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Comparative Pharmacokinetics and Injection Site Histopathology in Nude Mice Treated with Long-acting Buprenorphine Formulations

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Two long-acting formulations of buprenorphine are commercially available as analgesics for rodents. However, these drugs have not yet been studied in nude mice. We sought to investigate whether the manufacturer-recommended or labeled mouse doses of either drug would provide and sustain the purported therapeutic plasma concentration of buprenorphine (1 ng/mL) over 72 h in nude mice and to characterize the injection site histopathology. NU/NU nude and NU/+ heterozygous mice were subcutaneously injected with extended-release buprenorphine polymeric formulation (ER; 1 mg/kg), extended-release buprenorphine suspension (XR; 3.25 mg/kg), or saline (2.5 mL/kg). Plasma concentrations of buprenorphine were measured 6, 24, 48, and 72 h after injection. The injection site was examined histologically at 96 h after administration. XR dosing yielded significantly higher plasma buprenorphine concentrations than did ER dosing at every time point in both nude and heterozygous mice. No significant difference in plasma buprenorphine concentrations were detected between nude and heterozygous mice. Both formulations yielded plasma levels of buprenorphine of over 1 ng/mL at 6 h; XR sustained buprenorphine plasma levels above 1 ng/mL for over 48 h, whereas ER sustained this level for over 6 h. Injections sites of both formulations were characterized by a cystic lesion with a fibrous/fibroblastic capsule. ER induced more inflammatory infiltrates than did XR. This study indicates that while both XR and ER are suitable for use in nude mice, XR has a longer duration of likely therapeutic plasma levels and induces less subcutaneous inflammation at the injection site.

Abbreviations: ER, extended-release buprenorphine polymeric formulation; XR, extended-release buprenorphine suspension; nude, NU/NU nude mice; het, NU/+ heterozygous mice

MeSH terms: *buprenorphine*; *mice, nude*; *pathology*; *pharmacokinetics*

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Introduction

Analgesic duration of action is an important point of conversation in institutional animal care and use programs and in general veterinary practice. The ideal analgesic drug provides effective pain relief with minimal handling or manipulation of the animal. Long-acting analgesics that require less frequent administration reduce the dosing-related distress to both the animal and the technical staff. These formulations facilitate compliance with a prescribed dosing regimen and reduce the fluctuations in plasma concentrations associated with more frequent, repeated dosing.[6]

The most common opioid drug used in laboratory animal medicine is buprenorphine[17] with a reported duration of efficacy of 3 to 5 h in mice[9,11] and 6 to 8 h in rats.[9,18] Long-acting commercial formulations such as extended-release buprenorphine polymeric formulation (ER) and, more recently, extended-release buprenorphine suspension (XR), have become frequent components of analgesic protocols for mice and rats. ER is a patented, compounded extended-release buprenorphine preparation with a polymer base; it is available from a single distributor by veterinary prescription only.[22] ER has been shown to maintain plasma levels of buprenorphine above the commonly accepted therapeutic plasma concentration of 1 ng/mL[10] for up to 72 h in mice.[8,20] XR is an FDA-indexed extended-release buprenorphine preparation that is approved for subcutaneous use in mice and rats. XR is a lipid-encapsulated formulation that is pharmaceutical grade, can be ordered on a research DEA license, and is widely available from pharmaceutical distributors.[7] Similar to its ER counterpart, XR has been shown to maintain purported therapeutic plasma concentrations for up to 72 h in mice.[2,20,21]

Subcutaneous injection of ER and XR are both associated with injection site lesions in rodents.[1,6,8,13,21] The manufacturer of ER warns against the using the product in nude rodents based on alleged anecdotal local reactions to the novel polymer carrier. A case report of ER administration to nude rats reported a lack of buprenorphine absorption from the injection site in association with mild injection site inflammation.[15] The authors hypothesized that the lack of T cells and a cell-mediated immune response in these rats prevented dissolution of the vehicle and absorption of the buprenorphine. However, buprenorphine plasma levels were not measured in that case report. Further investigation is needed to understand the pharmacokinetics of buprenorphine using the extended-release polymer system in immunodeficient rodents, especially given evidence of

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strain-dependent opioid responses in mice.[19] The current study measured plasma buprenorphine concentrations after dosing of NU/NU mice (Crl:NU-*Foxn1*^{nu}) and their heterozygous controls (NU/+). NU/NU mice have a genetic mutation that causes the thymus to be absent or underdeveloped and thus the mice lack functional T cells. NU/+ mice were used as controls; these mice maintain normal thymic function and T cells.[5]

The objectives of the current study were 1) to determine whether the manufacturer- recommended and labeled mouse doses of ER and XR, respectively, provide a sustained plasma level of greater than or equal to 1 ng/mL for over 72 h in nude mice and 2) to characterize histopathological differences in inflammation at the injection sites of the 2 drugs. We hypothesized that the absorption and distribution of buprenorphine from the extended-release vehicles would be lower in the nude mice, based on the nude rat case report.[15] We further hypothesized that inflammatory reactions would be milder in the XR groups than the ER groups based on the difference in drug carrier.

Materials and Methods

Animals. A total of 36 (18 male, 18 female) NU/NU nude (nude) mice and 36 (18 male, 18 female) NU/+ heterozygous (het) mice were used as test subjects. The groups were balanced for sex to minimize the impact of any sex-related variability. Mice were 11 to 21 wk of age and weighed 33.4 ± 2.6 g for nude males, 27.2 ± 2.25 g for nude females, 38.2 ± 4.7 g for het males, and 28.4 ± 2.2 g for het females.

Mice were bred inhouse in a barrier facility using breeding stock originally obtained from Charles River Laboratories (Wilmington, MA). Based on dirty bedding exposure of sentinel animals followed by assessment of serology and PCR testing, mice were designated as SPF for mouse hepatitis virus, mouse minute virus, lymphocytic choriomeningitis virus, mouse rotavirus, Sendai virus, pneumonia virus of mice, ectromelia virus, reovirus 3, mouse adenoviruses, polyoma viruses, rat theilovirus, lactate dehydrogenase elevating virus, mouse parvovirus, mouse cytomegalovirus, hantaviruses, mouse thymic virus, *Mycoplasma*, *Corynebacterium kutscheri*, *Clostridium piliforme*, *Streptobacillus moniliformis* and *zooepidemicus*, *Salmonella*, *Rickettsia*, *Chlamydia*, *Filobacterium rodentium*, *Spironucleus*, *Citrobacter freundii*, β-hemolytic *Streptococcus*, *Bordetella bronchiseptica*, *Encephalitozoon cuniculi*, *Streptococcus pneumoniae*, *Pasteurella*, *Helicobacter*, *Pneumocystis*, mites, and nematodes. Four days prior to the study start date, mice were transferred to a conventional facility for the duration of the study.

Mice were housed in same-sex and same-genotype groups of 4 to 5 animals in autoclaved, ventilated microisolation cages with a reverse-osmosis automatic watering system (Tecniplast, West Chester, PA) and Sani-Chip bedding (Newco, Rancho Cucamonga, CA). The entire cage was changed 7 d after initial housing. The room was maintained on a 12:12-h dark:light cycle (lights on at 0600 and off at 1800) at 68 to 72 °F (20 to 22 °C) and 30 to 70% relative humidity. Mice received ad libitum access to irradiated Teklad LM-485 mouse/rat sterilizable diet 7912 (Envigo, Indianapolis, IN). Each cage received autoclaved paper towel for nesting material.

Mice were maintained in accordance with the University of California San Diego (UCSD) animal care program. The experimental protocol was approved by the UCSD IACUC. The UCSD animal care and use program is accredited by AAALAC International.

Study design. Nude mice (n = 36) and het mice (n = 36) were equally divided among 3 treatment groups: XR (3.25 mg/kg SC; Ethiqa XR, 1.3 mg/mL, Fidelis, North Brunswick, NJ); ER (1 mg/kg SC; Buprenorphine ER-LAB, 0.5 mg/mL, Zoopharm, Fort Collins, CO); or saline (2.5 mL/kg SC; 0.9% NaCl, Hospira, Lake Forest, IL). The labeled mouse dose was used for XR, and the manufacturer- recommended mouse dose was used for ER. Mice were anesthetized with isoflurane in an induction chamber, and the compounds were then administered once subcutaneously in the interscapular region using a 22-gauge needle. After injection, digital pressure was applied to the injection site for 5 s to prevent leakage of the drug. Body weights obtained the day before injection were used to calculate the individual dose volume for each mouse.

Blood collection. A blood sample of approximately 50 µL was obtained from the retroorbital sinus of each mouse at 6, 24, 48, and 72 h after compound administration. Blood was collected from isoflurane-anesthetized mice from alternating eyes by using an autoclaved and heparinized glass pipette. Blood samples were centrifuged to obtain plasma, which was then stored in individual microcentrifuge tubes at −80 °C until analysis.

Tissue collection. Mice were euthanized by carbon dioxide inhalation at 96 h after drug administration. Final body weight was then obtained. A 2-cm square sample of the interscapular skin was excised and preserved in 10% neutral buffered formalin for histopathologic evaluation of the injection site. The skin samples were routinely processed and embedded in paraffin, sectioned, mounted on glass slides, stained with hematoxylin and eosin, and microscopically evaluated by a pathology-trained veterinarian. The injection sites were evaluated for morphologic characterization that included the type and degree of inflammatory changes. During characterization, the inflammatory response was graded as absent, minimal, mild, moderate, or marked.

Plasma analysis. Quantitative determination of buprenorphine in mouse plasma was accomplished using high-performance liquid chromatography (HPLC) with tandem mass spectrometry detection (LC-MS/MS). Briefly, protein was precipitated out of 20 µL of plasma by using 60 µL of 100% acetonitrile (MeCN). Then 10 µL of supernatant containing buprenorphine was injected directly onto a C-18 reversed phase HPLC column (MacMod Ace-5, 2.1 × 150 mm). The LC mobile phase consisted of HPLC grade water with 0.1% formic acid (elute A) and MeCN with 0.1% formic acid (elute B) and eluted with a gradient program of 1 to 5 min/80% B; 5.50 to 10 min/20% B at a flow rate of 0.5 mL/min. MS/MS detection was performed in positive electrospray ionization mode at a mass transition of 468 → 55 m/z (buprenorphine) and 472 → 54 m/z (buprenorphine-D4 IS). Calibration standards were used to generate a curve using a linear regression algorithm to plot the peak area ratio compared with concentration with 1/x weighting, over the full dynamic range of analyte concentrations. This method had a dynamic range of 0.3 to 41 ng/mL.

Statistical analysis. Plasma buprenorphine concentration values below the limit of quantitation were counted as zeroes for statistical analysis. One 6-h plasma concentration data point from a female het mouse that received XR was biologically implausible and excluded from data analysis. Data were analyzed with Prism 9 (GraphPad Software, LLC, San Diego, CA). Plasma concentrations were analyzed with a mixed effects ANOVA followed by a Tukey's multiple comparisons test. Individual weight changes were analyzed with a one-way ANOVA followed by a Tukey multiple comparisons test. A P value of less than 0.05 was considered significant.

Results

Plasma buprenorphine concentrations. Buprenorphine was not detected at any time point in plasma from mice that were

dosed with saline. XR dosing yielded significantly higher plasma buprenorphine concentrations than ER dosing at every time point in both nude and het mice (for all comparisons, $P < 0.02$). Plasma buprenorphine concentrations did not differ significantly between nude and het mice for either formulation (for all comparisons, $P > 0.5$). In mice dosed with XR, the mean plasma buprenorphine concentration fell below 1 ng/mL between 48 and 72 h. In mice dosed with ER, the mean plasma buprenorphine concentration fell below 1 ng/mL between 6 and 24 h (Figure 1). A posthoc multiple comparisons analysis using a mixed effects ANOVA did not reveal any sex differences ($P > 0.7$).

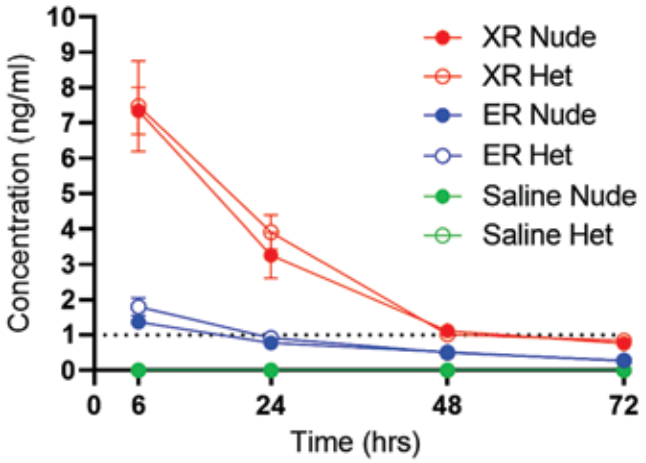

Figure 1. The mean plasma concentration of buprenorphine (ng/mL) over 72 h in pooled-sex nude and het mice administered either XR, ER, or saline with error bars indicating standard error of the mean. The dotted line represents the therapeutic threshold of 1 ng/mL.

Weight change. All groups showed a significant loss of weight over the course of the study ($P < 0.02$). All groups lost an average of 2.8 to 5.5% of their starting weight; this loss was not clinically apparent. The weight loss percentage was not statistically different among groups (for all comparisons, $P > 0.5$). The mice showed no observable clinical signs.

Macroscopic observations and injection-site histopathology. Euthanasia was performed at 96 h after injection. At this time, subcutaneous nodules were grossly visible in all nude mice that had been injected with XR or ER, but not with saline. Nodules were also present in het mice; although not visible through the fur, they were evident during tissue trimming of the injection site. Histologically, the injection sites of the 3 different compounds were easy to distinguish. The saline injection sites in both genotypes appeared normal, with no evidence of subcutaneous materials or inflammation. In contrast, both the XR and ER injections sites in both genotypes were characterized by a cystic lesion with a fibrous/fibroblastic capsule, infiltrated with variable numbers of mixed inflammatory cells, surrounding a thin to moderately thick layer of inflammatory/necrotic cells and granular material (Figures 2 and 3). Most of the cyst centers were clear, without cells, debris, or recognizable contents. The XR injection site cysts had a thinner capsule, containing more fibroblastic than mature fibrocytes.The mild inflammatory infiltrate consisted of neutrophils admixed with few lymphocytes, macrophages, and occasional mast cells (Figures 2 A and B, 3 A and B). The capsule of the ER injection site cysts had a marked inflammatory cell infiltrate with a large neutrophilic component (Figures 2 C and D, 3 C and D). The subcapsular layer of the

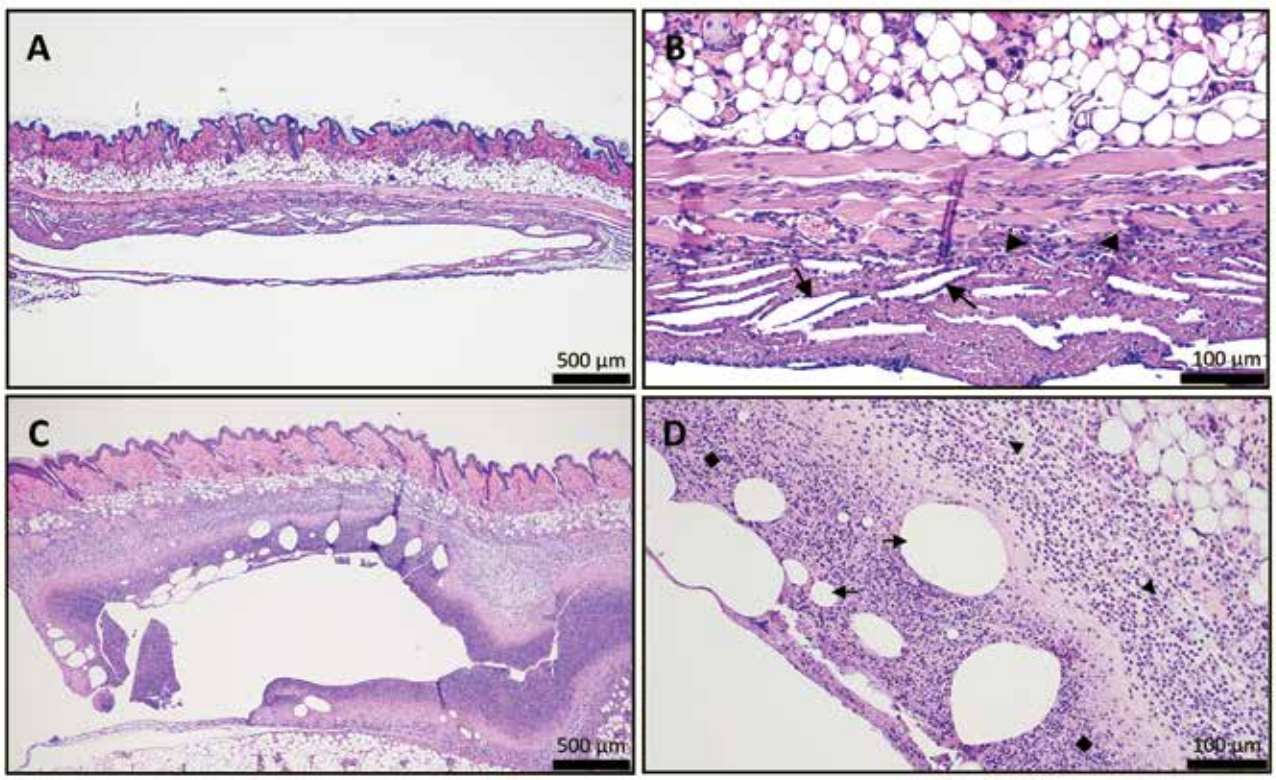

Figure 2. Histopathology of subcutaneous injection site in nude mouse administered XR at 4× magnification (A) and 20× magnification (B), and nude mouse administered ER at 4× magnification (C) and 20× magnification (D). In B, arrows denote cholesterol-type clefts in granular luminal material; arrowheads denote fibroblastic capsule. In D, arrows denote lipid-type spherules in luminal cellular debris; arrowheads indicate fibrous capsule with admixed neutrophilic inflammation; diamond indicates amorphous cellular debris.

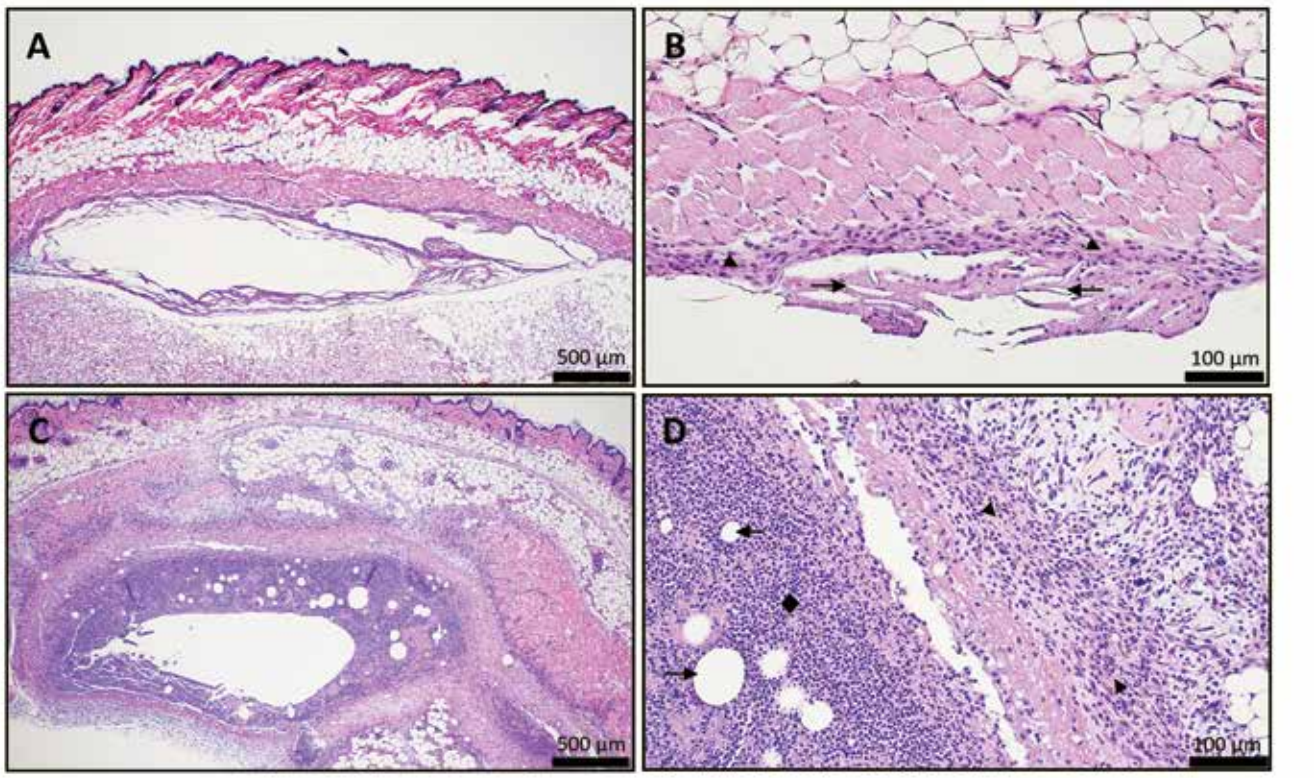

Figure 3. Histopathology of subcutaneous injection site in het mouse administered XR at 4× magnification (A) and 20× magnification (B), and het mouse administered ER at 4× magnification (C) and 20× magnification (D). In B, arrows denote cholesterol-type clefts in granular luminal material; arrowheads denote fibroblastic capsule. In D, arrows denote lipid-type spherules in luminal cellular debris; arrowheads indicate fibrous capsule with admixed neutrophilic inflammation; diamond indicates amorphous cellular debris.

XR cysts was acellular, with plump moderately large spindle-shaped clear spaces (cholesterol-type clefts; Figures 2 A and B, 3 A and B). In contrast, the subcapsular layer of the ER cysts was characterized by the presence of moderate to marked numbers of necrotic and intact neutrophils plus necrotic cellular debris, and small to moderately sized spherical vesicle-like spaces (lipid-type spherules; Figures 2 A and B, 3 A and B). No differences were detected between the lesions of nude and het mice that were injected with the same compound (Figures 2 and 3). No differences were detected between male and female mice injected with the same compound.

Discussion

The results of this study demonstrate that XR, an extended-release suspension of buprenorphine, can achieve the purported therapeutic level of greater than or equal to 1 ng/mL buprenorphine in plasma by at least 6 h after administration and can maintain this level for at least 48 h. ER, an extended-release polymeric formulation of buprenorphine, achieves a plasma level of 1 ng/mL by 6 h after administration and maintains this level for less than 24 h. These findings were consistent in both nude and heterozygous nude mice, suggesting that both XR and ER could be appropriate analgesics for both genotypes.

The duration of buprenorphine plasma levels greater than 1 ng/mL achieved by XR in the current study is similar to what has been previously reported,[14] although some previous studies report shorter[4] and longer[2,20,21] durations in mice. One study reported the duration of plasma levels greater than 1 ng/mL achieved by ER to be similar to our findings,[6] although several other studies report longer durations in mice.[2,6,12,14,20] Sample collection techniques, study design differences, or the stock/strain of the mice could account for these differences. The labeled dose of XR used in this study (3.25 mg/kg) is over 3 times higher than the manufacturer-recommended dose of ER (1 mg/kg); thus, a higher injected dose of ER may have sustained higher plasma concentrations of buprenorphine over a longer duration. Future studies using higher doses of both XR and ER could be conducted to determine the maximum duration achievable without clinical adverse effects.

Although we measured plasma levels in the same mice over the course of the experiment to reduce inter-animal variation, this design limited the volume of blood collected and number of time points evaluated. Future studies using a larger number of mice that are sampled at additional alternating time points could more closely determine the time point at which each drug formulation reaches and then drops below the purported therapeutic threshold. A previous study showed that XR injection in C57BL/6 mice led to plasma buprenorphine levels above 1 ng/mL as early as 30 min after injection.[4]

A growing body of evidence suggests that opioids may not be as effective in T cell deficient mice because these mice do not produce endogenous opioids of T cell origin. At times when NSG mice achieve the 1 ng/mL buprenorphine plasma level, mice of this strain still display mechanical hypersensitivity.[2] Nude mice given morphine have greater sensitivity to thermal and chemical pain than their heterozygous controls.[16] Future studies that combine pain assays and plasma drug levels are necessary to identify a true therapeutic buprenorphine plasma level in nude mice.

All 3 groups lost a significant amount of weight that was not clinically apparent. Because we found no difference between the drug groups and the saline group, we concluded that stress caused by daily anesthesia and blood collection was the likely

cause of weight loss. Other studies have similarly found no difference in weight loss between XR and control[2,15,21] or XR and ER.[2,20] This finding suggests that potential drug-induced weight loss is not a critical factor in selecting buprenorphine formulations for mouse studies. However, we did not follow the weight change past 96 h to investigate possible long-term differences.

The histopathologic analysis of the injection sites at 96 h after subcutaneous drug administration showed the presence of subcutaneous cystic lesions that had a greater degree of inflammation in mice that received ER as opposed to XR. Subcutaneous nodules have previously been reported in rats treated with ER and XR, and in mice treated with XR.[1,13,15,21] Two publications in rats found a milder inflammatory response to ER and XR than that found in the current study, but the lesions were evaluated at a later time point, between 8 d and 3 mo after injection.[13,15] One case report found subcutaneous nodules at the injection site of nude rats injected with ER and postulated that the drug was not absorbed systemically because buprenorphine was recovered from the nodules.[15] That case report did not evaluate plasma levels of buprenorphine. Although we did not try to recover buprenorphine from the subcutaneous skin nodules, our results indicate that buprenorphine was absorbed and reached purported therapeutic plasma levels.

Previous studies have observed cutaneous ulcerations at the injection site of mice treated with ER; such lesions are typically attributed to a topical reaction to ER leakage onto the skin.[2,3,6,8] The mice in the current study were injected under brief anesthesia, so leakage could be prevented by applying digital pressure around the injection site, and cutaneous lesions were not observed. The systemic effects of the varying levels of subcutaneous inflammation are not known and could be investigated in a future study; however, data from studies whose dependent variables include systemic inflammatory markers should be interpreted with caution if these drugs are used, and investigators should likely not use these drugs interchangeably. The inflammatory response in the subcutaneous tissue appeared the same between the two genotypes, suggesting the infiltrates are not due to an adaptive immune response involving T cells, but rather to an innate immune response to the compounds.

In conclusion, the labeled dose of XR maintained purported therapeutic plasma levels of buprenorphine (> 1 ng/mL) for at least 48 h in nude and heterozygous nude mice and may be an appropriate analgesic agent for these genotypes. In comparison, the manufacturer-recommended dose of ER maintained plasma levels greater than 1 ng/mL for less than 24 h and induced more inflammation at the site of injection. ER may be a reasonable choice for analgesia in procedures requiring shorter periods of pain relief in nude and heterozygous nude mice.

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