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Original Research

Effects of Extended-Release Buprenorphine on Mouse Models of Influenza

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Mice are widely used as small animal models for influenza infection and immunization studies because of their susceptibility to many strains of influenza, obvious clinical signs of infection, and ease of handling. Analgesia is rarely used in such studies even if nonstudy effects such as fight wounds, tail injuries, or severe dermatitis would otherwise justify it because of concerns that treatment might have confounding effects on primary study parameters such as the course of infection and/or the serological response to infection. However, analgesia for study-related or -unrelated effects may be desirable for animal welfare purposes. Opioids, such as extended-release buprenorphine, are well-characterized analgesics in mice and may have fewer immune-modulatory effects than other drug classes. In this study, BALB/c and DBA/2 mice were inoculated with influenza virus, and treatment groups received either no analgesics or 2 doses of extended-release buprenorphine 72 h apart. Clinical signs, mortality, and influenza-specific antibody responses were comparable in mice that did or did not receive buprenorphine. We therefore conclude that extended-release buprenorphine can be used to alleviate incidental pain during studies of influenza infection without altering the course of infection or the immune response.

Abbreviations and acronyms: ER-Bup, extended-release buprenorphine; HA, hemagglutinin; HAI, hemagglutination inhibition; IAV, influenza A virus

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Introduction

Influenza A virus (IAV) is a single-stranded RNA virus that has caused pandemics (such as in 1918) and seasonal epidemics with global effects on both human and animal health.[22] Mice are commonly used for influenza research, particularly BALB/c, C57BL/6, and DBA/2 strains.[5,18] Influenza pathogenesis in mice does not completely reflect that in humans. For example, many human influenza viruses require extensive adaptation to mice before they can efficiently infect mice, and mice do not transmit influenza virus after infection.[18] Nevertheless, mice can be used for preliminary assessments of influenza virus virulence.[3,4,28] Mice are also useful for assessment of immunologic responses to influenza virus infection and immunization, and the wide availability of well-characterized mouse reagents and mouse strains makes them particularly attractive for the analysis of immune responses.[28]

The mouse response to influenza A infection is strongly influenced by both the host and the virus strains. Mouse genetics can affect influenza A pathogenesis; for example, mouse strains such as DBA/2 are relatively susceptible to many strains of IAV, whereas the BALB/c mice are relatively resistant.[1,20,28,30,33] Both strains are susceptible, to different extents, to the 2009 H1N1 pandemic influenza strain. This virus, typified by A/California/07/2009 (H1N1pdm09), originated through extensive genetic reassortment among human, avian, and swine strains of IAVs in a swine host.[13,32] The altered virus entered the human population in 2009, causing a global pandemic in subsequent; this strain is now an endemic human seasonal influenza A strain for which mice are used as research models.[21]

Clinical signs of influenza-related disease in mice are important parameters for both describing the virulence of influenza virus strains and defining animal welfare and endpoint parameters. Mice infected with influenza show clinical signs that include weight loss, hypothermia, hunching, ruffled fur, lethargy, and death.[5,18,25]

The minimization of pain in research animals is a key goal of the refinement component of Russell and Birch's 3Rs.[31] However, although groups such as IACUCs, encourage analgesia for both expected and incidental (i.e., unrelated to protocol) pain that animals may experience while on study, researchers must consider how analgesic interventions might affect data integrity.[29]

Medications such as NSAIDs and opioids are commonly used to alleviate pain, including pain experienced by research animals both on and off study. However, the course of influenza disease in mice, including survival rate, survival time, and inflammatory responses, is modified by both steroids and NSAIDs.[23,35,39] Different classes of opioid drugs can vary in their ability to affect the immune system. Certain classes of opioids, such as full μ agonists, like morphine, alter the immune response to influenza virus when administered around the time of inoculation and during clinical manifestation of disease.[8,34] Partial μ agonists such as buprenorphine are less immune-modulatory or have minimal effects on animal immunologic responses.[9,26,37]

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The analgesic effects of buprenorphine are well characterized in research animals, including mice.[16] Buprenorphine is available in an extended-release formulation that is FDA-indexed for use in mice and rats.[11,36] Buprenorphine treatment, including extended-release buprenorphine (ER-Bup), can provide effective pain management for mice under various conditions and has been correlated with positive indicators of welfare and well-being such as nest building.[6,27] However, the effects of buprenorphine, particularly extended-release form, on the clinical signs associated with influenza infection in mice have not been specifically investigated. In addition, potential effects of buprenorphine on the serologic response to influenza have not been evaluated.

Maintaining pain management during a study while ensuring data integrity requires knowledge of effects of analgesics on animal models of infectious disease such as influenza. Although opioids are not a recommended treatment option for the clinical effects of influenza infection, they could offer relief for incidental conditions experienced by animals in a research setting for conditions such as fight wounds, tail injuries, or severe dermatitis. Based on the minimal effects of buprenorphine on the immune system in other studies, we hypothesize that ER-Bup administered at the time of inoculation and initial infection will not act as a confounder by altering the clinical signs, mortality, and antibody response of mice inoculated with influenza A. We therefore hypothesize that ER-Bup is a suitable analgesic drug to administer to mice during influenza serological studies.

Methods

Mice. Experiments using 7- to 9-wk-old female BALB/c and DBA/2 mice (Jackson Laboratories, Bar Harbor, ME) were performed in an AAALAC accredited facility at the Centers for Disease Control under a CDC IACUC-approved protocol. Mice were housed in groups of 5 in individually ventilated cages (GM500; Tecniplast, West Chester, PA) with corncob bedding (Teklad 7087C Inotiv; West Lafayette, IN); cages were changed every 2 wk. Mice had ad libitum access to pelleted food (Teklad 2018; Inotiv, West Lafayette, IN) and water supplied in water bottles. Environmental enrichment was provided in the form of red plastic huts (Bio-Serv, Flemington, NJ) and cotton squares (Ancare, Bellmore, NY) unless nesting scores were being evaluated, as described below. Environmental parameters were 68 to 75 °F, 30% to 70% humidity, and a 0600 to 1800 light:dark cycle. Mice were free of all pathogens described in FELASA recommendations as well as adenovirus, norovirus, and *Helicobacter* spp. as determined by the vendor.[24]

One day before influenza inoculation, mice were anesthetized with 4% isoflurane gas at 1 L/min oxygen flow and subcutaneous transponders (IPTT-300, BMDS; Avidity Biosciences, San Diego, CA) were implanted in the interscapular area per manufacturer's instructions, to identify individual mice and to measure temperatures.

Experimental overview and timeline. The experimental timeline and procedures are summarized in Figure 1. Experimental infections were performed in 2 replicates. Each replicate was composed of one cage of 5 mice per group (uninfected controls, infected without treatment, and infected with treatment [$n = 10$ mice per group total including both replicates]). All mice in a given cage received the same treatment. In each replicate, baseline temperatures and weights were recorded on day 0 and up to 100 µL of blood was obtained from the submandibular or facial vein of unanesthetized mice by using 4.5- to 5-mm lancets (Goldenrod; MediPoint, Mineola, NY). While under isoflurane anesthesia, mice were inoculated with the IAV designated as pdm09. Treatment groups were given ER-Bup at 0 and 3 d postinoculation.

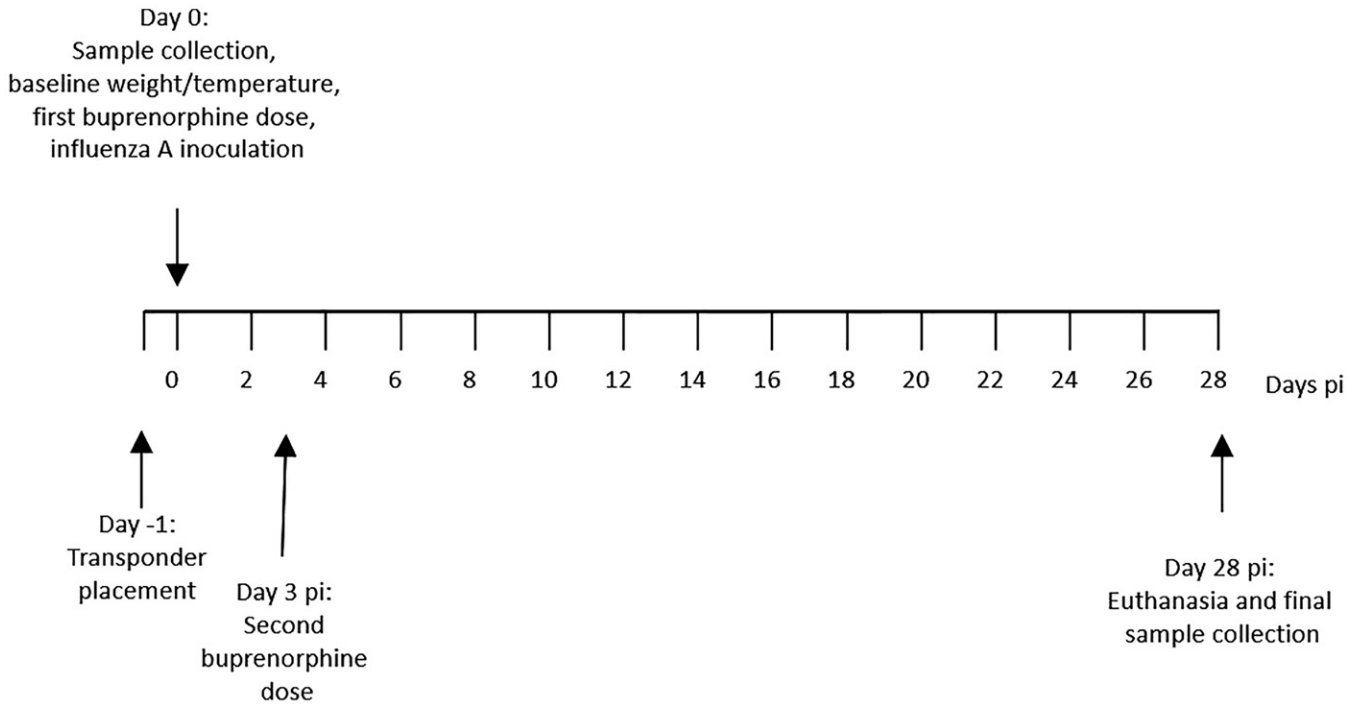

Figure 1. Timeline of experiment and procedures. BALB/c ($n = 20$) and DBA/2 ($n = 20$) mice were inoculated with human seasonal influenza A/New York/21/2009 (H1N1pdm09). $n = 10$ mice of both BALB/c and DBA/2 strains were uninfected controls. Physical parameters including weight, temperature, nesting scores, and clinical signs were tracked for 28 d. $n = 10$ of both BALB/c and DBA/2 mice were treated with ER-Bup on days 0 and 3. All mice were monitored at least once daily between days 0 and 14 and euthanized if indicated for humane reasons. Twenty-eight days after inoculation (or earlier, if euthanized for humane reasons), mice were euthanized and blood samples were collected via cardiac puncture for serological assessment. pi, postinoculation.

Body weight, temperature, and overall clinical scores, which included signs such as hunched posture, ruffled fur, lethargy, and respiratory signs, as described in Figure 2, were measured at least daily from 0 to 14 d after inoculation and at least every other day from day 16 to 28 after inoculation, with additional monitoring of mice that received clinical scores of 4 to 9. Mice that met endpoint criteria (score greater than or equal to 10) were euthanized with isoflurane overdose followed by cardiac exsanguination. No mice were permitted to naturally succumb to disease. Additional blood was collected via intracardiac collection at clinical endpoint or at the end of the study (28 d after inoculation).

Influenza virus. Influenza A/New York/21/2009 (H1N1pdm09) (pdm09) was isolated and then passed 3 times through embryonated chicken eggs. Genome sequencing and exclusivity testing was used to confirm the viral strain. All virus was kept at –80 °C until used in plaque assays and dilution for mouse inoculation. Virus plaque-forming units per milliliter were determined via a plaque-forming assay using MDCK-London cells. In brief, cells were plated at 800,000 cells per well in 6-well plates and allowed to grow overnight. Two hundred microliters of virus was diluted into 800 µL of infection media (500 mL DMEM [high glucose], 54,000 U penicillin/streptomycin, 14 mL 7.5% solution bovine serum albumin fraction, and 13.5 mL 1M HEPES buffer with TPCK-treated trypsin at 1.7 µg/mL) and serially diluted 1:10, 10 times. Five hundred microliters per dilution was plated per well and incubated for 1 h; plates were gently shaken every 15 min to ensure even infection. After an hour, infection media, sterile water, and 2.4% avicel were mixed at a ratio of 1:1:2, added over the cells, and incubated for 72 h at 37 °C. After 72 h, 10% neutral buffered formalin was used to fix the cells for 45 min. The contents of the wells were then removed and washed with water, followed by staining with crystal violet for 15 min. The crystal violet was washed out with water, plaques were counted, and plaque-forming units per milliliter were calculated. On the morning of inoculation, virus was diluted for use by suspension in cold, sterile PBS.

Infection with influenza. Mice were infected with influenza intranasally. In brief, mice were anesthetized with 4% isoflurane gas at a 1-L O_2/min flow rate in an induction chamber, removed, and held upright. Twenty-five microliters of influenza A inoculum suspended in PBS was administered in drops into each nostril with a 200-µL pipette as the drops were inhaled. Mice were then positioned in a dorsal recumbent position in its cage and monitored until recovery.

A pilot study was used to determine an appropriate dose of pdm09. In this study, BALB/c and DBA/2 mice were inoculated with varying doses of pdm09, ranging from 10^1 to 10^6 pfu per mouse. Doses that resulted in observable clinical signs of IAV infection without rapid mortality were selected. Based on these experiments, DBA/2 mice received 10^1 pfu and BALB/c received 5×10^3 pfu of pdm09.

Treatment with extended-release buprenorphine. Mice assigned to the treatment group received 0.05 mL extended-release buprenorphine (Ethiqa XR; Fidelis Animal Health, North Brunswick, NJ) subcutaneously at the interscapular area

Categories	0 points	1 point	3 points	5 points	10 points
Body weight as compared with baseline	No loss	≥10-<15% decrease	≥15-<20% decrease	≥20-<25% decrease	≥25% decrease
Appearance	Normal		Hunched; piloerection		
Temperature	Normal (>35°C)		30.1-35°C	27.1-30°C	≤27°C
Behavior	Normal		Mildly lethargic but responsive to mild stimulation; decreased ambulation or activity	Lethargic or responsive to persistent stimulation only	Unresponsive or moribund
Clinical signs	Normal	Ocular discharge		Moderate respiratory distress (i.e., labored breathing), ataxia, and/or mild to moderate dehydration	Severe respiratory distress and/or severe dehydration

Figure 2. Clinical score criteria. Mice were monitored at least daily for 14 d after inoculation with influenza A/New York/21/2009 and at least every second day between days 16 and 28 after inoculation. At each evaluation, each mouse was assigned a clinical score comprising the sum of each of the 5 criteria. Mice scoring 10 or higher were euthanized for humane reasons.

(3.25 mg/kg, with all mice weighing approximately 20 g). Mice that received extended-release buprenorphine on day 0 received the same dose again 72 h (i.e., 3 d after inoculation) based on the manufacturer's instructions.

Nesting scores. Previously published parameters were used to score nest building (0 to 5) for evaluation of the affective state of treated and untreated mice.[17] On the day of inoculation, mice were given 2 cotton squares (Ancare, Bellmore, NY) as their only enrichment. Nests were evaluated each morning before manipulation of the mice. Cotton squares were replaced on days 3 and 6 after inoculation, corresponding respectively with the second dose of extended-release buprenorphine and the time at which the drug was no longer pharmacologically active.

Serology. Serum was separated from whole blood using serum separator tubes (Sarstedt, Newton, NC) and was frozen at –20 °C until use. ELISA was performed to measure anti-HA IgG using homologous protein antigen (recombinant HA with a Histidine Tag from influenza A/California/04/2009 (H1N1pdm09)): (International Reagent Resource, FR-180) as the binding antigen, with modifications of previously described methods.[2] Serum was diluted 1:300 for an initial positive/negative screening via ELISA. Briefly, high-binding plates (Corning, Corning, NY) were coated with 100 µL of antigen at a concentration of 1 µg/mL. Plates were incubated at 4 °C overnight. The next day, plates were washed 3 times with 300 µL PBS-Tween 20 and blocked with 200 µL 1% BSA in PBS-Tween 20 (PBS-T) for 1 h at room temperature. Plates were washed 3 times with 300 µL PBS-Tween 20. 100 µL of samples were diluted 1:300 in 0.01% BSA in PBS-T and plated in duplicate. Samples were incubated for 1 h at room temperature (approximately 23 °C) and were then washed 4 times with 300 µL PBS-Tween 20. One hundred microliters of goat anti-mouse IgG-conjugated with peroxidase that was diluted 1:2,000 (Jackson ImmunoReseach Laboratories, West Grove, PA) in 0.01% BSA in PBS-T was incubated at room temperature (approximately 23 °C) for 30 min. Plates were washed 5 times with PBS-T and developed with 100 µL 3,3′5,5′-tetramethylbenzidine liquid substrate system for ELISA (catalog number t0440; Sigma-Aldrich, Burlington, MA) for 10 min. Development was stopped using 25 µL 2 M sulfuric acid, and wells were read at 450 nm using a BioTek Epoch2 microplate reader. ELISA results were recorded as the log of the titer of the highest dilution that was still positive based on our negative control cutoff.

Hemagglutination inhibition assays (HAIs) were performed as previously described.[7] Briefly, receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) was added 4:1 to serum and incubated at 37 °C for 18 to 20 h, followed by inactivation of receptor-destroying enzyme at 56 °C for 30 min. The receptor-destroying enzyme–treated serum was then treated with packed turkey red blood cells to remove nonspecific agglutinins. After treatment, the serum and blood were centrifuged for 5 min at 600 × *g*, and serum was pipetted into new tubes and stored at –20 °C until use. Influenza A/New York/21/2009 (H1N1) was diluted to 8 HA units per 50 µL in sterile PBS and confirmed via hemagglutination assay before using it in an HAI assay. Serum was serially diluted 2-fold in cold PBS from dilution of 1:20 to 1:2,560 in titer tubes and were then transferred to v-well plates. An in-lab anti-H1 monoclonal antibody was used as a positive control for the assay; PBS-only wells were used as negative controls. Serial dilutions of the virus were plated to confirm 8 hemagglutinating units per 50 µL. Twenty-five microliters of diluted mouse sera or the positive control or 50 µL PBS was transferred to v-bottom plate followed by 25 µL of virus per appropriate well. All wells were gently mixed and incubated at room temperature for 30 min. Fifty microliters of 0.5% Turkey red blood cells was added to each well and again gently mixed and incubated at room temperature for 30 min. HAI titers were then determined and recorded for analysis.

Statistical analysis. Preliminary power analysis was performed to estimate appropriate sample sizes, assuming a mean starting weight of 20 ± 1.4 g, $\alpha = 0.05$, and at least 80% power to detect a weight loss of 10%.[30] Mice were assigned to groups randomly, with all mice in a given age receiving the same treatment. Handlers were aware of the treatment and infection status. A linear mixed model with repeated measures was used to compare daily body weights, temperatures, and cumulative clinical scores (as described in Figure 2) between all groups. Serological results were compared using unpaired Mann-Whitney test between each combination of groups. Statistical analyses were performed using GraphPad Prism 9.3.1, Excel 2019, R version 4.1.3, and SAS 9.4.

Statistical analysis was conducted on both replicates of the experiment and on the combined samples. Results of the replicates were not significantly different. Therefore, the data shown represent total samples from both replicates. Statistical analyses were not performed on nesting scores because only one nest was present in each cage.

Results

Effects of extended-release buprenorphine on clinical presentation during influenza infection. Mice infected with influenza A/New York/21/2009 (H1N1pdm09) showed marked morbidity. For BALB/c mice, weight loss began around day 3 after inoculation and reached a nadir at day 7 (Figure 3A). Clinical signs that included hunched posture and piloerection were observed from days 3 through 10 in both treated and untreated infected mice (Figure 3B). Subcutaneous temperatures were not consistently altered by ER-Bup, although both groups of inoculated mice had significantly reduced body temperature around 6 and 7 d after inoculation (Figure 3C). Mortality before endpoint (i.e., euthanasia based on clinical signs before the 28-d scheduled endpoint) was relatively low (20% and 10% for untreated and ER-Bup treated, respectively), and the differences in early mortality between treated and untreated mice were not statistically different (Figure 3D). No obvious trend was seen in the nesting scores (Figure 3E).

DBA/2 mice, which are more susceptible to influenza A than the BALB/c strain,[1,20,28,30,33] received a lower viral dose (10^1 pfu compared with 5×10^3 pfu) but also showed significant weight loss between days 5 and 10, with a nadir at 8 d after inoculation (Figure 4A). DBA/2 mice that were infected and treated with ER-Bup had marginally but significantly lower weights than did infected nontreated mice on day 1 ($P = 0.0499$), but their weight trends were comparable for the remainder of the study (Figure 4A). Both nonspecific clinical signs, such as hunched posture and piloerection, and influenza-specific signs, such as labored breathing, were observed on days 7 through 13 (Figure 4B). Infection resulted in significantly lower temperatures in infected mice as compared with controls between days 7 and 10 for mice without treatment and on day 8 for mice that received ER-Bup (Figure 4C). The temperatures of the infected nontreated and treated groups were not significantly different except at 2 time points (days 8 and 9, $P = 0.006$ and $P = 0.0369$, respectively).

Up to 40% of infected DBA/2 mice were euthanized by day 9 or 10 based on clinical scores (Figure 4D) of both treated and untreated mice, with significant difference in mortality between the groups. The lowest nesting score was seen in ER-Bup–treated

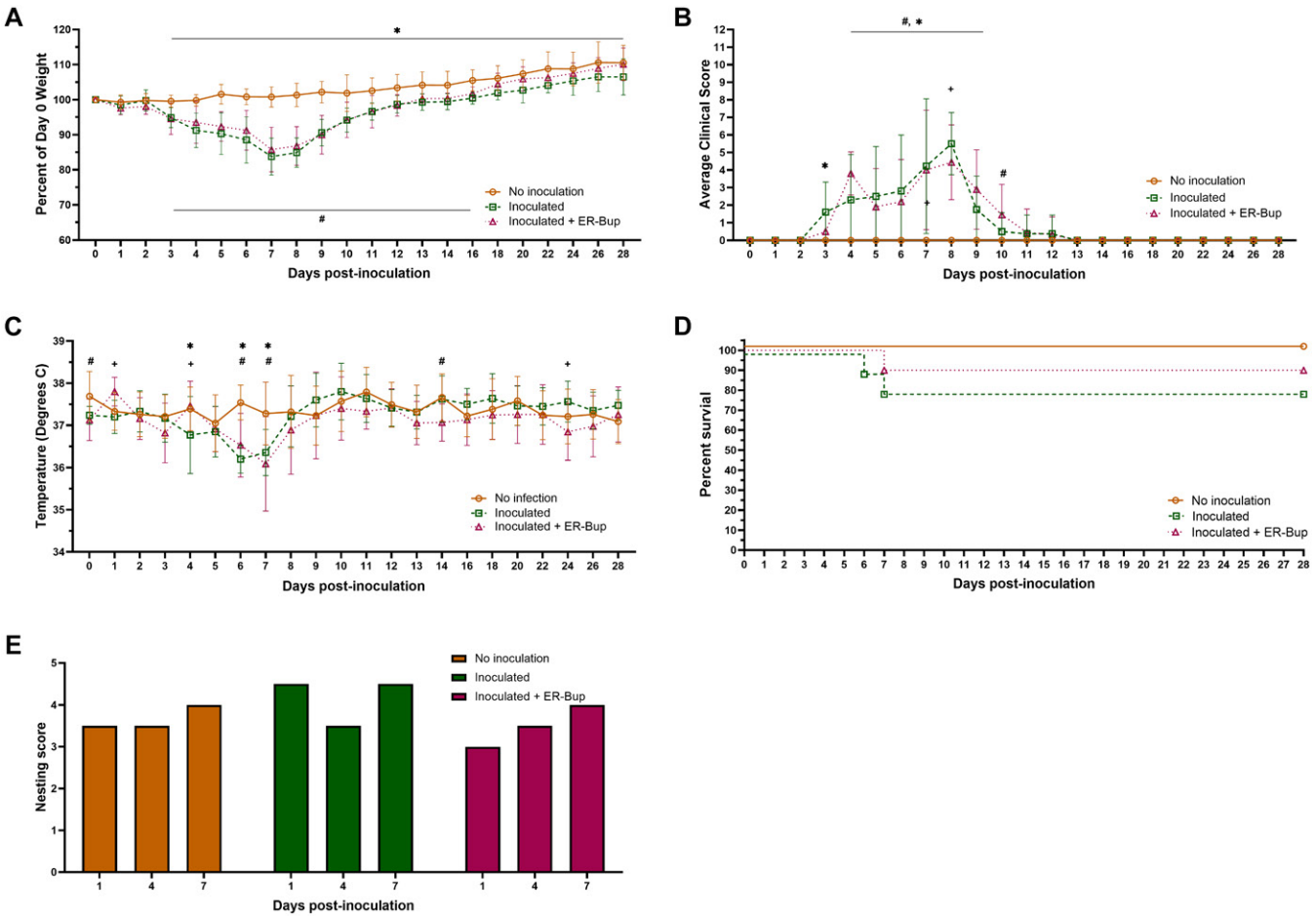

Figure 3. Influenza infection of BALB/c mice with and without extended-release buprenorphine treatment. BALB/c mice were inoculated with influenza A/New York/21/2009(H1N1pdm09) and monitored for 28 d after inoculation. (A) Body weights are shown as a percentage of the baseline weight measured on the day of but before inoculation (day 0). (B) Clinical scores determined as described in Figure 1. (C) Body temperatures (°C) were measured via a subcutaneous transponder. (D) Mortality, including mice euthanized for humane reasons, were as described in the Methods. (E) Nesting scores, as described in the Methods. In A to D, data are represented as mean ± SD. Statistically significant differences ($P \leq 0.05$) were determined using a linear mixed model with repeated measures in SAS. Symbols for specific comparisons are indicated as the follows *, inoculated mice compared with control; #, inoculated/treated compared with control; +, inoculated/untreated compared with inoculated/treated. Combined data from both replicates are shown.

mice on day 1 after infection (Figure 4E), at which mice showed few or no signs of infection (compare Figure 4A, B); during the period when weight loss and clinical signs of infection were apparent (over 5 d after infection), nesting scores were similar among all groups.

Effects of extended-release buprenorphine on serological responses to influenza infection. Based on the ELISA results, BALB/c mice infected with influenza had higher hemagglutinin (HA)–specific IgG titers as compared with untreated mice. No significant differences in IgG titers were detected between infected mice that received ER-Bup and those that did not (Figure 5A). Infected DBA/2 mice had significantly higher anti-HA titers than did uninfected controls, but IgG titers were not different between infected/treated and infected/untreated mice (Figure 5B). A possible confounder was that 2 of 10 infected/untreated and 4 of 10 infected/treated DBA/2 mice did not develop titers against influenza HA. Even when these mice were excluded from analysis, the titers of the infected/untreated and infected/treated groups were not significantly different from each other but were both significantly different from the uninfected/untreated mice (geometric mean titer: 4.5 ± 1.1 and 4.7 ± 1.2 for infected/untreated and infected/treated, respectively; $P = 0.86$).

Both groups of infected BALB/c mice had higher HAI titers than did the uninfected control mice (Figure 6A), but infected/untreated and infected/treated mice were not significantly different. Infected DBA/2 mice also had higher HAI titers than did uninfected mice (Figure 6B), with no significant differences in HAI titers of infected/treated and infected/untreated groups. When results were recalculated excluding infected mice that did not develop IgG titers, a significant difference was still not detected between the 2 infected groups (geometric mean titer: 7.3 ± 1.9 and 6.9 ± 2.0 for infected/untreated and infected/treated groups, respectively; $P = 0.42$).

Discussion

In this study, treatment of IAV-inoculated mice with 2 doses of ER-Bup did not significantly alter the clinical signs associated with infection or the serological response to infection. Data were comparable between treated and untreated groups of IAV-inoculated mice, with small variations at isolated time points. On day 8, untreated/inoculated DBA/2 mice had significantly higher clinical scores ($P = 0.007$) and on days 8 and 9 they had lower temperatures than inoculated/treated mice ($P = 0.006$ and 0.037, respectively). These differences were probably not caused by the ER-Bup because they did not change the overall

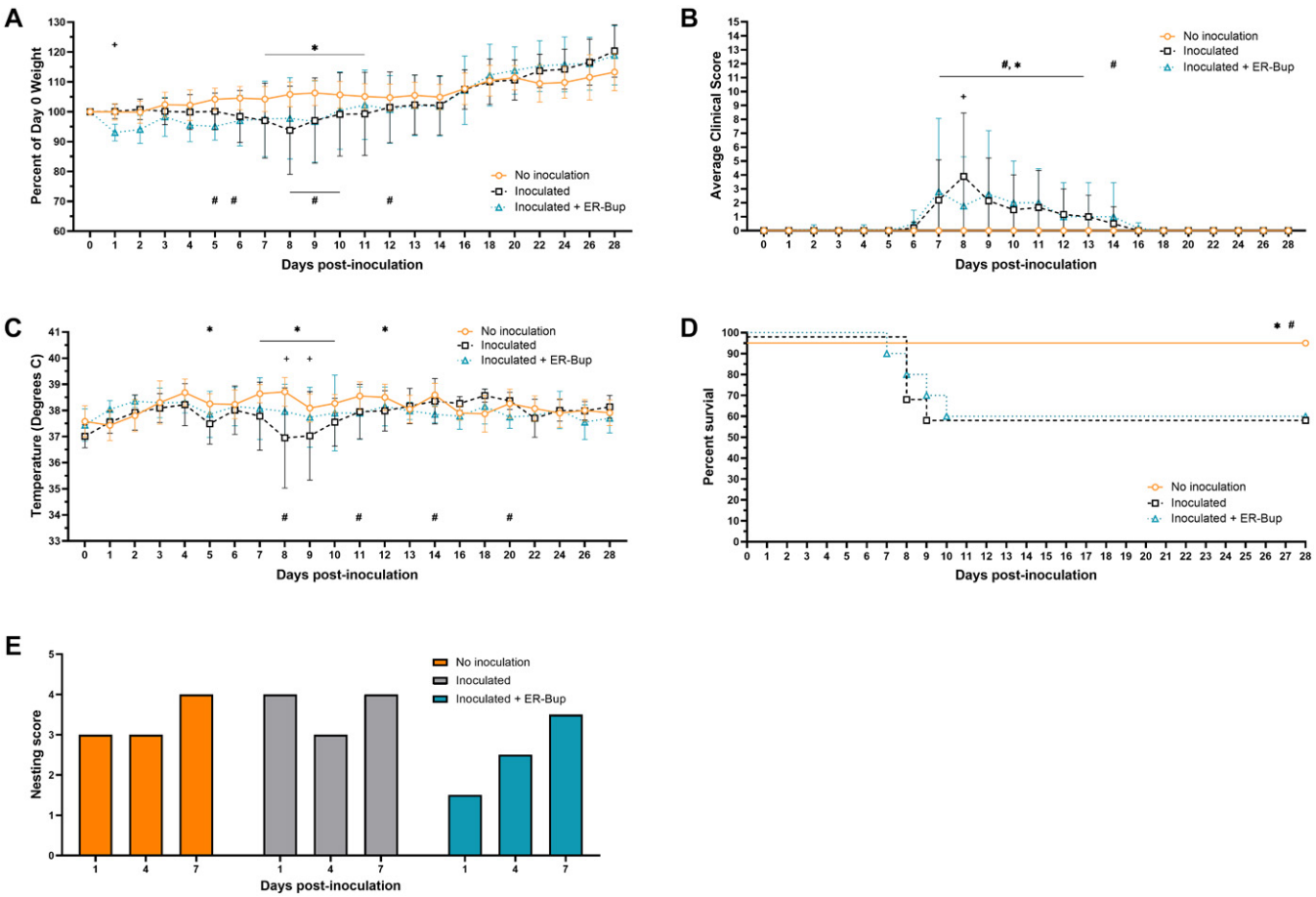

Figure 4. Influenza infection of DBA/2 mice with and without extended-release buprenorphine treatment. DBA/2 mice were infected with influenza A/New York/21/2009(H1N1pdm09) and monitored for 28 d after inoculation. (A) Body weights are shown as a percentage of the baseline weight before inoculation on day 0. (B) Clinical scores determined as described in Figure 1. (C) Body temperatures (°C) were measured via a subcutaneous transponder. (D) Mortality, including mice euthanized for humane reasons, were as described in the Methods. (E) Nesting scores were as described in the Methods. In A to D, data are shown as mean ± SD. Statistically significant differences ($P \leq 0.05$) were determined using a linear mixed model with repeated measures in SAS. Symbols for specific comparisons are indicated as the follows *, inoculated mice compared with control; #, inoculated/treated compared with control; +, inoculated/untreated compared with inoculated/treated. Combined data from both replicates are shown.

clinical and serological responses of the mice. Moreover, at this time point, the inoculated/treated mice still had higher clinical scores and lower temperatures as compared with noninoculated mice ($P = 0.008$ for day 8 clinical score differences and day 8 temperature $P = 0.02$, respectively).

The manufacturer of ER-Bup describes weight loss as a potential side effect of the drug.[12] ER-Bup treatment did not significantly alter weight loss in either DBA/2 or BALB/c mice, other than on day 1 in DBA/2 mice (Figure 4A). The reduction in weight of the treated mice could have been caused by the administration of ER-Bup on the preceding day, but weights were again not statistically different from other groups again until day 5 (Figure 4A). Weight loss was therefore likely caused by influenza A infection and not affected by ER-Bup. Also, in one case, treated DBA/2 mice had lower nesting scores 1 d after their initial treatment (Figure 4E); at this time, mice had shown no signs of influenza infection. Some studies report that ER-Bup can cause hyperactivity in mice,[27] which plausibly could reduce nest-building behavior. Alternatively, lethargy is a manufacturer-reported side effect of ER-Bup and could explain lower nesting scores.[12] However, poor nest building was inconsistent and did not occur in BALB/c mice, suggesting normal variability.

Vaccination against influenza virus is the key to preventing or reducing the widespread morbidity and mortality caused by this virus. Most influenza vaccines focus on inducing an antibody response to the influenza surface protein HA. Levels of antibody binding and HAI, which measures neutralizing antibody levels, are commonly used to determine vaccine efficacy and immune response after infection.[10,19,25,38] Immunomodulatory analgesics should therefore be avoided when studying vaccine efficacy. ER-Bup did not affect antibody production in mice inoculated with IAV, and thus our study indicates that this treatment would not confound IAV studies involving antibody production or vaccine efficacy.

A limitation of our study was the use of surrogate values (that is morbidity and mortality) rather than viral titers in the lung. Additional studies could measure viral titers in lung on days 3 or 5 after inoculation to directly measure effects of ER-Bup on viral replication.

Future work could also study the effects of ER-Bup on viral replication during active infection, respiratory tract changes, and other features of influenza immunopathogenesis. In our study, ER-Bup was administered at the time of inoculation to determine whether it would alter clinical signs and immune changes that develop several days after infection. Future work

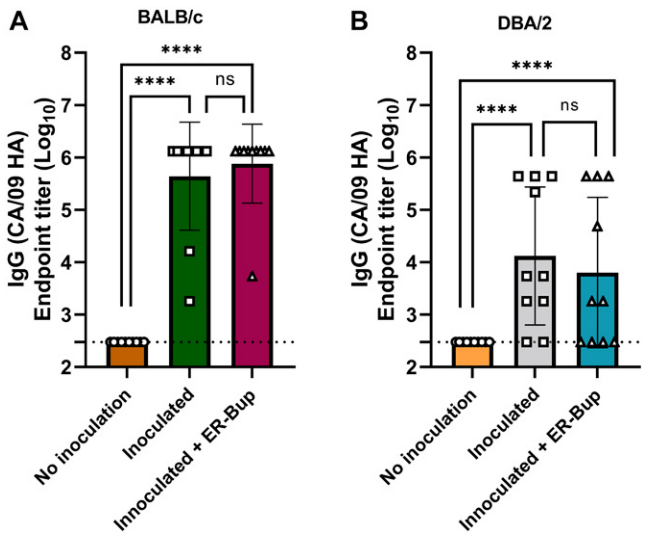

Figure 5. IgG specific for influenza A/California/04/2009. Serum from (A) BALB/c or (B) DBA/2 mice that had been inoculate with influenza and then did or did not receive treatment with ER-Bup. Sera were tested by ELISA to determine titers of antibodies specific for influenza A/California/04/2009. Results are shown as mean ± SD. Statistical analyses were performed using the unpaired Mann-Whitney test to compare each combination of groups: *, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant. Combined data from both replicates are shown.

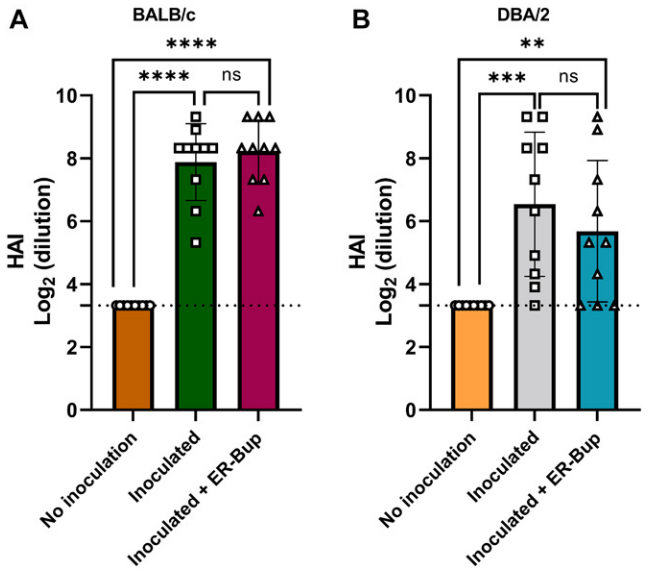

Figure 6. Hemagglutination inhibition titers in terminal sera from mice inoculated with influenza A/New York/21/2009. Serum from (A) BALB/c or (B) DBA/2 mice infected that were inoculated with influenza and either treated or not treated with ER-Bup was tested by hemagglutination inhibition assays to determine titers of antibodies specific for influenza A/California/04/2009. Results are shown as mean ± SD. Statistical analyses were performed using unpaired Mann-Whitney tests to compare each combination of groups. *, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant. Data shown are from both replicates.

could assess the effects of ER-Bup administered during active clinical signs.

Nesting scores in mice have been used to assess welfare in several models, but they have not been thoroughly investigated with regard to infectious disease.[6,14,15,17] Moreover, the nesting score used in our study was originally validated for singly housed mice or groups of 2; the use of nesting scores with up to 5 mice per cage has not been validated.[17] Future studies could also elucidate the direct benefit of social housing and environmental enrichment during infectious disease studies.

Although the use of ER-Bup may not be standard of care, it could alleviate some of the discomfort caused by IAV infection. Treated/infected BALB/c and DBA/2 showed no showed consistently significant differences in weight, temperature, clinical signs, or mortality as compared with the untreated/infected mice of the same strain. These data suggest that treating IAV-inoculated mice with ER-Bup did not reduce discomfort caused by the infection. However, ER-Bup could be used to treat mice experiencing unintended physical pain during an ongoing influenza study.[27] Mice that experience pain independent of the study design could have alterations to their immune response, such that pain therefore could be a confounding variable in immunologic studies.[9] The use of ER-Bup to reduce incidental pain should not confound data and may, in fact, reduce these physiologic variables. To alleviate the pain and distress directly caused by IAV, refinements such as valid and consistently applied pain scoring systems and endpoint criteria should be used. Based on the results of our study, we conclude that treatment with ER-Bup does not confound clinical or immunologic data in BALB/c and DBA/2 mice inoculated with IAV.

Acknowledgments

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