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Original Research

Effects of Buprenorphine Treatment on Influenza Pathogenesis in the Ferret (*Mustela putorius furo*)

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Ferrets are the gold-standard model for influenza A virus (IAV) research due to their natural susceptibility to human and zoonotic IAV, comparable respiratory anatomy and physiology to humans, and development of clinical signs similar to those seen in infected people. Because the presence and progression of clinical signs can be useful in infectious disease research, uncertainty in how analgesics alter research outcomes or compromise characteristics of disease progression have outweighed the concern regarding animal discomfort from these symptoms. Nonetheless, the principles of animal research require consideration of refinements for this important model for IAV research. Opioids offer a possible refinement option that would not directly affect the inflammatory cascade involved in IAV infection. Mirroring pathogenicity studies that use ferrets, 12 ferrets were inoculated intranasally with the A(H3N2) IAV A/Panama/2007/1999 and divided into 3 treatment groups (n = 4 each), of which 2 groups received buprenorphine treatments on different schedules and the third received a saline control. The duration and location of viral replication, lymphohematopoietic changes, and clinical signs were comparable across all groups at all time points. High quantities of infectious virus in nasal wash specimens were detected in ferrets from all groups through day 5 after inoculation, and peak viral titers from the upper respiratory tract did not differ between ferrets receiving buprenorphine treatments on either schedule. Compared with the saline group, ferrets receiving buprenorphine exhibited transient weight loss and pyrexia, but all groups ultimately achieved similar peaks in both of these measurements. Collectively, these findings support the continued evaluation of buprenorphine as a refinement for IAV-challenged ferrets.

Abbreviations: CW, conjunctival wash, IAV, influenza A virus; NW, nasal wash; RS, rectal swab

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Despite decades of international research and the availability of public health countermeasures, including vaccines and antivirals, influenza viruses remain a persistent threat to human and animal health.[26,35] Influenza A viruses (IAV) exhibit a diverse range of virulence, exist in several host reservoirs, and can show rapid rates of antigenic change.[26] As a result, IAV are associated with both seasonal epidemics and occasional pandemics in humans,[35] and animal infections with IAV have become key for understanding multifactorial traits that include pathogenicity, transmissibility, and vaccine efficacy. Due to their relatively small size, adaptability to the research setting, and similarities to human lung anatomy and physiology, ferrets provide an excellent model for respiratory diseases in humans and are a valuable small-animal model for such studies.[8,30] Data generated from ferrets are included in numerous risk-assessment rubrics evaluating the pandemic potential of novel and emerging influenza viruses, including those established by the Centers for Disease Control and Prevention and the World Health Organization.[14,51]

The study of influenza virus in ferrets dates back to the early 1930s, when this species was first found to be susceptible to influenza virus.[44] Ferrets are naturally susceptible to both human and zoonotic IAV.[47] After infection, ferrets present with clinical signs like those of humans; these signs are often not recapitulated in other species, such as mice and guinea pigs.[28,39,46] The severity and spectrum of clinical signs associated with influenza virus–inoculated ferrets can vary, depending on the virus strain, route and dose of inoculation, and various host parameters.[5] Whereas influenza viruses with low virulence in ferrets may cause only acute pyrexia and mild to moderate weight loss, isolates with high virulence can cause severe, systemic illness with gastrointestinal and neurologic symptoms.[4]

The 3Rs, replace, reduce, refine, encourage investigation of how research involving animals can be conducted in more humane ways.[2,13,37,41] Analgesia for symptoms of influenza in ferrets represents an opportunity for refinement, but this intervention could confound research assessing disease progression. NSAID and corticosteroids are often prescribed to treat the clinical signs associated with influenza in humans.[43] These interventions could alter the inflammatory cascade and subsequent pathophysiology of the disease, thus reducing the validity of studies designed to characterize and compare influenza viruses.[6,43] NSAID reportedly inhibit nuclear factor κB, a regulator of inflammatory processes that is involved in viral RNA synthesis.[25,27] In addition, NSAID have been found to increase survival rates in influenza virus-infected mice.[53] Therefore, the use of NSAID may be problematic in studies investigating the pathogenesis of influenza viruses.

Buprenorphine, an opioid, is an established analgesic in ferrets that can be administered either intravascularly, intramuscularly,

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or subcutaneously at 0.01 to 0.05 mg/kg with an analgesic duration of 6 to 12 h.[11,16,24,38,52] Historically buprenorphine has been described as a partial μ receptor agonist and κ and γ receptor antagonist,[22,29,40,48] but the drug recently was described to behave as a full μ agonist.[36] The ceiling effect of analgesia and the immunosuppressive effects reported with other opioids have not been documented to occur with buprenorphine.[15,36,42] However, the use of buprenorphine does have the possibility of adverse effects, including sedation, weight loss, constipation, and respiratory depression.[10,15,16,22,23,34,42] Nonetheless, buprenorphine is a commonly prescribed analgesic for numerous small mammalian species used in research settings.[20,22,40]

Given that influenza is an ongoing threat to human and animal health and because no replacement is available for data gained with the ferret model, pain mitigation options for research conducted in this species must be addressed. To date, concerns about altering the course of the disease have precluded the evaluation of refinements options in IAV-infected ferrets. The goal of the current study was to assess the effects of buprenorphine treatments on the pathogenesis of a seasonal IAV in ferrets; this assessment was achieved by comparing virus-inoculated ferrets that were either sham-treated or that received buprenorphine according to 2 different dosing schedules. We hypothesized that buprenorphine treatments would not affect experimental readouts, including morbidity, viral shedding, lymphopenia, and seroconversion in convalescent serum; these parameters are commonly measured during IAV research. Study results indicate that buprenorphine did not uniformly or significantly modulate disease progression, peak viral titers in the upper respiratory tract, or clinical responses used to characterize viral pathogenicity in ferrets.

Materials and Methods

Animals. Healthy male castrated and descented Fitch ferrets (n = 12; age, 9 mo; weight, 1.0 to 1.5 kg; Triple F Farms, Sayre, PA) were used in this study. Hemagglutination inhibition assays confirmed animals were serologically negative to currently circulating influenza A(H1N1)pdm09, A(H3N2), and B viruses prior to the start of the experiment. For the duration of the experiment, ferrets were housed in pairs in caging (Allentown Inc., Allentown, NJ) with grated flooring and were maintained in a Duo-Flo BioClean mobile environmental enclosure that provides HEPA-filtered air during 150 to 180 air-changes hourly (Lab Products, Seaford, DE). Water was provided ad libitum and pelleted feed (Lab Diet 5L14, St. Louis MO) was replenished twice a day. Ferrets were maintained under BSL2 conditions on a 12:12-h light:dark cycle, with a room temperature of 23 ± 3 °C and relative humidity of 35% to 50%. All experimental proceedings were IACUC-approved and conducted in an AAALAC-accredited vivarium.

Study design. Ferrets were randomly assigned into 3 groups of 4 animals each. Baseline blood samples, weights, and temperatures were collected at 3 d before virus inoculation. Temperatures were obtained via subcutaneous temperature transponders inserted into the dorsal space between the scapulae (via a 12-gauge applicator; 14 mm × 2 mm, IPTT-300, BMDS, Seaford DE). On the day of virus inoculation (day 0), group 1 received buprenorphine (0.04 mg/kg, 0.3 mg/mL, buprenorphine HCl, Phar Pharmaceuticals, New York, NY) treatments twice daily (0800 and 1600) for 5 d, from day 0 through day 4 after inoculation. Group 2 received buprenorphine treatments at the same dose and frequency from day 5 through day 9 after inoculation. Group 3 received sham treatments of sterile saline, at the same volume and frequency with 2 of the ferrets receiving treatments on days 0 through 4 and the remaining 2 ferrets receiving treatments on days 5 through day 9. All treatments were administered subcutaneously over the dorsal midline area by using 25-gauge needles and 1-mL syringes. Dosing schedules were centered around the acute phase of infection when clinical signs are most pronounced. Subcutaneous administration at 0800 and 1600 were chosen to mirror common vivarium treatment schedules and animal user proficiencies Observations regarding clinical signs, weight, and temperature were collected twice daily for the first 10 d after inoculation and then once daily through day 14, after which time ferrets continued to convalesce without research intervention until day 21. On day 21, final weights were collected, and ferrets were anesthetized for final blood collection and euthanasia (intracardiac administration of 1.0 mL/kg, 390 mg pentobarbital sodium and 50 mg phenytoin sodium per 100 mL, Euthanasia Solution, Med-Pharmex, Pomona, CA). Necropsy was only conducted on ferrets that developed significant clinical signs or adverse reactions during the study.

Ferrets were anesthetized for transponder placement, baseline sampling, virus inoculation, all blood collections, nasal washes (NW), conjunctival washes (CW), rectal swabs (RS), and euthanasia by using intramuscular combined ketamine (10 to 30 mg/kg; 100 mg/mL ketamine HCl, Zetamine, Vet One, Boise, ID) and xylazine (2 mg/kg; 20 mg/mL zylazine HCl, Anased, Akorn Animal Health, Lake Forest, IL). Assessment of general responsiveness to the environment (mentation) and observation of clinical signs were performed cage side prior to scheduled sedation. Treatment administration and weight and temperature measurements were performed on conscious ferrets, unless anesthesia for sample collection was planned, in which case these evaluations were conducted while ferrets were anesthetized.

Influenza virus challenge and assessment of viral load. The A(H3N2) influenza A virus A/Panama/2007/1999 (Panama/99) was propagated in the allantoic cavity of 10-d-old embryonated hens' eggs at 33.5 °C for 48 h as previously described.[45] Pooled allantoic fluid was clarified by centrifugation and stored in aliquots at –70 °C until use. Stock titer was determined by standard plaque assay in MDCK cells as previously described for titer determination.[45] All ferrets were inoculated intranasally under ketamine-xylazine anesthesia with 10^6 pfu of influenza virus diluted in 1-mL PBS.

Samples including NW, CW, and RS were collected on days 1, 3, 5, 7, and 9 pi for the determination of viral load via standard plaque assay, as previously described.[7,31,45] The limit of detection was 10 pfu/mL. Briefly, NW were collected by flushing the nares with 1.0 mL (0.5 mL/nostril) of sterile PBS containing 1% BSA and collecting the runoff in a sterile dish. CW were performed by flushing 0.5 mL sterile PBS BSA solution over the right eye 3 times and collecting the runoff. Immediately afterward, a sterile cotton-tipped applicator moistened in the sterile PBS–BSA solution was wiped gently over the right eye and placed into the collected CW runoff. Finally, RS were collected by using a sterile cotton-tipped applicator that was moistened in sterile PBS–BSA solution and inserted 1 to 2 in. into the rectum and then placed in a container filled with 1.0 mL of sterile PBS–BSA solution. All NW, CW, and RS samples were immediately placed on dry ice and subsequently stored at –70 °C until titration. On days –3 and 21, blood was collected from the cranial vena cava and via intracardiac puncture, respectively, and tested for antibodies against homologous virus via hemagglutination inhibition assays using 0.5% turkey erythrocytes.[45]

CBC analysis. Blood for CBC analyses was collected on days 0, 3, 7, and 14. A maximum of 1.0 mL of blood was collected via

Table 1. Clinical signs, virus replication, and seroconversion in buprenorphine-treated ferrets inoculated with IAV

Group[a]	Weight loss[b]	Temperature[c]	Respiratory symptoms[d]	Peak NW[e]	CW[e]	RS[e]	Seroconversion[f]
1	11.3 (6–8)	1.4	4/4	6.7 ± 0.5	4.3 ± 0.3 (2/4)	0 (0/4)	2560
2	14.1 (10–14)	1.5	3/4	6.4 ± 0.4	5.3 ± 0 (1/4)	1.5 (1/4)	2560
3	10.5 (6–9)	1.1	4/4	6.0 ± 0.4	3.4 ± 1.1	1.8 (1/4)	1250-2560

[a]Group 1, 0.04 mg/kg buprenorphine SC BID on days 0 through 4 after inoculation; group 2, 0.04 mg/kg buprenorphine SC BID on days 5 through 9; group 3, equal volume of saline SC BID on corresponding to buprenorphine treatments days.
[b]Percentage mean maximum weight loss during days 1 through 14 after inoculation with 10^6 pfu/mL of Panama/99 A(H3N2) IAV. Days on which maximum weight loss occurred are indicated in parentheses.
[c]Mean maximum rise in temperature (°C) above baseline temperature (37.9 to 39.3 °C) during observational period.
[d]Number of ferrets with observed respiratory symptoms (sneezing, nasal discharge).
[e]Maximum viral titer (recorded on days 1 through 5 for each animal; mean ± 1 SD) in ferret nasal wash (NW), conjunctival wash (CW), or rectal swab (RS) specimens, expressed as $\log_{10}$ pfu/mL (limit of detection, 10 pfu/mL; the number of ferrets with infectious virus detected is specified in parentheses when not 4/4).
[f]Seroconversion (range in titer from hemagglutinin inhibition assay is shown; ferrets in groups 1 and 2 had the same titer) to homologous virus collected on day 21. Serum from one ferret in group 2 was collected prior to euthanasia on day 17.

venipuncture of the cranial vena cava. Blood was immediately transferred to a 1.5-mL EDTA tube, gently inverted, and then kept cold until analyzed (Antech Diagnostics, Smyrna, GA) within 24 h of collection.

Clinical signs. Clinical presentation was observed throughout the study. Weight and temperature were recorded at least once daily through day 14. Cageside observation of clinical signs (lethargy, nasal or ocular discharge, sneezing, reduced feed intake, and diarrhea) were recorded at each check and before any scheduled anesthesia. Bright, alert, and responsive animals were considered to have normal mentation. Any ferret that lost more than 25% of its preinoculation body weight or exhibited neurologic dysfunction was euthanized.

Statistics. Descriptive values are presented as mean and standard deviation. Baseline clinical signs (body weight and temperature), viral titers, and hematologic values from individual ferrets were compared with respective time point values using 2-way ANOVA with the Tukey posttest; peak NW titers were log-transformed and analyzed by using 1-way ANOVA with the Tukey posttest. Significant differences were defined as those with a P value less than 0.05. Prism (version 7.05, GraphPad Software, San Diego, CA) was used for data analysis and figure development.

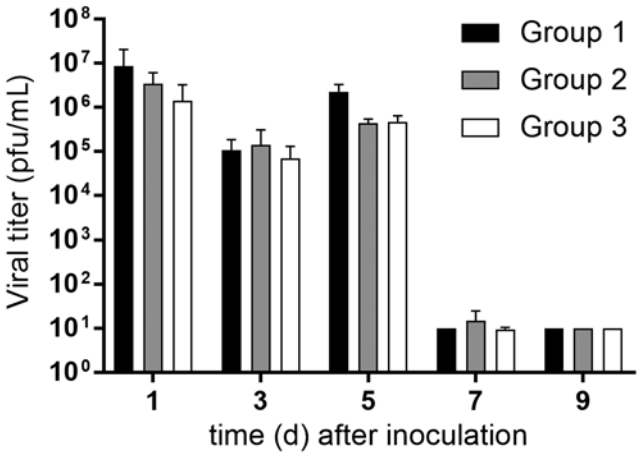

Figure 1. Replication of IAV in buprenorphine-treated ferrets. Ferrets (group 1, buprenorphine on days 0 through 4; group 2, buprenorphine on days 5 through 9; group 3, saline controls) were inoculated intranasally with 10^6 pfu of Panama/99 IAV. Nasal wash specimens were collected on alternate days after inoculation and titered for the presence of infectious virus by using a standard plaque assay. Data given as mean (bar, 1 SD) values from 4 ferrets per group per day of specimen collection. *, $P \leq 0.05$ by 2-way ANOVA. Limit of detection, 10 pfu/mL.

Results

Influenza virus replication in buprenorphine-treated ferrets. IAV-infected ferrets often develop transient fevers as early as 12 to 24 h after inoculation and can exhibit other morbidities thereafter; however, analgesics are often withheld due to concerns about altering research outcomes. Although NSAID are likely to significantly affect IAV disease progression, opioids pose no obvious confound to research design and offer a refinement to the model. For these reasons, we examined how buprenorphine treatment administered at the time of these expected morbidities might influence virologic outcomes. All ferrets were inoculated intranasally with the Panama/99 virus. Ferrets received buprenorphine twice daily for 5 d, during days 0 through 4 (group 1) or days 5 through 9 (group 2); control ferrets (group 3) received saline on the same schedule (Table 1). Ferrets in all groups were monitored twice daily after inoculation, and multiple specimens (NW, CW, and RS) were collected on alternate days throughout buprenorphine treatment for the assessment of viral replication in both respiratory and extrapulmonary sites.

The kinetics and magnitude of IAV replication were evaluated through measurement of viral replication and serologic titers. All inoculated ferrets were productively infected with IAV, and infectious virus was detected in all NW specimens on days 1 through 5. Mean viral titers in NW specimen were comparable between groups at all time points, with the exception of significantly ($P = 0.0095$) higher mean NW titers (compared with controls) on day 1 in ferrets that received buprenorphine on days 0 through 5 (Figure 1). However, peak NW titers did not differ among groups ($P > 0.05$, Table 1). Infectious virus was detected sporadically in CW and RS specimens on days 1 through 5 in all groups, independent of buprenorphine treatment schedule; 17% to 33% of CW specimens and 0% to 25% of RS specimens had detectable virus on days 1 through 5 (Table 1). Furthermore, comparable antibody titers to homologous virus were detected in convalescent serum collected on day 21 in all groups (Table 1). Collectively, these data indicate that viral replication and elicitation of humoral antibody responses was not substantially influenced by buprenorphine treatments administered during the acute phase of IAV infection.

Modulation of CBC parameters after IAV infection in buprenorphine-treated ferrets. Transient lymphohematopoietic changes associated with influenza virus infection are often used to characterize disease progression.[6,32,33] Lymphopenia during the acute phase of influenza virus infection is frequently observed in ferrets inoculated with seasonal IAV, typically resolving to preinoculation levels within 2 wk after viral challenge.

To assess whether buprenorphine treatment altered the kinetics or magnitude of CBC measurements, peripheral blood was collected prior to virus inoculation (day 0) and at several points after inoculation (days 3, 7, and 14). Absolute total WBC counts were determined (Table 2). All groups demonstrated the expected leukocytosis, with lymphopenia and neutrophilia, which resolved by day 14. Both groups of buprenorphine-treated ferrets showed significant differences in total WBC over time as compared with controls and with each other. These differences were not consistently associated with either treatment period or disease progression. Differences in the relative percentage of lymphocytes or neutrophils (expressed relative to total WBC values per group per day) were not statistically different between groups at any collected time points (Figure 2). Other hematologic values, including platelet and RBC counts, did not differ significantly between groups at any time point (data not shown). These findings indicate that WBC fluctuations did not differ markedly between virus-infected ferrets that received buprenorphine and those given saline only.

Clinical signs after IAV infection in buprenorphine-treated ferrets. Clinical signs in ferret IAV models are most pronounced during the acute phase of infection but typically resolve within 2 wk after inoculation. Assessments of IAV pathogenicity in this model include many of these parameters, but how buprenorphine treatment might modulate these features was unknown.

Through day 14, pyrexia, weight loss, sneezing, lethargy, nasal discharge, reduced feed intake, and diarrhea were observed to various degrees in all groups. A single ferret reached the weight-loss end point day 17 (described below); all other ferrets recovered without additional clinical intervention. Ocular discharge was not observed in any ferret. Clinical signs occurred predominantly during the first 10 d after inoculation, with individual and group variation (data not shown). All clinical signs were transient (less than 36 h), except for consistent sneezing on days 5 through 8 in a single ferret that received buprenorphine on days 0 through 4, and irregular lethargy on days 1 through 9 in the ferret that reached the weight loss endpoint. Sneezing, reduced feed intake, and lethargy were the most frequently observed symptoms; respectively, 92%, 75%, and 42% of all ferrets displayed these clinical signs, which occurred in 75% or more, at least 50%, and 25% to 50% of ferrets in each of the 3 groups. Normal mentation was observed in all ferrets, with lethargy ascribed to being slow to rise from sleep and showing reduced escape efforts during handing. Given the eating habits of ferrets and pair housing, unfinished food rations were considered to indicate reduced feed intake.

Ferrets from all groups exhibited moderate weight loss after IAV challenge (mean peak, 10.5% to 14.1%; Table 1, Figure 3 A) that returned toward baseline by the end of the study. Ferrets that received saline had a 10.5% mean maximal weight loss after inoculation with Panama/99 virus, in agreement with previous studies conducted without use of analgesics; weight loss in the buprenorphine-treated ferrets was also within the expected range for this well-studied virus.[18,54] However, some amount of weight loss occurred during buprenorphine treatment windows and in ferrets that received saline on the same schedule (Figure 3 B and C). One ferret that received buprenorphine on days 5 through 9 had a greater than 25% weight loss and was euthanized on day 17. This ferret was observed to be lethargic after inoculation, and its cage intermittently had remaining food, indicating reduced food intake. On gross pathologic examination, this ferret had hepatomegaly, with rounded edges and marked jaundice; otherwise, it was grossly normal. These findings, in conjunction with reduced feed intake, support a diagnosis of hepatic lipidosis likely secondary to influenza symptoms, buprenorphine treatments, or their combination. Given the lack of additional clinical signs and the gross findings and weight rebound of other members in this cohort, a primary cause for the hepatomegaly could not be identified. Aside from the continued weight loss, which did not reach humane endpoint criteria until after day 14 (after the acute phase of the infection), data from this ferret were comparable to others in the cohort. Because hepatic lipidosis often has a rapid progression, this condition was not likely to have influenced the collected samples. For these reasons, data from this ferret were included in all analyses.

Ferrets inoculated with seasonal IAV typically present with transient fever (defined here as 1 °C or more above

Table 2. CBC counts of IVA-infected ferrets that received buprenorphine

Cell type (reference interval; ×1000/μL)[19]	Time (d) after inoculation	Cell count (×1000/μL; mean ± 1 SD)[a]		
		Buprenorphine on days 0–4	Buprenorphine on days 5–9	Saline controls
WBC (3.0–16.7)	0	6.7 ± 1.4	4.1 ± 0.9	6.1 ± 1.3
	3	8.0 ± 1.2[b]	5.5 ± 0.7	7.0 ± 0.6[b]
	7	9.3 ± 0.9	7.0 ± 0.9	8.9 ± 0.8
	14	6.2 ± 4.1	5.5 ± 1.5	5.3 ± 0.5
Lymphocytes (0.6–10.5)	0	4.2 ± 1.3	2.6 ± 0.8	3.4 ± 1.0
	3	4.0 ± 1.0[b]	2.9 ± 0.3[c]	3.7 ± 1.4[b,c]
	7	3.2 ± 1.3	2.8 ± 0.8	4.2 ± 0.9
	14	3.9 ± 0.1	2.9 ± 1.6	3.3 ± 0.5
Neutrophils (0.9–7.4)	0	2.0 ± 1.3	1.2 ± 0.2	2.3 ± 0.6
	3	3.7 ± 0.9	2.4 ± 0.4	3.1 ± 0.8
	7	5.6 ± 0.9[b,c]	3.7 ± 1.1[b]	4.0 ± 1.2[c]
	14	1.9 ± 1.1	2.4 ± 0.7	1.6 ± 0.2
Monocytes (0.0–0.5)	0	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
	3	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
	7	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
	14	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Eosinophils (0.0–0.7)	0	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
	3	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
	7	0.2 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
	14	0.2 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
Basophils (0.0–0.2)	0	0.0 ± 0.1	0.5 ± 1.0	0.0 ± 0.0
	3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	7	0.0 ± 0.0	0.3 ± 0.6	0.0 ± 0.0
	14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]Values are from 4 ferrets per group, except for group 3, day 3 ($n = 3$ due to sample coagulation in one specimen prior to analysis). Significant ($P < 0.05$) between-group differences in WBC, lymphocytes, and neutrophils over time were determined through 2-way ANOVA with the Tukey posttest; similar superscript letters indicate significant differences between groups according to day.

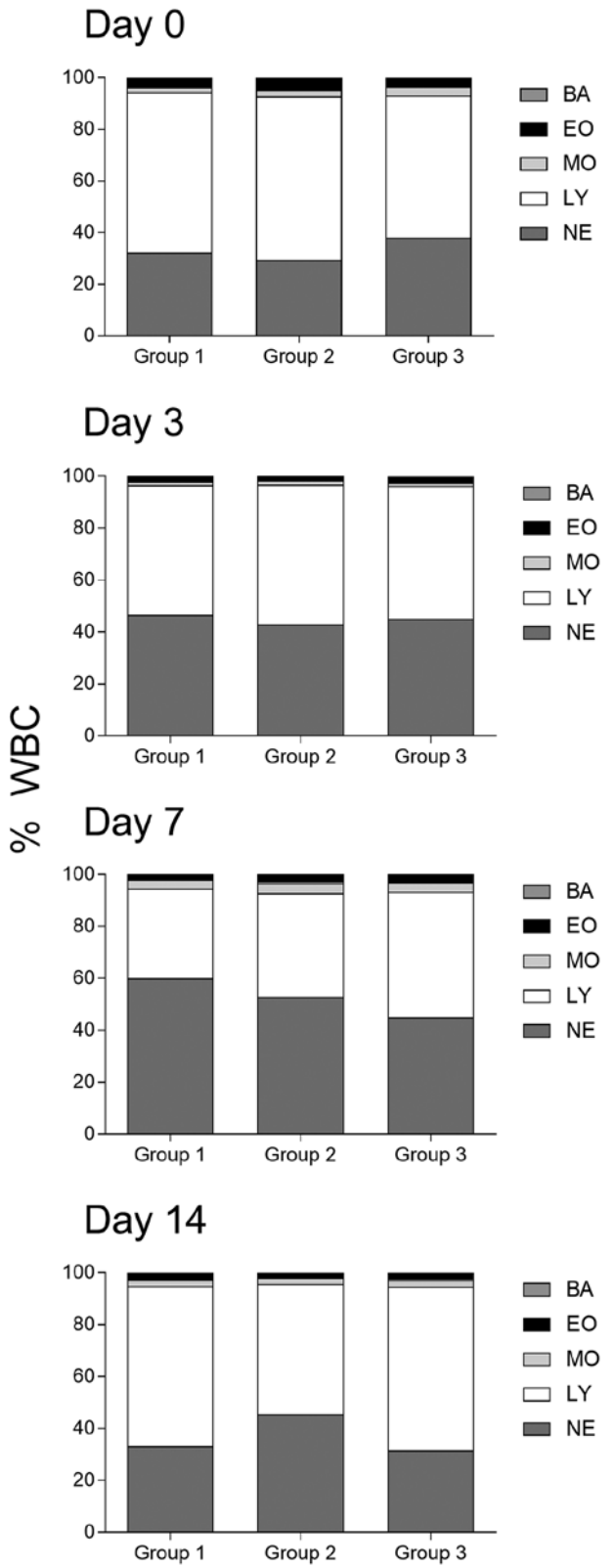

Figure 2. Kinetic analysis of circulating lymphocytes during IAV infection in buprenorphine-treated ferrets. Ferrets (group 1, buprenorphine on days 0 through 4; group 2, buprenorphine on days 5 through 9; group 3, saline controls) were inoculated intranasally with 10^6 pfu of Panama/99 IAV. Blood was collected in EDTA vacuum phlebotomy tubes on the days indicated and analyzed on a hematology scanner. Mean average percentages of lymphocytes (LY), neutrophils (NE), basophils (BA), monocytes (MO), and eosinophils (EO) in whole blood are shown (means from $n = 4$ ferrets per group except for group 3, day 3 [$n = 3$ due to sample coagulation in one specimen prior to analysis]).

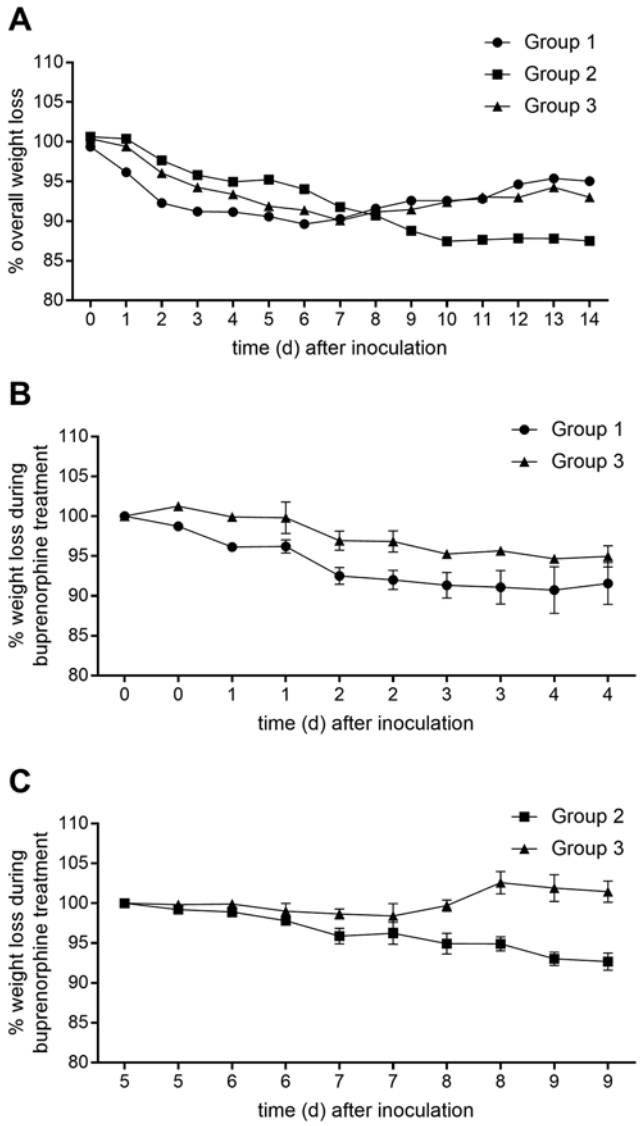

Figure 3. Weight loss in IAV-infected ferrets receiving buprenorphine-treatment or saline. Ferrets were inoculated intranasally with 10^6 pfu of Panama/99 IAV. (A) Daily mean weight change from preinoculation body weight for all groups ($n = 4$ per group). (B) Ferrets received buprenorphine treatment (group 1, $n = 4$) or saline (group 3, $n = 2$) days 0–4 pi; (C) Ferrets received buprenorphine (group 2, $n = 4$) or saline (group 3, $n = 2$) on days 5 through 9. (B and C) Body weights were collected twice daily (0800 and 1600), with the span of buprenorphine or saline treatment shown in each graph; body weight percentages were set at 100% on the first day of buprenorphine administration.

preinoculation baseline temperature) during the acute phase of infection, and infection with seasonal IAV may be associated with sneezing and other clinical signs. After inoculation with the H3N2 IAV, transient pyrexia was detected in all ferrets, with comparable temperature peaks among groups (Table 1), generally recorded within the first 72 h after inoculation. Similarly, expected clinical signs such as sneezing, nasal discharge, lethargy, and diarrhea were recorded across all groups (data not shown). Collectively, these findings indicate that buprenorphine treatment did not alter these assessments of virus pathogenicity in IAV-infected ferrets.

Discussion

Human and animal health is dependent on the continued research of influenza viruses using in vivo models. Although

ferrets provide invaluable data to IAV research and risk assessment,[4,8,30] concerns regarding the effects of analgesics on research outcomes have historically outweighed recommendations for pain mitigation. Because the information gained from ferrets cannot currently be replicated by using other models or in vitro methods, refinements for this model should be identified. Our study replicated routine IAV studies that evaluate viral shedding, associated morbidities, seroconversion in convalescent serum, and changes in peripheral blood cell values to determine whether buprenorphine treatment would alter these parameters. Our results indicate that buprenorphine treatments did not affect IAV replication or disease progression but had a modest and transient effect on weight loss. These findings support the use of buprenorphine in IAV ferrets without concerns regarding possible effects on research outcomes.

In the current study, intranasal inoculation of Panama/99 virus resulted in productive virus replication in the upper respiratory tract and convalescent serum titers in all ferrets. Collection and titration of NW specimens allows for the detection of viral replication in the nasal cavity and can be representative of replication in additional areas of the respiratory tract due to mucus propulsion associated with sneezing.[5] Our finding of high-titer virus in NW specimens, with sporadic infectious virus detection in CW and RW samples, is in agreement with prior studies evaluating Panama/99 virus and other seasonal IAV that are generally restricted to replication in the respiratory tract.[4] Except for a few IAV with enhanced mammalian virulence, NW titers traditionally peak in the early stages of infection (days 1 through 5 after inoculation) and then drop below levels of detection within 7 d of high-dose virus inoculation.[6,18,31] Mean viral titers in NW specimens from ferrets in all groups remained high (exceeding $10^{4.8}$ pfu/mL) through day 5, independent of buprenorphine administration or treatment schedule (Figure 1). NW titers on day 1 were significantly higher in ferrets that received buprenorphine on days 0 through 4 as compared with controls, but a significant titer elevation was not found in ferrets that received buprenorphine on days 5 through 9; mean day 1 titers exceeded $10^{5.8}$ pfu/mL and were within 1 log across all groups. Furthermore, the comparable NW titers between groups at all other times points examined suggest that this difference is likely due to interindividual differences among ferrets from an outbred stock and does not reflect treatment-specific confounders. Although similar studies using IAV in ferrets have not been conducted previously, our findings agree with a recent assessment of buprenorphine treatment in prairie dogs inoculated with monkeypox virus,[20] further supporting buprenorphine as an analgesic refinement that does not alter viral replication and kinetics.

Along with viral replication data, ferrets demonstrate lymphohematopoietic changes that are similar to those of humans during IAV disease progression.[6,39] All ferrets in our study developed expected and comparable leukocytosis, with lymphopenia and neutrophilia that resolved by day 14 (Table 2). At no time point did erythrocyte or platelet values vary significantly between groups ($P > 0.05$, data not shown). Significant differences in CBC counts occurred in all 3 comparisons (group 1 compared with 2, group 1 compared with 3, group 2 compared with 3) and occurred during, before, and after treatment. These sporadic differences between groups were not related to treatment schedules or disease progression. Given that Panama/99 virus is a low-virulence IAV, dramatic and nonresolving WBC trends were not expected; future studies with high virulence IAV, which are known to cause pronounced WBC changes, may help to clarify the effects seen in our study.[6,31,54] All ferrets in our study had comparable antibody titers to homologous virus in convalescent serum. These findings, in combination with our data on viral replication, support that buprenorphine treatments do not disrupt measurements of IAV pathogenicity.

Clinical signs and the magnitude of their effects in ferrets infected with IAV are often comparable to those in humans infected with the same strains. Although buprenorphine treatments were associated with greater weight loss in our ferrets, expected clinical signs (such as fever, sneezing, nasal discharge, lethargy, diarrhea, and reduced feed intake) occurred in all groups regardless of treatment schedule. Weight loss after buprenorphine treatment is well described in rodents but is not a regularly cited concern in other research animals.[1,17,34,40] When buprenorphine was studied as a refinement in monkeypox infection in prairie dogs, maximal weight loss was greater in infected prairie dogs that received buprenorphine treatments but patterns of weight loss were similar in infected untreated and uninfected treated animals.[20] Except for one ferret in group 2 that reached the humane endpoint due to weight loss, all groups had comparable weight loss that was trending back to baseline by day 14. This 25% weight loss in a single animal is unlikely to be due to buprenorphine treatment but rather due to an interindividual difference, given that no other ferrets approached this endpoint. In summary, peak measurements of weight loss and fever were comparable among all groups (Table 1), indicating that mild perturbation of these parameters due to buprenorphine treatment does not substantially affect summary measurements of IAV pathogenicity or introduce adverse reactions.

Although comparison of laboratory species is commonly based on size and model, species-specific factors such as diet and behavior should also be considered in determining appropriate comparisons. Prairie dogs infected with monkeypox represent an alternate animal infectious model, but a more applicable comparison to ferrets would be mink, which are from the same genus (*Mustela*). However, publications involving the use of buprenorphine in mink are limited to either short-term studies[21] or single-dose administration.[49,50] Given the recent COVID-19 outbreaks in commercial mink farms, studying the effects of buprenorphine in this genus could benefit future research models and agricultural animals alike. As with vendor production of ferrets, commercial breeding of mink may reduce genetic outliers in respective populations and thereby improve comparability.

Our findings that buprenorphine treatments do not confound research outcomes of IAV pathogenicity after infection with a seasonal H3N2 IAV open the door for future studies to explore these effects on a broader range of research applications, including evaluations of analgesia efficacy and administration refinement. Furthermore, future studies of influenza A challenge could focus on viral strains that have high virulence in ferrets, are capable of high-titer replication throughout and beyond the respiratory tract, elicit changes in respiratory depression, and more frequently lead to augmented clinical signs of infection in sham-treated ferrets. Similar evaluations of buprenorphine treatments in ferrets infected with a range of genetically distinct IAV would collectively provide more robust and informative data than would larger group sizes against a single challenge virus.[9] Considering the critical role of ferrets in the assessments of virus transmissibility, future studies to determine the influence of buprenorphine administration on transmission

would be valuable.[3] Our study administered treatments based on historical descriptions of peak clinical symptoms, but the clinical need for buprenorphine may differ by both time point and duration depending on the virus strain, dose, route of inoculation, and health status of the animal. Although our study recorded clinical symptoms, future studies that compare the duration and severity of symptoms before and after treatment would provide more guidance on clinical efficacy of these treatments. This need is apparent in the literature, because most publications that study buprenorphine administration in ferrets are pharmacologic reports rather than evaluations of efficacy. Pharmacokinetics and efficacy of several buprenorphine formulations have been documented in various other species. The standard buprenorphine formulation used in our study requires at least twice-daily administration. However, both extended-relief and buccal forms of buprenorphine have been used with success in other species.[12] In addition, single administration and needleless administration both could be useful for ferret challenge studies that involve zoonotic influenza viruses, which are conducted in BSL3 environments.

Using a seasonal H3N2 IAV, we found that buprenorphine treatments administered on 2 different schedules did not substantially modulate viral replication, disease progression, or clinical responses used to characterize viral pathogenicity in ferrets. Given the importance of this model for IAV research, the findings from this study and others that support the use of buprenorphine without confounding studies of viral pathogenesis. Collectively, these findings support buprenorphine treatments as a possible analgesic refinement in ferret IAV pathogenesis.

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