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Effects of Repeated Anesthesia Containing Urethane on Tumor Formation and Health Scores in Male C57BL/6J Mice

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Repeated injection of urethane (ethyl carbamate) is carcinogenic in susceptible strains of mice. Most recent cancer studies involving urethane-induced tumor formation use p53$^{+/-}$ mice, which lack one copy of the p53 tumor suppressor gene. In contrast, the same protocol elicits at most a single tumor in wildtype C57BL/6 mice. The effect of repeatedly injecting urethane as a component of a ketamine–xylazine anesthetic mixture in the highly prevalent mouse strain C57BL/6 is unknown. Male C57BL/6J mice (*n* = 30; age, 3 mo) were anesthetized once monthly for 4 mo by using 560 mg/kg urethane, 28 mg/kg ketamine, and 5.6 mg/kg xylazine. The physical health of the mice was evaluated according to 2 published scoring systems. The average body condition score (scale, 1 to 5; normal, 3) was 3.3, 3.3, and 3.4 after the 2nd, 3rd, and 4th injections, respectively. The visual assessment score was 0 (that is, normal) at all time points examined. Within 1 wk after the 4th injection, the mice were euthanized, necropsied, and evaluated histopathologically. No histopathologic findings were noteworthy. We conclude that repeated monthly injection with urethane as a component of an anesthetic cocktail does not cause clinically detectable abnormalities or induce neoplasia in C57BL/6J mice. These findings are important because urethane combined with low-dose ketamine, unlike other anesthetic regimens, allows for accurate recording of neuronal activity in both the brain and retina. Longitudinal neuronal recordings minimize the number of mice needed and improve the analysis of disease progression and potential therapeutic interventions.

Abbreviations: ERG, electroretinogram; VEP, visual evoked potential

Urethane is classified as a chemical carcinogen[15] and is used by many laboratories to induce tumor formation in mice. Critical parameters affecting urethane-induced tumor formation in mice include the mouse strain, urethane dose, and frequency of administration. For example, urethane induces tumors in albino strain A mice, which are naturally susceptible to tumor formation.[11,17,22] The delivery of urethane to Swiss or C57BL1 mice induces the formation of skin and lung tumors but only when a tumor-promoting agent, such as croton oil or X-ray irradiation, is given concurrently.[2-4,10,12,24] Within these strains, young mice appear to be more susceptible than are adults.[9,17]

One of the mouse lines most resistant to urethane-induced tumor formation is also the strain used most often in research studies: C57BL/6.[21] Weekly injections of 1000 mg/kg urethane for 10 wk are required to induce tumor formation in this strain.[21] The tumors typically induced in this model are pulmonary adenomas and hepatic hemangiomas or hemangiosarcomas.[8,9,11,13] Furthermore, 5 mo after the first injection, only half of the mice injected had developed tumors, at an average of 0.63 tumors per mouse.[21] Another study showed that the strain variability may be due to genetic alterations at 3 separate loci.[19] Despite the existence of more susceptible mouse strains, many studies use p53$^{+/-}$ mice on a C57BL/6 background to obtain reliable and consistent results.[8] Although these mice are inherently susceptible to tumor formation due to the lack of one copy of the p53 tumor suppressor gene, they still have to be injected repeatedly with high doses of urethane to induce tumor formation. For example, daily injection of 1mg/kg urethane for 180 d failed to induce tumor growth in p53$^{+/-}$ mice;[8] to achieve tumor formation in these mice, the urethane dosage must be increased to 10 to 100 mg/kg daily for at least 180 d.[8]

Another use of urethane is as an anesthetic. Electrophysiologists use urethane-containing anesthesia during the recording of electrical activity from the brain or retina of rodents, including that during vision testing through electroretinography (ERG) and a light-evoked encephalographic evaluation known as the visual evoked potential (VEP). Inducing anesthesia by combining urethane (560 to 1000 mg/kg) with ketamine (25 to 40 mg/kg) and xylazine (5.6 to 10 mg/kg) is ideal in this context because it avoids the confounding influences of higher doses of anesthetics on electrical responses, yet maintains a sufficient depth of anesthesia to obtain readable electrical signals.[5-7,18,25,27] However, in light of concerns regarding urethane-induced tumor formation, these recordings typically are only performed once in each rodent subject, just prior to euthanasia. This practice greatly limits the amount of information that can be obtained from a single animal. Longitudinal ERG and VEP from the same animal are needed to understand the progression of disease and therapeutic efficacy of various treatments.

Longitudinal assessments of vision in models of glaucoma, trauma to the eye or brain, or inherited retinal degenerations provide information on disease course and therapeutic windows (for review, see reference 29). In addition, these types of assessments would strengthen therapy studies by determining the duration of therapeutic efficacy.[29] Importantly, most studies of visual system degeneration use C57BL/6-based models, in which vision loss is induced either through mechanical or

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genetic manipulation. Although different mice can be assessed at each time point, this strategy decreases the power of statistical analysis and increases the total number of mice needed for each study. The hypothesis of the current study is that repeated injection of an anesthetic mixture containing 560 mg/kg urethane, 28 mg/kg ketamine, and 5.6 mg/kg xylazine does not decrease overall physical health according to 2 scoring systems or induce tumors in C57BL/6 mice.

Materials and Methods

Mice. Male C57BL/6J mice (n = 30) were purchased from Jackson Laboratories (Bar Harbor, ME) and acclimated for at least 3 d prior to beginning the study. Mice were housed in ventilated cages and racks (Super Mouse 70, Lab Products, Seaford, DE) containing corncob bedding (Enrich-o'Cobs, The Andersons Lab Bedding, Maumee, OH). Each cage had a disposable hut (Chinet StrongHolder, Huhtamika, Hopkinsville, KY). Mice were maintained on a 12:12-h light:dark photoperiod and had unlimited access to a commercial diet (5L0D, LabDiets, Land O'Lakes, St Louis, MO) and reverse-osmosis–purified (Edstrom Systems, Waterford, WI) water from an automatic system within the Vanderbilt University animal facility. Mice were maintained in an SPF environment free of mouse hepatitis virus, mouse parvovirus, lymphocytic choriomeningitis virus, Sendai virus, pneumonia virus of mice, epizootic diarrhea of infant mice, Theiler mouse encephalomyelitis virus, ectromelia virus, mouse adenovirus, mouse reovirus, *Mycoplasma pulmonis*, pinworms, and fur mites as determined by testing of sentinel mice. Murine norovirus and *Helicobacter* spp. are not routinely screened for or excluded in the facility and room where the mice were housed; however, given the study animals' procurement history, the use of ventilated cages, and the strict microisolation practices used in the room, the study mice were likely negative for these pathogens as well. All procedures adhered to AAALAC guidelines and the IACUC-approved protocol.

To undergo ERG, the mice received intraperitoneal injections of a ketamine–xylazine–urethane cocktail beginning at 3 mo of age and repeated once monthly for 3 mo for a total of 4 injections. The cocktail was made in the laboratory by combining 400 µL of 100 mg/mL ketamine (Wyeth, New York, NY), 80 µL of 100 mg/mL xylazine (Lloyd Laboratories, Shenandoah, IA), and 800 mg urethane (ThermoFisher, Waltham, MA) into 50 mL sterile double-deionized water. The cocktail was then filter-sterilized (Steriflips, Millipore, Billerica, MA) and transferred into an autoclaved bottle with a sterile stopper. A new needle was used each time anesthetic was retrieved from the bottle for injection. Each mouse received 14 µL/g of the cocktail, resulting in final doses of 28 mg/kg ketamine, 5.6 mg/kg xylazine, and 560 mg/kg urethane. ERG and VEP were performed after each mouse reached a stable surgical plane of anesthesia, assessed as the lack of response to toe or tail pinch. After the last round of ketamine–xylazine–urethane anesthesia and ERG, mice were anesthetized to areflexia and then perfused with PBS followed by 4% (v/v) paraformaldehyde in PBS.

Physical health assessments. Mice were weighed at baseline and then monthly at each anesthetic injection. In addition, within 1 wk after each dose of ketamine–xylazine–urethane anesthesia, mice were assessed independently by 3 of the coauthors. Physical health was also assessed according to a body condition scoring (BCS) system and a visual assessment scoring (VAS) system.[1,28] Mice were assessed by observers in the animal holding room. Assessments were made shortly after each cage was opened in a biosafety cabinet. Three examiners during the same session independently scored the health of the mice.

The BCS is a ranking of the mouse body condition on a scale of 1 to 5 with 3 representing a normal, well-conditioned mouse.[28] The score requires both observation and handling of the mice to determine the ease of detection of the spinal vertebrae. At BCS level 1 the skeletal structure is extremely prominent and vertebrae are clearly segmented. At the other end of the scale, a level 5 BCS represents a mouse that is smooth and bulky with no sign of bone structure even with firm palpitation.

As another assessment of overall mouse health, we used the VAS. The VAS combines the scores for 3 characteristics to result in an overall condition score.[1] The lowest score, 0, represents normal for each characteristic. Hair coat condition is ranked on a scale of 0 to 3, with 3 representing very rough hair coat or hair loss. The eyes are ranked from 0 (open, alert) to 2 (closed). Finally, the coordination and posture of the mouse is evaluated on a scale of 0 to 5. At a score of 1, the mouse is walking awkwardly or is slightly hunched but it still runs and moves around. With each increase in score the mouse's coordination and posture are progressively worse, until a score of 5 indicates that the mouse is hunched and not moving. Healthy mice have a VAS of 0.[1]

ERG recordings. Repeated ERG was performed in 10 mice as described previously.[5,6,18,25] Briefly, the eyes of dark-adapted, anesthetized mice were dilated by using 1% tropicamide (Acorn, Lake Forest, IL), and the mice were placed on a heated platform that is integrated with the ERG system (Diagnosys, Lowell, MA). Gold loop electrodes were gently placed directly onto the surface of the cornea, which was covered in eye drops (Refresh, Allergan, Irvine, CA). Platinum subdermal electrodes were placed into the tail and back of the head as ground and reference electrodes, respectively. The mice were exposed to 10 flashes of light at each of 4 light intensities, after which they were laid on a water-jacketed warming pad until they regained consciousness and were returned to their home cages. The total duration of anesthesia was not quantified but typically ranged between 30 and 60 min.

Histopathology. A full necropsy was performed on all mice used in the study. Tissues were collected and fixed overnight in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned at 4 microns and stained with hematoxylin and eosin. Sections of lung, liver, spleen, kidney, and reproductive tract were evaluated microscopically by an experienced veterinary pathologist.

Statistical analysis. All data are shown as mean ± 1 SD. To compare the results of the BCS and VAS over time in the same mouse, we used nonparametric repeated-measures ANOVA followed by a Friedman posthoc test. To compare mouse weight over time, we used nonparametric repeated-measures ANOVA followed by the Dunnett posthoc test. To compare the results from the ERG, 2-way ANOVA was performed followed by a multiple comparisons Tukey posthoc test. All statistics were calculated by using Prism software version 6 (GraphPad, La Jolla, CA). A P value less than 0.05 was considered statistically significant.

Results

Effects of repeated urethane anesthesia on BCS and body weight. Within 1 wk after the 2nd injection of urethane, the BCS (mean ± 1 SD) for the mice in this study was 3.3 ± 0.1 (Figure 1 A). The BCS remained at 3.3 ± 0.1 after the 3rd injection and increased to 3.4 ± 0.2 ($P < 0.05$) after the 4th injection of urethane. The increased score likely represents an aging-associated weight gain, in the weight measurements (Figure 1 B). The average starting weight for the mice was 27 ± 2 g; the mice

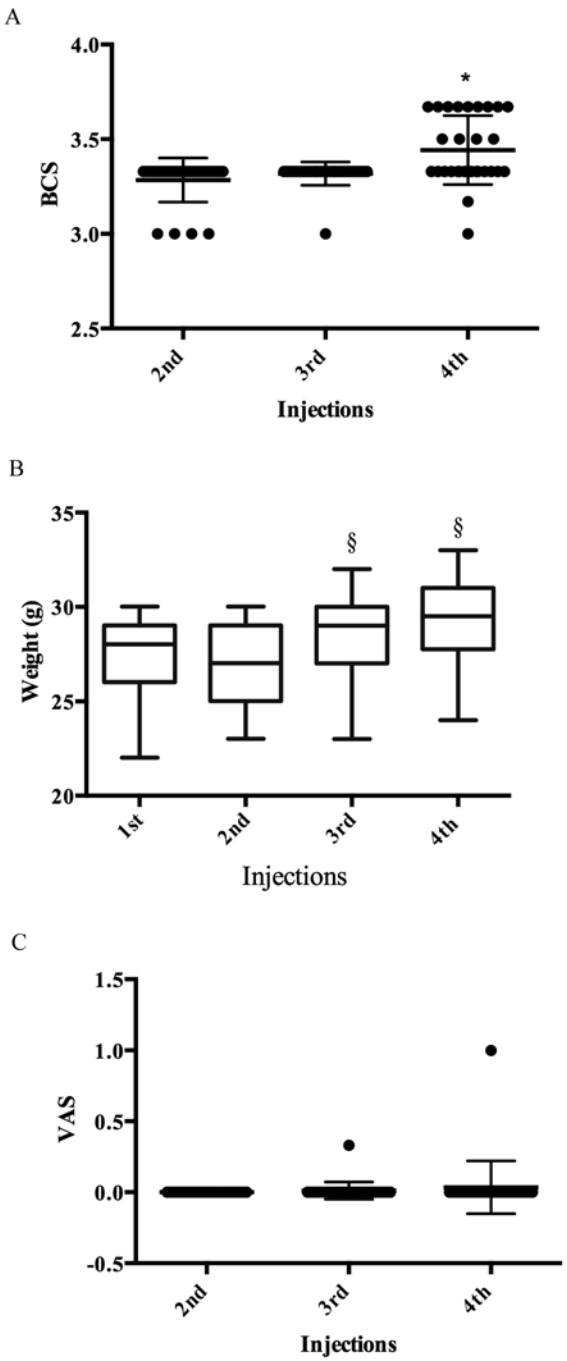

Figure 1. Repeated injection of ketamine–xylazine–urethane anesthetic cocktail into 30 male C57BL/6J mice had no adverse effect on the body condition score (BCS) and visual assessment score (VAS). (A) Average BCS. (B) Average VAS. Error bars represent 1 SD. *, $P < 0.05$; §, $P < 0.0001$

remained at this weight 1 mo later, at the second urethane injection. However, the average weight was increased at the time of the 3rd (29 ± 2 g) and 4th (29 ± 2 g) injections, thus indicating a slight (6%) but statistically significant ($P < 0.0001$) increase in body weight. Detecting a weight gain in male mice between 3 and 6 mo of age is unsurprising.

Effect of repeated urethane anesthesia on the VAS. The mice in this study had a VAS (mean ± 1 SD) of 0.0 ± 0.0, 0.0 ± 0.1, and 0.0 ± 0.2 within 1 wk after 2nd, 3rd, and 4th injections of urethane, respectively (Figure 1 C). There was no statistically significant difference between the groups.

Effect of repeated urethane anesthesia on the incidences of tumors and other pathologies. No noteworthy findings were detected grossly or by histologic exam. The histopathologic findings in the Harderian gland, liver, spleen, kidney, reproductive tract and mesenteric fat for all mice were within the normal spectrum of pathology for 6 mo old C57BL/6 mice. Focal mild perivascular cuffs of lymphocytes and plasma cells were detected in the lungs of 6 of the 30 mice examined (Figure 2 A). The liver of one mouse contained small pyogranulomas (Figure 2 B). In another mouse, the Harderian gland had a single focus of lymphoplasmacytic perivascular inflammation and focal minimal acinar dilation (data not shown).

Effect of repeated urethane anesthesia on ERG amplitude. The ERG of the mice was recorded while they were under ketamine–xylazine–urethane cocktail anesthesia. The same mice were followed after 3 additional once-monthly injections of the anesthetic cocktail; the ERG waveforms retained their expected shape at all 3 time points. The averaged waveforms from a 0 log cd*m/s^2 flash at each injection time point are shown (Figure 3 A). The amplitudes of the a wave (first hyperpolarization; Figure 3 A) and b wave (first depolarization; Figure 3 A) from each assessment were quantified (Figure 3 B and C). The amplitudes did not differ over time at any light intensity.

Discussion

Our current study supports previous results demonstrating that the C57BL/6 strain is resistant to urethane-induced tumor formation.[19-21] During one study, mice were injected twice with 1000 mg/kg urethane (6 wk between injections) and then evaluated 3 mo after the first injection to investigate the induction of lung tumors.[19] Using that paradigm, the authors detected an average of 1 tumor per C57BL/6 mouse by using visual examination alone. According to another regimen, C57BL/6 mice were injected weekly with 1000 mg/kg urethane for 8 contiguous weeks; 5 mo later, tumors were detected in only 50% of the mice, at the rate of 0.63 tumor per mouse.[20]

This study demonstrates that a dose of 560 mg/kg urethane is below the threshold dose necessary to induce tumor formation even when given according to a protocol similar to those used previously.[19,20] Specifically, we injected male C57BL/6 mice with 560 mg/kg urethane 4 times at 1 mo intervals and assessed for tumor formation 3 mo after the first injection. Unlike previous studies,[19-21] we did not detect tumors or neoplasms after this regimen. These results suggest that repeated use of 560 mg/kg urethane as part of an anesthetic cocktail does not induce tumors in the most common laboratory mouse strain, C57BL/6. Therefore, the use of urethane as an anesthetic in mice should not be restricted to endpoint assays. Urethane should always be used with appropriate safety measures to avoid the exposure of personnel to this chemical, which is classified by the International Agency for Research on Cancer as a group 2A carcinogen and thus considered "probably carcinogenic to humans."[15]

As with other anesthetics, high doses of urethane (that is, 1000 mg/kg and greater) attenuates the ERG b wave.[23] Therefore, low doses of multiple anesthetics typically are combined to avoid anesthetically driven alterations in the electrical responses. One such combination is ketamine–xylazine–urethane. High doses of ketamine alter the ERG and VEP in different and inconsistent ways making that approach unattractive. For example, although robust ERG responses are recorded from mice anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine,[5] this high dose of ketamine dramatically increases blood glucose levels (that is, exceeding 600 mg/dL for longer than 1 h).[7] Changes in blood glucose levels affect the ERG response in many species, includ-

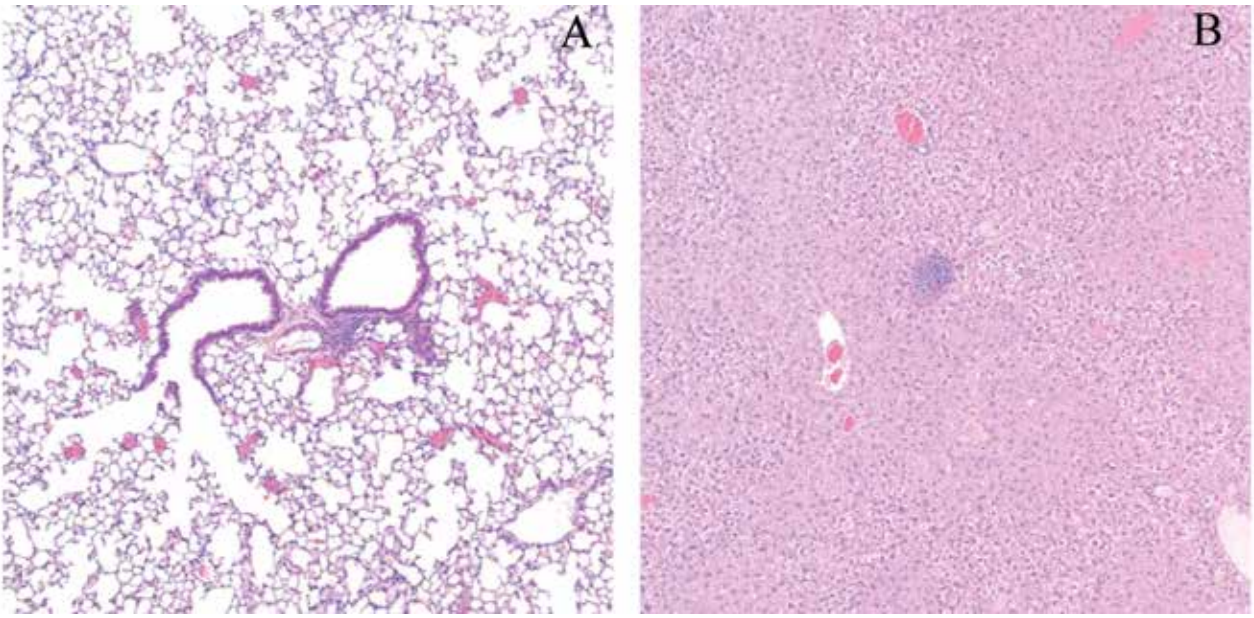

Figure 2. Repeated injection of ketamine–xylazine–urethane anesthetic cocktail into 30 male C57BL/6J mice did not induce tumor formation. Representative histopathology images from (A) lung, showing focal mild perivascular cuffs, and (B) liver, showing small pyogranulomas.

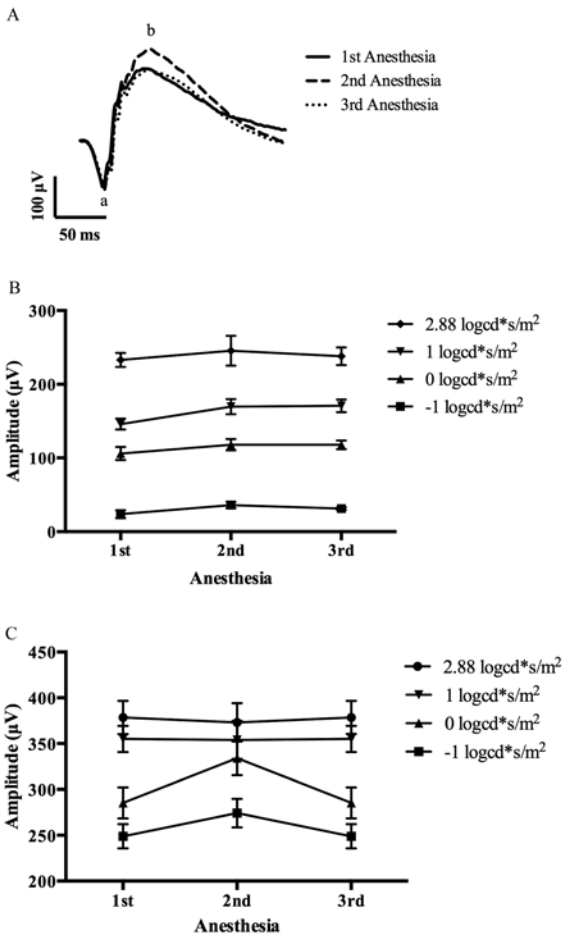

Figure 3. ERG waveforms and amplitudes are comparable at all time points assessed. (A) Averaged waveforms at the first, second, and third anesthesia after exposure to a 0 log cd*s/m^2 flash. The peaks of the a and b waves are indicated. (B) Quantification of the a wave amplitude at 4 light intensities (–1, 0, 1, and 2.88 log cd*s/m^2) at each anesthesia. (C) Quantification of the b wave amplitude at 4 light intensities (–1, 0, 1, and 2.88 log cd*s/m^2) at each anesthesia.

ing mice.[7] In addition, the VEP demonstrates dose-dependent effects due to ketamine–xylazine anesthesia.[16] For example, the co-injection of 130 mg ketamine and 14 mg/kg xylazine suppresses the VEP waveform; in contrast, the doses of 65 mg/kg ketamine and 7 mg/kg xylazine enhances the VEP at flash frequencies below 8 Hz but suppresses the response at higher frequencies.[16] Another group reported ketamine-induced effects on the VEP at doses of 50 and 100 mg/kg.[14] These findings were confirmed and expanded by another lab, noting an adverse and dose-dependent effect of ketamine on the VEP:[26] although a dose of 37 mg/kg ketamine had the least effect among the doses tested, the VEP of the anesthetized mice still differed statistically significantly from those of unanesthetized animals. An accurate and reproducible ERG and VEP and sufficient anesthesia to enable signal recording are achieved in mice when doses of 25 to 40 mg/kg ketamine and 8 to 10 mg/kg xylazine are combined with 560 to 1000 mg/kg urethane.[5,6,18,27]

Finally, the use of ketamine–xylazine–urethane had no apparent negative effects on the measures of health recorded in the current study. The body weight and BCS increased over time in our mice, VAS was unaffected, and no tumors or other pathologies were detected. We cannot exclude the possibility that our physical presence or the lack of an acclimation period prior to assessment may have masked subtle differences in VAS between time points. However, we believe the data are useful given that the observations were conducted consistently across all groups and likely would have revealed marked changes in the overall condition of the mice had such changes been present. Additional studies involving the use of more rigorous measures of health are needed to confirm our findings.

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