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The Effect of Acidified Drinking Water on Molar Tooth Enamel in C57BL/6 Mice

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The use of hydrochloric acid treatment of drinking water in many academic research rodent colonies and commercial vendors prompted us to investigate its effect on tooth enamel health in mice. Drinks with a low pH such as fruit juices and soft drinks have been demonstrated to cause demineralization of tooth enamel in humans and rodents. This study explored the hypothesis that acidified drinking water at the recommended range of 2.5 to 3.0 pH can lead to enamel erosion and compromised tooth integrity in mice. Specifically, we sought to quantify the effects of pH 2.5 or 3.0 daily drinking water exposure on molar enamel and bone mineral density over 1 and 3 mo. Methylene blue was used to quantify enamel erosion of the molar teeth, while dual-energy X-ray absorptiometry was used to quantify the bone mineral density of the mandible. After 1 mo of drinking water exposure, no statistically significant difference between the groups in enamel erosion or bone mineral density was observed. However, after 3 mo, a significant difference in enamel erosion for the pH 2.5 group compared with the other groups was identified, suggesting a potentially destructive process. There were no differences in bone mineral density between groups at any time point. These findings indicate that acidified drinking water of pH 2.5 may have deleterious effects on the enamel integrity of molar teeth in mice; however, drinking water of pH 3.0 seems safe for tooth enamel in mice during a short-term exposure of 3 mo. As this study only had a 1- to 3-mo exposure period, further study is needed to determine the effects of longer-term use of acidified drinking water at pH 3.0 in mice.

Abbreviations and Acronyms: BMD, bone mineral density; CC, Coca-Cola; CC-CaL, Coca-Cola with calcium lactate; CF, cystic fibrosis; DEXA, dual-energy X-ray absorptiometry; EE, enamel erosion; H_2SO_4, sulfuric acid; HCl, hydrochloric acid; MB, methylene blue

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Introduction

Hydrochloric acid (HCl; also known as muriatic acid) is a highly corrosive agent that is often used in the chemical industry for the neutralization of alkaline agents and in the production of chlorides.[1] Acidification of drinking water for rodents involves addition of HCl or other acids to the drinking water in small quantities to produce water with a pH associated with successful control of bacterial species, typically to a pH of 2.5 to 3.0.[2,3] Studies have shown that acidification with corrosive agents, like HCl, sulfuric acid (H_2SO_4), or chlorine, can reduce the presence of *Pseudomonas* species,[4–7] coliform bacteria[8] or other microbial contaminants.[9] Another study[10] revealed that acidifying water, along with other husbandry enhancements, resulted in increased survival in collagen VII-deficient mice. However, the acidification of drinking water for laboratory rodents is recognized as an important extrinsic variable. One study[11] revealed that acidified water changed the gut microbiota composition in mice, while a separate study[12] noted that acidified drinking water caused pronounced changes in neurologic functions. Hall and colleagues[13] noted that the number of bacterial species in the terminal ileum of mice decreased when drinking water was acidified with sulfuric acid. Despite the utility of HCl in inhibiting bacterial growth, little is known regarding the consequences of exposure to acidified water on the dental health of mice.[14]

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Many human subject studies[15–19] demonstrate an adverse effect of acidic soft drinks on dental health. One such study[20] concluded that the low pH of acidic soft drinks may be a contributing factor to dental erosion. Another study[21] used 6 soft drinks of varying pH and found there to be significant enamel erosion (EE) that correlated with pH as the 2 most erosive drinks had the lowest pH values. A different study[22] using human premolar teeth confirmed the corrosive potential of acidic drinks and juices as they noted cola soft drinks reduced the surface hardness of enamel and dentine, while orange juice and sports drinks reduced the surface hardness of the enamel.

Rats and mice have also been used to study the effect of acidic drinks such as fruit juice and soft drinks on tooth enamel.[23] These drinks vary in acidity between a pH of 2.4 and 4.54 and have been shown to result in EE in rodents in as little as 6 to 12 wk.[24–28] Previous research[29] on the effects of acidic drinking fluids in Osborne-Mendel rats, including fruit juice, soft drinks, sports drinks, yogurt, and soup, resulted in marked erosion on the lingual surface of the rat's lower molar teeth exposed to a sports drink of pH 3.2. Moreover, in a study[30] testing the popular soft drink Coca-Cola (CC) on Sprague–Dawley rats, all rats were pretreated with equal volumes of either CC, CC with calcium lactate (CC-CaL), or distilled water, then observed for food and fluid consumed, measured for weight gained, and erosion of teeth was scored after 5 total weeks of exposure. That study[30] showed that the mean erosion score was significantly greater for the CC groups as compared with the CC-CaL and distilled water groups. Furthermore, the erosive effect on mouse molar teeth was evaluated using 2 commercial drinking products: a sports drink and a cola drink. The authors concluded that the

mandibular molars were most eroded after exposure to acidic drinks.[31] Therefore, acidic pH can seemingly impact the enamel integrity in mice and rats after a relatively short exposure.

Mice (*Mus musculus*) and rats (*Rattus norvegicus*) are commonly used in dental research. A recent review[32] noted that rats are the most frequently used and with the highest average number of animals per study. However, mice remain a key model due to the ease of generating genetically modified lines and the wide availability of molecular reagents tailored to murine systems, making them particularly suitable for mechanistic studies in dentistry.[33–35] In addition, the continuously erupting incisors of mice and rats allow for the observation of all stages of enamel development and mineralization.[36] The iron-rich enamel of the mouse incisor teeth is also harder than that of humans.[37] A previous study[38] of pH regulation during enamel development in cystic fibrosis gene-knocked-out mice (CF mice) demonstrated that altered pH regulation during early enamel development leads to an altered enamel maturation and decreased mineralization of the incisor enamel in adult CF mice. There has been a lack of studies investigating the effect of acidified drinking water consumption on rodent EE. Karle and colleagues[39] noted significant EE in the molar teeth of rats with exposure to pH 2.0 drinking water for 24 wk. However, others have found that drinking water acidified to a pH of 2.7 did not have a significant effect on molar enamel in rats.[40] The observations from these studies indicate that there may be a pH-dependent erosive effect of acidified drinking water requiring further investigation. While several previous studies[41–44] have examined the effect of acidified drinking water on the immune system, behavior, and microbiome of mice, there is still a need for information regarding the effect of acidified drinking water on the tooth enamel in mice. This study evaluated the effects of exposure to HCl-treated drinking water in mice at pH 2.5 or 3.0, given for 1 or 3 mo. EE and mandibular bone mineral density (BMD) were analyzed using methylene blue (MB) staining and dual-energy X-ray absorptiometry (DEXA) after drinking water treatment. DEXA is a widely used technique that quantifies BMD in the central and peripheral skeleton by measuring the differential absorption of 2 X-ray beams passing through a sample.[45,46] MB is a dye used in dentistry that selectively penetrates porous or demineralized regions of tooth enamel, making it ideal for visualizing enamel defects and areas prone to acid damage.[47,48] We hypothesized that mice consuming HCl-treated drinking water at pH 2.5 for 3 mo would exhibit measurable alterations in tooth enamel integrity and mandibular bone density compared with the mice receiving neutral (pH 7.0) water.

Materials and Methods

Subjects. Young adult C57BL/6NCrl mice (7 to 9 wk old; n = 42; 21 male and 21 female) were purchased for the experiment (strain code 027; Charles River Laboratories [CRL]). The mice were sourced from CRL due to this vendor's abstention from using acidified water in their animal production colonies, thereby ensuring no prior exposure for the study animals.[49] CRL verifies that their mice are free of the following pathogens: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus, murine norovirus, Theiler disease virus, reovirus, rotavirus, lymphocytic choriomeningitis virus, ectromelia, mouse adenovirus of mice (types 1 and 2), murine cytomegalovirus, hantavirus, *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Helicobacter* spp., *Klebsiella* spp., *Mycoplasma pulmonis*, *Pasteurella multocida*, *Rodentibacter* spp., *Pseudomonas aeruginosa*, *Salmonella* spp., *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, β-hemolytic *Streptococcus* spp., Tyzzer disease, and all ectoparasites and endoparasites. Rodent health monitoring for our facility is performed on a quarterly basis using exhaust air dust media (SENTINEL; Allentown, Inc., Allentown, NJ) placed in the exhaust air plenum of each double-sided rack in the room. During the time of this study, *Helicobacter* spp., murine norovirus, and *Rodentibacter* spp., which are not excluded from this facility, were detected on the rack exhaust air duct media by PCR assay performed by a diagnostic testing laboratory (IDEXX Bioanalytics). Media were negative for the remaining pathogens tested: *Klebsiella* spp., *C. rodentium*, *Clostridium piliforme*, *Mycoplasma pulmonis*, epizootic diarrhea of infant mice, mouse adenovirus (types 1 and 2), mouse hepatitis virus, minute virus of mice, mouse parvovirus, Theiler murine encephalomyelitis virus, *Spironucleus muris*, pinworms, and furmites.

Mice were housed in microisolation ventilated cages (NexGen; Allentown, Inc., Allentown, NJ), within an AAALAC-accredited animal facility built to conform to the *Guide for the Care and Use of Laboratory Animals*.[50] All mice were housed in single-sex, 3 mice per group arrangements in cages containing hardwood maple bedding (Sanichips; Inotiv, West Lafayette, IN) changed weekly. Shredded paper nesting material was provided for enrichment (Bed-r'Nest; The Andersons, Maumee, OH). Rodent feed (5053 Irradiated Laboratory Rodent Diet; Purina, Richmond, IN) was provided ad libitum. Bottled purified water purchased from Allentown, either untreated or treated with HCl (VWR International; Radnor, PA), depending on the experimental group, was provided ad libitum and changed once weekly. Mice were housed on a 12-h-light:12-h-dark cycle, with room temperature set between 20 and 22 °C, and relative humidity maintained at 59% to 62%. An overview of the experimental design and water treatment timeline is illustrated (Figure 1). Mice were randomly distributed to the following groups: baseline control at pH 7.0 (n = 6; 3 male and 3 female), experimental group at pH 3.0 (n = 12; 6 male and 6 female), experimental group at pH 2.5 (n = 12; 6 male and 6 female), and control group at pH 7.0 (n = 12; 6 male and 6 female). Out of the pH 7.0 3-mo time point group, 1 male mouse died due to an unknown cause during the experiment and was therefore excluded from data analysis, reducing the total sample size to 41. A necropsy of the mouse was not performed. Six mice were analyzed at the baseline time point. Eighteen mice were analyzed at the 1-mo time point. Seventeen mice were analyzed at the 3-mo time point. The baseline group served as a reference for normal enamel and bone parameters before any water interventions and to control for age. The control group was carried throughout the study duration but received neutral (pH 7.0) water, acting as the negative control to compare against the acid-treated groups for any acute and chronic changes. Each mouse within the group was assigned an identifier (M1, M2, M3, for example, for males; F1, F2, F3, for example, for females). A random number generator was used to allocate the mice into either the 1-mo or 3-mo subgroup to ensure the assignment was unbiased. A power analysis was conducted to ensure the study had an adequate sample size to detect meaningful differences in tooth enamel outcomes between experimental groups. Based on previous research indicating moderate to large effects for similar enamel integrity measures, we aimed to detect an effect size of 0.95, which is classified as a large effect. Using nonparametric statistical tests at a significance level of 0.05 and a desired power of 99.98%, we employed G*Power software to calculate the sample size. Our analysis indicated that enrolling 6 mice per group would be sufficient to meet these parameters. All mice were naïve to experimental testing before this study. This research was approved by the IACUC at Tulane University (protocol no. 1911).

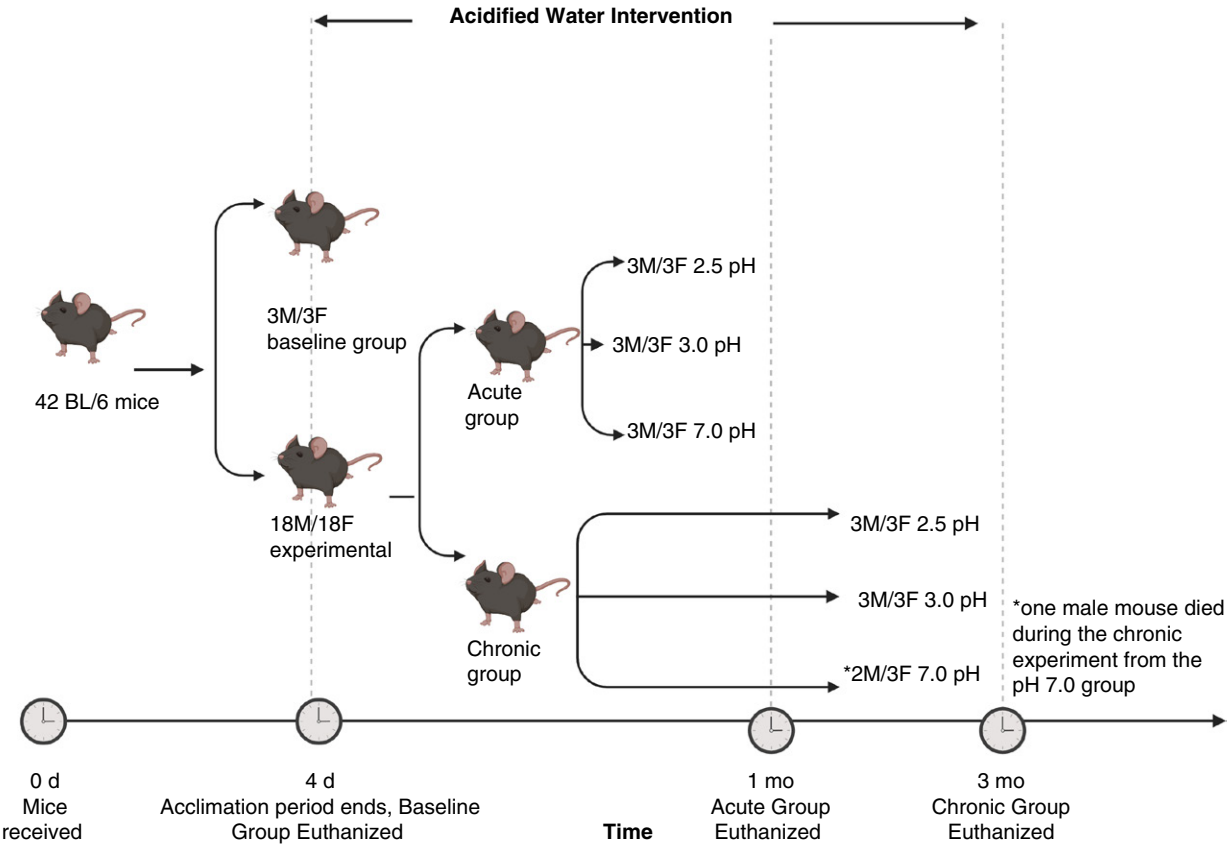

Figure 1. Schematic representation of the experimental timeline for 42 C57BL/6 mice (18 males [M] and 18 females [F] in the experimental groups, plus 6 mice in the baseline group). After a 3-d acclimation period, the baseline group (3 males and 3 females) were euthanized without HCl-treated drinking water intervention. The remaining 36 mice were divided into an acute group (18 mice) and a chronic group (18 mice). Each group was further allocated into 3 subgroups receiving HCl-treated drinking water at pH 2.5 or 3.0 or neutral water at pH 7.0 (3 males and 3 females per subgroup). The acute group was euthanized at 1 mo for data collection, while the chronic group continued HCl-treated water until 3 mo, at which point they were euthanized for data collection. Out of the pH 7.0 3-mo time point group, 1 male mouse died and was excluded from data collection. This design allowed for both short- and long-term assessment of acidified water exposure on tooth enamel and mandibular bone density. Created in BioRender. Coleman J. (2025) https://BioRender.com/q39g064.

Acidification of drinking water. All mice were acclimated for 3 d post-arrival to the facility with purified, nonacidified drinking water (Allentown). HCl drinking water solutions were prepared each week in 250 mL bottles of purified water (Allentown) and kept at room temperature. Using a micropipette, the appropriate amount of HCl was mixed into the water bottle to decrease the pH to a range of 2.45 to 2.54 or 2.95 to 3.04. For the 2 control groups, purified, nonacidified water (Allentown) was provided. The pH of each water bottle was verified using a calibrated pH meter (Mettler Toledo FiveEasy F20 pH/mV Meter; Mettler Toledo, Columbus, OH). The water bottles were then inserted into the mouse cages. After each week, the pH in the water bottles was retested to confirm that no change in pH had occurred. Water bottles with fresh drinking water solution were replaced weekly following the pH confirmatory testing. The volume of water remaining in the water bottles was noted weekly to ensure that mice in all experimental groups were consuming equal amounts of the acidified water. Water bottles were replaced with new water bottles and fresh drinking water solution in the first 8 h of the light cycle. In the middle of each week, the physical appearance of the mice was observed by laboratory staff during weekly cage changes to verify each mouse was healthy during the experimentation period. In addition to evaluating physical appearance, a veterinarian assessed the animal's body condition score weekly.

Mandible harvesting. Following the experimentation period, the mice were euthanized by CO_2 inhalation followed by cervical dislocation as a secondary method of euthanasia. The CO_2 flow rate was 30% to 70% displacement volume/minute in accordance with the 2020 AVMA Guidelines for the Euthanasia of Animals.[51] Following euthanasia, the carcass was weighed. Using scissors, the oral cavity was exposed, and the mandible was isolated. Excess tissue was trimmed from the mandible to expose the mandibular incisors and molar teeth. The mandibles were stored in distilled water in group-labeled specimen dishes at 4 °C for 1 h before analysis, which was consistent across all groups.

DEXA. BMD was measured by DEXA, calibrated using a reference phantom as described by the manufacturer (InAnlyzer2, Model S; Micro Photonics, Inc., Allentown, PA). Following dissection, mandibular bones from multiple animals were placed inside the main unit and DEXA scans were performed using the "mouse" sample type with the "quick" mode setting (scanning duration less than 30 s per group). Regions of interest were then drawn around each mandibular bone to ensure the most precise measurements, and BMD (g/cm^2) for each sample was quantified. DEXA scans were performed and analyzed by a single experienced partially blinded investigator (LDD). The mandibles were returned to their distilled water specimen dish post-scanning, to ensure the tissue would not become dry before staining.

MB staining. Post-DEXA scanning, the mandibular teeth were gently brushed with a 2% bleach solution followed by 1 rinse with 5 mL sterile saline to remove surface bacteria that could create stain artifacts. They were then photographed using a stereomicroscope at 10× magnification (Accu-Scope Ergo Tilting

Binocular Common Main Objective Stereoscope; ACCU-SCOPE, Commack, NY; Figure 2A and D). A 1% aqueous solution of MB dye (0.10 mL single-use MB; Vista-BLUE; Inter-Med) was applied to the mandibular molars and incisors using the dosing applicator for 10 s. Immediately post-MB application, the mandibles were returned to their distilled water specimen dish and refrigerated at 4 °C for approximately 24 h. After 24 h, the mandibles were photographed using the stereomicroscope. This staining method was adapted from the protocol described by Klaisiri and colleagues[52] (Figure 2B and E). An increase in MB staining corresponds to greater enamel damage. Specifically, the ratio of unstained (white) area to total tooth area decreases as the enamel sustains more damage, leading to an increase in MB staining.

Image processing and area determination. Quantification of MB dye penetration on the enamel surface of the incisors and molar teeth was conducted using the image analysis software ImageJ (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin). None of the incisor teeth from any animals exhibited MB staining, therefore, staining analysis was focused on the molar teeth only. After delimitation of the region of interest around the molars by a single experienced partially blinded investigator (JM), images were converted to 8-bit, and an automatic threshold was applied followed by a manual adjustment to the labial surface of the incisors to account for any nonspecific staining to the incisors and used as a baseline for intact enamel that is resistant to MB staining (Figure 2C and F). After measuring the total area of the molars, the unstained area of the molars was evaluated as the area above the manually set threshold to calculate the ratio of white, unstained tooth area to total tooth area.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0.1 (GraphPad Software; Boston, MA). The baseline group was included in the statistical analysis. Sex differences were analyzed for all variables. The Shapiro-Wilk test was run to determine the distribution of all measured outcomes, followed by outlier identification with the ROUT (Q = 1%) method. For 2-group comparison, a Student *t* test was performed. For 3-group comparison, ordinary 1-way ANOVA with Tukey posttest was used to compare the groups in normally distributed data, while the Kruskal-Wallis test with the Dunn multiple comparison test was used when 1 of the groups was not normally distributed. The BMD 3-mo group was the only group not normally distributed according to the Shapiro-Wilks test. The level of statistical significance was set at $P < 0.05$.

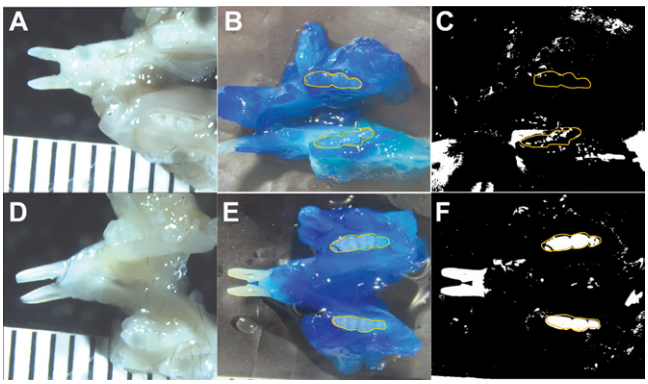

Figure 2. Representative gross anatomic and processed images of a C57BL/6 mouse mandible exposed to 3 mo of pH 2.5 acidified water or nonacidified purified water. (A and D) Gross anatomic view of the mandibles before methylene blue staining. (B and E) Gross anatomic view of the mandibles 24 h after methylene blue staining, highlighting significant uptake of the dye in areas of enamel erosion, primarily on the molars in mandibles exposed to acidified water. (C and F) ImageJ-processed view of the mandibles, illustrating the detection and quantification of blue or white variations in molar teeth. The area of unstained white compared with stained blue areas of enamel was quantified (magnification factor: 10×).

Results

Animal observations. No animals exhibited signs of illness, and all mice maintained a body condition score of 3 out of 5 for the study duration.

MB. We noted an absence of tooth staining of the incisor teeth for mice in all groups (data not shown), therefore for the MB test, only the total white area of the enamel and the stained blue area of the molar tooth occlusal surface, were quantified. There was no significant difference in EE between males and females throughout the study ($P = 0.6161$ at baseline; $P = 0.7377$ at 1 mo; $P = 0.7122$ at 3 mo; data not shown).

Interestingly, there was significantly less intact enamel in the baseline group without acidified water exposure compared with mice with 1 mo of exposure to pH 7.0, 2.5, or 3.0 drinking water ($P = 0.0077$, $P = 0.0127$, $P = 0.0053$; Figure 3). There was not a significant difference between EE of the 7.0 pH control group and pH group 2.5 or pH group 3.0 after month 1 ($P = 0.3092$; Figure 3). However, we did observe significantly more EE in pH group 2.5 at month 3 compared with month 1 ($P = 0.0022$; Figure 3). The amount of EE was not as pronounced in the pH 3.0 group, and there was no difference in EE between month 1 and month 3 in this group ($P = 0.4397$; Figure 3). There was also no change in enamel staining between month 1 and month 3 in pH group 7.0 ($P = 0.9975$; Figure 3).

DEXA. There was a small but statistically significant decrease in BMD between the baseline group and month 1 in pH group 2.5 ($P = 0.0221$; Figure 4) and an increase in BMD between month 1 and month 3 in pH group 2.5 ($P = 0.0020$; Figure 4). However, there was no difference in BMD between the baseline group and month 3 in pH group 2.5 ($P > 0.99$; Figure 4). There was no difference in BMD across time points for the pH 3.0 groups ($P > 0.99$; Figure 4). There was no significant difference in BMD between baseline and month 1 ($P = 0.2744$; Figure 4) or baseline and month 3 ($P = 0.1186$; Figure 4) in pH group 7.0. However, we did observe a significant increase in BMD between month 1 and month 3 in pH group 7.0 ($P = 0.0011$; Figure 4).

Although we did not observe a sex difference in BMD for pH group 3.0, there was a significant difference of BMD between males and females of pH group 7.0 ($P = 0.0411$; data not shown) and between males and females of pH group 2.5 ($P = 0.0021$; data not shown) at month 3. Females in both the pH 2.5 and pH 7.0 groups at month 3 exhibited greater BMD than males.

Discussion

This study aimed to evaluate the effects of a 1- or 3-mo acidified drinking water regimen on EE and BMD in C57BL/6 mice, using MB and DEXA, respectively.

Because DEXA is optimized for measuring bone density, it may be less sensitive to tissue depth changes.[53] To address this limitation, MB was used to provide a more direct assessment, often referred to as the MB penetration (or absorption) test.[52] While MB is relatively easy to perform and interpret, it does not offer the same quantitative analysis as DEXA, nor does it provide information about mineral composition. In addition, MB could stain tissues not of interest, therefore, post-MB application, the specimens were returned to their distilled water container to remove nonspecific staining by dilution in a neutral aqueous solution. A limitation of MB is that the intensity of staining can be subjective without a standardized analytical

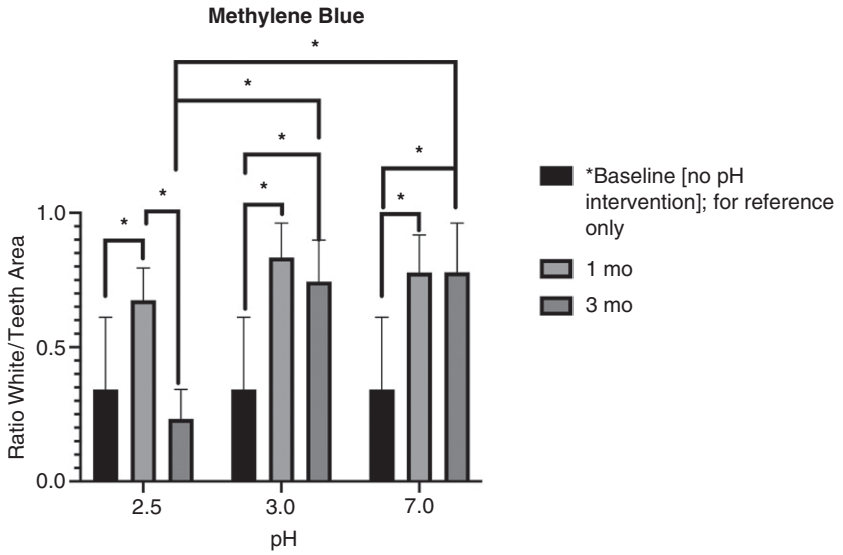

Figure 3. Effect of pH levels on the ratio of white-to-teeth area over time. The ratio of white-to-teeth area for 3 pH levels (2.5, 3.0, and 7.0) at 3 time points: baseline (black), month 1 (light gray), and month 3 (dark gray). The baseline data was not subjected to the pH conditions. For pH 2.5, significant differences were observed between baseline and month 1 ($P = 0.0127$) and month 1 and month 3 ($P = 0.0022$). For pH 3.0, significant differences were observed between baseline and month 1 ($P = 0.0016$) and baseline and month 3 ($P = 0.0053$). For pH 7.0, significant differences were observed between baseline and both month 1 and month 3. Cross-group comparisons show significant differences between pH 2.5 month 3 and pH 3.0 month 3 ($P = 0.0012$), as well as between pH 2.5 mo 3 and pH 7.0 mo 3 ($P = 0.0009$). Error bars represent SE. *, $P < 0.05$, statistical difference.

approach. Therefore, we used ImageJ software to quantify the extent of MB staining objectively. By measuring color intensity and distribution in digital images using image thresholding, ImageJ analysis reduced user bias and provided reproducible numerical data.[54] By combining these 2 methods, we were able to capture both the broader mineral density trends using DEXA and the localized enamel defects through MB staining, thus obtaining a more comprehensive evaluation of the tooth enamel and mandibular bone integrity.

Significant differences in EE were observed based on pH level and exposure duration, though no differences were noted between sexes. Molar staining was comparable across pH groups (7.0, 2.5, and 3.0) until the 3-mo mark, at which point mice in the pH 2.5 group showed significantly greater EE than those in the pH 3.0 and 7.0 groups. While more visible staining was observed on the molars of mice in the pH 3.0 group after 3 mo compared with 1 mo, this difference was not statistically significant. No significant differences were noted between pH 3.0 and pH 7.0 groups at any time point.

At the 3-mo time point, HCl-acidified drinking water had an erosive effect at 2.5 pH, observed as an increased uptake of MB. This rapid loss of enamel is consistent with a previous study in which erosive lesions were seen in nonobese diabetic mice exposed to CC drink for 6 wk.[55] Unexpectedly, baseline mice with no exposure to acidified water exhibited greater tooth staining compared with mice with 1 mo of exposure to pH 7.0, 2.5, or 3.0 drinking water. This may be due to the age difference, as the baseline group was euthanized shortly after arrival, while other groups were euthanized after 5 or 13 wk. Given that the mice were 8 wk old at the experiment's start, their enamel may have been thinner initially, aligning with published reports.[36,56,57] that mouse molar enamel undergoes post-eruptive maturation with increased thickness over time. One study[58] that investigated tooth development in B6 mice noted the major phases of mouse

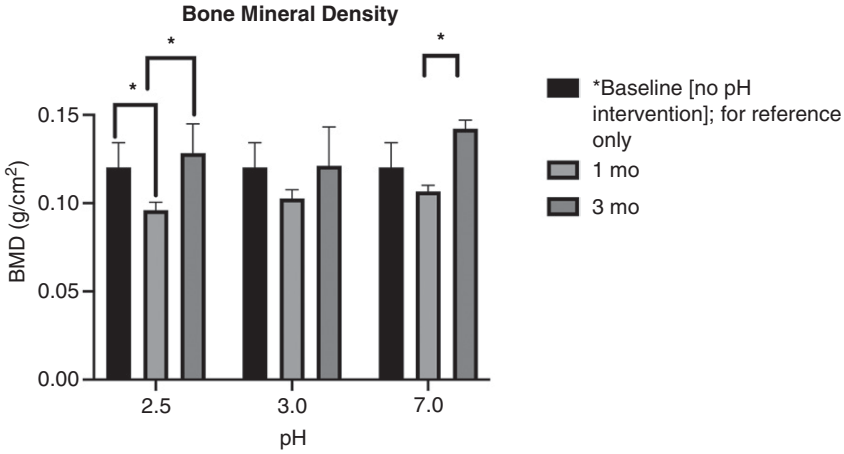

Figure 4. Effect of pH levels on bone mineral density (BMD) over time. The BMD (measured in g/cm^2) across 3 pH levels (2.5, 3.0, and 7.0) at 3 time points: baseline (black), month 1 (light gray), and month 3 (dark gray). The baseline data were not subjected to the pH conditions. Significant differences were observed for pH 2.5 between baseline and month 1 ($P = 0.0221$) and between month 1 and month 3 ($P = 0.0020$). For pH 7.0, a significant difference was noted between month 1 and month 3 ($P = 0.0011$). Error bars represent SE. *, $P < 0.05$, statistical difference.

tooth development do not reach completion until postnatal day 56, which is 8 wk. Our age-matched controls in the pH 7.0 group exhibited less tooth staining at the 1- and 3-mo time points compared with the baseline group which supports this theory.

While this study focused on acidified drinking water exposure starting at 8 wk of age, it is important to consider the potential effects of earlier exposure to acidified water. Mice are weaned at approximately 3 wk of age, and early exposure to low-pH water could have distinct effects on enamel formation and maturation. Future studies could investigate the impact of earlier exposure, particularly during critical periods of tooth development, to better understand how prolonged or early-life exposure to acidified water may influence tooth health and other physiologic processes.

Mice, like other rodent species, are characterized by their rapid and continuously growing incisors to compensate for the abrasive nature of their chewing activity.[42] With a continuous eruption rate of 2.8 mm/wk for the lower incisors, this results in a complete turnover of the incisor tooth in 35 d in mice.[59] The enamel of the incisor teeth of mice is also thicker, rich in iron, and has a higher structural complexity compared with the molar teeth.[56,57] As observed in our study as well as in previous studies of mice given sports and cola drinks, the incisor teeth of mice appear to have a natural resistance to the effects of acid exposure.[31,55] This finding is consistent with reports[60] of wear resistance in rabbits, which have hypsodont incisors, suggesting that the tooth morphology and histologic features shared among these animals may underlie their ability to withstand acidic conditions.

BMD findings revealed an initial decrease at the 1-mo time point in all groups compared with baselines, followed by an increase at 3 mo. As B6 mice, which began the study at 8 wk of age, do not reach skeletal maturity until between 12 and 42 wk, the observed increase likely reflects the natural increase in bone density over time.[61] This is supported by prior findings from Chen and colleagues,[62] who observed progressive increases in mandible bone volume and density in B6 mice over time. Our findings that young female mice had a greater mandibular BMD compared with males in the 3-mo exposure groups is consistent with a previous study in which female C57BL/6 mice reached peak BMD at 4 mo of age.[63] In a separate study[64] using BALB/c mice, female mice exhibited higher BMD than male mice until 7 mo of age when the BMD of males surpassed the females. Our results indicate that while the low pH drinking water affects the integrity of the tooth enamel, it does not appear to affect the mandibular alveolar bone. Additional research involving skeletally mature mice and extended exposure to acidified water would help clarify potential long-term effects on bone health.

In conclusion, our findings highlight time- and pH-dependent effects of acidified drinking water on tooth enamel and underscore the need to carefully consider widely adopted husbandry practices, such as water acidification, as extrinsic variables that can impact experimental outcomes. We recommend facilities perform regular pH monitoring and calibration of the pH meter to ensure that drinking water pH is maintained at a range effective against bacterial species while avoiding pH levels that can cause tooth damage.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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