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Effects of Maternal Fenbendazole on Litter Size, Survival Rate, and Weaning Weight in C57BL/6J Mice

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Fenbendazole is a broad-spectrum benzimidazole commonly used in laboratory animal medicine as an anthelmintic for elimination of pinworms. This drug is generally regarded as safe, with minimal side effects. Some data in rodent species indicate multiple physiologic effects of fenbendazole, including changes in immune parameters and behavior, but no studies to date have evaluated possible effects on reproduction in mice. The purpose of the current study was to determine the effects of several treatment regimens of fenbendazole on reproductive parameters in C57BL/6J mice. Uninfected mice were given fenbendazole-treated feed continuously or every other week until pups were born or weaned. This treatment also was combined with environmental decontamination. No significant differences in litter size, survival rate, or weaning weight were detected between groups. Under the conditions of this study, fenbendazole treatment does not affect reproduction in C57BL/6J mice.

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Pinworms of the order Oxyurina (*Syphacia obvelata*, *Syphacia muris*, and *Aspiculuris tetraptera*) are one of the most commonly reported parasites in the modern rodent facility.[3,4,7,11,15,17,18] A 2009 study indicated a pinworm prevalence rate of 0.25% among North American and European samples submitted to a large commercial diagnostic lab over a 5-y period.[48] A 1996 survey indicated that approximately 67% of the responding institutions from the top 100 institutional recipients of NIH funds reported previous pinworm infestation in their laboratory rodents.[26] Like many large institutions, our university has had pinworm outbreaks that require increased prophylactic testing and financial resources to prevent spread of the infestation. Although infestations typically are subclinical, heavy pinworm burdens can lead to rectal prolapse, enteritis, and intussusception in immunodeficient rodents or those that have other comorbidities.[8,14,15,56] Infested mice may show decreased weight gain, diminished growth rates, increased caloric demands for basal metabolism, and compromised overall nutritional status.[15,25,45,47] Parasite load can be influenced by animal age, weight, and sex.[4,7,8,12,14] Furthermore, pinworm infestation of mice may confound experimental results—most notably in immunologic research—by altering data collected from the mice.[1,15,24,28,29,36,37,45,47,53,59,62]

Pinworms have a direct life cycle: embryonated nematode eggs are ingested by the rodent host and then hatch and develop in the gastrointestinal tract.[7,8,15] Immature eggs are passed in the feces or laid on the hair surrounding the perianal region; transmission occurs through fecal–oral contact or fomites.[8,38] Pinworm ova are persistent in the environment and can recontaminate treated colonies.[8,14,21,22] Pinworms can be diagnosed by PCR analysis of feces, perianal cellophane tape testing for *Syphacia* spp., or fecal floatation for *Aspiculuris*.[8,14,34] Postmortem diagnostic testing is considered more reliable than premortem testing and involves direct examination of the colonic or cecal contents for adult worms.[35,48]

Several strategies have been used to eradicate pinworms, although treatments themselves can effect research outcomes.[8,47] Rederivation is the 'gold standard' for eliminating parasites from rodent colonies, but this approach is expensive and time-consuming.[28,40,47] The most common pharmacologic treatments for rodent pinworms include benzimidazoles, such as fenbendazole and avermectins, including ivermectin.[8,48] Benzimidazoles are versatile anthelmintics due to their wide range of effectiveness against gastrointestinal nematodes without reliance on systemic drug concentrations. In addition, benzimidazoles act through the inhibition of microtubule polymerization by binding to β-tubulin.[57] Benzimidazoles have adulticidal, larvicidal, and ovicidal properties that make these drugs an attractive treatment choice.[30,32] Many treatment regimens have been used; one such regimen for mice includes ad libitum diet that contains fenbendazole, fed continuously or every other week and combined with environmental decontamination.[5,9,20,22,63] Fenbendazole is often used for treatment because of its wide margin of safety, ease of administration, and documented effectiveness, although little is known about its effects on the physiology and behavior of rodents.[42]

Many facilities prophylactically treat incoming rodents with fenbendazole during the quarantine period regardless of their health status.[29] These mice are generally shipped to and from other institutions as part of a collaborative effort, typically with the intent to breed. At our institution, researchers are often concerned about the effects of fenbendazole on fecundity. To the best of our knowledge, only a few studies involving rats have been published,[2,28] and none exist regarding the reproductive effects of fenbendazole on mice. In general, toxic effects of fenbendazole have not been reported at therapeutic levels but potentially may alter or interfere with ongoing research experiments, such as effects on immune parameters.[6,17,43,61] Fenbendazole did

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not affect pain perception or behavior studies.[2,8,11,39,51,64] In an unpublished and non-peer-reviewed study referenced by WHO, rats showed reduced fertility and severe signs of toxicosis in pups (for example, decreased survival indices, decreased body weights at birth, and slower lactational growth) at a dose of 45 mg/kg.[64] A 1988 study found that mice given mebendazole (a drug in the benzimidazole family) had reduced litter size and female growth rate and increased incidence of kinked tails in the offspring.[3] Another study concluded that treatment with fenbendazole decreased litter size in Sprague–Dawley rats but not GEPR-9 rats.[28] However, fenbendazole did not affect pregnancy indicators in rats, including maternal weight gain, water consumption, number of pups born, and pup birth weights.[2]

The purpose of this study was to evaluate the effects of fenbendazole on fecundity in mice. To assess the effects of fenbendazole on reproductive parameters, we evaluated 2 common treatment schedules: an alternating schedule, during which mice received medicated diet for 1 wk followed by 1 wk of nonmedicated diet for a total of 5 treatments over a 9-wk period, and a 5-wk continuous application of treated feed. We chose to compare the effects of continuous feeding of the medicated diet as compared with an alternate-week approach regardless of treatment duration because of the breeding timeline. Continuous treatment provides a shorter treatment duration, greater cost effectiveness, and less labor.[19] Although using the alternating week regimen requires a longer duration of treatment, this regimen may be necessary for infestations of *A. tetraptera* because of their prolonged prepatent period.[19] Mice in the current study were not exposed to pinworms to reduce the likelihood of confounding effects and to isolate the pharmacologic effects of fenbendazole.

Materials and Methods

Ethical statement. All animal care and experimental procedures were in accordance with federal policies and guidelines governing the use of animals and were approved by the University of California–San Francisco's IACUC. The IACUC follows the guidelines in the 8th edition of *The Guide for the Care and Use of Laboratory Animals*.[10] The University of California–San Francisco has an AAALAC-accredited animal care and use program.

Subjects. C57BL/6J mice (age, 8 to 10 wk) were purchased (stock number 000664, Jackson Laboratory, Bar Harbor, ME). C57BL/6J mice were chosen for this study because of their common use in biomedical research. All mice were housed socially except for stud males, which were singly housed briefly while resting between mating sessions. Mice were housed in solid-bottomed cages containing autoclaved PaperChip (Shepard Specialty Papers, Watertown, TN) with a single cotton square (5.08 cm^2 pulp virgin cotton fiber, Ancare, Bellmore, NY). IVC (30.5 cm × 15.9 × 30 cm; 190.5 cm^2; Lab Products, Seaford, DE) and an air exchange rate of 35 to 40 ACH. Mice had continuous access to irradiated food (LabDiet Picolab 5053, PMI Nutrition, St. Louis, MO) and water purified by reverse osmosis and UV lighting.

The housing room was maintained at 19.5 to 23.3 °C with 30% to 70% relative humidity. Cages were changed every 2 wk. Food was replaced with fresh feed weekly, with either untreated or fenbendazole-containing pellets, depending on the experimental cohort. Mice were kept on a 12:12-h light:dark cycle. Cages were maintained in a SPF barrier facility from which dirty-bedding sentinel mice were tested quarterly by serology and fecal PCR analysis. All sentinels were seronegative for mouse hepatitis virus, pneumonia virus of mice, mouse parvovirus, minute virus of mice, epizootic diarrhea of infant mice, Theiler murine encephalomyelitis virus, and ectromelia and were free of ectoparasites and endoparasites. Animal care staff observed mice daily for clinical abnormalities.

Breeding. A pair breeding scheme was implemented with young, age-matched mice. All mice were acclimated for 1 to 2 wk after arrival before being paired for breeding. In an initial pair-mating test, we paired 100 virgin male mice with 100 virgin females with the expectation that 25% of the male mice would not copulate;[13,52,58] males that did not copulate were excluded from the study. However, 90 of the male mice successfully completed the test and were enrolled in the study. Successful mating was verified by the presence of a vaginal plug. After the initial mating test, male mice were singly housed and rested for 14 d; they were then randomly paired with a different virgin female and assigned to an experimental or control cohort (Figure 1). Offspring and adults used in this study were later used in other research projects or euthanized.

Experienced male and virgin female pairs were randomly allocated into 4 experimental cohorts and 1 control cohort with 18 pairs per cohort. The first was the control cohort and received standard, untreated feed (LabDiet Picolab 5053, PMI Nutrition, St. Louis, MO) until pups were weaned (Figure 2). This control cohort was used as the basis for comparison of data from treated cohorts (pups at born and pups weaned. The second cohort was placed on fenbendazole-medicated feed (LabDiet Fenbendazole 5053 with 150 ppm fenbendazole, PMI Nutrition, St. Louis, MO) continuously until pups were weaned. The third cohort received fenbendazole-medicated feed continuously until pups were born. The fourth cohort was fed fenbendazole-medicated feed during alternate weeks until pups were born. The fifth cohort was given fenbendazole-medicated feed during alternate weeks until pups were weaned. The experiment was designed to replicate common treatment regimens that used either 5 total weeks of fenbendazole provided either continuously or treatments given every other

Figure 1. C57BL/6J mice were tested for copulatory behavior. Successful male mice were then singly housed and rested for 2 wk before their placement with a virgin female mouse for the duration of the experiment. Experimental treatment was initiated when the experienced male mice were paired with a different female to breed. Pups were counted at birth and then weighed and counted at weaning (day 21).

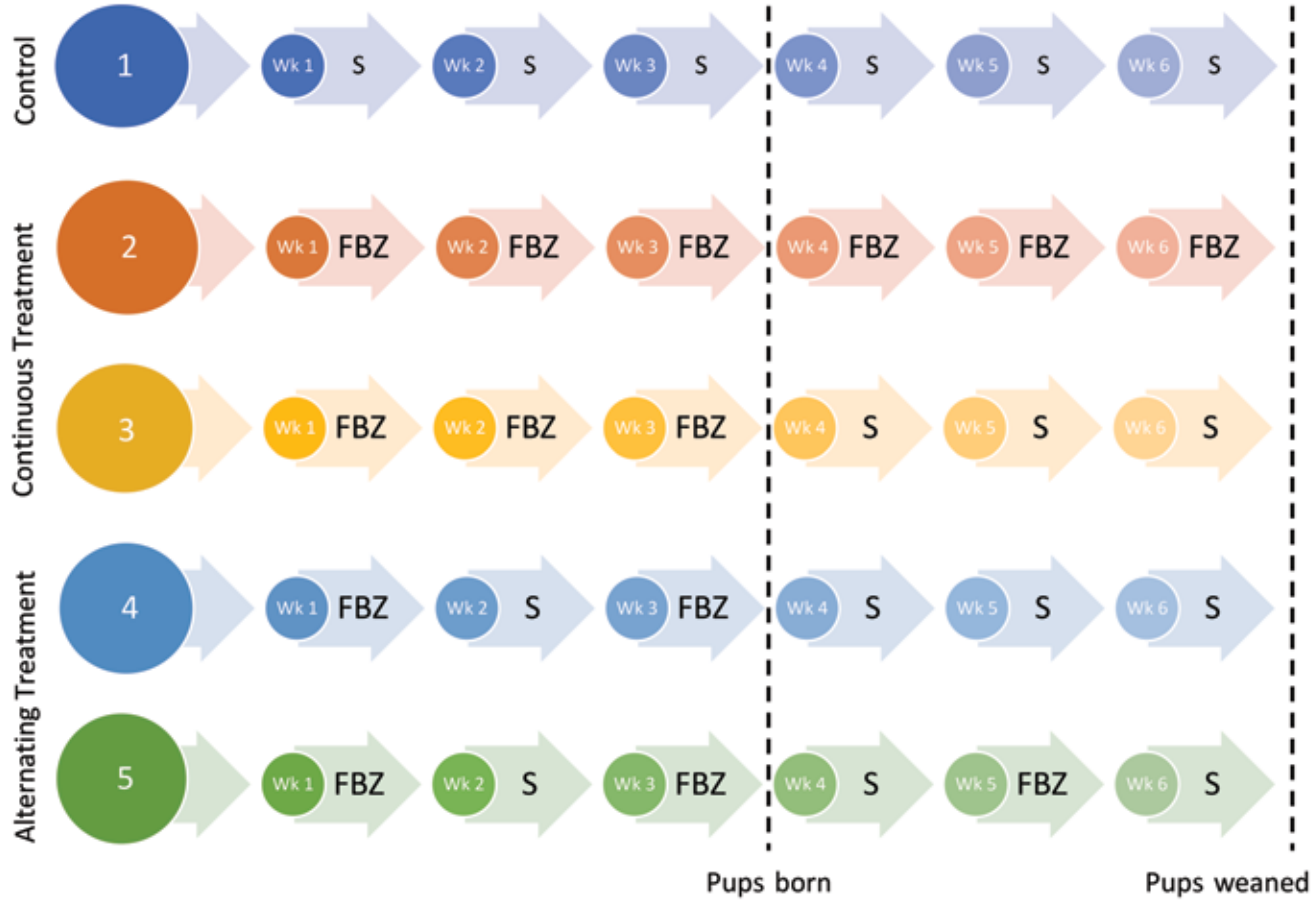

Figure 2. Experimental design. Cohort 1 was the control cohort that was fed standard unmedicated food continuously until pups were weaned. Cohort 2 was fed fenbendazole-treated chow continuously until pups were weaned. Cohort 3 was continuously fed fenbendazole until pups were born. Cohort 4 was fed fenbendazole-treated food during alternate weeks until pups were born. Cohort 5 was fed fenbendazole-treated food during alternate weeks until pups were weaned. All pups in each cohort were weaned at day 21 of the experiment. FBZ, fenbendazole-medicated food; S, standard unmedicated food.

week. The second cohort received 6 wks of fenbendazole because the drug was provided until the pups were weaned, which was a variable measured in this study. Neither of the alternating-treatment cohorts (4 and 5) received a total of 5 wks of fenbendazole, due to the breeding timeline.

Commercially available medicated rodent diets contain fenbendazole at 150 ppm or mg/kg, which is estimated to provide a target dosage of 8 to 12 mg/kg daily. For example, a 25 g mouse would need to consume 1.3 g daily of fenbendazole feed to reach our targeted minimum dose. Mice consume about 3-5 g of feed per day.[15] All mice were maintained on unmedicated feed after treatment was completed. The same 2 experienced personnel performed all procedures and husbandry care for all mice. All mating pairs were placed together on the same day; females were placed in the male cage, and all experimental mice were housed in the same room.

Reproduction. Breeding pairs were monitored daily between 0700 and 1000 for the birth of litters and remained together until the pups were weaned. All abnormalities and evidence of physical malformations, maternal aggression, or neglect were recorded. Pups were counted within 24 h of parturition; weanlings were counted, weighed, and weaned at 21 d of age. Newborn pups were counted by removing the cage from the IVC rack and opening the cage to briefly displace pups from the nest.

Statistical analysis. Statistical analyses were conducted by using STATA 14.1 (StataCorp , College Station, Texas). Differences in the number of pups at birth, pup weight at weaning, and pup survival to weaning among the 5 cohorts were analyzed by using the Kruskal–Wallis test. For all analyses, a P value of 0.05 or lower was considered to indicate a statistically significant difference between cohorts. Descriptive summary data are expressed as mean ± SD.

Results

No statistically significant differences in outcomes were detected among the 5 experimental cohorts. All litters were included in our primary outcome litter size. One litter from each cohort 1, 2, 4 and 5 and 2 litters from cohort 3 were excluded from the survival rate and weaning weight analysis because the litter size was smaller than 3 pups and no pups were alive at day 21. However, an initial analysis indicated that including values from all pups born would not have changed the conclusion of this study.

Litter size. No significant difference in litter size was detected among the 5 experimental cohorts (Figure 3 A). The control cohort had a litter size (mean ± 1 SD) of 6 ± 3 pups, whereas pairs with continuous treatment to birth had a litter size of 5.8 ± 2.8 pups, and those treated continuously until weaning had a litter size of 6.7 ± 2.0 pups. Pairs with intermittent treatment until birth or weaning had 6.2 ± 3.2 and 7.3 ± 2.3 pups, respectively. One pair in the control cohort was excluded from analysis because the adult male and several pups were found dead in cage.

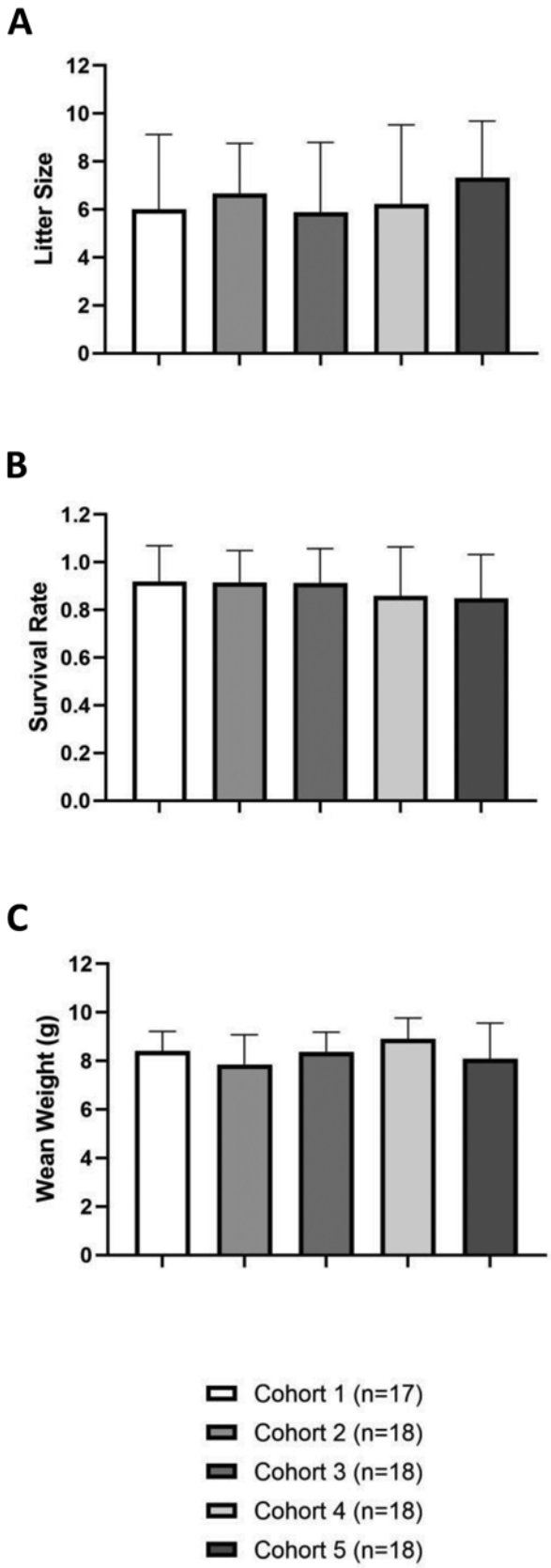

Figure 3. Reproductive parameters measured in response to fenbendazole in C57BL/6J mice. (A) Litter size: the number of pups at birth. (B) Survival rate: number of pups at weaning day 21 divided by the number of pups at birth. (C) Weaning weight: weight (g) of pups at day 21. The data are shown as mean ± 1 SD for 5 cohorts.

The analysis included 10 pairs for which pups were found dead; these pairs were in cohorts 1, 2, 4, and 5. Of these, only one pair had cannibalized pups (n = 2); for the other 9 pairs, pup bodies were intact. The pair with cannibalized pups had a total of 8 pups, 5 of which survived to weaning with weights within 1 SD of mean. One incident of a single stillbirth occurred (cohort 5): the pup was dead, and the dam was found in dystocia. This dam had a total of 8 pups in the litter, 7 of which survived to weaning with weaning weights within 1 SD of mean.

Survival. Pup survival did not differ (P = 0.9) among the 5 experimental cohorts (Figure 3 B). The survival rate was defined as the number of pups at weaning day 21 divided by the number of pups identified within 24 h of birth. Cohort 1 (controls; unmedicated feed only) had a survival rate of 90% ± 15% compared with 91% ± 14% for cohort 2 (fenbendazole-treated feed continuously until pups were weaned), 91% ± 15% for cohort 3 (fenbendazole-treated feed continuously until pups were born), 85% ± 21% for cohort 4 (medicated feed during alternate weeks until pups were born), and 85% ± 19% for cohort 5 (medicated feed during alternate weeks until pups were born). Pups were checked between 0700 to 1000 daily; pups were not included in the birth count when they were born after this check or when the dam was in active labor during daily checks. However, these pups were included in the count of pups weaned. This accounts for the pairs in cohorts 1 and 2 in which the survival rate exceeded 100%.

Weight at weaning. No significant difference (P = 0.1) in weanling body weight was identified among the 5 experimental cohorts (Figure 3 C). Weanling body weight in the control cohort was 8.4 ± 0.8 g. The weanling body weights of pups born to dams treated continuously until pup birth or weaning were 7.9 ± 1.2 and 8.4 ± 0.8 g, respectively. The body weights of weanlings from dams treated during alternate weeks until pup birth or weaning were 9 ± 0.7 and 8.1 ± 1.5 g, respectively.

Discussion

Fenbendazole-treated diets are one of the most common medicated feeds provided to laboratory rodents. Due to the high incidence of pinworm infestations in research mice, veterinarians frequently use fenbendazole-containing feed to treat mice with active infestation or as a preventative measure for rodents received from other institutions.[29] Our study is the first to address the reproductive effects of fenbendazole-treated feed on C57BL/6J mice. Our results indicate no significant differences in reproductive parameters between C57BL/6J mice fed standard chow or fenbendazole-medicated feed. Furthermore, no significant differences in reproductive indices, including litter size, survival rate, and weaning weight, were detected when C57BL/6J mice were exposed to fenbendazole continuously or intermittently. Our findings support the conclusion that fenbendazole does not influence major reproductive parameters in C57BL/6J mice. Fenbendazole's lack of effect in this regard is in contrast to an alternative drug, ivermectin, which affects reproduction and causes neonatal toxicity in rodents, especially mice.[33,47]

Environmental decontamination as part of a pinworm eradiation regimen is somewhat controversial and can depend on the species of pinworm.[47] Frequent cage changes could increase animal stress due to frequent handling and disturbance, including altering pheromones, olfactory cues, and nesting materials.[49] Some studies suggest pharmacological treatment alone can eradicate pinworm infections, thus potentially promoting more successful breeding and reducing the risk of preweaning pup

loss.[16,18,23,46,50] We recognize cage-change frequency as a possible limitation in our study; however, we found no evidence of stress with regard to poor breeding. A 2-wk cage-change frequency is optimal for animal health and practical husbandry techniques, but weekly cage changes could be considered for future studies.[50]

Weanling body weight is a useful but nonspecific indicator of reproductive success that has been used previously as a reproductive parameter.[46,59,64] Our data showed no differences in average weanling weights regardless of the length of exposure to fenbendazole. Cohort 2 had the longest exposure to fenbendazole-containing feed: a total of 6 wk (3 wk during gestation and 3 wk postpartum). In contrast, cohort 4 had the shortest exposure to fenbendazole-treated feed (total of 2 wk), yet weanling body weights were not significantly different between cohorts 2 and 4. In contrast, dams treated with ivermectin have 3 to 4 times higher drug concentrations in their milk than in plasma—a major contributing factor to ivermectin toxicosis in neonatal mice.[47]

Survival rate indicates the dam's maternal behavior and ability to lactate or provide pups with nutrients. When pups were found dead between the birth check and weaning on day 21, differentiating between stillbirth and cannibalization was difficult. Regardless, survival rate was consistent across cohorts, and the number of stillbirths did not vary significantly across groups in this study. Furthermore, the number of pups counted at birth occurred only during the 3-h time frame from 0700 to 1000. Pups born outside of this 3-h time frame and then consumed would not have been included in the birth count.

Our results are not consistent with the single similar study performed in rats, which suggested that fenbendazole treatment may reduce fecundity in rat breeding colonies.[28] These rats were reported to be historically poor breeders of an inbred strain, and sentinel rats in the GEPR9 colony were seropositive for Kilham rat virus, which is known to cause infertility, fetal resorption, abortion, and reduced litter sizes in rodents.[25,28,45] In addition, the rats had a natural pinworm infection, which could itself lead to reduced fecundity.[28] These previous results are difficult to compare with ours and confirm the need for future studies to further delineate fenbendazole's effect on rodents and to demonstrate whether mice differ from rats in this regard.

C57BL/6J mice are a relatively robust inbred strain, but additional studies are needed to further characterize the differences in rodent strains that are inbred, immunodeficient, or poor breeders. A future study could examine effects like parity, gestation length, and interlitter interval as an indicator of fecundity.[31,60] Cohort 2 had the longest exposure time to fenbendazole, but additional studies should consider the differences between continuous and intermittent regimens, given that a true 9-wk alternating regime potentially would allow for 2 litters to be exposed to fenbendazole during a given treatment period. In addition, the effects of pinworm infection alone on reproductive parameters are important to explore.

Early during the design of this study, breeding was a concern because many variables can influence breeding in different ways. Factors that substantially affect breeding include age, parity of the dam, genetic background, diet, light intensity and duration, temperature, noise, handling, and experimental conditions.[13] Limiting these types of confounding variables was a strength of this study. For example, background diet was consistent between unmedicated and medicated feed to reduce nutritional variation. Paper-chip bedding was used instead of corncob bedding, which can inhibit estrogen-dependent reproductive behavior in rodents.[44] The controlled animal facility environment and consistent seasonality were essential because various environmental factors can significantly affect several phenotypic characteristics in some mice.[41] Furthermore, virgin male and female mice were not screened for fertility prior to study enrollment, although a mating test was to verify copulatory behavior in the males[2,55]. The same 2 dedicated personnel collected data and provided husbandry support throughout the study, and all cohorts and breeding pairs were established on the same day during the same season. Lastly, our large sample size provided sufficient data for the valid calculation of statistically significant differences.

In summary, the data presented here demonstrate that C57BL/6J mice fed a fenbendazole-medicated diet either continuously or alternating weekly did not show reductions in litter size, survival rate, or weaning weight. Therefore, effects of fenbendazole on fecundity are not a likely problem when developing treatment plans for C57BL/6J mice with pinworms.

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