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Effects of Analgesics on Tumor Growth in Mouse Models of Prostate Cancer Bone Metastasis

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Murine models of tumor development often require invasive procedures for tumor implantation, potentially causing pain or distress. However, analgesics are often withheld during implantation because of concerns that they may adversely affect tumor development. Previous studies examining the effects of analgesics on the development and metastasis of various tumor lines show that the effect of analgesics depends on the tumor line and analgesic used. A blanket statement that analgesics affect the general growth of tumors is not adequate scientific justification for withholding pain relief, and pilot studies or references are recommended for each specific tumor cell line and treatment combination. In this study, we evaluated the effects of 2 commonly used analgesics on tumor growth in 2 models of prostate cancer (PCa) bone metastasis. We hypothesized that a one-time injection of analgesics at the time of intratibial injection of tumor cells would not significantly impact tumor growth. Either C57BL/6 or SCID mice were injected subcutaneously with an analgesic (carprofen [5 mg/kg], or buprenorphine [0.1 mg/kg]) or vehicle (0.1 mL of saline) at the time of intratibial injection with a PCa cell line (RM1 or PC3, *n* = 10 to 11 per group). Tumor growth (measured by determination of tumor burden and the extent of bone involvement) and welfare (measured by nociception, locomotion, and weight) were monitored for 2 to 4 wk. Neither carprofen or buprenorphine administration consistently affected tumor growth or indices of animal welfare as compared with the saline control for either cell line. This study adds to the growing body of literature demonstrating that analgesia can be compatible with scientific objectives, and that a decision to withhold analgesics must be scientifically justified and evaluated on a model-specific basis.

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Many murine models of bone metastasis rely on intratibial injections of tumor cells.[11] These injections are assumed to be painful for mice because any procedure considered painful in humans should be assumed to be painful in animals, regardless of their age or species.[28] In humans, bone biopsy, a similar procedure to intratibial injection, is considered painful, and both local and systemic analgesics are recommended during or after the procedure.[6]

Researchers have an ethical obligation to relieve experimental pain whenever possible. Pain can alter behavioral and physiologic responses, leading to immunosuppression, through glucocorticoid and catecholamine release.[33] Therefore, appropriate pain relief is both an ethical and scientific mandate; in many countries, the use of appropriate analgesics is legally required.[3,4] Investigators must therefore provide scientific justification if their experimental design requires the withholding of analgesics during painful procedures.[14,37]

Some protocols for intratibial injection in mice recommend administering a systemic analgesic before tumor cell injection,[8,24] although studies have shown that commonly used analgesics such as NSAIDs (nonsteroidal antiinflammatory drugs) and opioids can affect tumor progression.[16,26,36] NSAIDs reduce the chronic inflammation that has been linked to carcinogenesis,[39] while opioids can modulate immune response or cellular pathways that control cancer cell survival and migratory behavior.[2] Thus, researchers may claim the need to withhold analgesia during intratibial injection because it may interfere with establishing the tumor model. However, unrelieved pain should also be considered a significant research confounder. In one study, unrelieved surgical pain, independent of the tumor implantation procedure, increased tumor growth and metastasis, while administration of buprenorphine ameliorated these effects.[21] In human cancer patients, further research evaluating the potential adverse and protective effects of analgesics is necessary before making a recommendation either for or against the administration of such drugs.[23,26,30,40] Review articles examining the effect of analgesics on the development and metastasis of various tumors show inconsistent results that are highly dependent on the tumor model and analgesic characteristics such as route of administration, drug type, or dose.[2,33] The variability of how analgesia affects the general growth of tumors renders general statements inadequate as scientific justification for withholding analgesics; pilot studies or model-specific references are recommended for each specific scenario.[33]

In addition to inappropriately extrapolating the effects of analgesics from the literature, researchers may be reluctant to administer analgesics because they have not seen data regarding the use of analgesics in their specific model provided in the literature. A review article assessing the documentation of anesthesia and analgesia in research animals found that the majority of articles inadequately described the use of anesthetics and analgesics, failed to include an explicit statement that analgesics were withheld, or did not include discussion of how pain management or untreated pain would affect results.[9] This gap in literature may lead researchers to believe that analgesics cannot be used in their model or field of study.

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In the current study, we evaluated the effects of 2 commonly used analgesics (carprofen and buprenorphine) on tumor growth in 2 mouse models of prostate cancer (PCa) bone metastasis. We hypothesized that a single injection of an analgesic at the time of intratibial injection of PCa cells would not significantly alter tumor growth.

Materials and Methods

This study was approved by the IACUC of the University of Michigan (Ann Arbor, MI) and was conducted in an AAALAC-accredited facility in full compliance with the Guide for the Care and Use of Laboratory Animals.

To determine the effect of analgesics on tumor growth, mice were injected with either saline, carprofen, or buprenorphine, followed by intratibial injection of tumor cells. We initially used C57BL/6 mice to assess the effect of analgesics in an immunocompetent strain and then repeated the study using SCID mice to assess the effect of analgesics in immunodeficient mice.

Mice. Forty-five C57BL/6 (C57BL/6J, Jackson Labs, Bar Harbor, MI) and 43 SCID (Prkdc^{scid}, University of Michigan in-house breeding colony, Ann Arbor, MI) mice (*Mus musculus*) were used in this study. Mice were first sorted by body weight, and then assigned into treatment groups so that each group of mice had a similar range of body weights. Numbers of mice per group were consistent with previous similar studies,[7,21] and preliminary analysis demonstrated adequate power (83.8% power to detect a difference of 5% or greater change in tumor size with a mean:standard deviation ratio of 1.4.). Male mice (SCID mice: 14 to 26g, 13 to 14 wk at time of injection; C57BL/6: 22 to 30g, 12 wk at time of injection; n = 10 to 12 per treatment group) were negative for fur mites, pinworms, and the following viruses: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus, mouse norovirus, Theiler murine encephalomyelitis virus, reovirus, enzootic diarrhea of infant mice virus, lymphocytic choriomeningitis virus, ectromelia virus, adenovirus, mouse cytomegalovirus, mouse pneumonitis virus, polyoma virus, Hantaan virus, mouse thymic virus, and lactate dehydrogenase-elevating virus. Mice were socially housed in groups of 5 in individually ventilated cages (Allentown Caging, Allentown, NJ) on corncob bedding (Anderson's Bed-O'Cobs, Frontier Distributing, Maumee, OH) in a temperature and humidity-controlled room (72 ± 2 °F, 30% to 70% relative humidity) on a 12:12-h light:dark cycle with ad libitum feed (Laboratory Rodent 5001, PMI LabDiet, St Louis, MO) and reverse-osmosis water via automatic watering. All mice received one Enviropak (WF Fisher and Son, Somerville, NJ) per cage for enrichment and nest building purposes. Upon arrival, mice were acclimated for one week prior to experimental manipulation. We used mice at 12 to 14 wk of age (in contrast to previous studies[11,31] recommending that mice be inoculated at 4 to 8 wk of age) based on our experience that the tibias of 4 to 8 wk old mice are too small to tolerate the needle, increasing the chance of a failed injection.

Both syngenic allograft (immunocompetent mice [C57BL/6] with murine cancer cells [RM-1]) and xenogenic models (immunodeficient mice [SCID] with human cancer cells [PC3]) were used to represent the different types of mouse models used in PCa research. Immunodeficient mice are frequently used to study the growth of human cancer cells in mice. However, these mice may lack immune responses that could influence pain. Thus, we also used an immunocompetent strain so that a full immune response would be possible.

Experimental timeline. Baseline measurements of nociception, locomotion, and weight were taken at day 0. Mice received an intratibial injection and analgesic on day 1, as described below. On day 2, locomotion and nociception were reassessed, approximately 24 h after injection. All outcomes (bioluminescent imaging, radiographs, nociception, locomotion, and weight) were then measured weekly until the mice reached experimental endpoints described later, or at 4 wk (28 d).

Cell lines and cell culture condition. PC3-luc and RM-1-luc cell lines were used for this experiment. PC3, a human prostate cancer cell line that metastasizes to bone, was obtained from the American Type Culture Collection (ATCC), and transfected with the luc gene. RM-1, a mouse prostate cancer primary site cell line, was originally obtained from Dr Timothy C. Thompson (Baylor College of Medicine, Houston, TX), and transfected with the luc gene by Dr Lu Yi (Southern University of Science and Technology, Guandong, China). Cells were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA), and, for RM1-luc, 4ug/mL Blasticidin (InVivogen, San Diego, CA) supplemented with 10% FBS (fetal bovine serum) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA). All cells were maintained at 37 °C, 5% CO_2, and 100% humidity. Authentication of cell lines was performed every 6 mo using short tandem repeat (STR) profiling. All cells tested negative for mycoplasma.

Analgesics. C57BL/6 or SCID mice were injected subcutaneously with an analgesic (carprofen 5 mg/kg or buprenorphine 0.1 mg/kg) or vehicle (saline 0.1 mL) immediately before intratibial injection with PCa cell lines (RM-1-luc or PC3-luc) or a nontumor (saline) control. The treatment groups (n = 10 to 12 per group) were as follows for each intratibial injection and analgesic combination [tumor/analgesic)]: 1) tumor/carprofen, 2) tumor/buprenorphine, 3) tumor/saline, 4) saline/saline. Carprofen and buprenorphine were used because they are the 2 most commonly used analgesics for rodents at our institution.

Intratibial injection. For the intratibial injection, mice were anesthetized with isoflurane (2.5%, MWI, Meridian, ID) using a vaporizer (Surgivet Isotec 4, Smiths Medical, Minneapolis, MN). The right leg was shaved, prepped with 70% alcohol, and flexed at a 90-degree angle. A sterile 27G needle was used to drill a hole into the joint surface through the patellar tendon and tibial plateau to enter the intramedullary canal of the tibia as previously described.[11] A sterile 29G insulin needle was used to inject 20 uL of the cell solution or saline control into the created space (25,000 cells RM-1-luc, 200,000 cells PC3-luc). C57BL/6 mice only received RM-1 cells, while SCID mice only received PC3 cells.

Tumor measurements (tumor burden, bone involvement). ***Tumor burden (Bioluminescent imaging [BLI, photons/sec], weekly starting at 1 wk after injection).*** Mice were injected IP with 100 uL of (30 mg/mL) luciferase 10 min prior to imaging. Mice were imaged using the IVIS spectrum (IVIS Imaging Systems, PerkinElmer, Waltham, MA) while anesthetized with the attached isoflurane system (XGI-8 Gas Anesthesia System, IVIS Imaging Systems, PerkinElmer, Waltham, MA). Total flux (p/s) was calculated by manually defining regions of interest using Living Image Software (IVIS Imaging Systems, PerkinElmer, Waltham, MA).

Bone involvement (radiographs, weekly starting at 1 wk postinjection). Mice were sedated with 2.5% isoflurane before placement into the radiograph machine in sternal recumbency (Faxitron UltraFocus, Faxitron, Tuscon, AZ). Lower body (abdominal to hindlimb) radiographs were taken during recovery from isoflurane within the radiograph machine, and the right tibia was scored based on a previously established scoring system:[19] 0) normal, 1) lytic lesions present within the medul-

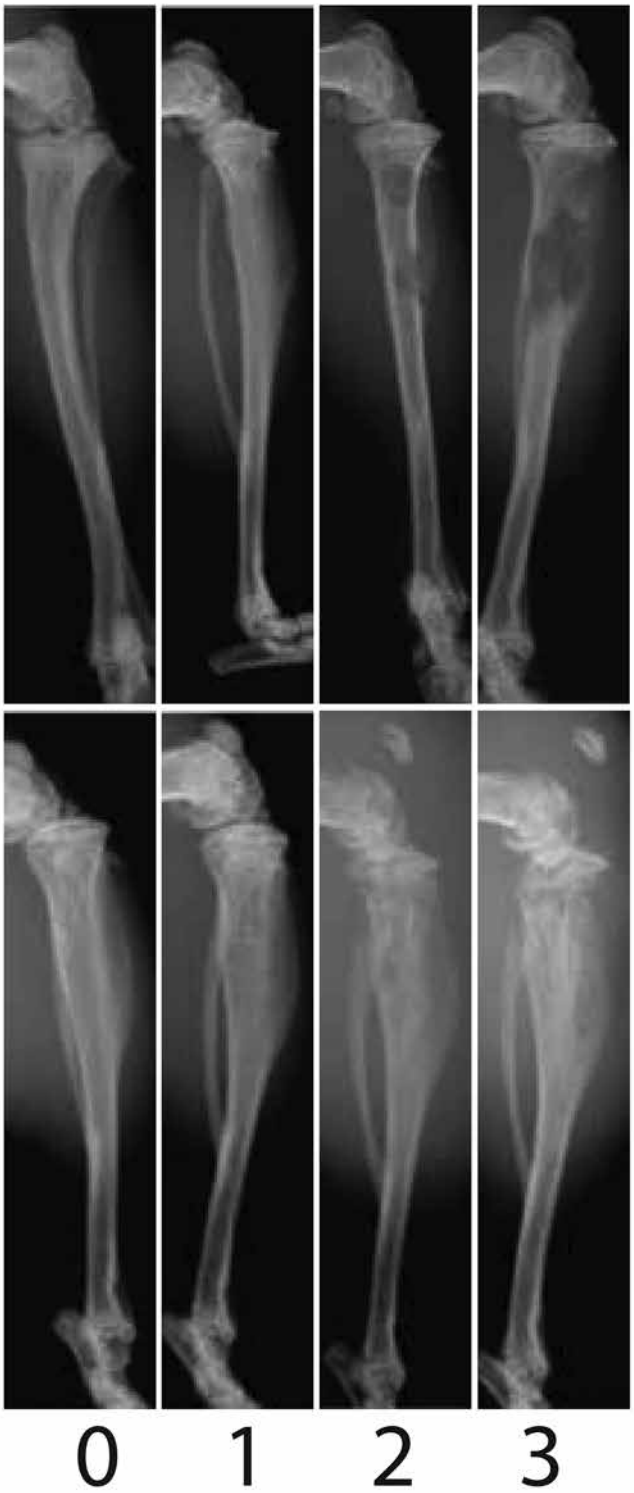

Figure 1. Representative radiographic scoring of C57BL/6 with RM-1-luc cells (top), and SCID mice with PC3-luc cells (bottom): 0) normal, 1) lytic lesions present within the medullary canal only, 2) involving one cortex; 3) involving 2 cortexes.

lary canal only, 2) involving one cortex, 3) involving 2 cortexes (Figure 1). The radiographs were evaluated by 2 independent observers who were blinded to the study groups at the time of scoring.

Welfare measurements (nociception, locomotion, weight). ***Nociception (von Frey [g], baseline [1 d before injection] and follow-up [24 h after injection], then weekly after).*** An electronic von Frey probe (IITC Life Science, Woodland Hills, CA) was used to measure mechanical sensitivity. Five mice at a time were individually placed in clear acrylic boxes on top of a wire floor that allowed the probe to pass through for evaluations. Mice were given approximately 5 min to acclimate to the boxes before measurements were collected. The Von Frey probe was applied perpendicular to the rear right paws of each mouse, and the response was recorded by an observer who was not applying the probe, thus helping to blind the assessment. The process was then performed on the hind left paw and was repeated twice more overall for each paw, for a total of 3 measurements on each rear leg. The values were averaged to provide a pain threshold score (peak force in grams) for each individual mouse. The average from the right (tumor bearing) foot was subtracted from the average of the left foot (nontumor control) to get a difference for each mouse. A lower Von Frey differential was interpreted to indicate increased nociception (suggesting increased pain) on the tumor bearing foot.

Locomotion (distance traveled [in], baseline [1 d before injection] and follow-up [24 h after injection], then weekly after). Mice were placed in a clean, empty mouse cage (Allentown Caging, Allentown, NJ) with an Everio video camera (JVCKENWOOD USA Corporation, Long Beach, CA) positioned on a tripod (Dolica, Rancho Cucamonga, CA) approximately 18 inches above the cage for an aerial view of the mouse's activity in the entire cage space. For the white coated SCID mice, black paper was placed under the cage to facilitate viewing during filming, and for the black coated C57BL/6 mice, white paper was placed under the cage. Mice were recorded in the cage for 15 min in the cage. Tests were conducted concurrently by 2 evaluators during each session. Distance traveled was calculated through a mixture of automated tracking and manual correction with the open-source tracking software Kinovea (https://www.kinovea.org/; version 0.8.15). The locomotion scorer was blind to treatment groups during the scoring process. The distance traveled was normalized for each mouse by dividing the distance traveled at each time point by the distance traveled prior to experimental treatment.

Weight ([g], baseline [1 d before injection], then weekly after). Mice were weighed using a scale (YA501, OHAUS Corporation, Parsippany, NJ). Weight was normalized by dividing the weight of a mouse at each time point by its baseline weight.

Experimental endpoints. Experimental endpoints necessitating early euthanasia included 1) presence of pathologic fractures on weekly radiographs and 2) a weight loss greater than 20% of baseline.

Additional monitoring. Consistent with institutional guidelines, mice were assessed daily by trained husbandry personnel for any signs of distress including but not limited to: tumor ulceration greater than 1/2 the surface area of the tumor, an ulceration that had effusion or appeared infected, tumors larger than 2 cm (tumor was not measured if obviously smaller than 2 cm), tumors that impaired the normal movement or behavior of the animal, lethargic presentation, hunched and scruffy presentation, or anything that could indicate a painful state such as reduced grooming or nest building. Mice that reached these institutional clinical endpoints were also euthanized.

Data analysis. Pairwise comparisons were made between groups for each time point using the Steel-Dwass test (nonparametric version of Tukey HSD) using JMP (SAS Institution, Cary, NC). Weighted κ values for interrater reliability between the radiographic scorers was calculated using the QuickCalcs web application (https://www.graphpad.com/quickcalcs/kappa1.cfm, GraphPad, San Diego, CA), and the average score between

the 2 raters was used for further analysis. Results were reported as mean ± SD, and significance was reported when $P < 0.05$.

Results

C57BL/6 mice. Due to the aggressive growth of the RM-1-luc cell line, all C57BL/6 mice had reached experimental endpoint by day 14, when pathologic tibial fractures were seen on routine monitoring radiographs. Three mice were removed from study (one each from tumor/saline, tumor/buprenorphine, and saline/saline groups) due to loss of ear tags from fighting. Likewise, some mice were singly housed due to fighting. No mouse lost more than 20% of baseline body weight, had apparent tumor ulceration, or was flagged for excessive lethargy or abnormal behavior.

In general, tumor measurements (tumor size, bone involvement) did not differ significantly between treatment groups (Figures 2 A and C). Tumor burden (measured through BLI) was higher in analgesic treatment groups as compared with the saline/saline group (-1.1e5 ± 1.8e5, -7.3e5, ± 9.0e5 p/s) as expected, but did not differ between the treatment groups themselves (tumor/buprenorphine [5.1e7 ± 8.2e7, 8.4e8 ± 1.3e9 p/s], tumor/carprofen [7.2e7 ± 1.3e8, 1.8e9 ± 2.7e9 p/s], tumor/saline [1.2e8 ± 3.1e8, 5.4e8 ± 6.3e8 p/s]) at any time point (days 7 and 14 respectively). Bone involvement scores (radiographic scoring) were higher on day 7 in the tumor/carprofen (0.79 ± 0.40) group as compared with the tumor/saline (0.67 ± 0.5, $P = 0.04$) and saline/saline group (0.41 ± 0.20, $P = 0.05$), but no differences were observed between any treatment groups by the next time point (day 14). Weighted κ scores measuring interrater reliability of radiographic scoring of C57BL/6 mice (0.505) indicated moderate agreement between raters.[15]

Welfare indices revealed sporadic differences between groups, but no consistent effects (Figures 2 B, D, and E). Nociceptive differences (measured using Von Frey filaments) between right and left legs and locomotion (distance traveled) were not significantly different from baseline at any time point. Some weight differences ($P = 0.02$) were observed on day 7 between tumor/saline (1.04 ± 0.03) and saline/saline (1.01 ± 0.01) groups, but these differences were not present on day 14.

SCID mice. The PC3-luc cell line was less aggressive than the RM-1 cells, so SCID mice were maintained until the experimental endpoint at day 28, with the exception of one mouse that was euthanized at day 21 due to pathologic fractures (possibly secondary to fighting). Some mice were singly housed due to fighting. No mouse lost more than 20% of baseline body weight, had apparent tumor ulceration, or was flagged for excessive lethargy or abnormal behavior.

Tumor measurements (tumor burden, bone involvement) generally did not differ significantly between treatment groups (Figures 3 A and C). The single exception is bone involvement at day 14, when saline/saline (0.15 ± 0.34) differed from tumor/buprenorphine (0.68 ± 0.40, $P = 0.04$) and tumor/saline (0.91 ± 0.70, $P = 0.02$), but not from tumor/carprofen (0.68 ± 0.56). After day 21, no significant differences in bone involvement were detected between treatment groups. Weighted κ scores measuring interrater reliability of radiographic scoring of SCID mice (0.613) indicated moderate agreement between raters.[15]

Welfare indices revealed sporadic differences between groups (Figures 3 B, D, and E). No significant differences in nociception were detected between any groups. For locomotion, on days 2, 7, and 14, tumor/buprenorphine was consistently lower than tumor/saline ($P = 0.02$, 0.04, 0.04 for each day) and saline/saline groups ($P = 0.04$ for all days) (Figure 3 D). The tumor/buprenorphine group (1.05 ± 0.07) weighed significantly more than the tumor/saline (0.98 ± 0.05) group only on day 28 (Figure 3 E).

Discussion

Overall, the results of this study suggest that a one-time dose of buprenorphine or carprofen has minimal impact on tumor development (based on tumor burden and bone involvement) in 2 mouse models of PCa. Tumor burden, quantitatively measured through bioluminescent imaging, was not significantly different between any treatment groups at any time point in any mouse model. However, radiographic scoring demonstrated a few minor differences between treatment groups. On day 7 in the C57BL/6 mice, tumor/carprofen bone involvement scores were higher than those of tumor/saline and saline/saline groups, although this difference was not observed the following week. In addition, on day 14, in the SCID mice, the saline/saline group had lower bone involvement scores than the tumor/buprenorphine and tumor/saline groups, but not the tumor/carprofen group. Additional studies at these times of tumor development may be warranted to see if these findings are clinically relevant or repeatable, as they were not observed the following week. Radiographic scoring is an ordinal measurement, and therefore may be prone to more variation and bias than objective measures such as BLI which did not differ at any time point in either mouse model.

The selected welfare tests (nociception, locomotion, weight) did not reveal any clear benefits to analgesic administration, perhaps due to problems with interpretation and study design. This study did not reveal any measurable differences in nociception between groups at any time point. One explanation is that for logistical reasons (time to equipment set up, recovery from procedure, and acclimation), the first assessment was made approximately 24 h after the intratibial injection. A previous study using a different model showed that the Von Frey difference was most pronounced at 3 to 6 h, and had mostly disappeared by 24 h.[7]

Locomotion differences occurred during days 2 to 14 in the SCID mice in that the tumor/buprenorphine group traveled consistently less than did the tumor/saline and saline/saline groups, but this difference disappeared by day 21. Increased distance traveled can be interpreted as either a positive or negative indicator of welfare. Decrease in distance traveled may be due to pain or physical tumor burden. Mice may travel less if they are in pain, lethargic, or unable to use the limb. However, distance traveled can also be increased by stress or pain due to anxiety or inability to find a comfortable resting position. A limitation of these tests is that they were conducted in the presence of humans and in an unfamiliar environment, which may have led to increased anxiety and exploratory behavior. Attempts were made to control for this by normalizing each mouse's distance traveled to its baseline score.

Sporadic changes in body weight were observed on day 7 in the C57BL/6 mice (tumor/saline was higher than saline/saline) and day 28 in the SCID mice (tumor/buprenorphine was higher than tumor/saline). However, interpretation of body weight in tumor models is challenging because mice can lose weight due to poor body condition or gain weight due to tumor growth.

Despite the sporadic differences and difficulty in interpreting welfare indicators, no consistent or definitive evidence indicated that mice treated with analgesics at the time of cell injection fared better than untreated mice. However, lack of definitive findings does not

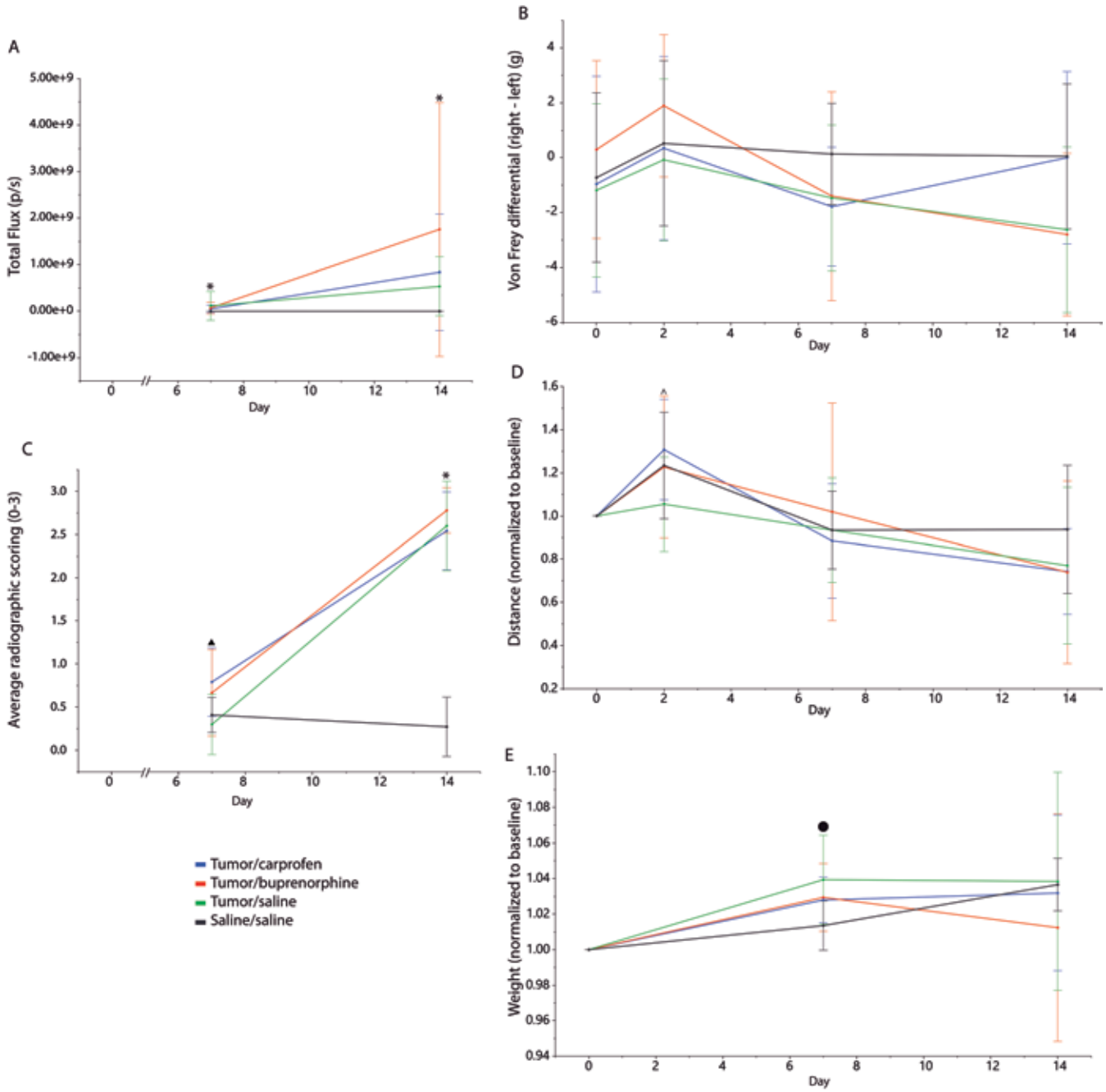

Figure 2. (A) Mean ± SD plots over the course of the study for tumor and welfare associated results for C57BL/6 mice. (A). Tumor Burden (BLI, p/s). (B). Nociception (Von Frey, g). (C). Bone Involvement (average radiographic scoring, 0-4). (D). Locomotion (Distance Traveled, in). (E). Weight (normalized). Key: * tumor treatment groups differ from the nontumor control but not from each other, ▲ tumor/carprofen differs from tumor/saline and saline/saline, ● tumor/saline differs from saline/saline. Pairwise comparisons made with a Steel–Dwass test, all reported comparisons have $P < 0.05$.

mean that analgesics are not warranted, but rather that additional measures (different tests or different time points) may be necessary to effectively gauge mouse welfare. Given that 1) analgesics did not affect tumor progression 2) our assessment of analgesic efficacy was limited and did not rule out the presence of unmeasured pain, 3) published literature[28] suggests that procedures involving "needle puncture" and "osteotomy" (with possible sequelae of "arthritis" and "periostitis") such as intratibial injections are painful, and 4) systemic analgesics are recommended for a similar procedure (bone biopsy) in humans,[6] and what is painful in humans should be assumed as painful in animals,[28] we recommend erring on the side of caution, and giving analgesics during these procedures unless demonstrated to be contraindicated for the specific model in use. Minimizing or alleviating animal pain in research is both a moral and regulatory imperative.[14,37]

This study had limitations in both study design (animals and tumors cell line) and in behavioral and tumor measurement tests. The first limitation in study design concerns the mice used. We used 12 to 14 wk old mice to increase the chance of successful injection. While other references[11,31] recommend injecting mice at 4 to 8 wk of age, in our experience, younger mice have smaller bones, which increases the risk of injection failure (cells

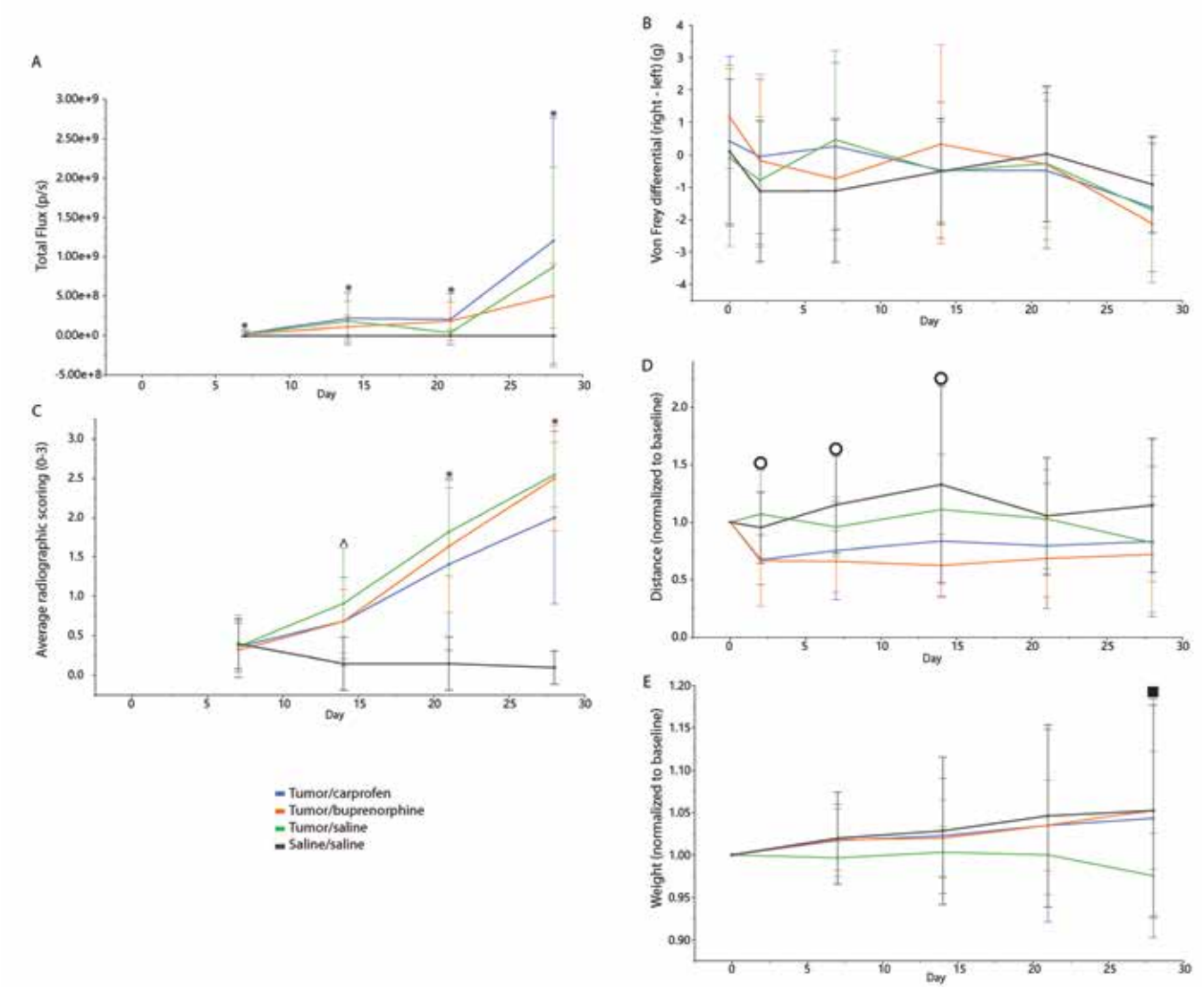

Figure 3. (A) Mean ± SD plots over the course of the study for tumor and welfare associated results for SCID mice. (A). Tumor Burden (BLI, p/s). (B). Nociception (Von Frey, g). (C) Bone Involvement (average radiographic scoring, 0–4). (D) Locomotion (Distance Traveled, in). (E) Weight (normalized). Key: * tumor treatment groups differ from the nontumor control but not from each other, ^ saline/saline differs from tumor/buprenorphine and tumor/saline, ○ tumor/buprenorphine differs from tumor/saline and saline/saline, ▪ tumor/buprenorphine differs from tumor/saline. Pairwise comparisons made with a Steel–Dwass test, all reported comparisons have $P < 0.05$.

grow outside the bone). However, age of inoculation may affect tumor growth kinetics. Although our method of alcohol prep for intratibial injection is consistent with previous published methods,[11] and our laboratory has not experienced adverse consequences associated with this method, further refinement may include a presurgical skin disinfectant like betadine or chlorhexidine to decrease the risk of joint infection. Our mice were housed on corncob bedding, which was the standard bedding at our institution at the time of this study. The phytoestrogens in corncob bedding may affect prostate cancer growth[41] and be a confounding influence in our study, even though all mice were housed with the same bedding. In addition, the mice used in our study were obtained from different vendors because all strains were not available from the same vendor. Variations in environmental features among vendors for the different strains of mice may contribute to microbiome differences, influencing pain perception.[22] Because our mice were acclimated for a week before experimental manipulation, the microbiome should have begun to stabilize around this time.[27] However, when analyzing our data, statistical analysis only compared mice of the same strain and source. Changing any of the variables (age, bedding type, mouse strain, or vendor) could affect outcomes.

The second limitation concerns the tumor cell line and injection process. The tumor cell line was not screened for rodent viruses other than mycoplasma. A variety of tumor cell line contaminants can have zoonotic potential or cause endemic infection that disrupts research outcomes, and endogenous retroviruses may affect some cancer studies.[32] In addition, we had a calculation error (assumed linear growth instead of logarithmic growth) in the initial number of cells for intratibial injection for RM-1. This resulted in earlier endpoint for C57BL/6 mice. Instead of the 25,000 cells initially administered, the starting cell number should have been around 1,000 to 1,500 cells to reach an endpoint at around 4 wk. Repeating this study with an appropriately screened cell line or a more appropriate starting number of cells may yield different results.

Third, we administered a single subcutaneous dose of carprofen (5 mg/kg) or buprenorphine (0.1 mg/kg), consistent with published references[34] and institutional guidelines, immediately before intratibial injection. Higher doses of analgesics may be necessary to see changes in behavioral parameters.[1,25] Due to the slower absorption of subcutaneous dosing (compared with intravenous, intraperitoneal, or intramuscular routes of administration),[20,35,38] we cannot be certain that we achieved adequate plasma levels for analgesia by the time the mouse woke up from isoflurane after intratibial injection was performed (several minutes after initial analgesic administration). To our knowledge, no information is available on the pharmacokinetics of subcutaneous buprenorphine or carprofen until 1 or 2 h respectively after administration.[10,15] Carprofen and buprenorphine are recommended to be administered every 12 h in mice,[34] so their effects could have disappeared by the time we performed our first set of welfare tests 24 h after intratibial injection. However, administering analgesics earlier or more frequently would result in increased handling of the mice, potentially introducing a stress confounder to our experiment. Given the amount of time necessary to perform intratibial injections and welfare testing on all of our mice, testing at an earlier time point than 24 h after analgesic injection would have been logistically difficult for this experiment.

Finally, our study only addressed acute pain caused by the intratibial injection, and results at later time points could be confounded by pain caused by the tumor. Based on our results, multidose or chronic administration of analgesics as previously described[13] could be useful.

Behavioral tests could be confounded by experimental variables as well as by limited tests. Mice hide signs of injury and suffering in human presence,[5] which occurred during testing. Additional tests for pain and distress could include more cage-side behavioral tests using a video camera such as nest consolidation,[29] grooming transfer,[29] and the mouse grimace scale,[18] potentially at earlier time points. However, many of these tests are validated for assessing pain immediately after a painful experience and would need further assessment for use in a long-term study such as ours. Locomotion and von Frey testing may not be appropriately sensitive for intraosseous pain, and additional readings (4 readings instead of the 3 taken in this study)[12] might improve sample size and statistical power.

Qualitative testing of tumors could also be expanded in future studies, including molecular tests for biomarkers of tumor progression, assessment at more frequent time points, and body composition data (through dual-energy X-ray absorptiometry [DXA] imaging or body condition scoring) to allow for better assessment of weight change from nontumor tissue compartments.

In conclusion, this study evaluated 2 different intratibial prostate cancer bone metastasis models: 1) the more aggressive mouse-derived allograft model (C57BL/6 mice + RM-1-luc cells) and 2) the less aggressive human-derived xenograft model (SCID mice + PC3-luc cells). Although the results of this study are specific to the mouse strains and cell type tested and should not be directly applied to other situations, other mouse strain and cell type combinations may be similarly unaffected by analgesics. Our investigation adds to a growing body of literature[13,33] suggesting that analgesics do not necessarily interfere with tumor growth and that scientific justification of withholding analgesia must be model-specific.

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