



Reproductive and developmental toxicity risk assessment for 4-methylimidazole

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ABSTRACT

4-Methylimidazole (4-MEI) is present in caramel color for food and beverages and as a by-product of the thermal treatment of food. A risk assessment for 4-methylimidazole reproductive and developmental effects was conducted using data from a National Toxicology Program Reproductive Assessment by Continuous Breeding and using 90th percentile dietary exposure data from the U.S. Food and Drug Administration. The National Toxicology Program reported reproductive and developmental toxicity in Sprague Dawley rats from high dietary exposure to 4-MEI. Using the benchmark dose method of modeling dose–response curves, the lowest margin of exposure (MOE) was 1489. Using the traditional no observed adverse effect level (NOAEL)/lowest observed adverse effect level (LOAEL), the margin of exposure at the 90th percentile human consumption was 735. MOEs of 100 or greater are considered to be of low concern. In conclusion, human exposure to 4-MEI in the diet in the United States is not expected to confer risk to reproduction or development.

1. Introduction

4-Methylimidazole (4-MEI; [Fig. 1](#)) is a constituent in caramel coloring classes III or IV, and the respective caramel can be used in carbonated beverages, pancake syrup, and barbecue sauce. 4-MEI also is a product of a Maillard reaction in the heating (e.g., roasting, toasting) of foods to produce non-enzymatic browning ([Casal et al., 2002](#); [Schlee et al., 2013](#); [Mottier et al., 2017](#); [Folmer et al., 2018](#)). The Maillard reaction is responsible for the production of melanoidins, brown substances that impart color to cooked foods and other compounds that impart characteristic aromas and flavors.

The National Toxicology Program (NTP) conducted a reproductive assessment by continuous breeding (RACB) for dietary 4-MEI that was published in 2020 ([Behl et al., 2020](#)), with additional data in an online repository ([Behl et al., 2025](#)). Based on the findings for prostate atrophy and delays in preputial separation/vaginal opening, 4-MEI was characterized as a reproductive and developmental toxicant with a lowest observed adverse effect level (LOAEL) of 50–60 mg/kg bw/day. Male reproductive toxicity in rats was shown previously for dietary 4-MEI in a 14-week NTP study ([Chan et al., 2004](#)). Testicular degeneration occurred in animals given 5000 or 10,000 ppm but not 2500 ppm. The present paper provides a risk assessment using the findings of the NTP RACB study and the estimated dietary intake of 4-MEI in human populations. A previously published "risk assessment" was focused on

carcinogenicity and used only exposure from soft drinks ([Smith et al., 2015](#)).

Given the prevalence of 4-MEI in the diet, a risk assessment for reproductive endpoints is important. The NTP study did not identify a NOAEL and due to toxicity in the high-dose (5000 mg/kg bw/day) group, many of the endpoints were obtained only for two dose groups plus the control, suggesting that mathematical modeling of the dose–response curve using benchmark dose methods may be helpful. The aim of this paper is to perform a reproductive risk assessment using benchmark dose analysis in addition to the NOAEL-LOAEL approach used by the NTP authors.

2. Methods

2.1. Reproductive assessment by continuous breeding

The RACB protocol was developed at NTP. The protocol used in the 4-MEI study was described by [Chapin and Sloane \(1997\)](#). The 4-MEI RACB was a two-generation study using dietary treatment at 0, 750, 2500, or 5000 ppm based on a dose-range finding study ([Behl et al., 2025](#)). Twenty-three pairs of adult Sprague Dawley male and female rats per dose group were dosed for two weeks prior to pairing. They then were bred to produce three successive litters (called F1a, F1b, and F1c). Pups from the first two litters were removed on postnatal day (PND) 4.

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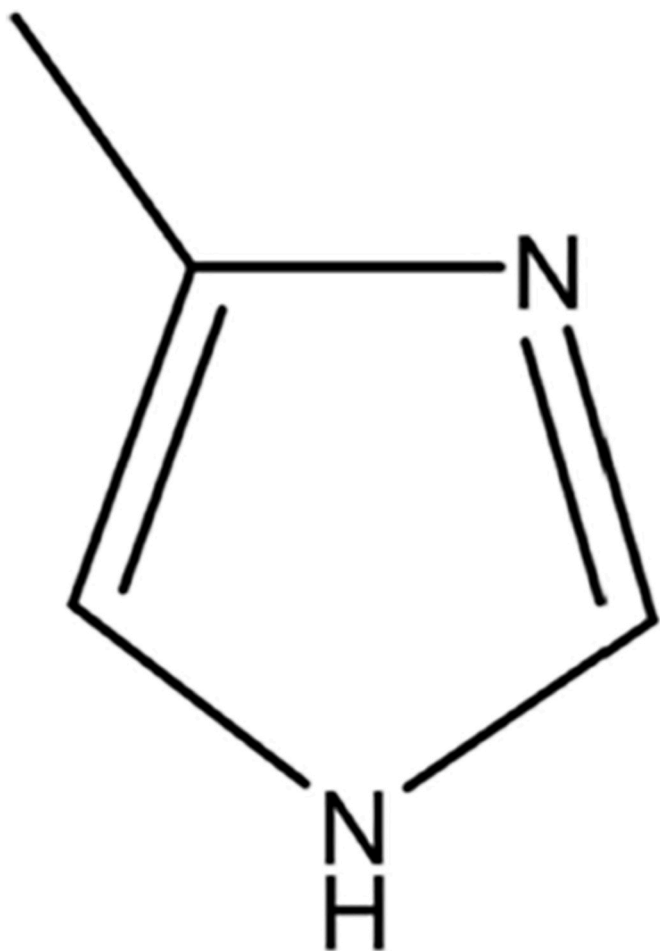


Fig. 1. Structure of 4-methylimidazole. From Folmer et al. (Folmer et al., 2018). There is no copyright.

F1c offspring were raised to weaning on PND 28 by their dams. F1c litters were standardized to 6 pups/sex/litter where possible. Beginning on PND 28, F1c offspring were given the diet consumed by their dams. On about PND 95, up to three F1c offspring/sex/litter were necropsied. About 40 F1c rats/sex/dose group were continued as parents of three successive F2 litters (called F2a, F2b, and F2c). F2a and F2b pups were removed on PND 4. F2c pups were necropsied on PND 28.

Crossover mating of treated F0 animals was used due to a decrease in litter size in that generation. Crossover mating involving F0 females in the 5000-ppm group was not possible due to moribundity in those animals. Due to F0 female morbidity and mortality, including difficulties in parturition in the production of F1a and F1b litters, these females were removed from study prior to the third mating, and there were no F1c litters. After production of the F1b litters, the highest dose level was 2500 ppm.

2.2. Exposure assessment

Human 4-MEI exposure levels were estimated in an FDA publication (Folmer et al., 2018). Foods evaluated were based on two databases that listed products containing added caramel color and based on product label verifications at grocery stores or on manufacturers' websites. Foods subject to thermal processing were also evaluated. Analyses were made by a contract laboratory using liquid chromatography and tandem mass spectrometry. Dietary intakes were derived from the National Health and Nutrition Examination Survey (NHANES) for 2009–2012, which estimated intake from two non-consecutive days, and from a commercial database that used data from a 10–14-day sample from the

same years. Exposure estimates are for foods only. For the current risk assessment, data from Table 7 of (Folmer et al., 2018) were used.

2.3. Risk assessment

Risk assessment was performed using methods employed by the U.S. Environmental Protection Agency summarized elsewhere in 1991 (U.S. Environmental Protection Agency 1991). An older method of quantitative risk assessment used the highest no-observed adverse effect level (LOAEL) as the “point of departure.” The point of departure was divided by uncertainty factors to arrive at a reference dose that was used to establish a regulatory level of exposure. For foods, a health-based guidance value (HBGV) is determined analogous to the reference dose. An alternative method for deriving a point of departure uses a benchmark dose (BMD). The BMD method was popularized 40 years ago and addresses limitations of the NOAEL/LOAEL method (Crump, 1984).

The BMD program is available from EPA (United States Environmental Protection Agency, 2025). BMD Software version 3.2 was used in the current modeling. For each endpoint, the BMD program recommends a model or will indicate that no optimized model exists. Data used for modeling were obtained from the NTP data repository (Behl et al., 2025). There are at least eight different dose–response models available, and the software ran all possible models. The optimal model recommended by the program was used in this report. For some datasets there were no adequate models due to failure of goodness-of-fit calculations to show satisfactory fitting of the model to the empirical data points. The lack of adequate models occurred more commonly when there were only two dose levels plus the control, as occurred in the 4-MEI study after litter F1b of the parental generation. When there were no acceptable models (i.e., no model fit the experimental data), risk assessment reverts to the NOAEL-LOAEL method.

Uncertainty factors applied to the point of departure can include up to an order of magnitude to account for differences between species (e.g., human vs. rat) and up to an order of magnitude to account for variability within a species (e.g., the most sensitive human compared to the least sensitive human). Other uncertainty factors can be selected to account for data quality, the completeness of the database, use of acute or subacute studies to model chronic exposures, or the use of a LOAEL as a point of departure when a NOAEL is not available. The use of typical uncertainty factors in risk assessment has been discussed (Renwick, 2004).

Based on analyses of data sets, including some studies from NTP, it has been determined that a 5 % response most closely matched the experimental NOAEL (Allen et al., 1994a, 1994b; Faustman et al., 1994). A benchmark response of 5 % is favored for developmental and reproductive endpoints and was used here for 4-MEI. The lower limit of the 95 % confidence interval around the modeled dose–response curve for a 5 % response is expressed as BMDL₀₅.

The risk assessment for the reproductive and developmental effects of 4-MEI was conducted using the BMD approach and the NOAEL/LOAEL approach. The purpose of this risk assessment was to estimate the likely health risk associated with 4-MEI exposures at FDA's 90th percentile high estimate scenario. The ratio of the BMDL₀₅ or NOAEL to the estimated human exposure is called the margin or exposure (MOE) (EFSA Scientific Committee et al., 2022). An acceptable MOE may reflect the uncertainty factors that would have been chosen to arrive at a HBGV. For example, an MOE of 100 suggests that uncertainty factors of 100 would be appropriate for the HBGV calculation and is the MOE recommended by EFSA for chemicals that are not genotoxic carcinogens.

3. Results

3.1. Results of the RACB study

The RACB study results are freely available online (Behl et al., 2020,

2025). The key results are summarized here for ready reference.

3.1.1. Adult toxicity

In the 5000-ppm dose group, 11 of 23 F0 females were found dead or were removed due to moribundity. In the 2500-ppm dose group, 4 of 23 F0 females died or were removed for moribundity. In the 0 and 750-ppm dose groups, one F0 female each died or was removed for moribundity. In the 2500-ppm dose group, removal of F0 females often occurred during parturition, raising a suspicion for dystocia. Convulsions occurred in F0 females in 39 % of the 5000-ppm group and in 16 % of F1 adult females in the 2500-ppm group. Convulsions after high dose 4-MEI exposure had been noted in a previous NTP toxicology study and were not considered related to reproductive toxicity.

Body weight gain in F0 adults showed dose-related decreases across all dose groups. F0 female body weight gains were decreased at 2500 and 5000 ppm. Some or all of the decrease in body weight gain may have been due to the decrease in litter size seen in these animals, but food consumption was decreased in the first week of gestation in these dose groups suggesting an effect independent of litter size. Body weights in F0 males was decreased in the 2500-ppm dose group by about 10 % and in the 5000-ppm dose group by about 13 % compared to the control group throughout most of the study.

3.1.2. Reproductive toxicity

Endpoints and effect levels are summarized in Table 1. In the 5000-ppm dose group, there was a decrease in mated F0 females per pair. Crossover mating of treated males with untreated females suggested decreased male fertility in this dose group. Crossover mating of treated females with untreated males was not performed due to parturition-associated moribundity in this dose group. Litter size was reduced at 2500 and 5000 ppm in F0 gestations. For the F1c mating, litter size was reduced across all dose groups by trend testing. Pup survival on PND 1–4 was reduced in the 2500-ppm matings in the first and second (F1a and

F1b) litters. For pup survival on PND 5–28, there were no alterations in the 750- or 2500-ppm dose groups.

F0 males had decreased sperm count per cauda epididymis and per gram cauda epididymis and decreased sperm motility after dietary treatment with 4-MEI 5000 ppm. F1 males had a decrease in sperm concentration per cauda epididymis in the 750- and 2500-ppm dose groups at the PND-95 interim necropsy but there were no alterations in these groups at the terminal necropsy. Estrous cycle alterations (extended diestrus or increased total cycle length) were seen in females at 2500 and 5000 ppm.

In F0 males, there were no alterations in testis weight in any dose group. Relative testis weight increased, consistent with the decrease in body weight across all dose groups. Epididymis weight decreased at 2500 and 5000 ppm. There were dose-related decreases in absolute and relative weight of the seminal vesicles with coagulating gland, the dorsolateral prostate, and the ventral prostate at all dose levels. There were decreases in absolute but not relative weight of the levator ani-bulbocavernosus complex at 2500 and 5000 ppm. In 2500-ppm F1 males, there was no change in absolute testis weight, a decrease in absolute weight of epididymis, seminal vesicles with coagulating gland, dorsolateral prostate, and ventral prostate.

On histopathological examination, there was atrophy of the ventral prostate in 9 of 23 males of the 750-ppm dose group, 20 of 23 males of the 2500-ppm dose group, and 23 of 23 males of the 5000-ppm dose group. Grading on a 4-point severity scale, the mean grades were 1.0 and 1.1 at 750 and 2500 ppm, respectively, and 2.4 at 5000 ppm. The findings of testicular degeneration and spermatid retention suggested a decrease in intratesticular testosterone. In F1 parental males, dorsal prostate atrophy was uncommon but ventral lobe atrophy occurred in 4 of 40 control animals, 10 of 44 males at 750 ppm, and 35 of 40 males at 2500 ppm. Testicular degeneration and spermatid retention were seen in 8 of 23 F0 males in the 5000-ppm group. Histopathological findings in females were less frequent and less severe.

Table 1
Reproductive endpoints in the 4-MEI RACB.

Endpoint	LOAEL	NOAEL	BMDL ₀₅	Model	AIC
F0 male endpoints					
% Motile sperm	2500	750	361 ppm (17 mg/kg bw/day)	Hill	641
Progressively motile	>5000	>5000	No acceptable model		
Epididymis weight	2500	750	No acceptable model		
Cauda sperm count	>5000	>5000	No acceptable model		
Sperm concentration	5000	2500	1155 ppm (54 mg/kg bw/day)	Polynomial, 2 or 3	1109
Prostate atrophy	750	none	170 ppm (8 mg/kg bw/day)	Multistage degree 2	107
F1 Nonparental male endpoints					
Epididymis weight	2500	750	862 ppm (46 mg/kg bw/day)	Exponential 2	-176
Sperm concentration	>2500	>2500	493 ppm (26 mg/kg bw/day)	Exponential 2	600
F1 Parental male endpoints					
% Motile sperm	2500	750	758 ppm (41 mg/kg bw/day)	Linear	378
Epididymis weight	2500	750	1198 ppm (60 mg/kg bw/day)	Polynomial, 2	-169
F1 Litter size (F0 parents)					
Litter A	2500	750	No acceptable model		
Litter B	2500	750	316 ppm (22 mg/kg bw/day)	Polynomial, 2	314
Litter C	2500	750	No acceptable model		
F2 Litter size (F1 parents)					
Litter A	750	none	No acceptable model		
Litter B	750	none	No acceptable model		
Litter C	>2500	>2500	395 ppm (25 mg/kg bw/day)	Exponential, 3	495
F1 pup weight gain PND 1–4					
Litter A	750	none	212 ppm (15 mg/kg bw/day)	Linear	364
Litter B	2500	750	165 ppm (10 mg/kg bw/day)	Linear	694
Litter C	2500	750	No acceptable model		
F1 pup weight gain PND 4–28 (litter C)					
Males	750	none	523 ppm (65 mg/kg bw/day)	Power	189
Females	750	none	567 ppm (71 mg/kg bw/day)	Power	204
F2 pup weight gain PND 4–28 (litter C)					
Males	2500	750	No acceptable model		
Females	2500	750	No acceptable model		

AIC Akaike information criterion, LOAEL lowest observed adverse effect level in ppm, NOAEL no observed adverse effect level in ppm. Data rounded to nearest whole number. Source documents are provided in the Supplementary material.

The NTP concluded that 4-MEI was associated with reproductive toxicity in rats at all dose levels tested based on reduced litter sizes, decreased male reproductive organ weights, and sperm endpoints. Based on food intake, the 750-ppm dose level was considered equivalent to 50–60 mg/kg bw/day and was considered the LOAEL. A NOAEL was not identified.

3.1.3. Developmental toxicity

Developmental endpoints included balanopreputial separation and vaginal opening. There were delays in balanopreputial separation and vaginal opening in offspring of the F1c and F2c generations (Table 2). There was a dose-related increase in age and body weight at sexual maturation, and the age-delay could not be explained by decrements in PND-28 body weight. After adjustment for PND-28 body weight, balanopreputial separation was delayed by about 2 days in the 750- and 2500-ppm dose groups, and vaginal opening was delayed by 3 days at 750 ppm and 6 days at 2500 ppm. In benchmark dose modeling, there was no model that fit the data for either endpoint. The NTP concluded that 4-MEI was associated with developmental toxicity manifested as delays in balanopreputial separation and vaginal opening in all dose groups.

3.1.4. Estimated effect level

Based on food intake, the 750-ppm dose level was described as equivalent to 50–60 mg/kg bw/day and was considered the LOAEL. A NOAEL was not identified. The published intake of 50–60 mg/kg bw/day was an average; however, there was a range of intakes depending on the phase of the experiment. In the 750-ppm females, the mean 4-MEI dose ranged from close to 100 mg/kg bw/day early in the study to about half that dose during pregnancy and 70–80 mg/kg bw/day during lactation. For this risk assessment, dose levels were retained as ppm. Conversion to mg/kg bw/day calculated was based on the food consumption data closest in time to the relevant endpoint.

3.2. Exposure assessment

Human exposure to 4-MEI is expected to occur through the consumption of foods and beverages with added 4-MEI from caramel coloring and with 4-MEI as a by-product of heating during processing or cooking. FDA authors reported the 4-MEI content of different foods and beverages and 90th percentile intake estimates for different age groups (Folmer et al., 2018). Intake estimates were given for estimated low, average, and high intakes. For the purposes of this risk assessment, high exposure scenarios and intake from combined consumption of caramel color and thermal treatment of foods were used (Table 3). The highest daily body weight-adjusted 90th percentile intake was 0.0049 mg/kg bw/day in children 2–5 years old. Use of the highest intake of the highest scenario was selected as a health-protective assumption.

Table 2

Age and body weight (litter mean \pm SEM) at signs of sexual maturation in rats exposed to 4-MEI. Modified from Table 6 in Behl et al. (2020).

	Dietary dose of 4-MEI, ppm		
	0	750	2500
F1c males, number of pups (litters)	99 (18)	115 (22)	61 (15)
Age at balanopreputial separation, days	43.5 \pm 0.4	46.2 \pm 0.4	47.2 \pm 0.6
Body weight at balanopreputial separation, g	195.8 \pm 2.7	205.6 \pm 2.9	190.4 \pm 3.4
F1c females, number of pups (litters)	96 (19)	111 (22)	67 (15)
Age at vaginal opening, days	33.8 \pm 0.2	37.2 \pm 0.3	49.4 \pm 1.8
Body weight at vaginal opening, g	106.2 \pm 2.0	117.2 \pm 2.0	117.1 \pm 1.8

Findings were statistically significant in all dose groups.

Table 3

Estimated daily intake of 4-MEI by age group for combined intake of caramel coloring and thermally treated foods in the high exposure scenario. Adapted from Folmer et al. (2018). There is no copyright. The highest exposure was in children 2–5 years old (bolded).

Population group	90th percentile daily intake of 4-MEI	
	mg/person	mg/kg bw
Infants up to 1 year old	0.026	0.0027
Children 1–2 years old	0.051	0.0046
Children 2–5 years old	0.084	0.0049
Children 6–12 years old	0.11	0.0033
Teenage boys, 12–18 years old	0.15	0.0024
Adults 18+ years old	0.20	0.0025
US population 2+ years old	0.18	0.0028

3.3. Quantitative risk assessment

Using the traditional NOAEL-LOAEL approach, a LOAEL of 50–60 mg/kg bw/day was derived from the RACB by Behl et al. (2020). No NOAEL was obtained. Although the study authors estimated intake in the 750-ppm dose group based on average feed consumption relevant to F0 prostate atrophy, estimated 4-MEI intake was provided in the data repository for individual 3-day or longer periods (Behl et al., 2025). The mean intake in the 750-ppm F0 males and females was about 36 and 37 mg/kg bw/day, respectively. For F1 males and females, higher doses were estimated. To be health-protective, a LOAEL of 36 mg/kg/day was used in this risk assessment. One approach to using the LOAEL to determine the point of departure when a NOAEL is not available is to use an uncertainty factor of 10 to estimate the NOAEL (U.S. Environmental Protection Agency (EPA), 2025). The estimated NOAEL, then, would be 3.6 mg/kg bw/day, the LOAEL divided by 10. The MOE would be 735, the estimated NOAEL of 3.6 mg/kg/day divided by the highest estimated human exposure level of 0.0049 mg/kg bw/day.

$$\text{LOAEL} = 36 \text{ mg/kg bw/d}; \text{ UF LOAEL to NOAEL extrapolation} = 10$$

$$\text{Estimated study NOAEL} = \text{LOAEL} \div 10 = 3.6 \text{ mg/kg bw/d}$$

$$\text{MOE} = \text{Estimated study NOAEL}$$

$$\div \text{Highest human exposure (mg / kg bw / d)}$$

$$\text{MOE} = 3.6 \text{ mg/kg bw/d} \div 0.0049 \text{ mg/kg bw/d} = 735$$

The BMD approach is well suited to data sets such as obtained in the Behl et al. study (Behl et al., 2020, 2025); rather than defaulting to an estimated NOAEL by dividing the LOAEL by 10, a BMDL₀₅ can be used to estimate a dose level at which there will be no toxicity (Allen et al., 1994a, 1994b; Faustman et al., 1994). A BMDL₀₅ was calculated for endpoints in the RACB study. The lowest of these values was used to estimate the margin of exposure.

3.3.1. Adult toxicity

An estimate of generalized adult toxicity was derived from the F0 body weight data. F0 animals began their exposure in adulthood, so there was no issue of developmental effects of the test article on the health of the animals. Weight in male F0 animals cannot be influenced by litter size as can weight or weight gain in pregnant F0 females. The BMDL₀₅ was calculated for the body weight gain of F0 males and females for the period prior to mating (Study Day 7) and for pregnant F0 females on GD 7 before the number of fetuses exerts an influence (Table 4). The most sensitive effect was a reduction in adult male body weight with a BMDL₀₅ of 335 ppm (24 mg/kg bw/day) using a best-fit exponential 4 model, AIC 822. The margin of exposure for adult toxicity is 24 mg/kg bw/day divided by 0.0049 mg/kg bw/day or 4898.

Table 4
Benchmark dose analysis of adult toxicity reflected in F0 body weight.

F0 weight affected	BMDL ₀₅
Adult males prior to mating	335 ppm (16 mg/kg bw/day)
Adult females prior to mating	584 ppm (35 mg/kg bw/day)
Females, gestation day 7	
Litter A	629 ppm (45 mg/kg bw/day)
Litter B	598 ppm (42 mg/kg bw/day)
Litter C	Not applicable ^a

^a No acceptable model.

3.3.2. Prostate atrophy

Prostate atrophy was considered by the Behl et al. (2020) investigators to be a key finding and was used to establish a LOAEL of 750 ppm. For prostate atrophy, the results were quantal rather than continuous, although the output of benchmark dose modeling appears continuous.

The benchmark dose software calls for a designation of extra risk or added risk. Extra risk and added risk modeling were the same because the control atrophy prevalence was 0 in the parental control males. Otherwise, the distinction between the two types of modeling is based on the risk attributed to the control group: extra risk treats the control risk as x and specifies the modeled dose at which risk becomes $x+5\%$ while added risk assumes that the control risk is x and the benchmark dose is modeled to correspond to $1.05x$. For the nonparental F₁ males, because no model was optimal and the benchmark dose calculations were similar to the other two groups, the analysis was not retained. The lowest BMDL₀₅ was 170 ppm (8 mg/kg bw/day), producing an MOE of 1632 (i.e., 8 mg/kg bw/day divided by 0.0049 mg/kg bw/day). This margin of exposure was the lowest margin using benchmark dose analysis of the Behl et al. data (Behl et al., 2020, 2025).

3.3.3. Developmental endpoints

The developmental endpoints included delays in balanopreputial separation and vaginal opening. There were no acceptable models for age at sexual maturation in male or female F₁ offspring. Balanopreputial separation was delayed about 2 days in males, but there was no dose-related difference between males in the 750 and 2500 ppm groups. Body weight at attainment was increased in the 750-ppm group and unchanged at 2500 ppm. Body weight at weaning was reduced in the 2500-ppm group but not in the 750-ppm group. The weight differences in male pups may have influenced puberty onset, which is sensitive to body weight, although adjustment for GD 28 body weight did not affect the delay. In females, vaginal opening was delayed and weight at attainment was higher, suggesting a genuine delay of sexual maturation not influenced by pup body weight. Models were not fit successfully by the benchmark dose program.

4. Discussion

4.1. Other studies

There have been few other studies of 4-MEI with reproductive endpoints. A 14-week NTP repeat dose toxicity study showed testicular degeneration in F344/N rats with dietary dose levels of 5000 or 10,000 ppm but not 2500 ppm (Chan et al., 2004). The difference in effect levels in this study compared to the RACB study may be related to the differences in rat strains.

Adult male Sprague-Dawley rats given 4-MEI in water by subcutaneous injection had a decrease in serum and testicular interstitial fluid testosterone concentration 2 h after a 10-mg/kg bw dose (Adams et al., 1998). The authors attributed this result to a decrease in hypothalamic gonadotropins or gonadal response to gonadotropins. Fertility was not assessed.

Three-week-old female ICI mice were given 4-MEI in water by gavage for 10 days at 0, 50, or 100 mg/kg bw/day (Lu et al., 2022).

Oocyte maturation *in vitro* was evaluated after removal of the zona pellucida. Other oocytes were fertilized *in vitro* and 2-cell embryos were evaluated. Germinal vesicle breakdown was inhibited by 100 but not 50 mg/kg bw/day, and extrusion of the first polar body was inhibited at both dose levels. Oocyte spindles were misshapen, aneuploidy was increased, and *in vitro* fertilization was decreased at 4-MEI 100 mg/kg bw/day.

Four-week-old male ICI mice were treated with 4-MEI in drinking water at 0, 250, or 500 mg/L resulting in estimated intakes of 0, 61.3, or 134.5 mg/kg bw/day (Lu et al., 2024). Testes were harvested after 8 weeks of exposure. There was no effect of treatment on body or testis weight. Epididymal sperm motility and testicular testosterone were decreased at the high-dose level. The testes from the high-dose group were described as showing an “irregular arrangement” of spermatogenic cells and vacuolation of Sertoli cells, but the photomicrographs were not of sufficient quality to evaluate these reported changes. Epididymal sperm concentration was not affected. *In vivo* fertilization was described as impaired, but the method of fertilization *in vivo* was not adequately described.

The RACB design has the advantage of permitting a large number of breeding events to be evaluated over two generations. Because pups in the first two litters in the F₁ and F₂ generations are removed from the dams in the first days of life, female fertility returns promptly. This design permits cumulative effects to be detected if there is a decrease in fertility or reproductive success over time. This RACB study used close to 20 adults of each sex in most of the dose groups and hundreds of offspring. This number of animals permits the identification of small changes in reproductive outcome and decreases the statistical variability associated with each endpoint. On the other hand, the large number of endpoints creates the opportunity for chance findings to appear to be statistically significant. The decreased food consumption observed in this study may reflect palatability challenges from 4-MEI in the diet or decreased appetite because of systemic toxicity, which may be particularly important during lactation and after pup weaning when 4-MEI doses could double.

4.2. Margin of exposure

In the RACB for 4-MEI, the margin of exposure for the most sensitive endpoint, prostate atrophy, was 735 using the LOAEL and 1469 using the BMDL₀₅ from benchmark dose analysis. The use of the LOAEL to estimate the NOAEL includes division by a factor of 10, giving rise to an estimated NOAEL of 3.6 mg/kg bw/day. The LOAEL is constrained by the experimenter's choice of dose levels. By contrast, benchmark dose analysis models the entire dose–response curve and permits a better-informed estimate of the NOAEL.

An acceptable margin of exposure has not been determined for all applications. For pharmaceutical products under review by FDA, a margin of exposure of 25 has been considered reassuring, albeit based on internal exposure metrics (U.S. Food and Drug Administration (FDA), 2011). EPA recommends calculation of a margin of exposure based on the uncertainty values that would apply in a risk assessment, usually 10 each for interspecies differences and intraspecies variability and 10 for going from a LOAEL to a NOAEL (U.S. Environmental Protection Agency (EPA), 2012). A review of 4-MEI by the International Agency for Research on Cancer (IARC) concluded that 4-MEI was possibly carcinogenic in humans based on studies in rats and mice but did not identify a genotoxic mechanism (International Agency for Research on Cancer (IARC)). The European Food Safety Authority (EFSA) recommends a margin of exposure of 100 for chemicals that are not genotoxic carcinogens (European Food Safety Authority (EFSA)).

4.3. Strengths and weaknesses

Strengths of this risk assessment include the availability of a well-designed two-generation study with an adequate number of animals

and the use of benchmark dose modeling, enabling consideration of the entire dose–response curve to explore model behavior below the range of the experimental dose levels. A detailed assessment of 4-MEI exposures in food in the U.S. was available.

A weakness of this risk assessment is the limitation to a consideration of only reproductive and developmental endpoints rather than inclusion of non-reproductive endpoints; however, an assessment of potential cancer risk based on soft drink exposure has been published elsewhere (Smith et al., 2015). The exposure assessment in this paper was based on a 2018 paper published by FDA authors based on diet data from 2009 to 2012 (Folmer et al., 2018). An updated exposure assessment on 4-MEI in the diet was not available in time for this publication.

In conclusion, based on the RACB, and the exposure assessment performed by FDA authors, human dietary consumption of 4-MEI is not expected to produce adverse reproductive or developmental effects.

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Declaration of competing interest

The author declares the following financial interests/personal relationships which may be considered as potential competing interests: Consultant, American Beverage Association.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yrtph.2025.105856>.

Data availability

The data are publicly available and a link is provided in the paper.

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