}CH_2CH_2OH$, CAS# 647-42-7,

* Corresponding author. Tel.: +1 302 366 5341; fax: +1 302 366 5211.
E-mail address: Pushkor.Mukerji@dupont.com (P. Mukerji).

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6:2 FTOH) as a key raw material, have been approved by regulators.

A substantial body of data describes 6:2 FTOH hazards, answering the call for more published information about alternatives [9]. The developmental and reproductive toxicity of 6:2 FTOH in CD rats as well as the acute, genetic, and subchronic toxicity in CD rats have been reported, including a benchmark dose of 5 mg/kg/day [10,11]. There was no portal of entry-specific toxicity observed for 6:2 FTOH when comparing repeated-dose oral and inhalation studies [10,12]. *In vitro* 6:2 FTOH metabolism in mouse, rat and human hepatocyctes and *in vivo* oral and inhalation single and repeated-dose metabolism and kinetics studies have been reported showing rapid metabolism and elimination [13–15]. Finally, the toxicity of two principal metabolism and environmental degradation products, perfluorohexanoic acid, PFHxA [16–18] and 5:3 acid, $C_5F_{11}CH_2CH_2COOH$ [19], have been reported.

The primary objective of this study was to evaluate the systemic oral repeated-dose and reproductive toxicity of 6:2 FTOH in CD-1 mice. The CD-1 strain was chosen for consistency with previously published toxicological evaluations of fluorotelomer alcohols and metabolites in mice [20–24]. The present evaluation of 6:2 FTOH in CD-1 mice was intended to provide a basis for comparison with previously published repeated-dose and reproductive effects of 6:2 FTOH in CD rats. Further, this evaluation encompasses the toxicological action and effects of the parent chemical and metabolites from its biotransformation, including PFHxA and 5:3 acid, resulting from 6:2 FTOH exposure in mice.

2. Materials and methods

2.1. Test substance and administration

The test substance, 1-octanol, 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-, also known as 6:2 fluorotelomer alcohol (6:2 FTOH; 99.7% purity; $C_6F_{13}CH_2CH_2OH$; CAS #647-42-7), was supplied by DuPont Chemicals and Fluoroproducts, Wilmington, Delaware, as a clear liquid. 6:2 FTOH was formulated in a vehicle of 0.1% Tween-80 in 0.5% aqueous methylcellulose. Formulations were administered by intragastric intubation to achieve dosage levels of 0, 1, 5, 25, and 100 mg/kg body weight/day (mg/kg/day). Control animals were administered the vehicle alone. All animals were dosed once each day at approximately the same time (±2 h) at a dose volume of 5 mL/kg body weight (calculated from the most recently collected body weight).

Analyses at five time points, including the beginning and end of the study, confirmed that the formulations for the 5, 25, and 100 mg/kg/day groups were homogeneous mixed at the targeted concentrations (±15% nominal) and stable until use. The concentration and stability of the formulation for the 1 mg/kg/day group could not be verified for the first six weeks of the premating period for P_1 males only, after which the formulation method was refined, such that concentration, homogeneity, and stability were consistently within the targeted range.

2.2. Test species and animal husbandry

Male and female Crl:CD1(ICR) mice were acquired from Charles River Laboratories, Inc. (Kingston, NY) and maintained in an AAALAC-accredited facility in accordance with the principles described in the Guide to Care and Use of Laboratory Animals [25]. Animals were housed individually in solid bottom caging with bedding and Nestlets™ as enrichment. Breeding pairs were housed together during the cohabitation period and litters were housed with their dams during the lactation period. Throughout the study, animals were fed PMI® Nutrition International, LLC (St. Louis, MO) Certified Rodent LabDiet® 5002 and provided with tap water *ad libitum*. Animal rooms were maintained on a 12-h light/dark cycle (fluorescent light), a temperature of 22 ± 4 °C, and a relatively humidity of 50 ± 20%.

Males were approximately 39 days old at arrival and 50 days old at study start. Females (nulliparous and not siblings of the males) were approximately 57 days old upon arrival and 75 days old at study start. Mice were released from quarantine for study use after an acclimation period of approximately 1 week based on adequate body weight gain and freedom from clinical signs of disease or injury.

All mice were weighed approximately weekly and cage-site examinations were performed at least twice daily to detect moribund or dead animals. All mice were also examined approximately 1–3 h after dosing for clinical signs of overt toxicity.

2.3. In-life and reproduction

The study was performed in compliance with GLP standards and in accordance with the OECD, Section 4 (Part 415) and U.S. EPA, OPPTS 870.3550 guidelines. Animals were divided by computerized, stratified randomization into five groups of 15 males and 15 females such that all animals were within 20% of the mean for the sex at study start. Parental (P_1) male mice were dosed for approximately 70 days prior to mating, in order to encompass the entire spermatogenic cycle; and throughout the cohabitation period (≤2 weeks), up until the day before scheduled euthanasia. P_1 female mice were dosed for approximately 14 days prior to mating, and throughout the cohabitation period (≤2 weeks), gestation, and lactation, up until the day before scheduled euthanasia. F_1 males and females that were selected for developmental landmarks were dosed from post-natal day (PND) 21 until the day before scheduled euthanasia.

Clinical observations, body weights, and food consumption data were collected on all P_1 generation mice approximately weekly throughout the premating period. During the cohabitation period, one male and one female mouse within each dose group were housed continually in the male's cage until successful mating was verified by an intravaginal copulation plug, at which time they were returned to individual housing. The day copulation was confirmed was designated as GD 0. For presumed pregnant females, clinical observations, body weight, and food consumption data were collected on GD 0, 4, 7, 11, 14, and 17. The day a litter was delivered was designated as

lactation day (LD) 0. For dams that delivered a litter, clinical observations and body weights were collected on LD 0, 7, 14, and 21, while food consumption data were collected on LD 0, 7, and 14. The number of live and dead pups and clinical observations for the pups were recorded once daily during the postpartum period, and pup body weights were collected on LD 0, 4, 7, 14, and 21. On LD 4, litters were randomly reduced to eight pups each (four per sex when possible).

For the 1, 5, and 25 mg/kg/day groups, offspring in the F_1 litters of each treatment level were randomly selected (one animal/sex/litter when possible) to be evaluated for collection of developmental landmark data. For the 100 mg/kg/day group, all offspring were euthanized as weanlings (PND 21) due to concerns about their ability to survive without the dam, based on low pup body weights and high pup mortality. Clinical observations, body weights, and food consumption data were collected on all surviving F_1 generation mice weekly from PND 21 until PND 40–43. The age and body weight at either vaginal opening or preputial separation were recorded.

2.4. Anatomic pathology

All P_1 mice and F_1 adults were euthanized by isoflurane anesthesia followed by exsanguination, after completion of the cohabitation period for P_1 males (on test days 107–109), on postpartum day 21 for P_1 females, on test day 59 for P_1 females that did not deliver a litter, and after developmental landmark achievement for all F_1 adults (on PND 40–43). F_1 weanlings designated for gross evaluations were euthanized by isoflurane anesthesia followed by CO_2 inhalation. The order of euthanasia was stratified across groups.

All P_1 mice, F_1 weanlings, and F_1 adults underwent an examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. The following tissues from P_1 mice were weighed and retained in an appropriate fixative: brain, liver (with gallbladder), and kidneys from males and females; testes and epididymides from males; and ovaries (with oviducts) and uterus (with cervix) from females. Relative organ weights (% of terminal body weight) were calculated. In addition, the following tissues from P_1 mice were retained in a fixative: nose (I & II) with teeth, pancreas; seminal vesicles, coagulating gland, and prostate from males; and vagina and mammary gland from females. F_1 pups that either died or were euthanized prior to the scheduled necropsy were examined grossly.

Microscopic examination of hematoxylin and eosin (H&E) stained paraffin sections were performed by a veterinary pathologist on all tissues collected from P_1 animals from the control and high-dose groups, from all P_1 pairs that failed to produce a litter, and from all parental animals dying spontaneously (*i.e.*, found dead) or euthanized prior to scheduled necropsy. In addition, the following organs from all P_1 adult mice from all dose levels were processed to slides and examined microscopically: liver, nose/teeth, ovaries, uterus, vagina, and mammary gland.

2.5. Clinical pathology

Each group of P_1 males and females was divided into two approximately equally sized subsets for hematology or clinical chemistry. Blood samples for hematology (5–8 animals/sex/group) or clinical chemistry (6–8 animals/sex/group) measurements were collected from the *vena cava* while each animal was under isoflurane anesthesia.

The following parameters were assessed with a Bayer® Advia 120 hematology analyzer: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular (cell) volume (MCV), mean corpuscular (cell) hemoglobin, mean corpuscular (cell) hemoglobin concentration (MCH), red cell distribution width (RDW), absolute reticulocyte count (ARET), platelet count, white blood cell count, and differential white blood cell count. The following parameters were assessed with an Olympus® AU640 clinical chemistry analyzer: aspartate aminotransferase (AST), alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), alkaline phosphatase (ALKP), total bilirubin (BILI), urea nitrogen (BUN), creatinine (CREA), cholesterol (CHOL), triglycerides, glucose, total protein, albumin, globulin, calcium, inorganic phosphorus, sodium, potassium, chloride, and total bile acids (TBA).

2.6. Statistical analyses

Litter survival and indices for mating, fertility, and gestation were compared using Fisher's Exact test [26] with a Bonferroni–Holm correction [27]. For all other continuous numerical data, transformations were applied when appropriate, based on preliminary tests for homogeneity of variance [28] and normality [29]; and a one-way analysis of variance [30] followed by Dunnett's test [31] were conducted. For all statistical analyses, significance was judged at $p < 0.05$.

3. Results

3.1. Mortality and clinical observations in P_1 mice

6:2 FTOH-related clinical abnormalities and mortality were observed in P_1 males and females at 100 mg/kg/day (data not shown). One male and two females at this dose level were found dead or humanely euthanized during the premating or gestation periods. Clinical signs of toxicity that preceded death in these animals included clonic and tonic convulsions, ataxia, tremors (head/upper body/forelimbs), hyperreactivity, increased muscle tone, lethargy, pallor, labored breathing, and gasping. Similar signs of toxicity were also observed in 3 additional mice (1 male and 2 females) in the 100 mg/kg/day dose group that survived to the scheduled euthanasia. When the clinical abnormalities did not result in death, they abated within approximately one week after the first occurrence. There were no 6:2 FTOH-related clinical observations or mortality in P_1 males or females at dose levels $\leq$25 mg/kg/day.

Table 1
P_1 in-life parameters.

Dose (mg/kg/day)	0	1	5	25	100
Males	14	15	15	15	14
Final body weight (day 105)	42.4 (2.9)	42.9 (3.2)	42.2 (2.9)	40.4 (2.2)	40.4 (3.2)
Body weight gain (days 0–105)	11.6 (2.3)	12.1 (3.7)	11.6 (2.2)	11.0 (1.7)	10.2 (2.9)
Daily food consumption (days 0–70)	5.8 (0.6)	5.9 (0.5)	5.8 (0.5)	5.6 (0.5)	5.4 (0.5)
Females – premating	15	15	15	15	14
Body weight (day 14)	27.3 (1.3)	27.7 (1.4)	27.7 (1.5)	27.9 (1.8)	27.4 (1.1)
Body weight gain (days 0–14)	−0.1 (0.9)	0.4 (0.9)	0.3 (1.7)	0.3 (1.2)	−0.1 (1.1)
Daily food consumption (days 0–14)	4.6 (0.5)	4.6 (0.5)	4.9 (0.5)	4.6 (0.5)	5.0 (0.5)
Females – gestation	14	14	14	14	10
Body weight (GD 17)	52.1 (4.0)	54.7 (5.5)	54.0 (4.1)	54.5 (4.6)	53.4 (3.9)
Body weight gain (GD 0–17)	25.2 (3.1)	27.6 (4.4)	26.9 (3.6)	26.7 (3.5)	24.4 (4.9)
Daily food consumption (GD 0–17)	6.4 (0.7)	6.3 (0.6)	6.5 (0.5)	6.3 (0.7)	6.3 (0.4)
Females – lactation	13	14	15	14	9
Body weight (LD 21)	39.2 (2.8)	41.5 (4.4)	39.9 (3.7)	40.4 (3.4)	35.7 (2.9)
Body weight gain (LD 0–21)	3.8 (3.2)	6.0 (3.1)	4.9 (2.8)	5.1 (3.3)	1.7 (2.9)
Daily food consumption (LD 0–14)	17.6 (2.3)	17.7 (1.5)	17.6 (1.6)	17.3 (1.4)	10.5 (1.7)[#]

Numbers of animals completing each phase are listed. Data are presented as mean (standard deviation). Units are grams (body weight parameters) and grams/day (food consumption).
[#] Significantly different ($p < 0.05$) from control by Dunnett's test.

3.2. Body weight and nutritional parameters in P_1 mice

Among P_1 males administered 100 mg/kg/day, mean values for final body weight (test day 105), body weight gain (test days 0–105), food consumption (test days 0–70), and food efficiency (test days 0–70) were 4.8%, 11.6%, 7.5%, and 12.6% lower than control means (not statistically significant), respectively, and were attributed to 6:2 FTOH (Table 1). There were no effects on body weight or nutritional parameters in P_1 males at dose levels ≤25 mg/kg/day.

For P_1 females during premating and gestation, there were no effects on body weight or nutritional parameters at any dose level (Table 1). During the lactation period, P_1 females administered 100 mg/kg/day exhibited statistically significantly lower body weight gain, food consumption, and food efficiency during the intervals LD 0–7 and LD 7–14, compared with controls, resulting in statistically significantly lower body weight on LD 7 and LD 14 (Table 1, Fig. 1). Compared with controls, mean body weights were 20% lower on LD 14 and mean food consumption was 51% lower from LD 7 to 14. There were no effects on body weight or nutritional parameters in P_1 females at dose levels ≤25 mg/kg/day.

3.3. Reproductive performance of P_1 mice

There were no effects in reproductive performance (mating index, gestation index, fertility index, precoital interval, gestation length, number of implantation sites, or post implantation loss) at any dose level (Table 2).

3.4. In-life data for F_1 offspring

Litters of dams administered 100 mg/kg/day exhibited 6:2 FTOH-related reductions in pup survival and body weight (Fig. 2), and clinical abnormalities associated with delayed maturation (data not shown). At 100 mg/kg/day, pup survival was statistically significantly lower than control on LD 14 and 21, and over the interval LD 4–21 (45% reduction in lactation index; Table 2). Mean pup body weights were statistically significantly lower than control on LD 4, 7, 14, and 21, achieving a decrement of 65% lower than control on LD 21. Pups in four litters at this dose level were observed with closed eyes on lactation day 21, due to delayed maturation. All F_1 pups at the 100 mg/kg/day dose level were euthanized on LD 21 due to concerns about their ability to survive without the dam. There were no differences in pup viability, survival, clinical observations, or body weight at any other dose level. No differences in live born or viability indices, litter size, or sex ratio were observed at any dose level.

Among F_1 males and females selected for further evaluation after weaning, there were no effects or statistically significant differences in the age at attainment of preputial separation or vaginal patency, body weight parameters, and nutritional parameters; and no clinical abnormalities or mortality, at dose levels ≤25 mg/kg/day, which was the highest level evaluated due to the early termination of the 100 mg/kg/day group (Table 3).

3.5. Hematology in P_1 mice

6:2 FTOH exposure at 100 mg/kg/day resulted in hematology findings for both red and white cell parameters in males and females (Fig. 3). Group mean red cell mass parameters (RBC, HGB, and HCT) were minimally to mildly decreased by up to 11 and 17%, in males and females, respectively (statistically significant for most parameters). In females, but not males, red cell mass changes were associated with an increase in ARET (132% above controls). Additional parameters reflecting these changes in red blood cell mass included minimal (less than 10% change compared to controls) but statistically significant decreased MCV in males (4% below control), increased RDW in males (8% above control), and decreased MCH in females (6% below control). Total white blood cells were increased by 41% (males) and 110% (females), and were

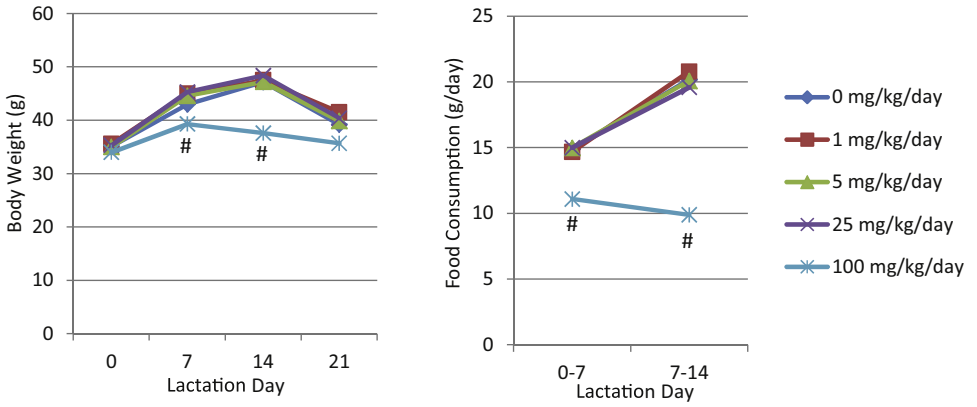

Fig. 1. P_1 in-life parameters during lactation. Mean values are displayed. #Significantly different ($p < 0.05$) from control by Dunnett's test.

Table 2
Reproductive parameters and F_1 litter data.

Dose (mg/kg/day)	0	1	5	25	100
Number of females paired	15	15	15	15	14
Number of females mated	14	15	14	15	11
Number of pregnant females	14	14	14	14	11
Number of females that littered	15	14	15	14	12
Mating index (%)[a]	100	100	100	100	100
Fertility index (%)[b]	100	93.3	100	93.3	100
Gestation index (%)[c]	100	100	100	100	100
Viability index (%)[d]	93.7	100	99.5	100	90.3
Lactation index (%)[e]	99.0	100	100	99.1	54.5[#]
Precoital interval (days)	2.0(1.2)[f]	2.0(1.1)	2.2(1.3)	2.6(1.8)	2.5(1.6)
Mean gestation length (days)	18.9(0.3)	18.8(0.4)	19.0(0.1)	19.0(0.3)	18.9(0.5)
Implantations	11.7(1.5)	12.1(1.6)	11.5(2.5)	12.1(1.9)	11.2(3.3)
Number of pups born	11.0(2.0)	11.5(1.5)	11.3(2.7)	11.1(1.7)	11.6(1.6)
Number of pups born alive	11.0(2.0)	11.5(1.5)	11.3(2.7)	11.0(1.8)	11.4(1.3)
Sex ratio (% males)	52.0(20.6)	51.6(8.5)	47.6(15.9)	40.7(14.6)	48.8(19.9)

[a] Number copulated/number paired.
[b] Number pregnant/number copulated.
[c] Number of litters with at least 1 live pup/number of litters.
[d] Number of pups alive on day 4/number of pups born alive.
[e] Number of live pups on lactation day 21/number of live pups on day 4 post-culling.
[f] Data are presented as mean (standard deviation).
[#] Significantly different ($p < 0.05$) from control by Dunnett's test.

associated with statistically significant increases in females (but not males) in absolute neutrophil, lymphocyte, and monocyte counts (data not shown).

No effects or statistically significant differences in hematology parameters were observed in males or females at dose levels ≤25 mg/kg/day.

3.6. Clinical chemistry in P_1 mice

Effects on liver-associated clinical chemistry parameters were observed in males and females administered 100 mg/kg/day (Fig. 4). Changes for AST, ALT, ALKP, SDH, and TBA were generally mild to moderate in males and

Table 3
F_1 in-life parameters.

Dose (mg/kg/day)	0	1	5	25
F1 males	13	14	15	14
Body weight gain (PND 21–40)	18.0(2.1)	18.0(2.3)	17.5(1.5)	18.2(1.7)
Daily food consumption (PND 21–40)	5.4(0.6)	5.7(0.5)	5.4(0.4)	5.4(0.5)
Age at preputial separation achievement	28.3(1.5)	27.5(1.2)	27.3(1.7)	27.9(1.5)
F1 females	13	14	14	14
Body weight gain (PND 21–40)	11.2(1.2)	11.0(1.4)	11.8(1.2)	11.2(1.9)
Daily food consumption (PND 21–40)	4.8(0.4)	4.9(0.4)	5.1(0.4)	4.9(0.5)
Age at vaginal patency achievement	29.8(2.2)	29.4(2.6)	29.2(2.8)	30.2(2.2)

Numbers of animals completing each phase are listed. Data are presented as mean (standard deviation). Units are grams (body weight gain), grams/day (food consumption), and post-natal days (preputial separation or vaginal patency achievement). There were no statistically significant differences ($p < 0.05$).

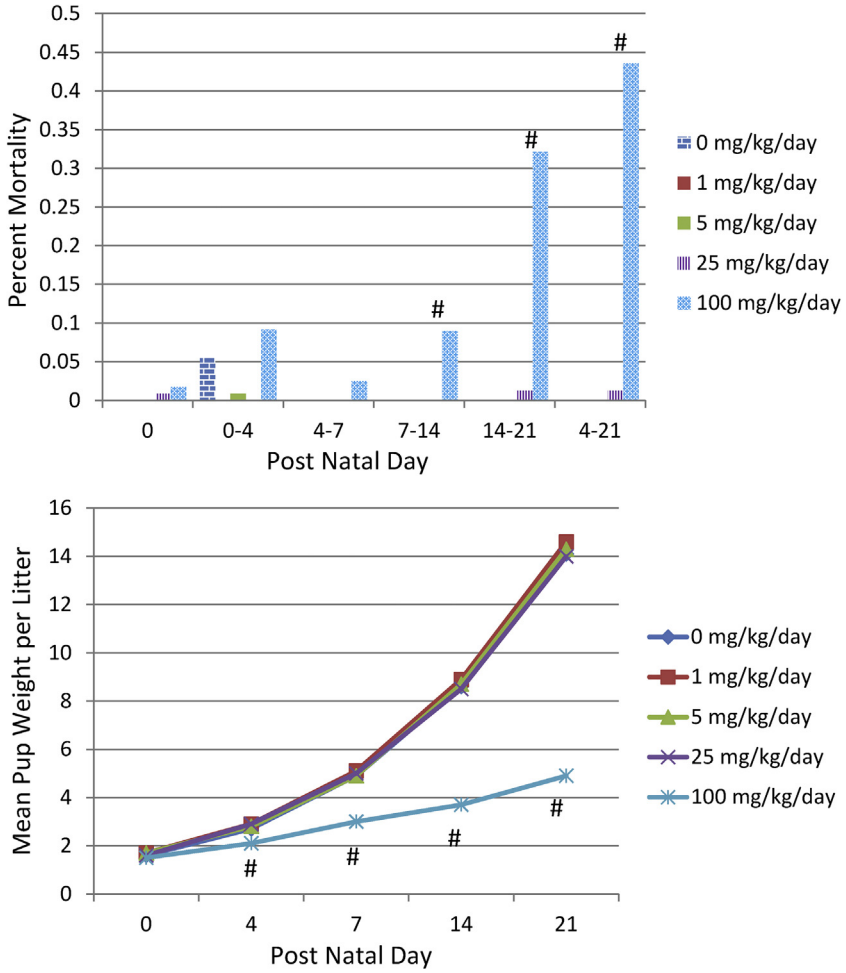

Fig. 2. F_1 mortality and pup weights during lactation. Mean values are displayed. #Significantly different ($p < 0.05$) from control by Dunnett's test.

moderate to severe in females, as group mean liver parameters were increased approximately 2.5- to 5-fold compared to controls in males, whereas in females, most parameters were increased by greater than 5-fold and by as much as 19-fold (TBA in females) compared to controls. Although not statistically significant, minimal increases in total BILI in these groups may also have been 6:2 FTOH-related. Elevations in some liver parameters (AST, ALT, SDH, and TBA, but not ALKP or BILI) in one 25 mg/kg/day female were associated with microscopic lesions in the liver and were attributed to 6:2 FTOH exposure.

Effects on other clinical pathology parameters in males and females at 100 mg/kg/day included decreases in the following (data not shown): BUN, decreased by 28% and 18% in males and females, respectively; CREA, decreased by 37 and 20%, respectively; and CHOL (males only; decreased by 35%). In addition, potassium was minimally higher in males and females at 100 mg/kg/day (23 and 15% above the controls, respectively; statistically significant in males only)

There were no effects on clinical chemistry parameters in males at dose levels ≤25 mg/kg/day, or in females at dose levels ≤5 mg/kg/day.

3.7. Organ weights in P_1 mice

Primary organ weight effects were observed in the liver of males and females at 100 mg/kg/day, and in the kidneys of males at 100 mg/kg/day (Tables 4 and 5). In addition, reproductive organ weight changes occurring secondary to body weight decrements were observed in male and female reproductive organs (Tables 4 and 5).

Group mean liver weight parameters were increased in both sexes (only liver weight relative to body weight was statistically significant), although these increases were greater in females compared to males (liver weight relative to body weight was increased 13% and 24% above control, in males and females, respectively).

Group mean kidney weight parameters were increased in males at 100 mg/kg/day (up to 25% above controls; statistically significant). These increases were not associated with relevant changes in kidney-related clinical pathology parameters or with microscopic changes in the kidney. Kidney weight relative to body weight was minimally higher in females at 100 mg/kg/day (11% above control; statistically significant). However, there were no statistically significant changes in other kidney weight parameters, and

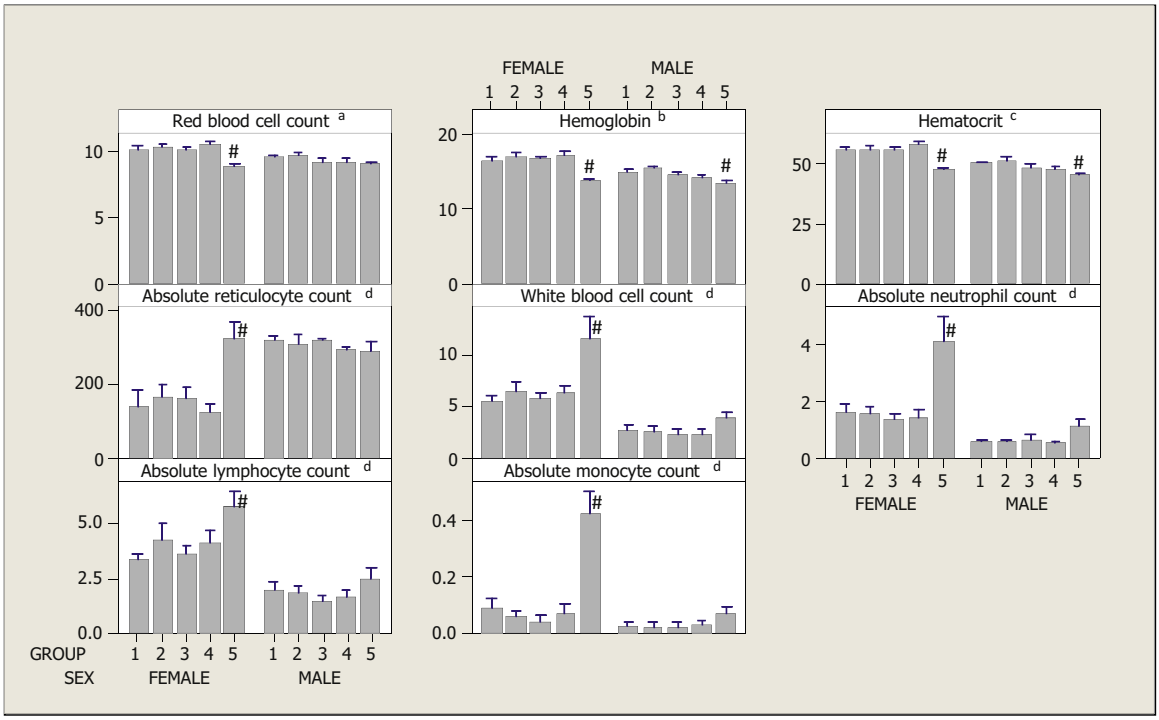

Fig. 3. P_1 hematology parameters. Groups 1, 2, 3, 4, and 5 correspond to doses of 0, 1, 5, 25, and 100 mg/kg/day, respectively. Mean values and standard error bars are displayed. Units: (a) $\times 10^6/\mu L$; (b) g/dL; (c) %; (d) $\times 10^3/\mu L$. #Significantly different ($p < 0.05$) from control by Dunnett's test.

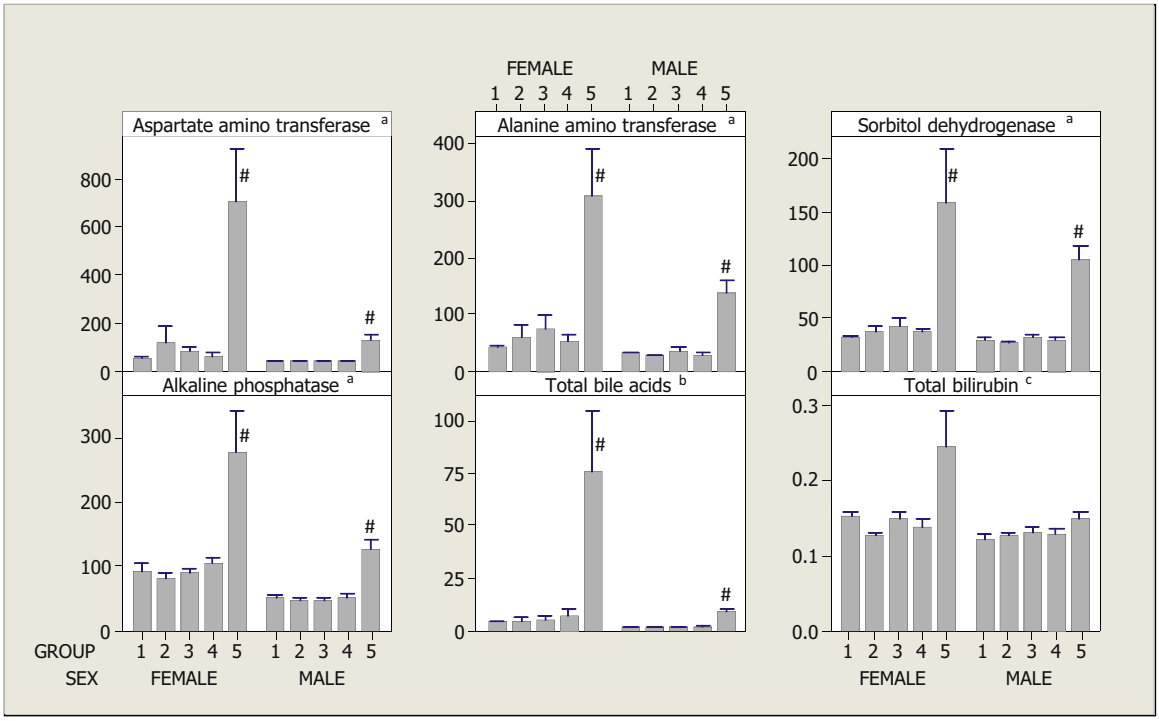

Fig. 4. P_1 clinical chemistry parameters. Groups 1, 2, 3, 4, and 5 correspond to doses of 0, 1, 5, 25, and 100 mg/kg/day, respectively. Mean values and standard error bars are displayed. Units: (a) U/L; (b) μmol/L; (c) mg/dL. #Significantly different ($p < 0.05$) from control by Dunnett's test.

Table 4
P_1 organ weights (males).

Dose (mg/kg/day) No. examined	0 (14)	1 (15)	5 (15)	25 (15)	100 (14)
Absolute					
Terminal body weight	41.9(3.1)	54.8(2.9)	42.0(2.6)	40.6(2.2)	39.4(2.9)
Liver	2.162(0.235)	2.140(0.219)	2.054(0.202)	2.093(0.162)	2.296(0.264)
Kidneys	0.659(0.117)	0.692(0.101)	0.6613(0.054)	0.710(0.089)	0.773(0.123)[#]
Epididymides	0.123(0.014)	0.125(0.014)	0.125(0.001)	0.121(0.011)	0.123(0.016)
Testes	0.235(0.027)	0.253(0.031)	0.258(0.040)	0.230(0.021)	0.254(0.028)
Relative					
Liver	5.159(0.402)	5.000(0.417)	4.903(0.483)	5.163(0.353)	5.817(0.368)[#]
Kidneys	4.564(0.207)	1.613(0.175)	1.576(0.191)	1.748(0.190)	1.959(0.260)[#]
Epididymides	0.296(0.046)	0.294(0.038)	0.299(0.031)	0.298(0.033)	0.313(0.048)
Testes	0.565(0.076)	0.593(0.085)	0.616(0.094)	0.568(0.066)	0.647(0.086)[#]

Data are presented as mean (standard deviation). Absolute weights are reported in grams; relative weights are reported as percent terminal body weight.
[#] Significantly different ($p < 0.05$) from control by Dunnett's test.

no treatment-related changes in kidney-related clinical pathology parameters or kidney histopathology. Therefore, this change in female mice was considered to be unrelated to 6:2 FTOH administration.

Organ weight changes occurring secondary to body weight decrements were observed in male and female reproductive organs. Absolute and relative ovarian and uterine weight parameters were moderately to markedly decreased in females at 100 mg/kg/day. Ovarian weights were decreased by up to 24% compared to controls, and uterine weights were decreased by up to 56% compared to controls (statistically significant for most weight parameters). Testes weight relative to body weight was statistically increased (15% above control) in males at 100 mg/kg/day.

There were no effects or statistically significant differences in organ weight parameters in males or females at dose levels ≤25 mg/kg/day.

3.8. Histopathology in P1 mice

3.8.1. Liver

Microscopic findings indicative of trophic and toxic effects on the liver were present in males and females at 100 mg/kg/day and in low incidences in females at 25 mg/kg/day (Table 6). These changes were generally more severe in females and included hepatocellular hypertrophy, oval cell hyperplasia, single cell necrosis of hepatocytes, and cystic degeneration (females only). Minimal microscopic hepatocellular hypertrophy was also present in males at 5 and 25 mg/kg/day and in females at 5 mg/kg/day.

Hepatocellular hypertrophy was most prominent in centrilobular regions and was characterized by enlarged hepatocytes containing increased amounts of pale to brightly eosinophilic granular cytoplasm and enlarged nuclei which often had prominent and multiple nucleoli. Hyperplastic oval cells, small spindiloid cells containing fusiform to oval nuclei and scant basophilic cytoplasm, radiated from periportal regions into the surrounding parenchyma and were often admixed with inflammatory cell infiltrates. Single cell necrosis (or apoptosis) of hepatocytes was most conspicuous in periportal regions and was characterized by individual shrunken, hypereosinophilic cells containing pyknotic nuclei. Focal to multi-focal areas of cystic degeneration, likely resulting from dropout of degenerate hepatocytes, were characterized by variably-sized cystic areas containing flocculent to homogenous eosinophilic material, often admixed with inflammatory cell infiltrates.

No microscopic effects were observed in the liver of males or females at 1 mg/kg/day.

Table 5
P_1 organ weights (females).

Dose (mg/kg/day) No. examined	0 (13)	1 (15)	5 (15)	25 (15)	100 (10)
Absolute					
Terminal body weight	39.2(2.8)	40.6(5.7)	39.9(3.7)	39.8(4.2)	35.4(2.9)
Liver	2.809(0.329)	3.070(0.605)	3.005(0.418)	3.048(0.526)	3.162(0.640)
Kidneys	0.514(0.027)	0.525(0.090)	0.516(0.038)	0.546(0.059)	0.515(0.051)
Ovaries	0.0316(0.0045)	0.0370(0.0088)	0.0342(0.0055)	0.0355(0.0095)	0.0244(0.0032)[#]
Uterus	0.264(0.131)	0.202(0.068)	0.198(0.058)	0.203(0.068)	0.118(0.069)[#]
Relative					
Liver	7.162(0.604)	7.512(0.774)	7.510(0.537)	7.624(0.911)	8.873(1.341)[#]
Kidneys	1.316(0.086)	1.293(0.113)	1.296(0.081)	1.376(0.114)	1.454(0.080)[#]
Ovaries	0.081(0.012)	0.093(0.025)	0.086(0.017)	0.089(0.023)	0.069(0.010)
Uterus	0.675(0.334)	0.515(0.211)	0.505(0.178)	0.518(0.198)	0.332(0.176)[#]

Data are presented as mean (standard deviation). Absolute weights are reported in grams; relative weights are reported as percent terminal body weight.
[#] Significantly different ($p < 0.05$) from control by Dunnett's test.

Table 6
P_1 histopathology of liver.

Dose (mg/kg/day): no. examined	Male					Female				
	0(15)	1(15)	5(15)	25(15)	100(15)	0(15)	1(15)	5(15)	25(15)	100(15)
Hypertrophy, hepatocellular	0	0	9	10	15	0	0	13	12	13
Mitotic figures, increased	0	0	0	0	5	0	1	0	1	10
Oval cell hyperplasia	0	0	0	0	15	0	0	0	2	12
Cystic degeneration	0	0	0	0	0	0	0	1	1	7
Single cell necrosis	0	0	0	0	12	2	1	1	1	12
Infiltrate, mononuclear (oval cell associated)	0	0	0	0	15	0	0	0	0	10
Pigment, increased	0	0	0	0	15	0	0	0	0	0

3.8.2. Teeth

6:2 FTOH-related changes in the incisor teeth, consistent with fluoride exposure, were present in males and females at 100 mg/kg/day. These changes included degeneration and atrophy of ameloblastic epithelium, accentuation of the normal laminar pattern of dentin and an increase in observed incomplete decalcification of enamel and/or dentin. Degeneration and atrophy of ameloblasts was characterized by segmental disorganization and attenuation of ameloblastic epithelium of the incisor teeth. Lamination of dentin was characterized by the presence of concentric basophilic rings within the dentin of these teeth. Incomplete decalcification of enamel and dentin was characterized by an increase in the observed presence of basophilic, mineralized debris in the enamel space of the incisor between the dentin and the gingiva. Normally, this material is lost during processing of slides and is not visible, but it is observed sporadically in control mice due to vagaries in processing. However, this finding was observed at a higher incidence and extent in males and females administered 100 mg/kg/day. Incomplete decalcification of nasal bones, also consistent with fluoride exposure, was observed in some animals at the 100 mg/kg/day dose level. There were no adverse changes in the teeth of males or females at dose levels ≤25 mg/kg/day.

3.8.3. Female reproductive tract and mammary gland

Eight of 11 nursing dams in the 100 mg/kg/day group that survived to the scheduled euthanasia at weaning were in anestrus. No dams in the control or other treated groups were anestral. Anestrus was characterized by marked thinning of the vaginal mucosal epithelium (which was often only a single cell layer thick), uterine involution and atrophy of ovarian interstitial cells. In the mammary glands of nursing dams in the 100 mg/kg/day group that survived to weaning, microscopic changes included decreased milk secretion (in 8/11 dams), characterized by lactational hypertrophy and hyperplasia of glandular epithelium consistent with an actively nursing dam, but with few or no alveoli containing proteinaceous secretion, and apparent mineralization (deep basophilic staining) of glandular secretion (in 11/11 dams).

No treatment-related changes in reproductive tract histology, estrus cyclicity or mammary glands were observed at dose levels ≤25 mg/kg/day, where body weight and nutritional parameters in both dams and offspring were similar to controls.

3.9. Gross pathology in F_1 mice

There were no 6:2 FTOH-related gross findings in F_1 pups or weanlings at dose levels ≤100 mg/kg/day, nor in F_1 adults at dose levels ≤25 mg/kg/day (the highest level evaluated due to the early termination of the 100 mg/kg/day group).

4. Discussion

The primary objective of this study was to evaluate the oral systemic repeated-dose and reproductive toxicity of 6:2 FTOH in CD-1 mice. Results from the present evaluation in mice, in conjuction with previously published results using CD rats [10,11], provide a basis for comparison between commonly used outbred strains of two rodent species.

4.1. Repeated-dose systemic and reproductive toxicity of 6:2 FTOH in mice

At 100 mg/kg/day, mortality was observed in 1/15 male and 2/15 female mice, as well as clinical signs of behavioral and physiological dysfunction, and reductions in body weight and nutritional parameters. Reductions in body weight were 5% in P_1 males and 20% in P_1 females. Alterations in red blood cell parameters at this dose level may be secondary to the body weight reductions [32]. In general, the alterations in red blood cell parameters were more severe in females than in males, and were associated with a regenerative response only in females, which corresponded to the sex difference in body weight reductions. Increased white blood cell counts at 100 mg/kg/day in males and females likely represent an inflammatory response to liver toxicity. Elevated clinical chemistry parameters indicative of hepatocellular injury (AST, ALT, ALP, SDH, and TBA) were greater in females than males at 100 mg/kg/day, and were also observed in one female (and no males) at 25 mg/kg/day.

Correspondingly, histopathologic alterations in the liver were more severe in females than males. While liver weights were increased in both sexes at 100 mg/kg/day, adverse microscopic liver effects (including singe cell necrosis, cystic degeneration, oval cell hyperplasia and increased pigment) were observed in females at dose levels ≥25 mg/kg/day and males at 100 mg/kg/day. In addition, decreases in BUN, CREA, and/or CHOL were observed in males and females at 100 mg/kg/day; the decreases may be secondary to the liver toxicity observed at this

dose level [33] but may also be in response to food consumption reduction and/or to hepatic enzyme induction [34]. Hepatocellular hypertrophy was observed at dose levels ≥5 mg/kg/day in both sexes, but was considered non-adverse when it occurred in the absence of correlative liver weight changes or histologic evidence of hepatotoxicity, based on industry standards and regulatory guidance [8,35].

The findings of hepatocellular hypertrophy were morphologically consistent with peroxisome proliferation and, along with increased mitotic figures, were consistent with the known trophic effects produced in the liver of rodents by peroxisome proliferator-activated receptor (PPAR) alpha agonists [36]. Microscopic evidence of hepatotoxicity included single cell necrosis and cystic degeneration. Hyperplastic oval cells likely represent a regenerative response to liver cell injury and are considered to arise from terminal ductule epithelial cells (canal of Hering cells) within periportal regions of the liver [37].

6:2 FTOH has been shown to rapidly metabolize with formation of fluoride [13,38]. Microscopic alterations of incisor teeth in both male and female mice at 100 mg/kg/day included degeneration and atrophy of ameloblastic epithelium (an adverse change), lamination of dentin, and incomplete decalcification of enamel and/or dentin. Changes in the ameloblastic epithelium and lamination of dentin have been previously described in the rat incisor following fluoride exposure [39–41]. Incomplete decalcification of teeth and bone are likely the result of fluoride-associated resistance to the acid decalcification procedure used in this study, as fluoride can reduce the acid solubility of mineral in teeth and bones [42,43] and as such, this finding was considered to be indicative of exposure to a metabolized fluoride, but non-adverse.

Reproductive organ weight parameters and/or histology were altered in males and females as secondary effects of decrements in body weight and nutritional parameters at 100 mg/kg/day. In males, an increased ratio of testes weight to body weight was attributed to body weight loss, as testes weights are known to be stable with moderate levels of body weight loss [44] and there were no correlative histologic findings in the testes. In females at this dose level, ovarian and uterine weight parameters were decreased, which corresponded to microscopic findings including anestrus with associated atrophic changes in the reproductive tract, and secretory depletion in the mammary gland. Anestrus and the correlative microscopic changes in the female reproductive tract are common nonspecific findings associated with reduced food intake and stress [45]. Secretory depletion in the mammary gland is likely the result of increased duration of nursing in the undernourished pups at 100 mg/kg/day, as undernourished rat pups from diet-restricted dams have increased duration of suckling compared to controls, especially toward the end of lactation [46].

The following changes were attributed to 6:2 FTOH exposure but non-adverse based on lack of association with organ injury or evidence of decreased function, as described above: increased kidney weights (100 mg/kg/day males), incomplete decalcification of enamel and dentin (25 mg/kg/day females), and minimal hepatocellular hypertrophy (5 mg/kg/day males and females; 25 mg/kg/day males).

Table 7
NOAELs of 6:2 FTOH in rats and mice.

	Mice	Rats[a]
Overall		
Systemic toxicity	5	5
Reproductive toxicity	>100[b]	>250[b]
Pup viability/growth	25	25
Individual parameters		
Mortality	25	25
Adverse clinical observations	25	25
Liver histopathology	5	5
Tooth histopathology	25	125
Hematology (red blood cell)	25	5
Hematology (white blood cell)	25	>250[b]
Clinical chemistry	5	5
Maternal body weight (lactation)	25	125
Pup survival	25	25
Pup body weights	25	25

[a] [10,11].
[b] Highest dose evaluated in the study.

Among F_1 pups, adverse effects at 100 mg/kg/day included mortality (45% reduction in lactation index), reduced body weights (65% reduction), and clinical signs of delayed maturation. The failure to thrive and delayed maturation of the pups were likely a result of the overt systemic toxicity observed in the dams.

For mice, the no-observed-adverse-effect level (NOAEL) for systemic repeated-dose toxicity was 25 mg/kg/day (males) and 5 mg/kg/day (females), based on effects at higher doses on mortality, clinical observations, body weight, nutritional parameters, hematology (red and white blood cell), clinical chemistry (liver-related), liver weights, and histopathology (liver, teeth, reproductive tract, and mammary gland). However, 6:2 FTOH was not a selective reproductive toxicant; any effects observed in offspring occurred at dose levels that induced mortality and severe toxicity in maternal animals. The NOAEL for reproductive toxicity was 100 mg/kg/day; no effects on reproductive outcome were observed at any dosage. The NOAEL for viability and growth of the offspring was 25 mg/kg/day, based on clinical signs of delayed maturation in pups, and reductions in pup survival and pup body weight during lactation at 100 mg/kg/day.

4.2. Comparison of 6:2 FTOH toxicity in rats and mice

The results of the present study can be compared with previous oral repeated-dose and reproduction studies in CD rats with 6:2 FTOH [10,11] and reflects the toxicological action and effects of 6:2 FTOH and its metabolites. The severity and range of effects were generally greater in mice, especially with regard to liver histopathology/function and pup growth/viability. However, the overall NOAELs for systemic toxicity, reproductive toxicity, and offspring viability/growth were consistent between species (Table 7), and 6:2 FTOH was not a selective reproductive toxicant in either species.

Among P_1 adults, the NOAEL for mortality and clinical observations was 25 mg/kg/day in both species (Table 7). However, the NOAEL for body weight loss during lactation

was higher in rats than mice, and the magnitude of the reduction at 100 mg/kg/day in mice exceeded that at 250 mg/kg/day in rats. The NOAEL for liver-related clinical chemistry changes and associated microscopic liver findings was 5 mg/kg/day in both species, although the extent of hepatocellular injury was more pronounced in mice. The magnitude of differences in clinical chemistry parameters and the incidences of histopathologic liver findings at 100 mg/kg/day in mice exceeded those at 250 mg/kg/day in rats. Correspondingly, white blood cell alterations, which may be associated with liver inflammation due to hepatotoxicity, were observed at 100 mg/kg/day in mice, but were not reported in rats at dose levels ≤250 mg/kg/day. Histopathologic evidence of liver toxicity in the rat (single cell necrosis, biliary and oval cell hyperplasia; hepatocyte vacuolation and peri-portal-associated inflammation) was reported for only a few animals (at ≥125 mg/kg/day in males and at ≥25 mg/kg/day in females), and generally with minimal severity ([10]; DuPont, unpublished data). In contrast, liver toxicity in the mouse (singe cell necrosis, cystic degeneration, oval cell hyperplasia, oval cell-associated mononuclear infiltrate and increased pigment) generally affected most animals of both sexes at 100 mg/kg/day with minimal to mild severity. Microscopic alterations in the incisor teeth were observed in mice at 100 mg/kg/day whereas similar effects were only reported at 250 mg/kg/day in rats. Microscopic findings in reproductive organs (anestrus with associated atrophic changes in the reproductive tract, and secretory depletion in the mammary gland) occurring secondary to decrements in body weight and nutritional parameters were observed at 100 mg/kg/day in mice, but were not reported in rats at dose levels ≤250 mg/kg/day. This difference between the two species was consistent with the greater difference in maternal body weight during lactation, in mice compared to rats.

Among F_1 pups, the NOAEL for mortality, clinical observations, and reduced body weights was 25 mg/kg/day in both species. The magnitude of the reductions in lactation index and pup body weight at 100 mg/kg/day in mice exceeded those observed in rats at 250 mg/kg/day, and is likely a result of the species difference in maternal body weight during lactation.

4.3. Comparison of 6:2 FTOH toxicity with other fluorotelomer alcohols and metabolites

Reproductive or systemic toxicity data are available for 8:2 FTOH; a fluoroalkylethanol mixture, $F(CF_2CF_2)_nCH_2CH_2OH$ (n = 3–6), including 30% (w/w) 6:2 FTOH; and PFHxA, a principal metabolite of 6:2 FTOH. Toxicity data for these related chemicals are consistent with data reported in both rats and mice for 6:2 FTOH with regard to NOAELs, the types of effects, and the lack of selective reproductive toxicity where applicable, *i.e.* any effects on offspring viability and growth were only observed at concentrations that were also associated with systemic toxicity. Reproductive evaluations of the fluoroalkylethanol mixture and PFHxA in CD rats yielded NOAELs of 20–50 mg/kg/day for systemic toxicity, greater than 250 mg/kg/day (the highest dose evaluated) for reproductive toxicity, and 25–100 mg/kg/day for offspring viability and growth ([47,11,16]). Subchronic evaluations of 8:2 FTOH, the fluoroalkylethanol mixture, and PFHxA in CD rats yielded NOAELs ranging from 5 to 25 mg/kg/day, based on effects at higher doses on body weight, clinical observations, liver and/or kidney weights, histopathology (liver, kidney, thyroid, or teeth), red blood cell and liver-related clinical chemistry parameters, and urinary or plasma fluoride [16,48,49].

8:2 FTOH has been evaluated for developmental and hepatotoxicity in mice (CD-1 or ddY strains) in addition to CD rats [20,22,49,50]. The studies in mice differed from those in rats with respect to basic study design elements (*e.g.*, number of exposures, diet *vs.* gavage), and they did not include a full evaluation of reproductive toxicity. Nonetheless, the differences between species for 8:2 FTOH toxicity were similar to those presently reported for 6:2 FTOH, *i.e.*, mice were generally more sensitive. Compared with rats, 8:2 FTOH exposure in mice resulted in hepatomegaly at a lower dose level and developmental toxicity (neonatal mortality and malformations in mice) with greater severity at a lower dose, although the effects on the fetus in both species were only observed at levels that induced maternal toxicity. The greater sensitivity of mice to hepatotoxic effects has also been demonstrated in evaluations of perfluorobutyrate (PFBA) and perfluorooctanoate (PFOA), which are metabolites of 6:2 FTOH and 8:2 FTOH, respectively. Exposure to PFBA and PFOA resulted in the same or lower NOAELs for hepatotoxicity in CD-1 mice compared with CD rats, while the severity of hepatomegaly/hypertrophy and the range of histopathologic effects (*e.g.* necrosis, increased mitotic figures) was generally greater in mice [21,23,24,51]. These comparisons are generally limited to one strain of each species (CD-1 mice and CD rats). The only other strain included in the discussion above was the ddY strain of mouse (Japan), which yielded results that were consistent with those from the CD-1 strain [50].

Mechanisms underlying the greater sensitivity of mice to hepatotoxic effects are not certain, but several proposed explanations include possible species differences in peroxisome proliferation or in kinetic properties of metabolites. Induction of peroxisome proliferation in rodents has been confirmed for 8:2 FTOH, PFBA, and PFOA [21,23,50], and may be presumed for 6:2 FTOH on the basis of histologic effects described in Section 4.1 and beta oxidation measurements in one of its principal metabolites, 5:3 acid [19]. To the extent that peroxisome proliferation may be responsible for any species differences in 6:2 FTOH toxicity, the relevance for humans may be limited, because humans tend to be resistant to such effects, in contrast to rats and mice [52]. Loveless et al. [21] noted that species differences in PFOA toxicity may be a result of additional mechanisms as well, due to the inconsistent correlation between liver weight increases and peroxisomal beta oxidation.

Henderson and Smith [22] suggested that the sensitivity of mice to 8:2 FTOH may be attributed to the role of terminal metabolites such as perfluorooctanoic acid (PFOA). This assertion formed a basis for proposed hazard classifications for 8:2 FTOH which were not adopted by the ECHA Committee for Risk Assessment [53,54], due to a wider body of kinetic, molecular, and epidemiologic data that did not

support this claim [55–62]. In addition, these classification arguments pertaining to longer chain fluorochemicals are not directly applicable to 6:2 FTOH, because of differences between short chain and longer chain metabolites. PFHxA (the analogous 6-carbon metabolite from 6:2 FTOH) is eliminated from plasma 40 to 80 times faster than PFOA in humans, rats, and mice [15,63,64], and the hepatotoxicity is correspondingly lower in rats and mice as well [16,65].

Species differences in FTOH toxicity may also be due to possible kinetic differences in other metabolites such as fluorotelomer unsaturated aldehydes (FTUALs), which are potential precursors to perfluorocarboxylic acids (PFCAs) such as PFOA and PFHxA. Evaluations of toxicokinetics, protein reactivity, cytotoxicity, and acute aquatic toxicity have indicated that FTUALs are generally more toxic than the PFCAs, and have been suggested to be responsible for rat liver lesions following FTOH exposure ([66–70,72]). Further evaluation would be required to determine species differences in kinetics of FTUALs and other FTOH metabolites. Repeated-dose evaluations of intermediate metabolites may not be possible because of their relative instability.

5:3 acid is another 6:2 FTOH metabolite that has been evaluated in mammalian and aquatic species for biopersistence, acute oral toxicity, and two week repeated-dose toxicity. The extent of acute and systemic toxicity was considered consistent with previously reported data for 6:2 FTOH and indicated low potential for bioaccumulation in mammals [19]. Although 5:3 acid has not been specifically evaluated for reproductive or subchronic toxicity, the results of the present evaluation of 6:2 FTOH represents the combined effects of the parent chemical and all metabolites, including the principal metabolites PFHxA and 5:3 acid, and intermediates such as FTUALs.

5. Conclusions

CD-1 mice were generally more sensitive than CD rats with respect to systemic toxicity from oral administration of 6:2 FTOH, and this was likely responsible for the more severe effects observed in the offspring (*i.e.*, pup growth and viability) at doses where systemic effects were observed. The liver was the primary target organ in both species, evidenced by increased liver size, trophic and toxic histopathologic effects, and elevations in clinical chemistry parameters that were consistent with hepatotoxicity. Mortality, clinical abnormalities, red blood cell changes, and reductions in male body weights were consistent across species, but the reduction in P_1 female body weight during lactation was markedly greater in mice than in rats. Correspondingly, changes in the female reproductive tract and mammary gland secondary to body weight decrements were observed only in mice, and the magnitude of the reductions in lactation index and pup body weight were greater in mice than in rats. While the severity of the effects was generally greater in mice than rats, the overall NOAELs were identical in both species, 5 mg/kg/day for systemic toxicity and 25 mg/kg/day for offspring viability/growth. 6:2 FTOH was not a selective reproductive toxicant in either species; no effects on reproductive outcome occurred at any dose level, and any effects observed in offspring occurred at dose levels that induced mortality and severe toxicity in maternal animals. The present evaluation of 6:2 FTOH in CD-1 mice corroborates the benchmark dose of 5 mg/kg/day previously reported for 6:2 FTOH in CD rats. Together, these studies reflect the toxicological action and effects of 6:2 FTOH and its metabolites in rats and mice.

Conflict of interest

The authors are employed by DuPont, which is a manufacturer of 6:2 FTOH.

Transparency document

The Transparency document associated with this article can be found in the online version.

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