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A two-generation reproductive toxicity study of sodium molybdate dihydrate administered in drinking water or diet to Sprague-Dawley rats

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ABSTRACT

In an OECD Test Guideline 416 multigenerational study, groups of 24 male and 24 female Sprague-Dawley rats were administered sodium molybdate dihydrate at 0, 5, 17, or 40 mg molybdenum (Mo)/kg bw/day in the drinking water or 40 mg Mo/kg bw/day in the diet over two generations to assess reproductive toxicity. No adverse effect on reproductive function was observed at any dose level in either generation as indicated by no significant dose-related effect on estrus cycles, sperm parameters, mating, fertility, gestation, litter size, pup survival, growth or postnatal development. Systemic toxicity, including decreased body weight, food consumption (males only) and water consumption, was observed among both sexes given 40 mg Mo/kg bw/day in the diet. Serum levels of Mo and copper were increased in a dose-related manner. The No Observed Adverse Effect Levels (NOAEL) are 17 mg Mo/kg bw/day for systemic toxicity and 40 mg Mo/kg bw/day for reproductive toxicity.

1. Introduction

Molybdenum (Mo), a key constituent of several important enzymes (e.g. sulfite oxidase, xanthine oxidase, aldehyde oxidase, nitrogenase), is an essential element for plants, animals and humans [1,2]. Trace levels are found in a wide variety of foods, with the highest concentrations in grains, legumes, nuts and dairy products. Human exposure to Mo occurs primarily via diet and drinking water from Mo compounds that dissociate at physiological pH to form molybdate ions. Occupational exposure to Mo may also occur from mining and industrial processes, such as the manufacture of structural steel and stainless steel.

The toxicity of excessive dietary exposure to Mo is well known in cattle and sheep, causing a condition known as “teart” or molybdenosis, which is characterized by diarrhea, weight loss, discolored coats, and ultimately death if not treated [3]. This condition in cattle and sheep is associated with reduced bioavailability of copper (Cu) due to high levels of sulfide in the rumen that interacts with molybdate to form thiomolybdates [4]. Tetrathiomolybdate is a potent Cu chelating agent, and it is used in humans for the treatment of Wilson’s disease, which is associated with high levels of Cu in the body [5].

In humans, the toxicity of Mo is observed to be low, indicating that the findings in ruminants do not appear to be relevant to humans or monogastric animals [6]. In an older (1961) environmental epidemiological study, an increased incidence of gout and elevated uric acid levels were reported among residents living in an area with high levels of Mo in the soil [7]. However, serious methodological difficulties, analytical problems in the assessment of blood and urinary copper levels, and the complete absence of any method validation, mean that the results of that study are widely questioned for their plausibility [2]. No alterations in uric acid levels were observed in a controlled study in male volunteers given 500 or 1500 μg per day of Mo orally in the form of ammonium molybdate in sorghum [8].

The reproductive and developmental toxicity of Mo has been evaluated in GLP-compliant studies in laboratory animals. In an OECD Test Guideline (TG) 414 developmental toxicity study, no evidence of prenatal developmental toxicity was observed among the offspring of pregnant Sprague-Dawley rats given sodium molybdate dihydrate in the diet at dose levels up to 40 mg Mo/kg bw/day on gestation days (GD) 6–20 [9]. No effect on semen quality, estrus cycles, or the histopathology of male or female reproductive organs was observed in a guideline (OECD TG 408) 90-day study with additional reproductive

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toxicity evaluations in Sprague-Dawley rats fed diets containing sodium molybdate dihydrate at dose levels up to 60 mg Mo/kg bw/day [10].

Developmental and reproductive effects have been reported in earlier studies in animals exposed to Mo; these studies, which were not conducted according to regulatory guidelines or GLP, exhibit various limitations. Fungwe et al. [11] reported increased resorptions and decreased fetal body weight among the offspring of Sprague-Dawley rats given sodium molybdate dihydrate in the drinking water at concentrations up to 100 mg Mo/L for at least 8 weeks prior to mating and through gestation day 21 (GD 21). However, it is not possible to determine the exact dose levels of the test material consumed by the rats in this study since neither the water intakes nor body weights are reported. This and several other significant limitations of this study are further addressed in the Discussion section.

Pandey and Singh [12] reported adverse effects of sodium molybdate on sperm count, motility and morphology, as well as decreased organ weights (testes, epididymis, seminal vesicles, ventral prostate), among male Druckery rats administered sodium molybdate by gavage at dose levels of 12 and 20 mg Mo/kg bw/day for five days per week for 60 days. However, this study was poorly reported with no clear description of the effects on the testes or the actual numbers of animals affected, and there were apparent inconsistencies in the statistical analyses.

Lyubimov et al. [13] reported reduced epididymal weights, sperm counts, and sperm motility, as well as histologic changes in the testis and epididymis in CD rats (unreported strain) given 4.4 mg Mo/kg bw/day as ammonium tetrathiomolybdate for approximately 60 days, but not at lower doses. There were no effects on the estrus cycle or reproductive indices among female rats at any dose level. Because tetrathiomolybdate is a potent chelator of Cu used to treat Wilson's disease and is chemically unlike any other molybdate, it is not representative of the soluble Mo compounds to which the general population is exposed that dissociate at physiological pH to form molybdate ions.

Su-ling et al. [14] reported decreased live fetus rate and fetal body weight and increased malformations among the offspring of pregnant Wistar rats given up to 40 mg/kg bw/day of "molybdenum acid ammonium salts" by gavage on gestation day (GD) 7-16. No further information (e.g., molecular formula, CAS number, source, purity, stability) on the test material is provided, yet several different ammonium molybdates exist. The publication is quite brief, and the experimental procedures are not described in sufficient detail for a full evaluation.

Zhai et al. [15] evaluated the effects of sodium molybdate dihydrate administered to ICR male mice in the drinking water at concentrations of 0, 12.5, 25, 50, 100, or 200 mg/l L for 14 days. The authors concluded that Mo had a beneficial effect on sperm parameters at a moderate dose (25 mg/L) but had a detrimental effect at higher doses ($\geq$100 mg/L). Body weights and food and water consumption were not reported.

Wang et al. [16] reported changes in semen parameters in male Kunming mice fed a copper deficient diet, plus Mo and Cu alone or in combination, in the drinking water. The authors concluded that testicular tissues of mice were adversely affected by high Mo and exacerbated by a Cu- deficient diet. However, the study has numerous serious flaws. For example, the actual dose levels of Mo and Cu in mg/kg bw/day were not provided. The chemical form and source of Mo were not identified. All animals were maintained on an unconventional low copper diet, which was composed of "wheat, corn, and soybean grown in a Cu-deficient region in the Guan Village," and the Cu deficient diet was also deficient in Mo ($<$ 0.17 ppm Mo).

Given the uncertainties in the published literature about the reproductive toxicity of Mo, the purpose of the current study was to assess the potential reproductive toxicity of sodium molybdate dihydrate when given in the drinking water or diet to male and female Sprague-Dawley rats. This study was conducted in accordance with OECD Test Guideline 416 [17].

2. Materials and methods

2.1. Test material and formulations

Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) with a purity of 99% was supplied by Climax Molybdenum Company, Phoenix, AZ. Sodium molybdate dihydrate was selected as a source of molybdate ion $[MoO_4]^{2-}$ that is representative of the broader class of soluble molybdenum(VI) compounds, since at physiological pH in biological systems, soluble molybdenum compounds exist in the form of molybdate ion [18–20]. For administration in the drinking water, sodium molybdate dihydrate was formulated in reverse-osmosis deionized water at least every 14 days and stored at room temperature. For administration to the animals in the diet, sodium molybdate dihydrate was formulated in Certified Rodent Diet #5002 (PMI Nutrition International) at Charles River Laboratories, Inc. (Horsham, PA). The formulations in diet were prepared as needed based on 42-day stability and stored at room temperature. All samples of feed and water to be analyzed were shipped overnight to the Diagnostic Center for Population and Animal Health, Michigan State University, Lansing, MI. Analyses were performed by inductively coupled plasma - mass spectroscopy (ICP-MS). The amount of test substance used each week to prepare dosed water and diet was adjusted weekly based on estimated average body weight and food and water consumption for the next week of study, to achieve a daily exposure of 0, 5, 17 or 40 mg Mo/kg bw/day from the drinking water or 40 mg Mo/kg bw/day from the diet.

2.2. Animals and housing

The study was conducted at Charles River Laboratories, Inc. (Horsham, PA), where the animal facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). Two hundred sixty (130 males and 130 females) Crl:CD(SD) Sprague-Dawley rats were received from Charles River Laboratories, Inc. (Raleigh, NC). The rat is the preferred species for testing according to the OECD 416 Guidelines for the two-generation reproductive toxicity study. At receipt, the males and females were approximately 45 and 37 days of age, respectively. Each animal was acclimated for 7 days prior to assignment to dose groups based on computer-generated (weight-ordered) randomization procedures to ensure no significant weight variation among the groups. Each rat was uniquely identified by a metal ear tag.

All cage sizes and housing conditions were in compliance with the *Guide for the Care and Use of Laboratory Animals* [21]. The rats were individually housed in solid-bottomed cages, except during the cohabitation and postpartum periods and during urine collection. During cohabitation, each pair of rats was housed in the male rat's nesting box. During the postpartum period, each dam and delivered litter were housed in individual nesting boxes with ground corncob nesting material (Bed-o'Cobs, The Andersons, Industrial Products Division, Maumee, OH) until weaning. For urine collection, the rats selected for collection were individually housed in a stainless steel, wire bottom metabolism cage. Animal rooms were maintained at a temperature of 22 °C ± 3 °C with a relative humidity of 50% ± 20% and 10 or greater air changes per hour. A 12-hour light/12-hour dark cycle was maintained.

2.3. Study design

The study was designed in accordance with OECD 416 guidelines [17] and US EPA OPPTS 870.3800 [22] for reproductive toxicity studies. In addition, the study was conducted in compliance with Good Laboratory Practices (GLP) as described in the United States Code of Federal Regulations, Title 40, Parts 160 and 792, Good Laboratory Practice Standards. Dose formulation analyses and elemental analyses of blood, urine and tissues were performed at Michigan State University

(Lansing, MI) according to established Standard Operating Procedures (SOPs), controls, and approved test methodologies to ensure integrity and validity of the results generated. These analyses were not conducted in compliance with the GLP regulations; however, this laboratory is fully accredited by the American Association of Veterinary Laboratory Diagnosticians Accreditation Committee.

Groups of 24 male and 24 female parental (P) generation rats were administered sodium molybdate dihydrate in the drinking water to provide dose levels of 0, 5, 17, or 40 mg Mo/kg bw/day. An additional group of 24 males and 24 females was given sodium molybdate dihydrate in the diet to provide a dose level of 40 mg Mo/kg bw/day. The dose levels were chosen based on a range-finder study in rats given sodium molybdate dihydrate in the drinking water or diet. The drinking water route of administration was chosen as the main route of exposure for the current study because it allows comparison with the Fungwe et al. [11] study and because the range-finder study suggested a possible decrease in the pregnancy rate (6/10) at 40 mg Mo/kg bw/day in the drinking water, but not in the diet. Human and animal exposure to molybdenum, which is an essential trace element, occurs *via* both diet and drinking water.

All rats were administered the diet and drinking water *ad lib*. The concentrations of sodium molybdate dihydrate in the diet and water were adjusted periodically, usually weekly, to account for changes in body weight and food or water consumption. The male rats were directly exposed to the test or control diet or drinking water for at least 10 weeks before cohabitation, during the cohabitation, and continuing through to the day of euthanasia (Fig. 1). The female rats were directly exposed to the test or control diet or drinking water for at least 10 weeks before cohabitation, during the cohabitation, gestation, littering and post-partum periods (lactation period) and continuing through to the day of euthanasia. Concentrations of the test substance in the drinking water and diet were offered to the F1 generation rats in the same way as for the P generation described above, and the mg Mo/kg bw/day doses consumed were calculated based on the body weight and food or drinking water consumption observations starting after weaning on Postnatal Day (PND) 22. A computer-generated randomization procedure was used to assign 24 male and 24 female F1 generation rats to cohabitation within each dose group, one male rat per female rat avoiding sibling pairings.

F1 and F2 generation pups were directly exposed to the test and/or carrier control substances after they began consuming food/water (approximately on PND 15). Pups may also have been exposed to the test and/or carrier control substances during maternal gestation (*in utero* exposure) or via maternal milk or excreta during the lactation period. After weaning (beginning on PND 22), the selected F1 generation pups were directly exposed to the test or control diet or drinking water for at least 10 weeks before cohabitation, during the cohabitation, gestation, littering and post-partum periods (lactation period) and continuing through to the day of euthanasia. All F2 generation pups were terminated at weaning.

2.4. Parental observations

Throughout the study, all animals were observed for general health, mortality and moribundity twice daily. Clinical observations were recorded at least weekly during the acclimation period, on the day of randomization, daily during the dose period, and on the day of scheduled euthanasia.

Male rats were individually weighed on the day after arrival, at least weekly during the acclimation period, at least weekly during the dose period and on the day of scheduled euthanasia. Female rats were individually weighed on the day after arrival, at least weekly during the acclimation period, at least weekly during the dose period, on Gestation Days (GD) 0, 7, 10, 14, 20, and on PND 0, 4, 7, 14, 21, 28, 35, 42, 49, 56 and 58 and the day of scheduled euthanasia.

Food consumption was measured for male rats at least weekly during the dose period and for female rats at least weekly during the dose period, on GD 0, 7, 10, 14, 18, 20 and PND 0, 4, 7, 10, 14, 21, 28, 35, 42, 49, 56 and 58, and on the day of scheduled euthanasia. Water consumption values were recorded daily during exposure periods and prior to euthanasia. During cohabitation, when two rats occupied the same nesting box with one food jar and one water bottle (using the lower concentration available at the time, as applicable), any replenishment of the food jars and water bottles was documented; individual values were not recorded or tabulated. Food and water consumption was not tabulated after PND 14, when it was expected that pups would begin to consume maternal food and water.

2.5. Mating procedures, reproductive performance and estrus cycles

Each female was mated with one allocated male of the same dosage group, avoiding sibling pairings, until copulation occurred, or the mating period elapsed. The cohabitation period consisted of a maximum of 10 and 17 days in the P and F1 generations, respectively. Female rats with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ* were considered to be at Day 0 of Presumed Gestation (GD 0) and assigned to individual housing. F1 females that did not mate within the first 10 days of cohabitation were assigned alternate males (same dose group) and remained in cohabitation for a maximum of 7 additional days. P and F1 female rats not mated after completion of the cohabitation period were assumed to be at GD 0 on the last day of cohabitation.

Estrus cycles were evaluated in the P and F1 generation females by examining the vaginal cytology of samples obtained by vaginal lavage. Samples were collected for 14 consecutive days before pairing, during the cohabitation period until spermatozoa were observed in a smear of the vaginal contents and/or a copulatory plug was observed.

All P and F1 generation pregnant females were allowed to deliver naturally and rear their young to weaning (PND 21). Female rats were evaluated for adverse clinical signs (including any indication of prolonged parturition), duration of gestation (GD 0 until the time the first pup was observed), litter size and viability at birth (number of live and dead pups at birth). Male and female mating and fertility indices, days

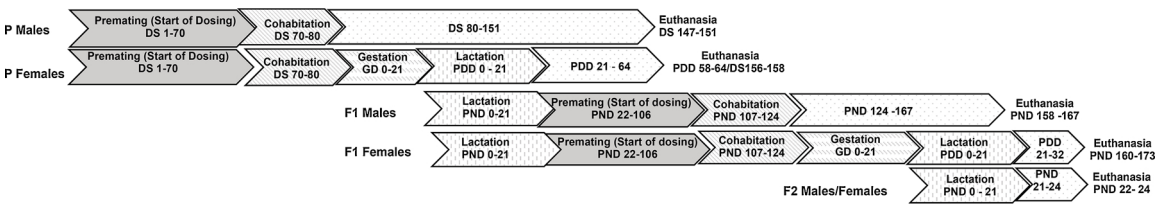

Fig. 1. Schematic diagram of study design.
DS: Day of Study. From start of Dosing P Generation Males and Females.
PND: Post Natal Day. FI Generation Males and Females and F2 Pups. Start of Dosing for F1 generation PND22.
GD: Gestation Day. P Generation Dams and F1 Dams.
PDD: Post Delivery Day: P Generation Dams and F1 Dams.

in cohabitation, and female conception rates were calculated.

2.6. Litter data

Litters were observed for dead pups at least twice daily and the pups in each litter were counted once daily during the preweaning period. Pups that were found dead before examination of the litter for pup viability (PND 0) were evaluated for vital status at birth. The lungs were removed and immersed in water. Pups with lungs that sank were identified as stillborn; pups with lungs that floated were identified as liveborn and to have died shortly after birth.

Pups that died before scheduled termination were examined for gross lesions and the cause of death as soon as possible after the observation was made. Clinical observations of the pups were recorded daily. Individual pup body weights were recorded on PND 0, 4, 7, 14 and 21.

2.7. Developmental landmarks including anogenital distance and sexual maturation

All F1 and F2 pups were observed daily for pinna unfolding beginning on PND 2, hair growth beginning on PND 7, incisor eruption beginning on PND 9, and eye opening beginning on PND 12. The number of pups meeting the criterion was recorded on each day of testing. Testing continued until the day the criterion was attained by all pups in the litter or until the day of scheduled euthanasia. Anogenital distance was measured for all male and female F2 pups on PND 4. Sexual maturation was evaluated daily for vaginal opening among females beginning on PND 27 and for preputial separation beginning on PND 34 in the F1 generation. Body weights were recorded on the day the criterion for sexual maturation was achieved.

2.8. Elemental analyses of urine, serum, and tissues

Just prior to or at scheduled euthanasia, urine, blood, liver and kidney samples were collected from P and F1 males and females for analysis for Mo, Cu, manganese, cobalt, zinc, iron, selenium and sulfur. Blood and tissue samples were also collected from F1 and F2 pups around Day 23 postpartum.

For the P generation rats, a single urine sample was collected over approximately 24 h from 12 rats/sex/group for males on days 30, 31, 32 or 33 after the first day of dosing (DS) and on DS 106, 108, 109 or 110, and for females on DS 30, 31, 32 or 33, and after delivery on Postdelivery Day (PDD) 25, 29, 30, 31, 32, 33, 34, 35 or 36, or for females that mated and did not become pregnant by 30 days after the day of mating. For the F1 generation rats, a urine sample was collected over approximately 24 h from 12 rats/sex/group on PND 48, 49, 50 or 51 (males and females), and on PND 144, 145 or 146 (males) and on PDD 23, 24, 25, 26, 27 or 28 (females), or 47 or 48 days after mating (females that mated but did not become pregnant). The same animals were utilized for both urine collections. Food and water remained available *ad libitum* during the collection interval. Samples were maintained at ambient conditions until the collection was complete, at which time the samples were frozen immediately.

On the day before scheduled euthanasia (for the same P and F1 generation animals selected for urine collections), blood samples (0.5 ml) were collected from the jugular vein. Blood samples were collected in the morning and in the afternoon. In addition, blood samples were collected under isoflurane/oxygen anesthesia from the inferior vena cava from 2 pups/sex/litter (when possible) on Day 23 ± 2 days postpartum from the F1 and F2 generation pups not selected for continued observation. At euthanasia, the liver and right kidney were frozen on dry ice until stored in a freezer set to maintain −80 °C until shipment for tissue analysis.

Blood samples were transferred into serum separator tubes and allowed to clot for at least 30 min at ambient temperature. The samples were centrifuged for approximately 10 min in a refrigerated centrifuge at 3500 rpm. The resultant serum was separated, transferred to uniquely labeled clear polypropylene tubes, and frozen immediately over dry ice until stored in a freezer set to maintain −20 °C, as applicable.

The serum, tissue, and urine samples were frozen and shipped on dry ice to the Diagnostic Center for Population and Animal Health, Michigan State University, Lansing, MI, for analysis. Serum, tissue and urine samples were analyzed for concentration of Mo, Cu, manganese, cobalt, zinc, iron, selenium, and sulfur. Elemental analysis was performed by inductively coupled plasma - mass spectroscopy (ICPMS) [23]. Serum and tissue samples were diluted 20-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2% propanol and germanium, scandium, rhodium, indium and bismuth as internal standards. Elemental concentrations were calibrated using a linear curve of the analyte-internal standard response ratio. NIST standard reference materials, along with in-house serum pools, were used as quality controls.

2.9. Termination/necropsy: P and F1 generation adults

A gross necropsy of the thoracic, abdominal and pelvic viscera was performed at scheduled euthanasia and for all rats that were found dead or euthanized prior to scheduled termination. P and F1 males were euthanized by carbon dioxide asphyxiation and necropsied on DS 147–151 and PND 158–167, respectively, after parturition of paired females. Similarly, P and F1 females were euthanized by carbon dioxide and necropsied after weaning of their pups on PDD 58–64 for the P dams, and PDD 24–34 for the F1 dams, respectively. Organ weights were recorded for all scheduled euthanasia animals, including brain, epididymis (total and left cauda), adrenal glands, thyroid glands, pituitary gland, kidney, liver, ovaries, oviducts, prostate gland, seminal vesicles with coagulating glands (with and without fluids), spleen, thymus, testes, and uterus. Organ weights relative to body weights were calculated. Tissues, except for the testes, were routinely processed, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. All testes were placed in Davidson's fluid for between 24 and 48 h and were then rinsed and fixed in neutral buffered formalin prior to sectioning and staining with hematoxylin and eosin. The following organs were examined microscopically in the adult animals: cervix, epididymis, adrenal, parathyroid, pituitary, prostate, seminal vesicles (including coagulating gland), thyroid, kidney, lungs, ovaries, oviduct, testes, uterus, and any gross lesions/masses. The testes underwent a detailed qualitative evaluation to assess the progression of stages on the spermatogenic cycle, cell associations, and proportions expected to be present during spermatogenesis, along with assessment of interstitial and supporting cell types (Leydig cells, macrophages, vasculature, and rete testis). Any cell or stage specific findings were noted.

2.10. Termination/necropsy: F1 and F2 generation pups

All pups not selected for continued observation on PND 24 ± 2 days were euthanized and examined for gross lesions; gross lesions were preserved in neutral buffered 10% formalin. Pups that died before scheduled termination were examined for gross lesions and the cause of death or condition as soon as possible after the observation was made. The presence or absence of milk in the stomach was determined. The brain, spleen, and thymus were weighed at necropsy from one arbitrarily selected F1 pup not selected for further evaluation per sex/litter at scheduled euthanasia (when possible). For the F2 pups, organ weights (brain, epididymis, adrenal, pituitary, prostate, thyroid, seminal vesicles, kidney, liver, ovaries, spleen, thymus, testes, and uterus) were recorded at necropsy for one arbitrarily selected pup/sex/litter (when possible) for the scheduled euthanasia animals. The same organs were evaluated microscopically in the F2 pups as in the adults.

2.11. Sperm evaluation: P and F1 generation adults

At the time of necropsy of the P and F1 adult males, an assessment of the male reproductive system was performed. A similar proportion of males from each group for the assessment of the male reproductive system, as appropriate, were euthanized on any one day. Sperm motility was evaluated using computer-assisted sperm analysis (CASA). Motility was evaluated following dispersion, into an appropriate medium, of sperm from each vas deferens. A homogenate was prepared from the left cauda epididymis for evaluation to determine sperm concentration (sperm per gram of tissue weight). Sperm concentration was evaluated using CASA. The remaining portion of the left cauda epididymis was used to manually evaluate sperm morphology. Sperm morphology evaluations included the following: 1) determination of the percentage of normal sperm in a sample of 200, where feasible; and 2) qualitative evaluation of abnormal sperm. The left testis was used for evaluation of testicular spermatid concentration via CASA. The left testis was weighed (both before and after removal of the tunica albuginea) and then homogenized. A sample from the resulting homogenate was stained with an IDENT Stain Kit from Hamilton Thorne Research, and a slide was prepared for analysis. Ten fields were analyzed to determine testicular spermatid concentration (spermatids per gram of tissue weight).

2.12. Ovarian follicle counts and uterine examinations: P and F1 generation adults

At the time of necropsy of both P and F1 generation females, the reproductive tract was dissected from the abdominal cavity. The number and distribution of implantation sites was recorded. Entire left and right ovaries for the P and F1 generation were embedded in separate paraffin blocks. Consistency in orientation of each ovary was imperative, such that each ovary was placed into its own designated block. Each ovary was sectioned beginning at 200 µm within the ovary. The first section was collected and designated as section 1 for follicle counts. After advancing the microtome 100 µm, the next section was collected as section 2. This process continued until a total of five sections from each ovary were collected for follicle counts, plus one section collected (between follicle count sections 3 and 4) on a separate slide for routine histopathological evaluation by the study pathologist. The five sections were placed on one slide for each ovary.

Each section was quantitatively evaluated for primordial follicles to include small growing follicles. Presence or absence of corpora lutea was recorded for each animal. Group means and standard deviations were calculated for each animal (left, right, and both ovaries combined). Where the sample size was appropriate (n > 2), group mean values were compared in order to detect any significant differences in the number of primordial follicles in left ovaries, right ovaries, and both ovaries combined. Uteri of apparently nonpregnant rats were examined while being pressed between glass plates to confirm the absence of implantation sites.

2.13. Statistical analyses

Means, standard deviations and percentages were calculated, as appropriate. Adult data were evaluated with the individual rat as the unit measured. Litter values were used in evaluation of pup data, as appropriate. Clinical observation incidence data, as well as other proportional data were analyzed as contingency tables using the Fisher's Exact Test [24].

Continuous data, including variables with interval or ratio scales of measurement, such as body weights, food consumption and percent motility were analyzed as follows: Bartlett's Test of Homogeneity of Variances was used to test the hypothesis that all dose groups had equal variance [25]. A nonsignificant result ($p > 0.001$) indicated that an assumption of homogeneity of variance was appropriate, and the data were compared using the Analysis of Variance (ANOVA) [26]. If that test was significant ($p \leq 0.05$), the groups given the test substance were compared with the control group using Dunnett's Test [27].

If Bartlett's Test was significant ($p \leq 0.001$), the ANOVA was not appropriate, and the data were analyzed as follows: The Kruskal-Wallis Test [28] was used to analyze the data, and in the event of a significant result ($p \leq 0.05$), Dunn's Test [29]was used to compare the groups given the test substance with the control group. Count data, such as days in cohabitation, the day a developmental landmark appears or number of implantation sites, were analyzed using the Kruskal-Wallis Test [28] and in the event of a significant result ($p \leq 0.05$), Dunn's Test [29] was used to compare the groups given the test substance with the control group.

3. Results

3.1. Analysis of drinking water solutions and diet

Stability analyses demonstrated that the test substance is stable in the carrier control substances (drinking water and diet) for at least 14 days, when prepared and stored under the same conditions used for this study. All water and diet formulations that were analyzed were found to be close to the planned concentrations of the test material and thus appropriate for use. The amount of test substance used each week to prepare dosed water and diet was adjusted weekly based on estimated average body weight for the next week of study, to achieve a daily exposure of 0, 5, 17 or 40 mg Mo/kg bw/day from the drinking water or 40 mg Mo/kg bw/day from the diet. This weekly adjusting of concentration was continued throughout the premating period for both males and females, throughout gestation and lactation for the females, and until termination for the males.

3.2. Consumed average dosages

The average consumed doses of Mo, based on body weights and water/diet consumption, were generally close to the targeted dose levels, as shown in Table 1. However, during lactation, the average consumed doses were higher than the intended doses by18-35% and 2–20% among the P and F1 generation females, respectively.

3.3. Clinical signs and mortality (P and F1 generations)

There were no deaths attributed to the test material in the P or F1 generation. One P generation male rat given 40 mg Mo/kg bw/day in the diet was found dead on DS 62; this death appeared to be secondary to aspiration of the bedding material, and no other deaths were observed among high dose males. Among the P generation females, one control female and one 40 mg Mo/kg bw/day diet female were euthanized due to injury unrelated to the test material (swollen limb on PDD 23 and fractured palate on PDD 43, respectively). One F1 generation control group male rat was euthanized on PND 158 with hindlimb paralysis, and one F1 generation male rat in the 17 mg Mo/kg bw/day drinking water group was euthanized on PND 44 due to severe eye damage. One F1 generation female 17 mg Mo/kg bw/day drinking water dose group rat died during blood collection on PDD 30. All other P and weaned F1 generation rats survived to scheduled euthanasia.

A small but statistically significantly increased number of P male rats given 40 mg Mo/kg bw/day in the drinking water had “vocalization to touch” (7/24) compared with the controls (1/24). Dietary administration of 40 mg Mo/kg bw/day produced “vocalization to touch” in 5/24 (NS) P males. Otherwise, no adverse clinical observations related to the test substance occurred in the P or weaned F1 male rats or female rats during the premating, gestation or lactation periods.

Table 1
Summary of average consumed doses (mg Mo/kg bw/day) among P and F1 generation male and female rats exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
P Generation males					
Male Rats N	24	24	24	24	24
Premating to termination DS 1-143	0	5.1 ± 0.4	18.0 ± 1.3	39.7 ± 3.2	41.3 ± 2.7
P Generation females					
Female rats N	24	24	24	24	24
Premating to cohabitation DS 1-71	0	5.4 ± 0.4	17.0 ± 1.2	41.4 ± 3.5	40.8 ± 3.0
Gestation GDs 0-20	0	5.1 ± 0.7	16.9 ± 2.0	42.0 ± 5.1	44.7 ± 2.2
Lactation (PDD) 0-14	0	5.9 ± 0.8	20.0 ± 2.9	54.0 ± 13.2	49.4 ± 9.5
F1 Generation males					
Male Rats N	24	24	24	23	24
Premating to termination PND 22-162	0	4.9 ± 1.1	17.4 ± 4.1	39.0 ± 9.2	41.3 ± 7.8
F1 Generation females					
Female rats N	24	24	24	24	24
Premating to cohabitation PND 22- 106	0	5.0 ± 1.3	16.9 ± 4.5	40.3 ± 10.8	42.6 ± 7.1
Gestation GD 0-20	0	4.9 ± 0.7	16.3 ± 2.3	39.6 ± 4.2	40.5 ± 4.4
Lactation (PDD) 0-14	0	6.0 ± 0.7	19.1 ± 2.2	46.7 ± 4.7	40.7 ± 7.2

DS = days after the first day of dosing for the P generation.
GD = day of gestation for P and F1 generations.
PND = postnatal day – days on study for the F1 generation.
PDD = postdelivery day – applies to both P and F1 dams.

3.4. Body weights (P and F1 generations)

Body weights and body weight gains in P-generation male rats were reduced or statistically significantly reduced in the 40 mg Mo/kg bw/day diet group compared to the concurrent control group, at each weekly interval and throughout the study period (Fig. 2a). On DS 71 (last weight prior to cohabitation) and on DS 143, the average body weight was 94.1% and 91.4%, respectively, of the controls group values for the 40 mg Mo/kg bw/day diet group. Exposure to sodium molybdate dihydrate at dose levels up to 40 mg Mo/kg bw/day in drinking water had no overall significant effect on P-generation male body weights or body weight gain.

Among the P-generation females given 40 mg Mo/kg bw/day in the diet, the average body weight was 4% less than controls on DS 71 (the last day recorded prior to cohabitation) and statistically significantly reduced (6–7% decrease) during pregnancy on GD 7, 10 and 14 compared to the concurrent control group values, and body weight gains were statistically significantly reduced (22% decrease) for GD 0 to 7 (Fig. 2b). Lactation body weights were reduced or statistically significantly reduced for all days postdelivery evaluated from PDD 0 to 56; lactation body weight gains did not differ statistically significantly from the control group values for any interval evaluated. Body weights and body weight gains in P-generation female rats given sodium molybdate dihydrate in the drinking water were generally comparable to controls during the premating, gestation and lactation periods.

Among F1 males given 40 mg Mo/kg bw/day in the diet, body weights were lower or statistically significantly lower compared to controls for each weekly interval from PND 22 to 106 (last weight prior to cohabitation) and post cohabitation until euthanasia (Fig. 2c); on PND 106 and 162, body weight was 92.9% and 92.4%, respectively, of the control values, and body weight gains were statistically significantly reduced from PND 22 to 162. Body weights of F1 males given sodium molybdate dihydrate in the drinking water were generally comparable to the concurrent control group throughout the study. Body weight gains for PND 22 to 106 (last weight prior to cohabitation) were statistically significantly lower in the 40 mg Mo/kg bw/day F1 male drinking water group compared to the controls. For the F1 females administered 40 mg Mo/kg bw/day in the diet, body weights and body weight gains were comparable among the groups for the pre-mating period. Body weights for PND 85 and 99 and body weight gains for PND 22 to 106 were statistically significantly lower compared to the control values. Gestation body weights in the 40 mg Mo/kg bw/day diet dose group were statistically significantly reduced (7–8% decrease) for GD 0, 14 and 20 compared to the concurrent control group values. Lactation body weights were statistically significantly reduced for most days of lactation evaluated; lactation body weight gains differed statistically significantly from controls from PDD 14 to 21. Among F1 females given sodium molybdate dihydrate in the drinking water, body weights were generally comparable to the controls for each week during the premating, gestation and lactation periods (Fig. 2d). Compared to controls, statistically significant reductions and/or body weight losses ($p \leq 0.05$ to $p \leq 0.01$) occurred in the 40 mg Mo/kg bw/day drinking water group for gestation body weight gain (GD 0 to 20), PDD 21 and PDD 14 to 21 and 0 to 21.

3.5. Food and water consumption (P and F1 generations)

Although food consumption over the entire exposure period did not differ among the groups, a slightly reduced but statistically significant decrease in food consumption was observed at about half the weekly intervals measured among the P generation males given 40 mg Mo/kg bw/day in the diet, but not in the drinking water. Among P generation females, food consumption was not statistically significantly affected during pre-mating, gestation or lactation by administration of 40 mg Mo/kg bw/day in the drinking water or diet.

Among F1 animals, food consumption was statistically significantly decreased in males from PND 56 to 106 in the 17 mg Mo/kg bw/day (drinking water) and 40 mg Mo/kg bw/day (drinking water and diet) groups. Among F1 females, no significant effect on food consumption was observed.

Slight decreases in water consumption were observed among P-generation males given 40 mg Mo/kg bw/day in the diet, but not in the drinking water; these decreases were statistically significant at only a few weekly intervals. Water consumption was not statistically significantly affected by administration of up to 40 mg Mo/kg bw/day among P-generation females during pre-mating, gestation or lactation. Among the F1 generation, water consumption was not statistically significantly different from controls among males or females given up to 40 mg Mo/kg bw/day in the drinking water or diet.

3.6. Reproductive performance (P and F1 generations)

Administration of sodium molybdate dihydrate in the drinking water or diet had no significant effect on reproductive performance at dose levels up to 40 mg Mo/kg bw/day in either the P or F1 generation (Table 2). The average number of estrus cycles observed during the 14-day period of vaginal cytology was not altered by exposure to the test material in the drinking water or diet among either generation of female rats. The number of females exhibiting persistent estrus or persistent diestrus was not affected by exposure to the test material. The fertility index and pregnancy rate were not statistically significantly different from controls among the P and F1 generation male and female groups exposed to up to 40 mg Mo/kg bw/day in the drinking water or diet. All pregnant females had liveborn pups.

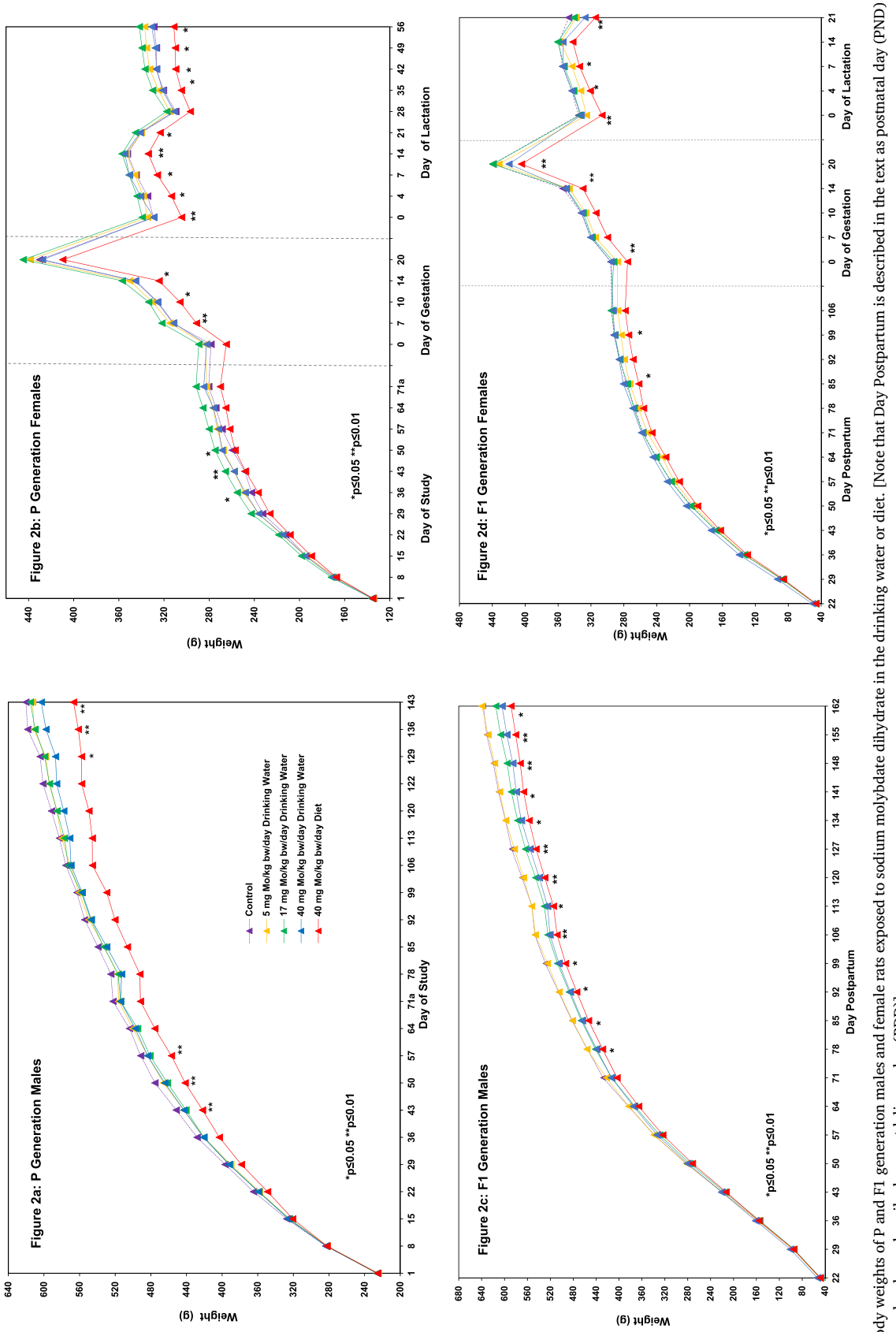

Fig. 2. Body weights of P and F1 generation males and female rats exposed to sodium molybdate dihydrate in the drinking water or diet. [Note that Day Postpartum is described in the text as postnatal day (PND) and Day of Lactation has been described as postdelivery day (PDD)].

Table 2
Reproductive performance of P and F1 generation male and female rats exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
P generation males					
Rats in cohabitation, N	24	24	24	23	24
Days in cohabitation, mean ± SD	2.9 ± 2.0	3.2 ± 1.7	2.8 ± 1.9	2.8 ± 1.1	2.7 ± 1.2
Mating Index[a], N/N (%)	23/24	23/24	24/24	23/23	24/24
	(95.8)	(95.8)	(100.0)	(100.0)	(100.0)
Fertility Index[b], N/N (%)	22/23	22/23	23/24	22 /23	19/24
	(95.6)	(95.6)	(95.8)	(95.6)	(79.2)
F1 generation males					
Rats in cohabitation, N	24	24	22	24	24
Days in cohabitation, mean ± SD	2.7 ± 2.0	2.5 ± 1.7	2.3 ± 1.0	2.9 ± 2.4	3.0 ± 2.2
Mating Index[a], N/N (%)	24/24	24/24	22/22	22/24	23/24
	(100.0)	(100.0)	(100.0)	(91.7)	(95.8)
Fertility Index[b], N/N (%)	22/24	23/24	21/22	19/22	22/23
	(91.7)	(95.8)	(95.4)	(86.4)	(95.6)
P generation females					
Rats evaluated, N	24	24	24	24	24
Estrus cycles/14 days, mean ± SD	3.4 ± 0.6	3.4 ± 0.6	3.4 ± 0.6	3.1 ± 0.6	3.3 ± 0.7
Females with persistent estrus, N (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Females with persistent diestrus, N (%)	0 (0.0)	1 (4.2)	0 (0.0)	2 (8.3)	0 (0.0)
Rats in cohabitation, N	24	24	24	24	24
Days in cohabitation, mean ± SD	2.9 ± 2.0	3.2 ± 1.7	2.8 ± 1.9	3.0 ± 1.2	2.7 ± 1.2
Mating Index[a], N/N (%)	23/24	23/24	24/24	24/24	24/24
	(95.8)	(95.8)	(100.0)	(100.0)	(100.0)
Fertility Index[b], N/N (%)	22/23	22/23	23/24	23/24	19/24
	(95.6)	(95.6)	(95.8)	(95.8)	(79.2)
F1 generation females					
Rats evaluated, N	24	24	24	24	24
Estrus cycles/14 days, mean ± SD	3.1 ± 0.7	3.3 ± 0.5	3.3 ± 0.6	3.1 ± 0.6	3.2 ± 0.6
Females with persistent estrus, N (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Females with persistent diestrus, N (%)	1 (4.2)	0 (0.0)	1 (4.2)	1 (4.2)	1 (4.2)
Rats in cohabitation, N	24	24	24	24	24
Days in cohabitation, mean ± SD	2.7 ± 2.0	2.5 ± 1.7	2.4 ± 1.3	3.2 ± 3.6	3.3 ± 3.4
Mating Index[a], N/N (%)	24/24	24/24	24/24	24/24	23/24
	(100.0)	(100.0)	(100.0)	(100.0)	(95.8)
Fertility Index[b], N/N (%)	22/24	23/24	23/24	21/24	22/23
	(91.7)	(95.8)	(95.8)	(87.5)	(95.6)

*Statistically significantly different from control value, $p < 0.05$ (None found).
**Statistically significantly different from control value, $p < 0.01$ (None found).
[a] Number of rats that mated/number of rats in cohabitation.
[b] Number of pregnancies/number of rats that mated.

3.7. Litter data (F1 and F2 litters)

The average number of liveborn pups per litter was not statistically significantly different from controls in any exposed group in either generation. Pup survival indices and gender ratios were not affected by exposure to sodium molybdate dihydrate in the drinking water or diet (Table 3). Mean pup weight per litter was not statistically significantly different from the control values for any exposed group in the F1 or F2 generations (Table 4). There were no adverse clinical or necropsy observations attributable to the test material among the F1 or F2 pups in any group exposed to up to 40 mg Mo/kg bw/day in the drinking water or diet.

3.8. Developmental landmarks (F1 and F2 pups)

Administration of up to 40 mg Mo/kg bw/day in the drinking water or diet did not affect the onset or development of eye opening, hair growth, incisor eruption, or pinna unfolding among the F1 and F2 generation pups (Table 5). The average anogenital distances for male and female F2 pups on Day 4 postpartum did not differ statistically significantly compared to controls among any exposure group. Preputial separation and vaginal patency were not statistically significantly altered in the F1 generation litters by exposure as high as 40 mg Mo/kg bw/day in the drinking water or diet; the average body weights on the day that the sexual maturation biomarker was achieved was not statistically significantly different from the controls for any group exposed to the test material.

3.9. Sperm evaluation (P and F1 generations)

Exposure to up to 40 mg Mo/kg bw/day in either the drinking water or diet did not produce a statistically significant decrease in sperm density (cauda epididymal), spermatid density (testicular) or sperm motility in the P and F1 generations (Table 6). There was no statistically significant effect on the mean percentage of normal or total abnormal sperm. In the two groups of P generation males given 40 mg Mo/kg bw/day, the standard deviation for total abnormal sperm was larger compared to the other groups; in each group, the increase in the standard deviation was due to a single male with 52% and 94% abnormal sperm in the groups given 40 mg Mo/kg bw/day in drinking water the diet and, respectively. In contrast, there was no evidence of a similar increase in the standard deviation for total abnormal sperm in any group in the F1 generation.

The percent of sperm with no head was statistically significantly increased in the P generation males given 40 mg Mo/kg bw/day in the diet compared to the control value. Among the P generation males given 40 mg Mo/kg bw/day in the drinking water, a slight increase (not statistically significant) was observed in the percent of no head sperm. These increases were not considered treatment-related because (1) in both cases, they were largely attributable to one male each (the same individual males with the high incidences of abnormal sperm mentioned in the previous paragraph)

Table 3
Natural delivery observations among P and F1 generation females exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
P generation females and litters					
Number Pregnant/Number Tested, N (%)	22 /24 (91.7)	22/24 (91.7)	23/24 (95.8)	23/24 (95.8)	19 /24 (79.2)
Delivered litters, N (%)	22 (100.0)	22 (100.0)	23 (100.0)	23 (100.0)	19 (100.0)
Duration of gestation (days), mean ± SD	22.5 ± 0.5	22.6 ± 0.5	22.6 ± 0.5	22.6 ± 0.5	22.6 ± 0.5
Implantation sites/delivered litter, mean ± SD	15.8 ± 2.9	16.1 ± 1.9	16.6 ± 2.0	15.4 ± 2.4	15.9 ± 1.5
Gestation Index[a], %	100	100	100	100	100
Litter size (live + stillbirths), mean ± SD	15.2 ± 3.0	15.2 ± 2.1	15.5 ± 1.9	14.6 ± 2.4	15.3 ± 1.7
Liveborn, mean ± SD	15.1 ± 3.0	15.1 ± 2.2	15.3 ± 2.0	14.5 ± 2.3	15.1 ± 1.8
Stillborn, N (%)	1(0.3)	3(0.9)	4(1.1)	3(0.9)	3(1.0)
Viability Index[b], %	98.5	99.1	99.4	97.9	99.3
Lactation Index[c], %	98.2	98.8	99.1	99.4	99.6
% Male pups/litter on PND 0, mean ± SD	50.2 ± 15.6	50.6 ± 13.0	52.3 ± 14.0	53.0 ± 14.2	54.2 ± 14.8
% Male pups/litter on PND 21, mean ± SD	50.5 ± 15.8	50.5 ± 14.0	52.4 ± 13.8	52.8 ± 14.3	53.9 ± 15.4
F1 generation females and litters					
Number Pregnant/Number Tested, N (%)	22/24 (91.7)	23/24 (95.8)	23/24 (95.8)	21/24 (87.5)	22/24 (91.7)
Delivered litters, N (%)	22 (100.0)	23 (100.0)	23 (100.0)	21 (100.0)	22 (100.0)
Duration of gestation (days), mean ± SD	22.9 ± 0.3	23.0 ± 0.2	22.9 ± 0.3	23.0 ± 0.3	22.9 ± 0.4
Implantation sites/litter, mean ± SD	14.8 ± 1.4	15.5 ± 1.8	14.9 ± 3.4	15.1 ± 2.1	15.3 ± 2.4
Gestation Index[a], %	100	100	100	100	100
Litter size (live + stillbirths), mean ± SD	14.3 ± 1.3	15.4 ± 2.2	14.3 ± 3.8	14.5 ± 1.9	14.9 ± 2.2
Liveborn, mean ± SD	14.1 ± 1.4	15.4 ± 2.2	14.0 ± 3.8	14.5 ± 1.9	14.9 ± 2.2
Stillborn, N(%)	4 (1.)	1 (0.3)	6 (1.8)	(0.0)	0 (0.0)
Viability Index[b], %	97.7	98.3	97.8	99.3	97.9
Lactation Index[c], %	99.0	98.8	98.4	99.7	98.8
% Male pups/litter on PND 0, mean ± SD	47.8 ± 16.6	52.7 ± 17.8	53.7 ± 13.8	53.7 ± 12.2	54.3 ± 15.3
% Male pups/litter on PND 21, mean ± SD	47.5 ± 16.6	53.4 ± 17.3	53.1 ± 13.0	54.3 ± 12.5	53.8 ± 15.0

*Statistically significantly different from control value, $p < 0.05$ (None found).
**Statistically significantly different from control value, $p < 0.01$ (None found).
[a] Number of rats with live offspring/number of pregnant rats.
[b] Number of live pups on Day 3 postpartum/number of liveborn pups on Day 0 postpartum.
[c] Number of live pups on Day 21 (weaning) postpartum/number of live pups on Day 3 postpartum.

with approximately 24% and 27% sperm with no head in the diet and drinking water groups, respectively, (2) no evidence of an increase in the percent sperm with no head was observed among F1 males given up to 40 mg Mo/kg bw/day in the diet or drinking water, and (3) the percent sperm with no head was within the historical control range. In summary, there was no clear evidence of any compound-related effect on sperm concentration, spermatid concentration, sperm motility or morphology at dose levels up to 40 mg Mo/kg bw/day by either route of administration in either generation.

Apparent differences in sperm density and motility, and spermatid density between the two generations (including in the controls) was due to an upgrade in the CASA software. After revalidation, the gating parameters used for the second generation differed from the first generation resulting in lower density measurements and higher motility counts in the first generation and higher density counts with lower percentage motility in the second generation. The gating affects what the software will identify as a sperm vs. debris, and with more debris identified as sperm, the motility will appear lower.

Table 4
Litter observations among F1 and F2 generations exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
F1 litters					
Litters with one or more liveborn, N	22	22	23	23	19
Live litter size on PND 0, mean ± SD	15.1 ± 3.0	15.1 ± 2.2	15.3 ± 2.0	14.5 ± 2.3	15.1 ± 1.8
Live litter size on PND 21, mean ± SD	14.6 ± 3.0	14.8 ± 2.2	15.1 ± 1.9	14.1 ± 2.4	14.9 ± 1.8
Pup weight/litter on PND 0, g, mean ± SD	6.5 ± 0.7	6.7 ± 0.5	6.7 ± 0.7	6.8 ± 0.6	6.4 ± 0.6
Pup weight/litter on PND 21, g, mean ± SD	42.0 ± 8.4	42.6 ± 5.4	42.4 ± 7.2	45.4 ± 7.6	41.3 ± 4.9
F2 litters					
Litters with one or more liveborn, N	22	23	23	21	22
Live litter size on PDD 0, mean ± SD	14.1 ± 1.4	15.4 ± 2.2	14.0 ± 3.8	14.5 ± 1.9	14.9 ± 2.2
Live litter size on PDD 21, mean ± SD	13.6 ± 1.1	15.0 ± 2.0	13.5 ± 3.4	14.4 ± 1.8	14.4 ± 1.7
Pup weight/litter on PDD 0, g, mean ± SD	7.0 ± 0.6	6.8 ± 0.5	7.1 ± 0.8	7.0 ± 0.5	6.6 ± 0.4
Pup weight/litter on PDD 21, g, mean ± SD	48.0 ± 6.7	43.9 ± 6.4	48.6 ± 11.2	47.4 ± 8.2	45.6 ± 5.0

*Statistically significantly different from control value, $p < 0.05$ (None found).
**Statistically significantly different from control value, $p < 0.01$(None found).

Table 5
Developmental landmarks among the F1 and F2 generation pups exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
F1 generation pups					
Litters evaluated, N	22	22	23	23	19
Average day of eye opening[a], mean ± SD	14.7 ± 0.9	14.6 ± 0.7	14.6 ± 0.9	14.4 ± 0.8	14.8 ± 0.7
Average day of hair growth[a], mean ± SD	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0
Average day of incisor eruption[a], mean ± SD	11.6 ± 0.8	11.7 ± 0.6	11.6 ± 0.9	11.2 ± 0.9	11.9 ± 0.9
Average day of pinna unfolding[a], mean ± SD	3.5 ± 0.6	3.5 ± 0.7	3.4 ± 0.5	3.2 ± 0.4	3.3 ± 0.5
Average day of preputial separation[a], mean ± SD	44.6 ± 1.7	44.8 ± 2.6	44.7 ± 1.9	44.2 ± 2.3	45.0 ± 2.0
Average day of vaginal patency[a], mean ± SD	31.0 ± 1.5	31.0 ± 1.4	31.8 ± 2.2	31.2 ± 2.7	31.3 ± 2.0
F2 generation pups					
Litters evaluated, N	22	23	23	21	22
Average day of eye opening[a], mean ± SD	13.0 ± 0.9	13.3 ± 0.6	13.1 ± 0.6	12.8 ± 0.9	13.3 ± 0.7
Average day of hair growth[a], mean ± SD	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0
Average day of incisor eruption[a], mean ± SD	10.1 ± 0.8	10.3 ± 1.1	10.3 ± 1.3	9.8 ± 0.7	10.3 ± 1.1
Average day of pinna unfolding[a], mean ± SD	2.3 ± 0.4	2.3 ± 0.4	2.3 ± 0.5	2.2 ± 0.4	2.2 ± 0.4
Male anogenital distance on PDD 4, mean ± SD	4.77 ± 0.62	4.97 ± 0.47	4.89 ± 0.72	4.50 ± 0.51	4.70 ± 0.42
Female anogenital distance on PDD 4, mean ± SD	2.30 ± 0.21	2.30 ± 0.22	2.13 ± 0.28	2.16 ± 0.28	2.18 ± 0.24

*Statistically significantly different from control value, $p < 0.05$ (None found).
**Statistically significantly different from control value, $p < 0.01$ (None found).
[a] The average day postpartum that at least 50% of the pups tested in the litter had the developmental measure present.

Table 6
Semen evaluation of the P and F1 generation male rats exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
P generation males					
Rats evaluated, N	24	24	24[a]	23	24
Sperm density[b] cauda epididymis, mean ± SD	644 ± 377	675 ± 303	672 ± 391	756 ± 396	608 ± 314
Spermatid density[c] testes, mean ± SD	51 ± 51	52 ± 46	52 ± 47	54 ± 44	47 ± 34
% Motile sperm, mean ± SD	89 ± 7	89 ± 10	90 ± 6	88 ± 5	87 ± 14
% Normal morphology sperm, mean ± SD	96 ± 2.8	95 ± 5.2	94 ± 4.4	93 ± 10	91 ± 17
% Abnormal morphology sperm, mean ± SD	3.7 ± 2.8	3.9 ± 2.5	5.6 ± 4.4	6.8 ± 10	8.8 ± 17
% Detached head sperm, mean ± SD	2.2 ± 2.0	3.1 ± 3.9	2.8 ± 1.4	3.4 ± 5.0	5.1 ± 12
% No head sperm, mean ± SD	1.2 ± 0.9	1.1 ± 1.3	1.3 ± 1.3	2.6 ± 4.9	3.1 ± 5.5*
% Broken flagellum sperm, mean ± SD	0.4 ± 0.5	0.7 ± 0.7	0.9 ± 2.3	0.7 ± 0.9	0.9 ± 1.5
F1 generation males					
Rats evaluated, N	23[d]	24	22[d,e]	24	24
Sperm density[b]cauda epididymis, mean ± SD	1652 ± 485	1649 ± 388	1747 ± 648	1784 ± 544	2011 ± 578
Spermatid density[c] testes, mean ± SD	406 ± 195	350 ± 116	316 ± 92	331 ± 150	280 ± 90
% Motile sperm, mean ± SD	68 ± 16	67 ± 10	64 ± 12	66 ± 18	68 ± 11
% Normal morphology sperm, mean ± SD	96 ± 1.9	95 ± 1.8	97 ± 2.2	97 ± 2.0	97 ± 2.4
% Abnormal morphology sperm, mean ± SD	3.9 ± 1.9	5.0 ± 1.8	3.1 ± 2.2	3.2 ± 1.9	3.2 ± 2.4
% Detached head sperm, mean ± SD	2.2 ± 1.1	3.0 ± 1.3	1.9 ± 1.7	1.9 ± 1.3	2.3 ± 1.8
% No head sperm, mean ± SD	1.5 ± 1.3	1.4 ± 0.8	1.1 ± 1.0	0.9 ± 0.8	0.9 ± 0.8
% Broken flagellum sperm, mean ± SD	0.2 ± 0.4	0.5 ± 0.7	0.2 ± 0.4	0.3 ± 0.6	0.04 ± 0.14

*Statistically significantly different from control value, $p < 0.05$.
**Statistically significantly different from control value, $p < 0.01$ (None found).
[a] Two males in the 17 mg Mo/kg bw/day group were excluded from the summaries of the sperm morphology because fewer than 200 sperm were available for evaluation. One male had zero sperm in the sample for morphology; the other had 10 sperm in the sample.
[b] Sperm density expressed as million sperm/mL was calculated by dividing the sperm count by the volume in the image area and adjusting for dilution.
[c] Spermatid density expressed as million spermatid/mL was calculated by dividing the spermatid count by the volume in the image area and adjusting for dilution. The difference in sperm and spermatid counts and % motile between the P and F1 generations is discussed in the Text Section 3.9.
[d] Excludes one rat that was euthanized due to adverse clinical observations.
[e] Excludes one rat that was discovered to be a female on PND 35.

Table 7
Terminal body and organ weights (mean ± SD) of the P and F1 generation male rats exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
P generation males					
Rats evaluated, N	24	24	24	23[a]	24
Terminal body weight (g)	628 ± 72	618 ± 57	621 ± 63	608 ± 57	570 ± 60**
Liver (g)	21.1 ± 3.3	20.5 ± 2.6	20.2 ± 2.7	19.4 ± 2.4*	17.7 ± 2.1**
Kidney, left (g)	2.18 ± 0.24	2.11 ± 0.25	2.04 ± 0.23	2.09 ± 0.33	1.99 ± 0.28
Brain (g)	2.29 ± 0.10	2.25 ± 0.10	2.27 ± 0.08	2.25 ± 0.14	2.25 ± 0.11
Spleen (g)	0.98 ± 0.18	0.94 ± 0.15	1.01 ± 0.14	1.00 ± 0.13	0.94 ± 0.12
Thymus (g)	0.25 ± 0.07	0.25 ± 0.07	0.26 ± 0.09	0.25 ± 0.06	0.22 ± 0.07
Testis, left (g)	1.91 ± 0.18	1.88 ± 0.19	1.92 ± 0.15	1.90 ± 0.15	1.88 ± 0.15
Epididymis, left (g)	0.87 ± 0.11[b]	0.83 ± 0.07	0.87 ± 0.07	0.86 ± 0.09	0.82 ± 0.08
Cauda epididymis, left (g)	0.39 ± 0.05[b]	0.39 ± 0.04	040 ± 0.04	0.39 ± 0.06	0.38 ± 0.06
Seminal vesicles w fluid (g)	2.12 ± 0.41[b]	2.08 ± 0.39	2.09 ± 0.38	2.22 ± 0.41	2.15 ± 0.30
Prostate (g)	1.61 ± 0.19	1.49 ± 0.30	1.44 ± 0.25	1.42 ± 0.28	1.46 ± 0.24
Adrenals, paired (mg)	69 ± 8	72 ± 10	70 ± 10	71 ± 15	66 ± 12
Thyroid/parathyroid (mg)	32 ± 6	32 ± 5	30 ± 5	33 ± 11	39 ± 13
Pituitary (mg)	16 ± 3	17 ± 4	17 ± 4	16 ± 5	17 ± 3
F1 generation males					
Rats evaluated, N	23[c]	24	22[d]	24	24
Terminal body weight (g)	648 ± 65	646 ± 64	623 ± 62	613 ± 50	598 ± 61*
Liver (g)	22.8 ± 3.2	21.8 ± 3.1	21.1 ± 3.1	20.7 ± 2.9	19.6 ± 2.3**
Kidney, left (g)	2.15 ± 0.24	2.11 ± 0.22	2.06 ± 0.23	2.03 ± 0.25	2.02 ± 0.21
Brain (g)	2.31 ± 0.11	2.24 ± 0.11	2.28 ± 0.09	2.25 ± 0.09	2.24 ± 0.10
Spleen (g)	0.97 ± 0.13	0.95 ± 0.16	0.97 ± 0.18	0.98 ± 0.18	0.95 ± 0.14
Thymus (g)	0.28 ± 0.05	0.27 ± 0.08	0.28 ± 0.07	0.24 ± 0.06	0.28 ± 0.9
Testis, left (g)	1.85 ± 0.14	1.89 ± 0.15[b]	1.90 ± 0.13	1.88 ± 0.16	1.83 ± 0.24
Epididymis, left (g)	0.80 ± 0.06	0.81 ± 0.07[b]	0.83 ± 0.06	0.82 ± 0.06	0.78 ± 0.07
Cauda epididymis, left (g)	0.32 ± 0.03	0.33 ± 0.03[b]	0.33 ± 0.03	0.34 ± 0.04	0.33 ± 0.04
Seminal vesicles w fluid (g)	2.21 ± 0.29	2.25 ± 0.39	2.25 ± 0.32	2.37 ± 0.47	2.12 ± 0.29
Prostate (g)	1.40 ± 0.25	1.51 ± 0.28	1.30 ± 0.24	1.46 ± 0.27	1.38 ± 0.19
Adrenals, paired (mg)	64 ± 13	72 ± 12	66 ± 9	71 ± 12	68 ± 9
Thyroid/parathyroid (mg)	45 ± 6	48 ± 8	46 ± 7	49 ± 8	46 ± 8
Pituitary (mg)	16 ± 4	17 ± 4	16 ± 4	17 ± 4	18 ± 5

* Statistically significantly different from control value, $p < 0.05$.
** Statistically significantly different from control value, $p < 0.01$.
[a] Excludes male rat found dead on day 63 of the study.
[b] Excludes left testis, epididymis, and cauda epididymis weights for one control P male and one 5 mg Mo/kg bw/day F1 male with small left testis, epididymis, and cauda epididymis; appearance and weight on right side were normal in both males.
[c] Excludes one male euthanized due to adverse clinical observations.
[d] Excludes one male euthanized due to adverse clinical observations and one rat discovered to be a female on PND.

3.10. Ovarian evaluation (P and F1 generations)

No treatment related effects were observed on ovarian weights or histopathology in any of the P or F1 treated groups. In the P generation, a statistically significant increase was noted in the average number of primordial follicles in the left ovary (but not the right ovary) of females administered 17 mg Mo/kg bw/day in the drinking water and 40 mg Mo/kg bw/day in the diet compared to the control group. In the F1 generation, statistically significant increases in the number of primordial follicles in the right, left, and combined ovaries were observed in groups given 17 or 40 mg Mo/kg bw/day in the drinking water or 40 mg Mo/kg bw/day in the diet. Only a decrease in primordial follicles would be considered adverse. The increased primordial follicle counts were not considered toxicologically important or adverse because: 1) no effect on fertility, ovarian histology, or ovarian weight was seen in either generation, 2) all values were within the historical control range, and 3) no clear dose-response relationship was observed.

3.11. Necropsy, organ weights and histopathology (P and F1 generations)

No exposure-related macroscopic findings were observed at the scheduled necropsies of the P and F1 rats of either sex. Some changes in organ weights were observed, but these were not considered adverse because: (1) there were no histological changes in any organ evaluated, (2) the increases in organ weights relative to body weights were a reflection of decreased terminal body weights, and (3) the few statistically significant decreases observed were inconsistent (across generations and sexes) and not dose-related.

The absolute and relative liver weights were statistically significantly decreased compared to controls among P generation males given 40 mg Mo/kg bw/day in the drinking water or diet (Table 7). Among the F1 males, the absolute liver weight was statistically significantly decreased at 40 mg Mo/kg bw/day in the diet, but not in the drinking water; relative liver weights were not statistically significantly different from controls among the F1 males given the test material in the drinking water or diet.

In males given the test material in the drinking water, the relative (but not absolute) weights of the left and right epididymis, left testis minus the tunica albuginea, paired adrenals, and thyroid/parathyroid were statistically significantly increased in the 40 mg Mo/kg bw/day F1 group compared to controls. In the group given 40 mg Mo/kg bw/day in the diet, the relative weights of the thyroid/parathyroid (P and F1 generations) and brain (P generation only) were statistically significantly increased compared to the controls. The significant increases in relative organ weights observed in P and F1 males were not considered adverse since they reflected the lower terminal body weights in the 40 mg Mo/kg bw/day groups.

In females, the absolute and relative weights of the thyroid/parathyroid were statistically significantly reduced compared to controls given 17 or 40 mg Mo/kg bw/day in the drinking water in the P

Table 8
Terminal body and organ weights (mean ± SD) of the P and F1 generation female rats exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
P generation females					
Rats evaluated, N[a]	21	22	22	23	18
Terminal body weight (g)	332 ± 22	342 ± 29	346 ± 25	334 ± 36	316 ± 17
Liver (g)	11.6 ± 1.0	11.9 ± 1.2	12.2 ± 1.7	11.6 ± 1.4	10.9 ± 1.1
Kidney, left (g)	1.21 ± 0.10	1.18 ± 0.11	1.25 ± 0.10	1.17 ± 0.12	1.20 ± 0.10
Brain (g)	2.12 ± 0.08	2.07 ± 0.08	2.14 ± 0.11	2.09 ± 0.09	2.10 ± 0.11
Spleen (g)	0.66 ± 0.08	0.70 ± 0.08	0.70 ± 0.10	0.72 ± 0.10	0.69 ± 0.09
Thymus (g)	0.27 ± 0.08	0.27 ± 0.04	0.29 ± 0.07	0.24 ± 0.07	0.26 ± 0.09
Ovaries, paired (mg)	121 ± 25	123 ± 24	120 ± 26	119 ± 20	121 ± 26
Adrenals, paired (mg)	92 ± 12	90 ± 10	91 ± 15	85 ± 13	86 ± 14
Thyroid/parathyroid (mg)	36 ± 7	36 ± 7	30 ± 8*	26 ± 5**	32 ± 6
Pituitary (mg)	25 ± 7	24 ± 4	23 ± 5	23 ± 5	25 ± 6
F1 generation females					
Rats evaluated, N[a]	22	23	22	21	22
Terminal body weight (g)	321 ± 18	318 ± 23	324 ± 20	317 ± 27	303 ± 22*
Liver (g)	14.5 ± 1.6	13.7 ± 1.5	13.9 ± 1.5	13.9 ± 2.2	13.1 ± 1.3
Kidney, left (g)	1.33 ± 0.09	1.27 ± 0.10	1.28 ± 0.08	1.27 ± 0.14	1.28 ± 0.12
Brain (g)	2.06 ± 0.11	2.05 ± 0.08	2.05 ± 0.08	2.01 ± 0.10	2.02 ± 0.11
Spleen (g)	0.68 ± 0.09	0.68 ± 0.08	0.69 ± 0.08	0.68 ± 0.09	0.65 ± 0.08
Thymus (g)	0.30 ± 0.07	0.31 ± 0.08	0.30 ± 0.09	0.27 ± 0.08	0.29 ± 0.7
Ovaries, paired (mg)	146 ± 19	167 ± 68	136 ± 19	139 ± 23	144 ± 23
Adrenals, paired (mg)	94 ± 8	93 ± 15	89 ± 12	86 ± 14	88 ± 14
Thyroid/parathyroid (mg)	43 ± 9	46 ± 11	42 ± 10	38 ± 9	41 ± 6
Pituitary (mg)	19 ± 4	20 ± 5	18 ± 6	18 ± 5	20 ± 5

* Statistically significantly different from control value, $p < 0.05$.
** Statistically significantly different from control value, $p < 0.01$.
[a] Females that had not become pregnant or that died or were euthanized prior to scheduled termination were excluded in the terminal organ weight summaries.

generation, but not in the F1 generation (Table 8). No significant effect on thyroid/parathyroid weights was observed in either generation in the group given 40 mg Mo/kg bw/day in the diet. When the test material was administered in the diet, the relative weight of the spleen was statistically significantly increased (12%) among the P generation (but not F1) females given 40 mg Mo/kg bw/day. No other difference in organ weights was observed among P or F1 females given the test material in the drinking water or diet.

The histological findings observed in the organs were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence and severity in control and treated animals and, therefore, were considered unrelated to ingestion of the test material. The microscopic evaluation of the testes produced no clear evidence of a treatment-related effect on spermatogenesis. Among the P generation males, minimal or mild degeneration of the seminiferous tubules was observed in 3 of 24 rats in the 40 mg Mo/kg bw/day diet group, and in 1 of 24 rats in the 17 mg Mo/kg bw/day water dosed group. Severe degeneration of the seminiferous tubules was observed in an additional rat in the 17 mg Mo/kg bw/day water dosed group. No histologic lesions were observed in the testes in any rats in the 0, 5 or 40 mg Mo/kg bw/day water dosed groups. Among the F1 males, minimal or mild degeneration of the seminiferous tubules was observed in 2 of 24 rats in the 40 mg Mo/kg bw/day diet group. Severe degeneration of the seminiferous tubules was observed in 1 of 24 rats in the 40 mg Mo/kg bw/day water dosed group. Mild granulomatous inflammation was observed in the testis of 1 of 24 control rats. No histologic lesions were observed in the testes in any rats in the 5 or 17 mg Mo/kg bw/day water dosed groups. All F2 male pups necropsied on PND 22 were sexually immature; as expected, microscopic examination revealed no active spermatogenesis in the testes.

3.12. Necropsy, organ weights and histopathology (F1 and F2 generation pups)

No adverse clinical or necropsy observations occurred in the F1 or F2 pups at doses of the test substance as high as 40 mg Mo/kg bw/day for either the diet or water exposed groups. No necropsy gross lesions or clinical observations were considered exposure-related among pups of either generation as none occurred in more than one to four litters and/or the observation was not dose dependent.

All F1 pups necropsied at weaning appeared normal except for two pups from the same litter in the 40 mg Mo/kg bw/day water group that had dilation of the lateral ventricles of the brain. Similarly, all F2 pups necropsied at weaning appeared normal except for 2, 1, 3, 0 and 0 pups each from a different litter in the 0, 5, 17, 40 Mo/kg bw/day dosed water and 40 Mo/kg bw/day dosed diet group pups, respectively. Two pups, each from a different litter, in the control group had clear fluid filled cysts in the kidney, the pup in the 5 mg Mo/kg bw/day dosed water group had situs inversus of the abdominal viscera, and two of three pups from three different litters in the 17 mg Mo/kg bw/day dosed water group had slight dilation of renal pelvis and the third pup had a clear fluid filled cyst in the kidney.

Necropsy of the F1 and F2 dead/stillborn pups revealed no structural abnormalities. The incidence of unfed pups (no milk in their stomach) was 1 or 2 in each of the F1 groups and 2, 3, 2, 0 and 5 pups in the 0, 5, 17 and 40 mg Mo/kg bw/day dosed water groups and 40 mg Mo/kg bw/day diet groups, respectively, of the F2 generation.

No effect on absolute or relative organ weights (brain, spleen, and thymus) was seen among the F1 male and female pups at doses of the test substance as high as 40 mg Mo/kg bw/day for either the diet or water exposed groups (Table 9). A statistically significant decrease in the absolute weights of the brain, spleen, and thyroid/parathyroid compared to controls was observed among female F2 pups (but not male pups) exposed to 5 mg Mo/kg bw/day, but not higher doses, in the drinking water. These reductions were not considered to be exposure related because they were not dose-dependent. There was a statistically significant reduction in the thyroid/parathyroid weight among the F2 female pups in the 40 mg Mo/kg bw/day diet dose groups in comparison with the control group value. This reduction was not considered to be test substance related because it was comparable to the reduction observed in the 5 mg Mo/kg bw/day water dose group indicating that it was within the range of normal variation and was only observed in the

Table 9
Organ weights (mean ± SD) among the F1 and F2 generation pups of rats exposed to sodium molybdate dihydrate in the drinking water or diet.

	Dose Group (mg Mo/kg bw/day)				
	0	5 (water)	17 (water)	40 (water)	40 (diet)
F1 generation male pups					
Rats evaluated, N	22	22	23	23	19
Brain (g)	1.52 ± 0.07	1.51 ± 0.08	1.50 ± 0.10	1.51 ± 0.08	1.48 ± 0.12
Spleen (g)	0.21 ± 0.6	0.24 ± 0.7	0.23 ± 0.11	0.25 ± 0.09	0.22 ± 0.07
Thymus (g)	0.21 ± 0.5	0.22 ± 0.04	0.21 ± 0.07	0.23 ± 0.05	0.20 ± 0.07
F1 generation female pups					
Rats evaluated, N	22	22	23	22	19
Brain (g)	1.46 ± 0.10	1.46 ± 0.10	1.46 ± 0.07	1.48 ± 0.09	1.44 ± 0.08
Spleen (g)	0.21 ± 0.06	0.23 ± 0.05	0.23 ± 0.09	0.24 ± 0.09	0.21 ± 0.06
Thymus (g)	0.20 ± 0.05	0.22 ± 0.04	0.20 ± 0.05	0.24 ± 0.06	0.21 ± 0.06
F2 generation male pups					
Rats evaluated, N	22	23	22	21	22
Liver (g)	3.00 ± 0.27	2.79 ± 0.50	2.98 ± 0.68	2.90 ± 0.43	2.78 ± 0.46
Kidneys, paired (g)	0.84 ± 0.10	0.76 ± 0.10	0.81 ± 0.18	0.81 ± 0.13	0.78 ± 0.11
Brain (g)	1.59 ± 0.08	1.53 ± 0.07	1.56 ± 0.11	1.55 ± 0.08	1.56 ± 0.07
Spleen (g)	0.32 ± 0.05	0.28 ± 0.06	0.33 ± 0.10	0.31 ± 0.06	0.30 ± 0.08
Thymus (g)	0.27 ± 0.05	0.25 ± 0.06	0.27 ± 0.09	0.29 ± 0.07	0.27 ± 0.06
Testis, left (g)	0.18 ± 0.03	0.16 ± 0.03	0.18 ± 0.04	0.17 ± 0.04	0.16 ± 0.04
Epididymides, paired, (g)	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
Seminal vesicles (g)	0.05 ± 0.02	0.06 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.05 ± 0.02
Prostate (g)	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02
Adrenals, paired (mg)	23 ± 6	24 ± 6	22 ± 8	25 ± 6	24 ± 7
Thyroid/parathyroid[a] (mg)	9 ± 2	9 ± 3	8 ± 2	9 ± 2	8 ± 2
Pituitary (mg)	3 ± 2	3 ± 2	3 ± 2	3 ± 2	3 ± 2
F2 generation female pups					
Rats evaluated, N	22	23	23	21	22
Liver (g)	2.88 ± 0.42	2.50 ± 0.52	2.81 ± 0.65	2.83 ± 0.39	2.68 ± 0.41
Kidneys, paired (g)	0.81 ± 0.09	0.73 ± 0.13	0.80 ± 0.17	0.79 ± 0.10	0.76 ± 0.09
Brain (g)	1.54 ± 0.05	1.47 ± 0.10**	1.54 ± 0.10	1.51 ± 0.07	1.50 ± 0.06
Spleen (g)	0.30 ± 0.04	0.26 ± 0.07*	0.32 ± 0.08	0.30 ± 0.05	0.29 ± 0.06
Thymus (g)	0.27 ± 0.04	0.25 ± 0.06	0.27 ± 0.09	0.29 ± 0.06	0.27 ± 0.06
Uterus, cervix, oviducts (g)	0.09 ± 0.03	0.08 ± 0.03	0.08 ± 0.04	0.10 ± 0.04	0.08 ± 0.03
Ovaries, paired (mg)	30 ± 6	26 ± 9	27 ± 12	29 ± 8	28 ± 7
Adrenals, paired (mg)	22 ± 4	23 ± 7	21 ± 6	21 ± 6	23 ± 8
Thyroid/parathyroid[a] (mg)	10 ± 2	8 ± 2**	10 ± 2	10 ± 2	8 ± 3**
Pituitary (mg)	3 ± 1	2 ± 1	3 ± 1	3 ± 1	2 ± 1

* Statistically significantly different from control value, $p < 0.05$.
** Statistically significantly different from control value, $p < 0.01$.
[a] Fixed weight.

female pups. No other effect on organ weights (brain, spleen, kidney, liver, thymus, pituitary, adrenals, testes, epididymis, prostate, seminal vesicles, ovaries, uterus) was observed among male or female F2 pups. No treatment-related histologic effects were observed among the F2 pups.

3.13. Elemental analyses of serum, urine and tissues

The levels of Mo in the serum of blood samples taken just before termination show a clear, dose-related increase over the entire dose range (Fig. 3). In general, the serum Mo concentrations were slightly higher in males and females given 40 mg Mo/kg bw/day in the diet compared to males and females given the same dose in the drinking water. The serum levels of Mo among the F1 adult males and females were similar to those observed in the P generation. As in the P generation, administration of the test material produced lower serum levels of Mo in F1 adult females than in the F1 adult males. Serum levels of Mo among the F1 and F2 pups sampled at weaning were increased in a dose-related manner. In contrast to the sex-related difference in serum Mo levels in adults, the serum Mo levels were similar in male and female pups in the same dose group.

The only other element measured which showed a dose-related change at increasing doses of sodium molybdate dihydrate was Cu (Fig. 3). Serum Cu levels increased up to 5-fold in males and less than 2-fold in females exposed to 40 mg Mo/kg bw/day. A 700-fold increase in the mean serum Mo concentration at 40 mg Mo/kg bw/day in the diet was associated with a 5-fold increase in mean serum Cu in P generation males; in P generation females, the smaller increase in serum Mo was associated with a proportionately smaller increase in serum Cu. A similar pattern of dose-related increases in serum Cu was observed among F1 adult males and females, as well as among the F1 and F2 pups.

Urinary concentrations of Mo were increased in a dose-related manner in both the P and F1 generations, and the increased levels of Mo in the urine (expressed as mg Mo/24 h/kg bw) were more pronounced in males than in females (Table 10). Based on calculations of the percentage of the dose excreted in the urine in 24 h, the difference in urine Mo levels observed between males and females appears to be due to greater absorption of Mo in males compared to females. Both the adult P and F1 males excreted a mean of 68% of the administered dose in the urine in 24 h, whereas the P and F1 females only excreted about 28% and 44%, respectively, of the administered dose in 24 h. The gender difference was less pronounced among the pups; the male and female pups excreted about 84% and 69%, respectively, of the administered dose in the urine in 24 h.

Liver and kidney levels of both Mo and Cu were also increased in a dose related manner, with the highest levels being observed in the kidneys, which were presumably affected by the contribution of Mo in residual urine (Fig. 4). The concentrations of Mo in these organs were consistently higher among the male rats compared to the females.

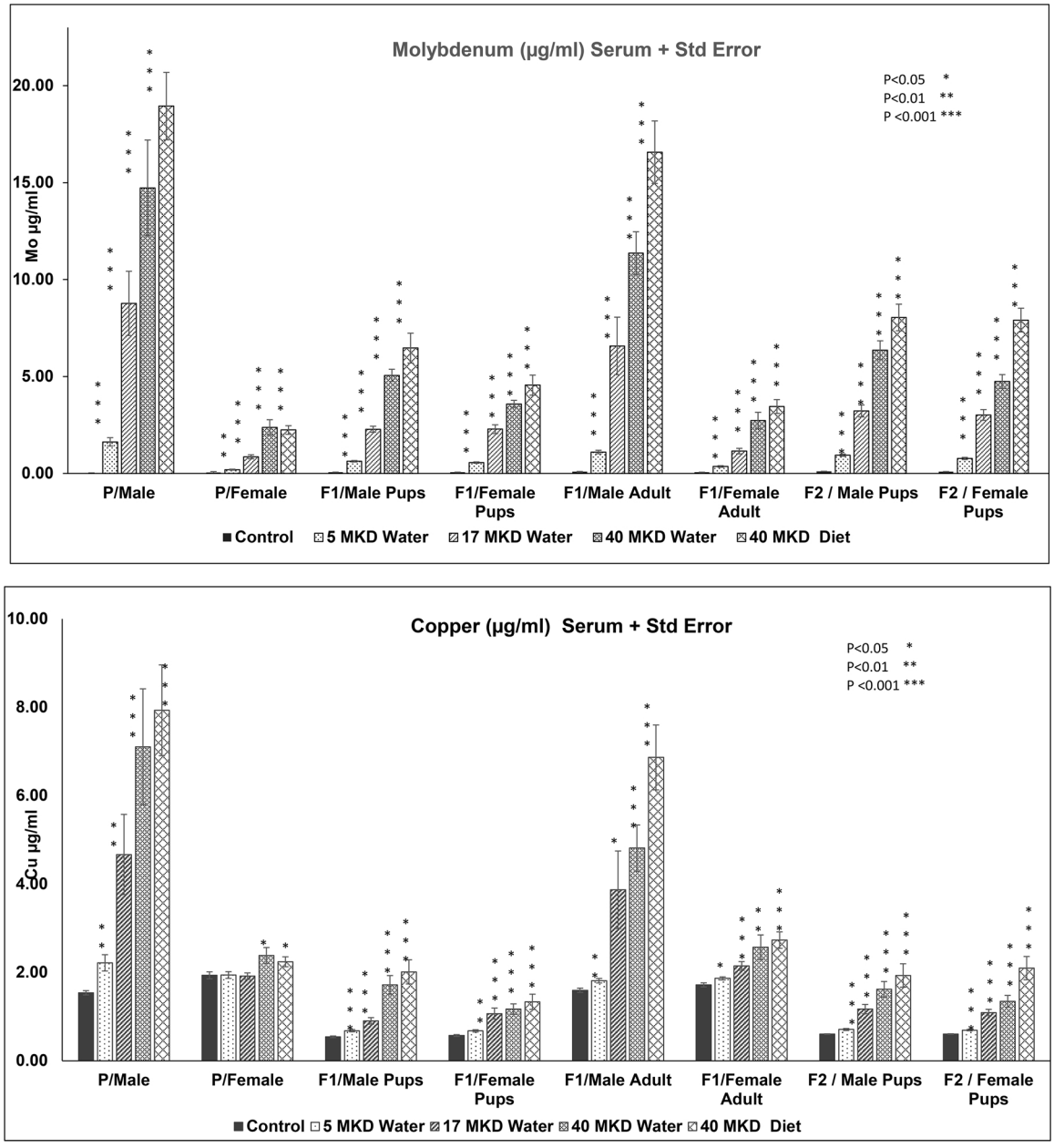

Fig. 3. Serum concentrations of molybdenum (µg/ml) and copper (µg/ml) among P and F1 generation male and female rats exposed to sodium molybdate dihydrate in drinking water or diet (Serum was sampled the day before euthanasia in all cases).

4. Discussion

This study is the first published two-generation reproductive toxicity study in animals of ingested Mo, administered as sodium molybdate dihydrate, performed according to current testing guidelines (OECD TG 416) and under GLP conditions. Administration of up to 40 mg Mo/kg bw/day in the drinking water or diet produced no significant reproductive toxicity in either generation. Parameters evaluated included mating and fertility indices, estrus cycle, ovarian primordial follicles, sperm count, motility and morphology, reproductive and other organ weights and histopathology, gestation length, parturition, litter size, pup weights and postnatal survival and developmental landmarks. No dose related significant adverse effects were observed in any of these parameters.

Systemic toxicity was observed at the high dose (40 mg Mo/kg bw/day), particularly among the males given the sodium molybdate dihydrate in the diet. In both generations, statistically significantly lower body weights were observed consistently throughout the study among the high dose males receiving the test material in the diet. At the time of scheduled necropsy, the body weights of the P and F1 males receiving 40 mg Mo/kg bw/day in the diet were approximately 91% and 92%, respectively, of the control body weights. Among the females, statistically significantly lower body weights, particularly during gestation and lactation, were observed among P and F1 rats given the test material in the diet, but the percent decrease in body weight gain was less in females than in males. In comparison, administration of up to 40 mg Mo/kg bw/day in the drinking water had no statistically significant effect on body weight or body weight gain in either sex in either generation. The body weight results in the current study are consistent with the statistically significant decreases in male and female body weights in rats given 60 mg Mo/kg bw/day in the diet in an earlier guideline 90-day repeated dose toxicity study by the current study authors [10]. In the 90-day study, compared to controls, statistically significant decreases in body weight gain and in food conversion efficiency were

Table 10
24 h urine molybdenum concentrations (mg Mo per 24 h/kg bodyweight) and percentage of oral dose excreted in urine in.24 h

	Dose Group (mg Mo/kg bw/day)					Mean % of dose excreted in urine
	0	5 (water)	17 (water)	40 (water)	40 (diet)	
	Molybdenum in urine, mg Mo/24 hours/kg bw (% of dose in 24-hour urine)					
P Male	0.07	3.17	13.35	19.84	32.24	68.4
DS 106-110		(63.4)	(78.5)	(49.6)	(80.6)	
P Female	0.08	1.48	4.72	11.29	10.55	28.0
PDD 26-37		(29.6)	(27.8)	(28.2)	(26.4)	
F1 Male Pups	0.12	4.30	13.71	36.64	31.54	84.2
PND 49-51		(86.0)	(80.6)	(91.6)	(78.9)	
F1 Female Pups	0.10	4.17	11.07	25.01	25.98[a]	69.0
PND 50-52		(83.4)	(65.1)	(62.5)	(64.9)	
F1 Male	0.05	2.84	13.91	27.99	24.91	67.7
PND 145-147		(56.8)	(81.8)	(70.0)	(62.3)	
F1 Female	0.23	2.47	7.82	17.40	14.51	43.8
PDD 25-28		(49.4)	(46)	(43.5)	(36.2)	

DS = days after the first day of dosing for the P generation and days after birth for the F1 generation.
DL – Day of Lactation for P Generation.
PND = postnatal day covers postpartum and lactation.
PDD = postdelivery day for P and F1 dams.
[a] One outlier excluded from calculation.

observed early during the treatment period.

The greater degree of systemic toxicity in males than females may be explained by a gender-related difference in Mo absorption in the rat. Administration of sodium molybdate dihydrate in the drinking water or diet produced a dose-related increase in serum levels of Mo in males and females of both generations. However, serum Mo concentrations were generally higher in adult males than in adult females in both generations (Fig. 3). For example, in the P generation, the average serum Mo concentration was 6.2 and 8.6 times greater in males than females when administered 40 mg Mo/kg bw/day in the drinking water and diet, respectively. Similarly, in the F1 generation, the average serum Mo level was 4.2 and 4.8 times greater in males than females given 40 mg Mo/kg bw/day in drinking water and diet, respectively.

The most likely explanation for the gender-related difference in Mo levels in serum, as well as in liver, kidney and urine, is reduced oral absorption of Mo in females compared to males. Analysis of the 24-hour urine samples revealed that, in adult female rats, only about 28–44% of the administered dose was found in the urine compared to 68% in adult male rats. The gender-related difference in serum Mo concentrations was much smaller (1.1 to 1.2-fold greater in males than females) in a 90-day subchronic toxicity study when serum samples were collected from male and female Sprague-Dawley rats after 12 weeks of exposure to up to 60 mg Mo/kg bw/day in the diet, and the females were never pregnant [10].

In both generations of pups, serum levels of Mo at the time of weaning (PND 22) were also increased in a dose-related manner. In contrast to adults, however, the serum Mo levels were comparable in male and female pups in the same dose group. This finding suggests that whatever factor(s) is responsible for the gender-related difference in serum Mo levels in adults, it is not present at the time of weaning.

Increasing doses of sodium molybdate dihydrate in either water or diet were associated with increases in Cu levels in serum, liver, kidneys and urine among males and females. The Cu/Mo interaction, which was observed in both adults and pups in both generations in the current study, was noted previously in 90-day repeated dose toxicity and developmental toxicity studies by the current study authors, of orally

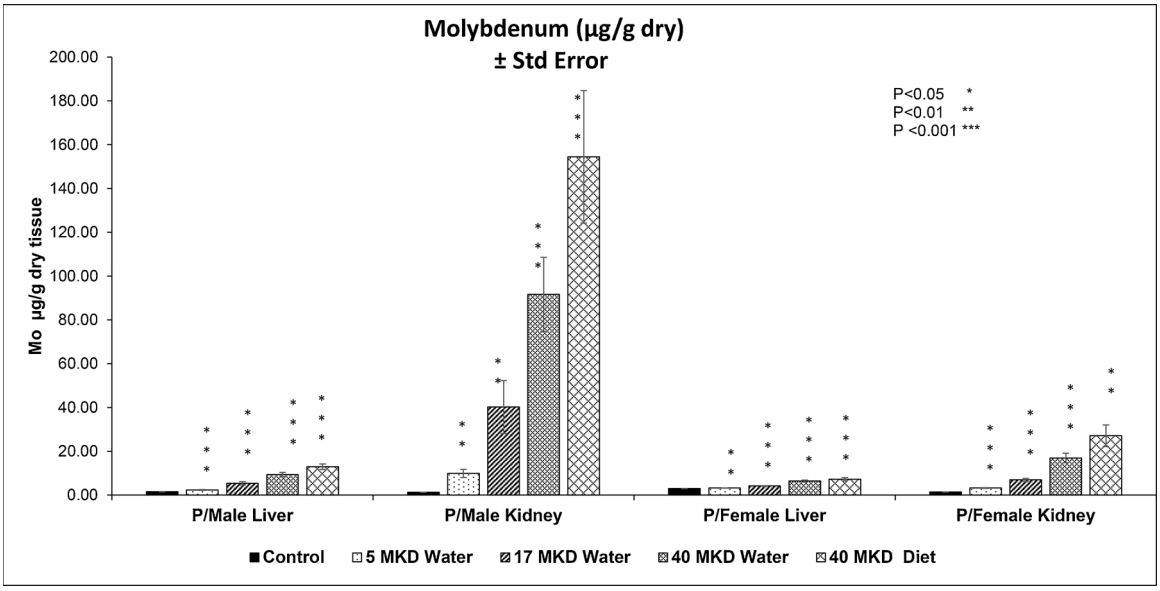

Fig. 4. Terminal concentrations of molybdenum (µg/g dry weight) in liver and kidney of P generation male and female rats exposed to sodium molybdate dihydrate in drinking water or diet.

Table 11
Comparison of molybdenum and copper concentrations in the liver of female rats in the 2-generation reproduction, developmental toxicity, 90-day repeated dose, and Fungwe et al. (1989) studies.

Study	Dose (mg Mo/kg bw/day)				
2-Generation reproductive toxicity study, P generation	0	5 (water)	17 (water)	40 (water)	40 (diet)
2-Generation reproductive toxicity study, F1 gen.	0	5 (water)	17 (water)	40 (water)	40 (diet)
Developmental toxicity study [9]	0	3 (diet)	10 (diet)	20 (diet)	40 (diet)
90-Day repeated dose toxicity study [10]	0	5 (diet)	17 (diet)	_	60 (diet)
Fungwe et al. (1989) study [30]	0	0.9 (water)	1.6 (water)	8.3 (water)	16.7 (water)
Molybdenum concentration in liver of female rats (μg/g dry)					
2-Generation repro. study, P generation, mean ± SD	2.96 ± 0.04	3.18 ± 0.04	4.10 ± 0.16	6.48 ± 0.43	7.23 ± 0.75
2-Generation repro. study, F1 generation, mean ± SD	2.79 ± 0.06	3.26 ± 0.07	4.57 ± 0.27	7.84 ± 0.59	8.38 ± 0.56
Developmental toxicity study, mean ± SD [9]	2.47 ± 0.14	3.43 ± 0.80	6.14 ± 1.64	9.71 ± 2.20	17.8 ± 2.63
90-Day repeated dose study, mean ± SD [10]	2.46 ± 0.28	3.51 ± 0.45	4.92 ± 0.62	_	13.0 ± 3.65
Fungwe et al. (1989) study, mean ± SE [30]	2.38 ± 0.11	4.50 ± 0.21	5.59 ± 0.37	10.7 ± 0.16	13.2 ± 0.14
Copper concentration in liver of female rats (μg/g dry)					
2-Generation repro. study, P generation, mean ± SD	14.0 ± 0.24	13.4 ± 0.28	14.0 ± 0.35	20.0 ± 5.0	20.4 ± 3.8
2-Generation repro. study, F1 generation, mean ± SD	12.2 ± 0.42	13.4 ± 0.22	13.9 ± 0.30	15.1 ± 0.83	17.2 ± 0.87
Developmental toxicity study, mean ± SD [9]	12.8 ± 0.68	13.0 ± 1.9	14.7 ± 1.5	17.0 ± 4.6	19.5 ± 3.5
90-Day repeated dose study, mean ± SD [10]	19.2 ± 2.1	23.8 ± 3.6	25.2 ± 5.1	_	36.3 ± 7.0
Fungwe et al. (1989) study, mean ± SE [30]	101 ± 3.1	112 ± 5.4	108 ± 6.3	131 ± 9.5	159 ± 10.3

administered sodium molybdate dihydrate [9,10]. In the present study, serum Cu levels increased up to 5-fold in males and less than 2-fold in females exposed to 40 mg Mo/kg bw/day compared to controls. In the 90-day study, serum Cu levels among rats given 60 mg Mo/kg bw/day in the diet increased 4-fold in males and 3.4-fold in females. Many essential elements interact with other essential elements. Based on the constant ratio of Cu/Mo across dose levels in males and females, it is hypothesized that the interaction between Cu and Mo may neutralize each other in order to prevent toxic effects due to either excess or deficiency provided a sufficient supply of each of these essential elements is maintained. The changes in Cu levels observed in the present studies at doses less than 40 mg Mo/kg/day are considered to represent a physiological adaptation, not a toxic response, since neither symptoms of toxicity attributable to the test material nor signs of copper excess or deficiency were observed at these doses.

The results of the present study do not confirm earlier reports of reproductive toxicity effects on male and female rats administered Mo. The results of the current study contrast with those of the Fungwe et al. [11] study, in which female rats were exposed to sodium molybdate dihydrate in drinking water containing 0, 5, 10, 50, or 100 mg Mo/L from weaning at three weeks of age until mating around eleven weeks of age, and through pregnancy until sacrifice on GD 21. Fungwe et al. [11] reported increased resorptions and decreased fetal body weight among the offspring of pregnant rats exposed to sodium molybdate dihydrate in the drinking water at estimated doses ≥1.6 mg Mo/kg bw/day. The magnitude of the reported decreases in fetal body weight were significant, but not dose-related (i.e., 32% and 30% decreases at the estimated doses of 8.3 and 16.7 mg Mo/kg bw/day, respectively). In contrast, in the current study, there was no evidence of a decrease in pup weight at birth or a reduction in the number of live pups per litter at doses of up to 40 mg Mo/kg bw/day in the drinking water or in the diet. Similarly, no effect on either resorptions or fetal body weight was observed in a recent developmental toxicity study by the current study authors in which pregnant rats were given sodium molybdate dihydrate in the diet at doses up to 40 mg Mo/kg bw/day on GD 6–20 [9].

Fungwe et al. [11] also reported statistically significant prolonged estrus cycles in rats exposed to Mo in the drinking water for six weeks at estimated doses of 1.6, 8.3, and 16.7 mg Mo/kg bw/day. In contrast, in the current study, no effect on the length of the estrus cycle or the number of females with persistent estrus or persistent diestrus was found at doses of up to 40 mg Mo/kg bw/day in the drinking water or diet in either generation. Similarly, in a recent OECD guideline-compliant, GLP 90-day repeated dose toxicity study by the current study authors, no effect on the estrus cycle was found in rats given up to 60 mg Mo/kg bw/day in the diet [10].

The inability of the current study to confirm the effects reported by Fungwe et al. [11] is concerning because that study has been used historically as the critical study for risk assessments of Mo [2,6]. A major deficiency of the Fungwe et al. [11] study is that the actual dose levels are not identified. The publication only states the concentrations of Mo added to the drinking water, and it does not report the dose levels actually consumed in mg Mo/kg bw/day. In fact, it is not possible to accurately calculate the dose levels since neither the water consumption nor the body weights of the rats were reported. The publication states that the Mo intake by the pregnant rats from water per week in the 0, 5, 10, 50 and 100 mg Mo/L dose groups were “0, 0.82, 1.65, 7.85, and 17.64 mg/L.” However, the intake units of “mg/L” is clearly incorrect, so it is unclear whether these values relate to intake per rat, per 100 g, or per kg body weight. Vyskocil and Viau [6], in a review of the Fungwe et al. [11] publication, made a variety of assumptions about body weights and water consumptions in order to calculate dose levels of 0, 0.9, 1.6, 8.3, and 16.7 mg Mo/ kg bw/day. Subsequently, and in the absence of OECD test guideline compliant GLP studies until recently, these assumed dose levels have been used extensively worldwide by regulatory agencies in setting NOAEL and LOAEL values for Mo.

In a separate publication, Fungwe et al. [30] reported hepatic concentrations of Mo and Cu among pregnant female rats exposed to the same drinking water concentrations under the same conditions as in the Fungwe et al. reproductive toxicity study [11]. Table 11 compares the liver concentrations of Mo and Cu in female Sprague-Dawley rats in the Fungwe et al. study [30] with those observed in the current study and two other recent guideline studies: prenatal developmental [9] and 90-day repeated dose toxicity [10] studies (i.e., “the guideline studies”). The liver concentrations were determined at the termination of each of the four studies, including the current study. In the case of the Fungwe study [30] and the guideline developmental toxicity study [9], the liver samples were collected on GD 21 and GD 20, respectively. In the current study, the liver samples were collected after the lactation period. In the 90-day study, the liver samples were collected at the termination of

the study in females that were never mated. A comparison of serum concentrations of Mo and Cu between the Fungwe et al study and the guideline studies is not possible because Fungwe et al. [30] did not analyze serum for Mo or Cu.

The concentrations of Mo in the liver were comparable at similar dose levels in the three guideline studies, as shown in Table 11. In comparison, Fungwe et al. [30] reported a similar range of hepatic concentrations of Mo as observed in the guideline studies, but at much lower dose levels of Mo than in the guideline studies. For example, at the presumed Lowest Observed Adverse Effect Level (LOAEL) of 1.6 mg Mo/kg bw/day in the Fungwe study [11], the liver concentration of Mo was similar to those observed at dose of 17 or 40 mg Mo/kg bw/day in the guideline studies. This suggests that the actual dose levels administered in the Fungwe study [11] were considerably greater than the dose levels estimated by Vyskocil and Viau [6].

Of note, the Fungwe et al. [11] study used a semi-purified diet with a low Mo content of only 25 ppb whereas the present study used a standard commercial diet containing 1800 ppb of Mo. It is noteworthy that, although Fungwe [30] gave a diet deficient in Mo, the liver concentration of Mo in the control group in the Fungwe study was similar to that observed in the control groups of the guideline studies. Since the control group in the Fungwe study was given a diet deficient in Mo, it is unclear why the Mo concentration in the liver of the control group was not lower in the Fungwe study than in the guideline studies.

The Cu concentration was lower in the semi-purified diet (6.3 ppm) used by Fungwe et al. [11] compared to the commercial diet (approximately 12 ppm) used in the current study. A minimum dietary Cu concentration of 8 ppm is recommended for pregnant rats [31]. Based on this difference in diets, it has been hypothesized that the low Cu content of the semi-purified diet used by Fungwe et al. [11], together with deficiencies of other constituents or nutrients of such a diet, may have contributed to the observed toxicity in the Fungwe et al. [11] study. However, an evaluation of the Cu levels observed in tissue in the various studies does not support this hypothesis, as discussed below.

The Cu concentrations in the liver of the females at termination in the four studies are presented in Table 11. Importantly, the Cu concentrations in the liver were higher at all dose levels in the Fungwe study [30] than at any dose level in the guideline studies. Notably, the Cu concentration in the liver of the control group of the Fungwe study was nearly 10-fold higher than observed in liver of the control groups in the guideline studies. This observation is also concerning since the controls in the Fungwe study were reportedly given a lower Cu diet than were the control groups in the guideline studies. If Cu deficiency was responsible for the effects reported by Fungwe et al. [11], lower Cu liver concentrations would be expected in the Fungwe et al. [30] study compared to those observed in the guideline studies. The data show just the opposite. Therefore, it is unlikely that the difference in results is related to Cu deficiency since the liver Cu concentrations were actually higher in the Fungwe study than in any of the guideline studies. Although Fungwe et al. [11,30] did not measure Cu in serum, the guideline studies have shown that the concentration of Cu in liver correlates with the Cu concentration in serum and other tissues.

Notably, the Cu concentration in the liver increased at ascending dose levels of Mo in all four studies (Table 11). In the Fungwe et al. elemental analyses [30], the Cu concentration in the liver increased 57% across the dose range of Mo studied. Similarly, in the guideline developmental toxicity study, the liver Cu concentration increased 52% across the dose range of Mo. In the current study, 46% and 41% increases in hepatic Cu levels were observed across the dose range of Mo in both the P and F1 generations. And finally, in the 90-day repeated dose toxicity study (the study with the highest dose of 60 mg Mo/kg bw/day), the Cu concentration in the liver increased 89% across the dose range of Mo.

Another major deficiency of the Fungwe et al. study [11] is the composition of the diet. The semi-synthetic diet used by Fungwe et al. was the American Institute of Nutrition rodent diet AIN-76 A. Due to numerous nutritional and technical problems encountered with AIN-76 A, this diet was revised in 1993 by the American Institute of Nutrition [31,32]. For example, AIN-76 A was found to be deficient in the sulfur-containing amino acids because casein, which is low in sulfur amino acids, was the sole source of protein. Animal studies have shown that the balance of a sulfur amino acid, methionine, relative to other amino acids in the maternal diet is critical, as fetal growth is not only retarded by diets that are deficient but also by those containing excess [33]. The replacement rodent diet, AIN-93 G, was developed for growth, pregnancy and lactation [31,32]. Major differences in the new formulation of AIN-93 G compared with A1N-76 A include: L-cystine was substituted for DL-methionine as the amino acid supplement for casein; the amount of phosphorus was reduced to help eliminate the problem of kidney calcification in female rats; manganese concentration was lowered to one-fifth the amount in the old diet; the amounts of vitamin E, vitamin K and vitamin B-12 were increased; and molybdenum, silicon, fluoride, nickel, boron, lithium and vanadium were added to the mineral mix [31]. Thus, the Fungwe et al. [11] study was conducted using a diet that is no longer considered appropriate for studying reproduction and development.

The Fungwe et al. [11] study has many additional significant limitations. First, the group size (12–14 pregnant rats/dose group) was inadequate to meet current guidelines. Second, it is not clear whether the fetus or litter was used as the statistical unit; the correct statistical unit is the litter. Third, the source and purity of the test material was not provided. Fourth, the drinking water solutions were not analyzed for Mo. Fifth, food and water consumptions were not measured in pregnant animals; food and water consumption from small groups of non-pregnant animals (n = 6) were used as surrogates for pregnant animals. Sixth, the evaluation of the fetuses was unconventional (no soft-tissue or skeletal exam). Seventh, the incidence of resorptions was unusually low in the control group, and no historical control data was provided. Eighth, the number of animals evaluated for estrus cycles was small (n = 6).

The results of the current study are also inconsistent with those of Pandey and Singh [12], who reported adverse effects on sperm count, motility and morphology in Druckery rats exposed to sodium molybdate by gavage at dose levels of 12 and 20 mg Mo/kg bw/day for five days per week for a 60-day period. In contrast, no effect on sperm count, motility, morphology, fertility, reproductive organ weight or histopathology was observed in the current study at doses up to 40 mg Mo/kg bw/day for a longer duration. Similarly, no adverse effects on sperm counts, motility or morphology were observed in a recent 90-day study of rats given up to 60 mg Mo/kg bw/day in the diet [10]. Possible factors contributing to the discrepancy in findings between the two guideline studies and the Pandey and Singh [12] study include the difference in group size (24 vs. 10 males/group), the different strains of rats (Sprague-Dawley vs. Druckery), and the different routes of exposure (drinking water and diet vs. gavage), although no significant difference in toxicity was observed between diet and gavage administration in a 28-day range-finder study for the 90-day study of sodium molybdate dihydrate [10].

The NOAEL for reproductive toxicity of 40 mg Mo/kg bw/day in the current two-generation reproductive toxicity study is approximately 20,000–40,000 times higher than the range of average human dietary intake of 0.001 to 0.002 mg Mo/kg bw/day [4]. The typical human serum concentration of Mo is less than 1 ng/ml [4,34,35]. In comparison, at 40 mg Mo/kg bw/day, the serum level of Mo in the P male rats is at least 15,000-fold (drinking water) and 19,000-fold (diet) greater than the typical serum Mo level in humans and in the P female rats, at least 2,400-fold (drinking water) and 2,200-fold (diet) greater than the typical serum Mo level in humans.

5. Conclusion

Administration of sodium molybdate dihydrate in the drinking

water and diet to male and female rats at doses up to 40 mg Mo/kg bw/day through two generations did not result in any adverse effects on reproductive function or development as indicated by no significant dose-related effect on estrus cycles, sperm parameters, mating, fertility, gestation, litter size, pup survival, growth or postnatal development across generations. Systemic toxicity, evident by statistically significantly lower body weight, food consumption (males only) and water consumption, was observed at 40 mg Mo/kg bw/day among males and females given the test material in the diet. Higher serum levels of Mo were observed in males than females following administration of sodium molybdate dihydrate, and based on the 24 h urine concentrations, this appeared to be primarily due to reduced absorption of molybdenum in adult females compared with males. The NOAELs are 17 mg Mo/kg bw/day for systemic toxicity and 40 mg Mo/kg bw/day (the highest dose tested) for reproductive toxicity in this study.

Conflicts of interest

FJM, FMS, and SAH are consultants to IMOA. SC is an employee of IMOA.

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