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Efficacy of 3 Buprenorphine Formulations for the Attenuation of Hypersensitivity after Plantar Incision in Immunodeficient NSG Mice

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Buprenorphine is perhaps the most prescribed analgesic for management of postoperative pain in mice. Although various buprenorphine formulations are effective in commonly used immunocompetent mouse strains, a knowledge gap exists regarding its efficacy in immunodeficient mice. Here we used a plantar incision to evaluate the efficacy of 3 buprenorphine formulations for attenuating postoperative mechanical and thermal hypersensitivity in the immunodeficient NSG mouse strain. We also characterized the pharmacokinetics of these formulations over a 72-h period. We hypothesized that all 3 buprenorphine formulations evaluated—the standard preparation and 2 extended-release products (Bup-HCl, Bup-ER, and Bup-XR, respectively)—would attenuate postoperative mechanical and thermal hypersensitivity resulting from a plantar incision in NSG mice. Male and female NSG mice (*n* = 48) were allocated to 4 treatment groups: saline (0.9% NaCl, 5 mL/kg SC once); Bup-HCl (0.1 mg/kg SC, BID for 2 d); Bup-ER (1.0 mg/kg SC once); and Bup-XR (3.25 mg/kg SC once). Mechanical and thermal hypersensitivity assessments were conducted 24 h before surgery and at 4, 8, 24, 48, and 72 h afterward. All groups of mice showed mechanical and thermal hypersensitivity within the first 24 h after surgery. Behavioral pain indicators (guarding, toe-touching [intermittent partial weight bearing], licking the incision, vocalizations) were observed in some mice from each group at every postoperative time point. Plasma buprenorphine was measured in a separate group of mice and concentrations surpassed the suggested therapeutic level (1.0 ng/mL) for less than 4 h for Bup-HCl, for at least 24 h for Bup-ER, and for 72 h for Bup-XR. Our results indicate that at the dosages studied, these buprenorphine formulations do not adequately attenuate postoperative mechanical and thermal hypersensitivity in the plantar incisional model in NSG mice. These findings support the need for strain-specific analgesic protocols for mice used in research.

Abbreviations: Bup-HCl, buprenorphine hydrochloride; Bup-ER and Bup-XR, 2 extended-release buprenorphine formulations; *Il2rg*, IL2 receptor common gamma chain; NOD, nonobese diabetic; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ

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Introduction

Providing analgesia for rodents undergoing potentially painful experimental procedures is both an ethical and legal obligation.[22] Furthermore, the quality of data and validity of preclinical research hinges on effective nociception treatment in research animals.[23] The response to analgesics varies depending on species, strain, sex, drug formulation, and dose.[41,49] Although tailoring the analgesic protocol to the species is standard in veterinary medicine (in both clinical and research settings), a growing body of research highlights the importance of strain-specific analgesia as an experimental refinement.[35,41,42]

Buprenorphine is a partial μ-opioid receptor agonist frequently used in research for treating moderate to severe pain in mice.[41] Its popularity over other opioids is largely due to its proven efficacy in a variety of mouse strains and pain models,[10,19,24,36,53] its comparatively longer duration of action, and its wide safety margin.[19,26,31,41,60] A major limitation of the standard formulation, buprenorphine hydrochloride (Bup-HCl), is the need for repeated dosing (typically 2 or 3 times daily), resulting in handling-related stress for mice and additional time requirements for researchers.[2,24,44] Several studies have shown that even these dosing frequencies may be insufficient, with nadirs falling below the therapeutic threshold.[4,18,24,26,27] To reduce the need for redosing, extended-release (Bup-ER, previously labeled as sustained-release buprenorphine [Bup-SR]) and extended-release injectable suspension (Bup-XR) formulations are now available, and a growing body of literature supports their sustained analgesic clinical efficacy in a variety of rodent species, strains, and models.[1,24,27,36] In addition, pharmacokinetic studies have suggested that plasma buprenorphine concentrations achieved with these formulations can remain above the suggested therapeutic threshold of 1.0 ng/mL[19] for 24 to 72 h or more.[24,26,29] However, because most research is conducted using immunocompetent strains,[49] a gap in knowledge exists regarding buprenorphine's efficacy and pharmacokinetics in immunodeficient mouse strains.

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice are a highly immunodeficient strain and are used in numerous research fields, including immunology, oncology, and infectious disease.[7,45,46] Innate immunity in the background strain, nonobese diabetic (NOD), is naturally impaired due to functional defects in NK,

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macrophage, and dendritic cell populations in conjunction with the absence of hemolytic complement.[48] These defects were combined with adaptive immune dysfunction through backcrossing of the *scid* mutation from the CB17-*scid* strain onto the NOD background, resulting in a NOD-*scid* strain that also lacks mature T and B lymphocytes.[48] The NSG strain was generated by backcrossing a null allele of the IL2 receptor common γ chain (*Il2rg*) onto the NOD-*scid* strain, which eliminated NK cell development, further impaired lymphocyte development, and disrupted cytokine signaling through *Il2rg* by IL2, IL4, IL7, IL9, IL15, and IL21 (that is, γc family cytokines).[30,46,47] This genotype renders NSG mice severely deficient not only in both innate and adaptive immunity but also in some aspects of cytokine-mediated cellular communication.

Although the unique biology of NSG mice makes them a valuable model for a multitude of biomedical research applications, their unique immunologic profile may have important implications for pain processing and analgesic efficacy. A large body of evidence implicates the immune system in modulating inflammatory pain, likely through the release of endogenous opioid peptides by immune cell populations.[6,21,32,40,51] A recent study conducted in immunocompromised nude mice suggests a critical role for functional immune cells (notably T lymphocytes) in exogenous opioid-mediated analgesia, although more research is needed to further elucidate the mechanisms of these interactions.[40] Furthermore, alterations in complex cytokine signaling due to *Il2rg* knockout and the absence of functional immune cells—many of which are major producers of cytokines[30]—may also alter pain processing pathways in NSG mice. IL2, for example, has a wide range of biologic functions,[30] including intrinsic antinociceptive activity, likely mediated through μ-opioid receptors.[50] Disruptions at the neuroimmune interface, the ever-increasing use of mice with natural or genetically engineered immunodeficiencies for biomedical research, and the moral obligation to minimize pain and distress in research animals collectively generate the need to clinically evaluate buprenorphine efficacy in immunodeficient animals.

To our knowledge, the efficacy of buprenorphine for attenuation of postoperative nociception has not been evaluated previously in immunodeficient mice. The aim of this study was to evaluate the efficacy of 3 formulations of buprenorphine (Bup-HCl, Bup-ER, and Bup-XR) at attenuating mechanical and thermal hypersensitivity after plantar incisional model in NSG mice. We hypothesized all 3 buprenorphine formulations would attenuate mechanical and thermal hypersensitivity. We also characterized the pharmacokinetic profiles of these 3 buprenorphine formulations in NSG mice over a 72-h period.

Materials and Methods

Mice. Adult male and female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (*Mus musculus*) were used to test hypersensitivity (n = 48) and to measure buprenorphine plasma concentrations (n = 48). At the time of surgery for hypersensitivity testing, the mean age was 63 d (range, 47 to 126 d), and mean weight was 24 g (range 18 to 33 g). Mice used for plasma collection had a mean age of 52 d (range, 42 to 62 d) at the time of drug administration, with a mean weight of 23 g (range, 17 to 35 g).

Mice were bred inhouse in a barrier facility using breeding stock originally obtained from The Jackson Laboratory (Bar Harbor, ME). Breeder mice were not regularly replenished, so experimental mice could be a substrain of the initially purchased mice. Sentinel mice in the barrier facility were free of mouse parvovirus, minute virus of mice, mouse hepatitis virus, mouse rotavirus (EDIM), Theiler's murine encephalomyelitis virus, Sendai virus, murine adenovirus 1 and 2, ectromelia virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, reovirus, murine norovirus, *Helicobacter* spp., *Rodentibacter pneumotropicus* (*Pasteurella pneumotropica*), *Mycoplasma pulmonis*, endo- and ectoparasites, and pinworms.

After weaning, mice were maintained in the barrier facility until several days before the study start date, at which time they were transferred to a conventional facility that housed the equipment required for daily hypersensitivity testing. The room housed only NSG mice for the duration of the study. Mice were housed in same-sex groups in individually vented cages containing irradiated, prefilled corncob bedding (Innovive, San Diego, CA). They received ad libitum access to autoclaved commercial rodent diet (Teklad Global 18% Protein Rodent Diet 2018SX, Envigo, Indianapolis, IN) and acidified water (Aquavive, Innovive, San Diego, CA) and were provided with Enviro-dri (Lab Supply, Fort Worth, TX) nesting material. Rooms were maintained on a 12:12-h light:dark cycle (lights on, 0700, fluorescent lighting, 100 to 400 lux) at 20 to 23 °C and 30% to 70% relative humidity. All experimental procedures were approved by the Stanford University IACUC (Administrative Panel for Laboratory Animal Care), and mice were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*.[22]

Hypersensitivity testing. ***Study design.*** Mice (n = 48) were acclimated to the housing and experimental room for at least 72 h before baseline testing. All mice underwent baseline testing at 72, 48, and 24 h before surgery, as described below. Data from the first 2 baseline sessions were not used. Data collected at 24 h before surgery (day –1) was used as the baseline for subsequent comparisons. On the morning of surgery (day 0), each mouse was randomly assigned to one of 4 treatment groups: saline (Saline; n = 12 [7 males, 5 females]; 5 mL/kg SC administered once; 0.9% sodium chloride, Hospira, Lake Forest, IL); buprenorphine hydrochloride (Bup-HCl; n = 12 [7 males, 5 females]; 0.1 mg/kg SC BID; buprenorphine hydrochloride 0.3 mg/mL, Par Pharmaceutical, Chestnut Ridge, NY); extended-release buprenorphine (Bup-ER, n = 12 [6 males, 6 females]; 1.0 mg/kg SC administered once; buprenorphine ER-LAB 0.5 mg/mL, ZooPharm, Fort Collins, CO); and buprenorphine extended-release injectable suspension (Bup-XR; n = 12 [6 males, 6 females]; 3.25 mg/kg SC, administered once; Ethiqa-XR 1.3 mg/mL, Fidelis, North Brunswick, NJ). Saline and Bup-HCl were administered using a 25-gauge needle; Bup-ER and Bup-XR were administered using a 22-gauge needle. All treatments were administered subcutaneously over the left shoulder immediately prior to making the skin incision. Bup-HCl was redosed twice a day (once in the morning and once in the afternoon) on days 0 and 1 such that repeat doses were administered approximately 8, 24, and 32 h after initial preoperative dose. The doses given at 8 and 24 h were administered approximately 15 min before hypersensitivity testing sessions. After injection, digital pressure was applied at the injection site for 5 s to prevent leakage. Mechanical and thermal hypersensitivity testing were performed at 4, 8, 24, 48, and 72 h after surgery by the same experimenter, who was blind to the treatment of the mice. Hypersensitivity testing was performed between 0700 and 1130, except for the 4- and 8-h time points, which occurred between 1100 and 1530 and between 1500 and 2000 h on day 0, respectively. Mice were returned to the home cage for at least 1 h between the 4- and 8-h assessments. Upon completion of the final (72 h) hypersensitivity assessment, mice were euthanized by carbon dioxide asphyxiation, followed by cervical dislocation. All mice underwent postmortem examination to assess gross pathology.

Surgery. Anesthesia was induced by using 4% to 5% isoflurane delivered in 100% O_2 in an induction chamber. Once induced, anesthesia was maintained with 0.5% to 3% isoflurane delivered using a nose cone on a nonrebreathing circuit. Sterile ophthalmic lubricant was applied to both eyes, and mice were kept on a circulating warm-water blanket. Cefazolin (30 mg/kg SC once; West-Ward Pharmaceutical, Eatontown, NJ) and 0.9% saline warmed to 32 to 33 ºC (5 mL/kg) were administered subcutaneously between the shoulders just before making the skin incision. Mice were positioned in sternal recumbency with the left hind leg extended and secured with tape to optimize exposure of the plantar surface, which was then aseptically prepared with 3 alternating passages of povidone–iodine swabs (Povidone–Iodine Swabsticks, PDI, Orangeburg, NY) and alcohol.

The plantar incisional surgery was performed as previously described.[14,38] Briefly, a 5-mm longitudinal incision was made along the plantar aspect of the foot, beginning 3 mm from the tibiotarsal joint, and extending distally. Care was taken to avoid incising the paw pads. By using curved forceps, the underlying plantaris muscle was gently elevated, and a no. 15 scalpel blade was used to create a stab incision through the center of the muscle. Caution was used to avoid severing of any muscle attachments, transecting muscle fibers or damaging underlying structures. While the muscle was elevated, a second pair of fine-tipped forceps was inserted through the stab incision and used to apply gentle lateral traction to the muscle for approximately 10 s. Saline was applied to the surgical site and absorbed with a sterile cotton-tipped applicator prior to closure. The skin was closed in a single horizontal mattress pattern using 4-0 silk suture. Topical antibiotic ointment (Neosporin, Johnson and Johnson Consumer, Skillman, NJ) was applied to the incision site. Total surgical time for each mouse was less than 10 min. Mice were monitored in a recovery cage with thermal support until conscious and were fully ambulatory when returned to a clean home cage.

Mechanical hypersensitivity testing. Responses to mechanical stimuli were evaluated by using the von Frey monofilament nociceptive assay. Mice were placed in bottomless, acrylic enclosures (10.1 × 10.1 × 12.5 cm) that were positioned on an elevated mesh platform (Electronic von Frey Mesh Stand, IITC Life Science, Woodland Hills, CA). Mice were acclimated to this environment for at least 15 min before testing. A von Frey monofilament (0.4 g, Asthesio, DanMic, San Jose, CA) was calibrated and then used to apply 0.4 g of bending force to the plantar aspect of the left hind paw for a total of 10 trials per mouse. After each application of the monofilament, a period of at least 10 s was permitted before a subsequent trial was performed on a given mouse. The monofilament was directed at various locations adjacent to the incision site. The monofilament was applied for 1 to 2 s before being withdrawn, and the mouse's response was recorded. Any clear nocifensive behavior (for example, withdrawal, shaking, or licking of the stimulated paw) was considered a positive response; absence of such behaviors was considered a negative response. Trials in which the behavioral response could not be clearly interpreted were omitted and repeated. Mechanical hypersensitivity was defined as a significant increase in the frequency of positive responses as compared with baseline (the values obtained at 24 h before the incision). Mechanical hypersensitivity testing preceded thermal hypersensitivity testing on each day.

Thermal hypersensitivity testing. Responses to thermal stimuli were evaluated by using the Hargreaves nociceptive assay. Each mouse was placed in a bottomless, acrylic enclosure as described for mechanical hypersensitivity testing. The enclosure was placed atop a raised, tempered-glass surface preheated to 29 °C (Plantar Analgesia Meter, IITC Life Science). Mice were acclimated to this environment for a minimum of 15 min before data collection. Once mice were acclimated, focal (4 × 6 mm) radiant heat from a 50-W light bulb with a beam intensity of 30% was directed at the plantar surface of the left hind paw, and the latency to withdraw the paw was recorded. A cut-off time of 20 s was used to prevent burns or other tissue damage. Four trials were performed on the left hind paw of each mouse, with a minimum of 3 min between trials. Mean withdrawal latency (thermal latency) for each mouse was calculated from the last 3 trials, omitting the first trial in all cases. In some cases, high activity levels precluded the completion of 4 successful trials, and the mean was calculated using the second and third trials, as available. The criteria used for positive and negative responses were the same as those for the mechanical hypersensitivity assay. Thermal hypersensitivity was defined as a significant decrease in thermal latency relative to baseline (the values obtained at 24 h before the incision).

Clinical observations. Clinical observations for abnormal behavior or clinical signs (for example, behavioral pain indicators, altered activity, ease of acclimation to study environment) were recorded daily during hypersensitivity assessments. Mice were weighed preoperatively to ensure optimal accuracy for drug dosing, and daily after completion of hypersensitivity testing. Observations were made by a single experimenter who was blind to the experimental group. After euthanasia, a gross postmortem evaluation was performed.

Statistical analysis. Data were analyzed by using repeated-measures ANOVA, followed by the Duncan Multiple Range Test for assessment of significance of differences in withdrawal responses within groups over time. Body weights were compared between baseline (−24) and 72 h by using a one-sided, paired *t* test. Data are expressed as mean ± SEM. A *P* value less than 0.05 was considered statistically significant. All analyses were performed by using R software.[39]

Buprenorphine plasma concentration experiment. ***Study design.*** Buprenorphine was administered to mice as described above for hypersensitivity testing. Plasma concentrations were determined at 4, 8, 24, 48 and 72 h, consistent with the hypersensitivity assessment time points. Mice (n = 48) were randomly assigned to one of the same 4 treatment groups used for the hypersensitivity experiment. Mice were anesthetized with isoflurane and injected subcutaneously with saline (n = 3), Bup-HCl (n = 15), Bup-ER (n = 15), or Bup-XR (n = 15). All mice also received subcutaneous fluid supplementation (0.9% NaCl, 5 mL/kg SC). The mice were allowed to recover in a warm recovery cage before being returned to their home cage. Terminal blood collection occurred at 4, 8, 24, 48, or 72 h after injection (n = 3 per time point, per treatment group, except for saline control [n = 3, with collection occurring at 4 h]). Both sexes were represented at each time point within each group. Mice in the Bup-HCl group were dosed twice a day for 2 d to mirror the dosing regimen used for the hypersensitivity experiment. Therefore, mice in the Bup-HCl group euthanized at the 8- and 24-h time points had been injected approximately 15 min before terminal blood collection. Mice euthanized at the 48-h time point had received their final dose approximately 16 h before terminal blood collection.

Plasma collection. Anesthesia was induced with 4% to 5% isoflurane delivered in 100% O_2 using an induction chamber. An adequate plane of anesthesia was determined before retroorbital blood collection based on absence of pedal withdrawal in response to a toe pinch. Death under anesthesia was confirmed

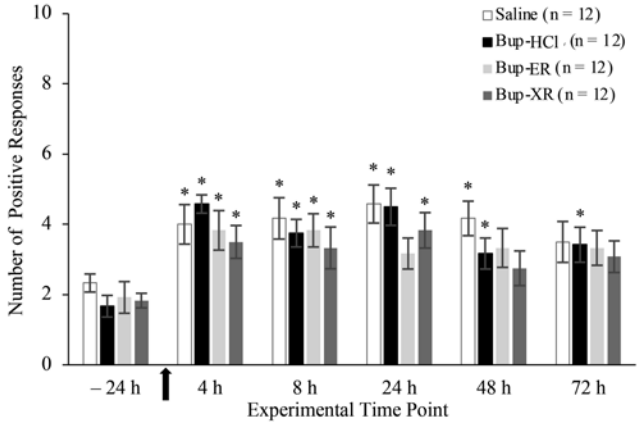

Figure 1. Mechanical hypersensitivity (number of positive responses, mean ± SEM) of the left hind paw of NSG mice. The arrow indicates hour 0, the time of plantar incision. Mechanical hypersensitivity was present for at least 48, 72, 8, and 24 h in the saline, Bup-HCl, Bup-ER, and Bup-XR groups, respectively. *, significant ($P < 0.05$) hypersensitivity relative to baseline (−24 h) within the respective treatment group.

by cervical dislocation. Whole blood was collected via nonheparinized capillary tubes into lithium-heparin microtainers (BD Microtainer Tubes with LH, Becton, Dickinson and Company, Franklin Lakes, NJ) and centrifuged at 2500 rpm for 20 min. Plasma was separated, placed in cryogenic tubes, labeled, and stored at −80 °C until analyzed.

Plasma buprenorphine concentration analysis. Plasma buprenorphine concentrations were measured by the Pharmaceutical Sciences Research Institute at the McWhorter School of Pharmacy (Samford University, Birmingham AL) via liquid chromatography–tandem mass spectrometry. Individual samples had a minimum volume of 50 µL and were shipped overnight on dry ice. Buprenorphine standard spiking solutions were prepared in 50:50 deionized water:acetonitrile to yield plasma concentrations ranging from 0.2 to 200 ng/mL. The buprenorphine plasma samples and standards (100 µL) were fortified with internal standard (50 ng/mL terfenadine). Acetonitrile (1 mL) was added to precipitate the plasma proteins, and the mixture was vortexed and centrifuged. The organic layer was transferred to a clean test tube and evaporated to dryness under nitrogen in a 50 °C water bath. The samples were reconstituted in dilution solvent and analyzed by liquid chromatography–tandem mass spectrometry. Matrix-matched standards and quality-control samples were prepared by using blank control plasma.

Results

Hypersensitivity testing. No significant differences were found in mechanical or thermal hypersensitivity testing based on sex, so male and female data were combined for further analysis.

Mechanical hypersensitivity. Mechanical hypersensitivity between groups did not differ at baseline. All 4 experimental groups showed mechanical hypersensitivity after surgery (Figure 1, Table 1). Mice in the saline control group had significantly ($P < 0.05$) increased mechanical hypersensitivity at 4, 8, 24, and 48 h as compared with baseline. Mechanical hypersensitivity in the saline control group was not significantly different between measurements made at 4, 8, 24, 48, or 72 h after surgery. Mechanical hypersensitivity of the Bup-HCl group was significantly ($P < 0.05$) increased at all postoperative time points (4, 8, 24, 48, and 72 h) as compared with baseline. In the Bup-HCl group, mechanical hypersensitivity at 4 and 24 h was significantly ($P < 0.05$) greater than at 48 h, but the 8- and 72-h time points were not different from any of the postoperative time points (Table 1). Mechanical hypersensitivity in the Bup-ER group was significantly ($P < 0.05$) higher at 4 and 8 h after surgery as compared with baseline. Mechanical hypersensitivity in the Bup-ER group was not significantly different between any of the postoperative time points (4, 8, 24, 48, 72 h). Mice in the Bup-XR group had significantly ($P < 0.05$) greater mechanical hypersensitivity at 4, 8, and 24 h after surgery as compared with baseline. Mechanical hypersensitivity in the Bup-XR group was not significantly different between any postoperative time points (4, 8, 24, 48, and 72 h).

Thermal hypersensitivity. Thermal hypersensitivity between groups did not differ at baseline. All 4 experimental groups showed thermal hypersensitivity after surgery (Figure 2, Table 1). Mice in the saline control group had significantly ($P < 0.05$) reduced thermal latency at 4, 8, 24, 48, and 72 h as compared with baseline. In the saline group, thermal latency was not significantly different between the measurements taken at 4, 8, 24 and 48 h after surgery, but thermal latency was significantly ($P < 0.05$) longer at 72 h as compared with the other postoperative time points. In the Bup-HCl, Bup-ER and Bup-XR groups, thermal latency was significantly ($P < 0.05$) reduced at all postoperative time points (4, 8, 24, 48 and 72 h) as compared with their respective baseline values. In addition, the Bup-HCl, Bup-ER

Table 1. Mechanical and thermal hypersensitivity in NSG mice

	Time point					
Group	–24 h (baseline)	4 h	8 h	24 h	48 h	72 h
Mechanical hypersensitivity (no. of positive responses; mean ± SEM)						
Saline	2.3 ± 0.3[a]	4.0 ± 0.6[b]	4.2 ± 0.6[b]	4.6 ± 0.5[b]	4.2 ± 0.5[b]	3.5 ± 0.6[a,b]
Bup-HCl	1.7 ± 0.3[a]	4.6 ± 0.3[c]	3.8 ± 0.4[b,c]	4.5 ± 0.5[c]	3.2 ± 0.4[b]	3.4 ± 0.5[b,c]
Bup-ER	1.9 ± 0.5[a]	3.8 ± 0.6[b]	3.8 ± 0.5[b]	3.2 ± 0.4[a,b]	3.3 ± 0.6[a,b]	3.3 ± 0.5[a,b]
Bup-XR	1.8 ± 0.2[a]	3.5 ± 0.5[b]	3.3 ± 0.6[b]	3.8 ± 0.5[b]	2.8 ± 0.5[a,b]	3.1 ± 0.5[a,b]
Thermal hypersensitivity (s; mean ± SEM)						
Saline	15.8 ± 0.8[a]	2.8 ± 0.5[b]	2.3 ± 0.4[b]	3.3 ± 0.4[b]	2.8 ± 0.4[b]	5.6 ± 1.2[c]
Bup-HCl	15.9 ± 0.9[a]	5.6 ± 1.4[b]	5.1 ± 1.6[b]	4.1 ± 0.5[b]	5.2 ± 0.9[b]	6.2 ± 1.3[b]
Bup-ER	16.2 ± 0.8[a]	5.0 ± 0.9[b]	4.9 ± 0.9[b]	5.1 ± 1.0[b]	4.3 ± 0.6[b]	5.4 ± 0.8[b]
Bup-XR	15.5 ± 0.8[a]	6.3 ± 1.0[b]	6.3 ± 0.8[b]	4.6 ± 0.7[b]	6.1 ± 1.1[b]	5.5 ± 1.0[b]

Different superscripted letters within a treatment group indicate statistically significant ($P < 0.05$) differences between values at respective time points.

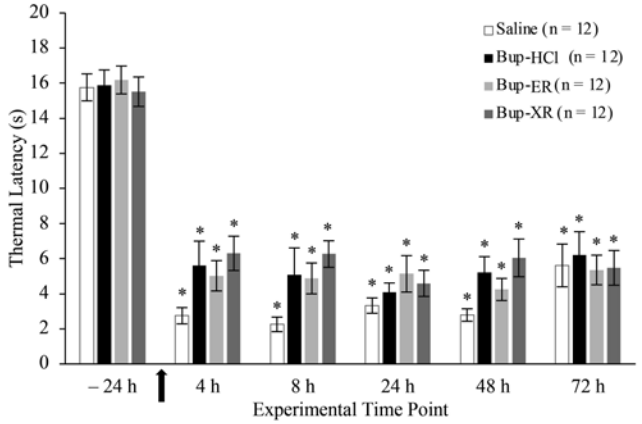

Figure 2. Thermal hypersensitivity (withdrawal latency [s], mean ± SEM) of the left hind paw of NSG mice. The arrow indicates hour 0, the time of plantar incision. Thermal hypersensitivity was present for at least 72 h in all treatment groups. *, significant ($P < 0.05$) hypersensitivity relative to baseline (–24 h) within the respective treatment group.

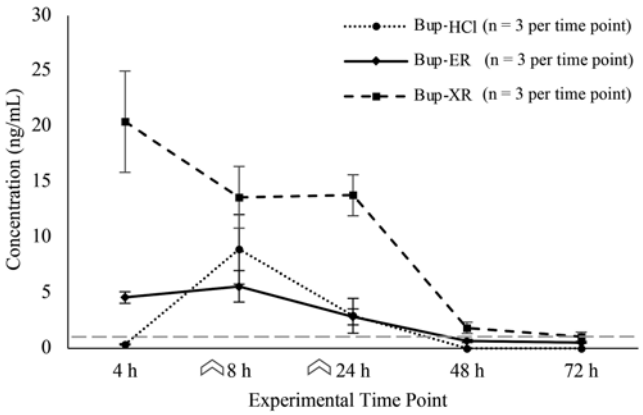

Figure 3. Plasma buprenorphine concentration (ng/mL, mean ± SEM) in NSG mice treated with Bup-HCl, Bup-ER, and Bup-XR ($n = 3$ per group, per time point). Arrowheads indicate redosing of Bup-HCl at 15 min prior to the 8- and 24-h collection time points. The dotted horizontal line at 1.0 ng/mL represents the suggested therapeutic level for buprenorphine in mice.[19]

and Bup-XR groups showed no significant differences between any of the postoperative time points (4, 8, 24, 48 and 72 h).

Body weight. Baseline body weights were not different between treatment groups. Body weights of male and female mice were not significantly different between treatment groups or at any of the time points, though males weighed significantly more than females throughout ($P < 0.05$).

Clinical observations. Behavioral indicators of pain (for example, guarding, toe-touching [intermittent partial weight bearing], vocalizations) were not seen in any group during baseline testing but were seen in some mice in each group at all postoperative time points. Painful behaviors peaked at different time points in different treatment groups (saline: 50% of mice at 48 h; Bup-HCl: 33% at 8, 24, and 48 h; Bup-ER: 58% at 24 h; Bup-XR: 50% at 48 h). Furthermore, locomotor activity was subjectively increased in all the buprenorphine-treated groups after surgery, particularly during thermal hypersensitivity assessments. Hyperactivity was not observed in the saline control group. No other abnormal behaviors were noted.

Gross pathology. Postmortem examination was performed on all mice after completion of the final hypersensitivity assessment (72 h after surgery) and revealed no gross abnormalities in the saline and Bup-HCl groups. Two Bup-ER and one Bup-XR mice had small (diameter, 1 to 2 mm), well-circumscribed, crusted cutaneous lesions suggestive of partial-thickness dermal ulceration at the presumed buprenorphine injection site. These lesions were nonpalpable and lacked grossly identifiable inflammation. In addition, 7 of 12 mice that had received Bup-XR had subcutaneous accumulation of an oily substance around the injection site. These lesions ranged from poorly to well-circumscribed, lacked an obvious capsule or gross inflammatory signs, and were not visible or palpable through the skin. No other gross abnormalities were observed.

Plasma buprenorphine concentrations experiment. Plasma buprenorphine was not detected in any of the saline controls. At the earliest time point (4 h), the Bup-HCl group had a plasma buprenorphine concentration of 0.4 ± 0.1 ng/mL. In this group, plasma concentrations above the suggested therapeutic level (1.0 ng/mL) were detected at the 8- and 24-h time points (8.9 ± 3.1 and 2.9 ± 1.6 ng/mL, respectively), with samples that had been collected 15 min after Bup-HCl redosing. Plasma buprenorphine levels in mice treated with Bup-ER were above 1.0 ng/mL at 4 h (4.6 ± 0.5 ng/mL) and peaked at 8 h (5.6 ± 1.4 ng/mL). This value fell below 1.0 ng/mL by 48 h (0.7 ± 0.1 ng/mL). Plasma buprenorphine concentrations in the Bup-XR group peaked at 4 h (20.4 ± 4.5 ng/mL) and remained above the suggested therapeutic level for at least 72 h (1.1 ± 0.4 ng/mL; Figure 3).

Discussion

To our knowledge, this study is the first to investigate the ability of buprenorphine (including sustained-release and extended-release formulations) to attenuate hypersensitivity in immunodeficient (NSG) mice experiencing incisional pain. More specifically, we determined whether 3 commonly used buprenorphine formulations attenuated postoperative mechanical and thermal hypersensitivity in NSG mice in a clinically relevant context by using a plantar incision to incite a robust inflammatory response. Mechanical hypersensitivity was assessed using the von Frey monofilament tests, and the Hargreaves test was used to evaluate thermal hypersensitivity. Our group has extensive experience with this surgical pain model and with both of these methods for assessing hypersensitivity.[1,5,11,25,36,44,61]

Our findings indicate that during the first 24 h after surgery, none of the 3 formulations prevented postoperative mechanical or thermal hypersensitivity in this model. Despite achieving plasma buprenorphine concentrations above the suggested therapeutic level (1.0 ng/mL),[19] mechanical hypersensitivity was observed in all treatment groups at 4 and 8 h after plantar incision, and thermal hypersensitivity was observed in all treatment groups at the 4-, 8-, and 24-h time points.

Previous research has established that the duration of hypersensitivity induced in this incisional pain model depends on both the species[8,38] and mouse strain.[35,42] A previous study from our group using the incisional model in C57BL/6 mice revealed that both mechanical and thermal hypersensitivity lasted for only 24 h after surgery in saline-treated mice.[36] In the current study, mechanical and thermal hypersensitivity in saline-treated controls appeared as early as 4 h after surgery and lasted for at least 48 and 72 h, respectively. These results indicate that in this model, NSG mice have greater mechanical and thermal hypersensitivity than do immunocompetent C57BL/6 mice.

The postoperative hypersensitivity observed in NSG mice in this study relative to their baseline values may be linked to their immune status—specifically the strain's T cell deficiency. A recent study found enhanced baseline sensitivities in several

other T cell–deficient strains (CD1 *nude*, *Rag1*-null mutant, and *Cd4*-null mutant) compared with immunocompetent (C57BL/6 and CD-1) controls.[40] The authors of that study suggested their finding was due to an inability to produce endogenous opioids (of T cell origin) involved in stress-induced analgesia.[40] Their outcome was consistent with another report of greater pain sensitivity in T cell–deficient nude mice compared with BALB/c controls in a model of experimentally induced inflammation.[6] In both studies,[6,40] adoptive transfer of functional T lymphocytes to the immunodeficient strains effectively reduced the sensitivity of immunodeficient mice to levels comparable to immunocompetent controls. However, important differences exist between the current and previously mentioned studies (including the mouse strains and hypersensitivity assays used), making direct comparisons and conclusions untenable. Nonetheless, future research using NSG mice and similar adoptive cell transfer techniques may help to further elucidate the relationship between the immune system and surgically induced hypersensitivity.

Consideration of immune system perturbations may also explain why buprenorphine did not attenuate postoperative hypersensitivity in the current study. In addition to differences in basal sensitivity discussed above, strain-specific differences in analgesic responsiveness are well described in the literature.[40,42,49] Opioid efficacy has been reported with regard to numerous immunocompetent mouse strains, but relatively few assessments have been performed in immunodeficient strains. Reduced antinociceptive response to morphine and enkephalin was reported in the immunodeficient *beige-J* strain, and subsequent studies pointed to its B cell deficiency as an underlying factor.[28,33] The same group that demonstrated increased sensitivity of T cell–deficient strains (discussed above) also found that, as compared with immunocompetent CD1 and C57BL/6 mice, T cell–deficient mice showed less analgesia after morphine administration in the tail-withdrawal test.[40] Adoptive transfer of $CD4^+$ T cells from immunocompetent CD1 mice resulted in morphine analgesia equivalent to that of the donor strain.[40] The mechanism by which this effect occurs remains undetermined, but the authors of the study speculated that morphine administration may alter gene expression and thus increase opioid receptor number or function.[40] Here again, differences in strain, drug, and hypersensitivity assessment modality preclude direct comparisons. In addition, the potential influence of disrupted cytokine signaling on nociceptive processing in NSG mice warrants further evaluation, given the substantial body of evidence linking immunomodulatory activity of cytokines to inflammation and pain processing,[17,54,55] including the antinociceptive activity of IL2, which is believed to interact with μ-opioid receptors.[50,58,59] Furthermore, a study comparing 2 analgesic protocols in women undergoing hysterectomy reported that patients who experienced less postoperative pain had higher levels of IL2, providing additional support for the cytokine's involvement in pain perception.[3] These studies suggest that immune cell populations and signaling molecules play integral roles in mediating exogenous opioid-induced analgesia, thus perhaps explaining the lack of hypersensitivity attenuation observed in buprenorphine-treated NSG mice.

Bup-HCl, Bup-ER and Bup-XR are frequently used postoperative analgesics in mice. In the present study, mechanical hypersensitivity was observed in all groups as soon as 4 h after surgery, with no attenuation observed until the 24- and 48-h time points for the Bup-ER and Bup-XR groups, respectively (Table 1). Mechanical hypersensitivity persisted throughout all postoperative assessments for the Bup-HCl group. In contrast, in our group's previous study with C57BL/6 mice, equivalent dosages of Bup-ER and Bup-XR effectively attenuated mechanical hypersensitivity as early as 4 h and for at least 24 h after surgery.[36] In the current study, thermal hypersensitivity was observed in all groups by 4 h after surgery and persisted throughout the observation period, with no return to baseline (that is, no attenuation) observed in any group (Figure 2). The presence of mechanical and thermal hypersensitivity in NSG mice during the first 24 h after surgery implies a need for alternative analgesic regimens for the clinical management of postoperative nociception.

The current study evaluated both male and female NSG mice. Despite documented sex-associated differences in nociceptive and analgesic sensitivity,[15,34,41] we did not find any differences between sexes in any treatment group or at any time point. Similarly, immunodeficient nude mice lacked sex-specific differences during a tail flick test after morphine administration.[40] However, sex-associated differences are often subtle, and larger sample sizes may be required to detect them.[34]

Plasma buprenorphine concentrations of 1.0 ng/mL or greater are generally accepted as sufficient to yield antinociceptive effects in rodents.[19] In the current study, the mean plasma buprenorphine concentrations of mice treated with Bup-ER and Bup-XR exceeded 1.0 ng/mL by 4 h and remained above this threshold for at least 24 h in the Bup-ER group and 72 h in the Bup-XR group. Bup-ER did not attenuate mechanical or thermal hypersensitivity at the 4- and 8-h time points, despite achieving mean plasma buprenorphine concentrations (4.6 and 5.6 ng/mL, respectively) above the expected therapeutic level. Similarly, Bup-XR failed to attenuate mechanical and thermal hypersensitivity at 4, 8, and 24 h, despite mean plasma buprenorphine concentrations of 20.4, 13.6, and 13.8 ng/mL, respectively. Therefore, our results do not corroborate the notion that buprenorphine is efficacious at plasma levels exceeding 1.0 mg/mL in NSG mice. These results contrast with our previous study in C57BL/6 mice, in which a plasma buprenorphine concentration of 2 to 3 ng/mL provided clinically effective attenuation of mechanical hypersensitivity.[36]

In the current study, mechanical hypersensitivity in the Bup-ER and Bup-XR groups was attenuated beginning at 24 and 48 h, respectively (Figure 1, Table 1), when plasma buprenorphine concentrations measured 2.8 and 1.8 ng/mL, respectively. We speculate that these results most likely show that the incisional pain became less severe at later time points. As discussed above, our previous research supports this claim: C57BL/6 mice treated with saline demonstrated mechanical and thermal hypersensitivity for just 24 h after the same surgery.[36] These earlier data were used to determine the Bup-HCl dosing regimen for the current study, in which mice received their fourth (and final) dose of Bup-HCl approximately 32 h after surgery.

The Bup-HCl group surpassed 1.0 ng/mL only at 8 and 24 h, that is, 15 min after scheduled redosing. Redosing in this group was performed before plasma collection to mimic the dosing schedule of the hypersensitivity experiment (in which mice received repeat doses of Bup-HCl before the 8 and 24 h testing sessions) and to assess correlations between behavioral responses and pharmacokinetic data. At 4 h (without redosing), the mean plasma concentration in the Bup-HCl group (0.4 ± 0.1 ng/mL) was well below the suggested therapeutic level. These findings indicate rapid uptake and efficient Bup-HCl clearance in NSG mice and add to a growing body of evidence in other mouse strains to suggest that standard Bup-HCl dosing regimens (that is, every 8 to 12 h) may be inadequate.[18,24,26,27] In addition, despite plasma concentrations exceeding the suggested 1.0 ng/mL threshold at the 8- and 24-h time points in the

Bup-HCl group, mechanical and thermal hypersensitivity were not attenuated at any time point. Again, these results suggest the therapeutic level of 1.0 ng/mL does not accurately reflect buprenorphine's effectiveness in NSG mice, thus implying a clinical need for strain- and construct-specific analgesia.

The literature on determining efficacious doses of buprenorphine in mice varies widely in findings across studies, with some variation likely related to the strain, model, and assessment modalities employed.[9,24,26,36,42,53,57] The dosages that we used for the current study (Bup-HCl, 0.1 mg/kg; Bup-ER, 1.0 mg/kg; Bup-XR, 3.25 mg/kg) are within the reported effective dose ranges according to previous studies[9,24,36] or manufacturers' recommendations.[16] The dosages used were efficacious in immunocompetent mice with intact cytokine signaling pathways and immune cell populations (notably T lymphocytes) that can produce endogenous opiates.[6,40,52] The intricacies of the NSG genetic construct may be accompanied by an inability to produce endogenous opiates, as has been suggested for other immunodeficient strains.[40] For example, nude mice require significantly higher doses of morphine than do CD1 mice to yield equivalent analgesia scores in 2 distinct nociceptive assays.[40] Therefore, the buprenorphine dosage needed for clinical efficacy in NSG mice may be higher than those previously reported for other strains.

Although buprenorphine is considered to have a wide safety margin,[13,19,31,56] adverse effects have been reported in a variety of species and include respiratory depression,[13,19,20,56] sedation,[11,44,56] weight loss,[20,24] pica,[12] injection-site nodules or lesions[1,9,37] and increased activity.[13,20,24,36,42,57] In the current study, body weight was not different between any treatment groups at any time point. Marked hyperactivity was noted in all buprenorphine-treated groups as early as 4 h after injection, comparable to previous reports in C57BL/6 mice.[36] This effect may present a practical challenge for hypersensitivity assays that require subjects to remain stationary for a short period of time (for example, the Hargreaves test).[43] We encountered this problem in the current study, and future experiments evaluating the efficacy of opioids in mice should account for this adverse drug effect. In addition, over half of the mice in the Bup-XR group (7 of 12) had accumulations of an oily substance—presumably residual vehicle—in the subcutaneous space near the injection site. Their presentation differed from a recent case report that described cystic structures in athymic nude rats treated with Bup-ER.[37] Those authors speculated that the strain's impaired cell-mediated immunity may have contributed to the development of chronic lesions at the injection site; these lesions contained detectable levels of buprenorphine, suggesting incomplete absorption of extended-release vehicles in some rodent strains.[37] In the current study, plasma buprenorphine concentrations indicated that both Bup-ER and Bup-XR were absorbed into circulation by as early as 4 h after injection. A previous study from our group did not reveal any serious (that is, life-threatening) adverse effects associated with Bup-XR administered at twice the label dose (6.5 mg/kg) in C57BL/6 mice,[36] suggesting that higher doses may be practical in future studies using NSG mice.

In conclusion, none of the 3 formulations of buprenorphine evaluated (Bup-HCl, Bup-ER, Bup-XR) attenuated mechanical hypersensitivity prior to the 24-h postoperative time point in immunodeficient NSG mice. Similarly, none of the formulations attenuated thermal hypersensitivity at any postoperative time point up to 72 h. Our results align with the existing literature regarding opioid efficacy in immunodeficient mouse strains, implicating a potential role for immune cell populations and cytokine signaling in opioid-mediated analgesia[6,40,52] and, by extension, a need for strain- or construct-specific analgesic protocols for rodents used in research. Future studies should evaluate higher buprenorphine doses, alternative analgesic drug classes (for example, NSAID, local anesthetics), and multimodal analgesia in NSG mice. In addition, other pain and hypersensitivity assessment modalities should be evaluated. The results of this study likely extend to other immunodeficient rodent strains and genetic constructs, so further research using other immunodeficient animals is warranted.

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