Impact of Analgesics on Disease Pathogenesis in a Murine Model of Crimean-Congo Hemorrhagic Fever Virus

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Citation

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Abstract
In the development of infectious disease models, no suitable replacement for the use of animals that are capable of modeling such complex disease processes are currently available. There is a scientific imperative to ensure that animal models are relevant and translatable while upholding the moral and ethical obligation to relieve pain in animals. The use of Hazara virus (HAZV) in B6.129S2-Ifnar1tm1Agt/Mmjax (IFNAR) mice is currently being used as a BSL-2 surrogate model of Crimean-Congo hemorrhagic fever virus (CCHFV) infection. We hypothesized that discomfort associated with Hazara virus infection in female mice genetically lacking type I interferon signaling (IFN-I) could be treated with acetaminophen treated drinking water or sustained release buprenorphine (Bup-SR) without significantly altering disease pathogenesis. Our study found that neither of these low labor analgesics were acceptable treatment options. Acetaminophen treated water resulted in inadequate dosing due to decreased consumption making it an unsuitable analgesic for this model. Subcutaneously administered Bup-SR did not significantly alter disease pathogenesis but had unacceptable side effects in the form of neuroexcitation following administration. Results of this research study will be used to guide future considerations regarding the use of analgesics in CCHFV and other infectious disease models.

INTRODUCTION
On institutional animal care and use committee (IACUC) protocols, a common justification for withholding analgesics is a concern that these compounds may confound results. Such concerns must be balanced against a moral obligation to perform humane research, the potential confounding effects of unrelied pain, and consistency with pain-relieving practices in human medicine. Historically, analgesics have been withheld in many infectious disease models due to their potential impact on the immune response. In addition, pain and response to analgesic treatment can differ between sex, age, and strain of rodent making it difficult to predict potential experimental effects or interactions. For these reasons, research studies are needed to assess the impact of analgesics on specific infectious disease models before concluding that use of analgesics would have an unacceptable impact on study results.

Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the Nairoviridae family within the order Bunyavirales, is an important human pathogen that is both endemic and emerging throughout Asia, Africa, and Europe. A common feature of acute disease is liver injury ranging from mild to fulminant hepatic failure. The processes through which CCHFV induces severe liver injury are unclear, mostly due to the limitations of existing small animal systems. Historically, the only small-animal model in which CCHFV consistently produces severe liver damage was mice lacking type I interferon (IFN-I) signaling through STAT-1 gene deletion, disruption of the IFN-1 receptor gene, or antibody-mediated Type I interferon blockade. Recently Hazara virus (HAZV), another member of the Nairoviridae family within the order Bunyavirales and closely related to CCHFV, has shown promise as a BSL-2 surrogate model for CCHFV infection in mice lacking type I interferon. This murine infection model recapitulates the CCHFV-mediated hepatic injury observed in humans with elevated liver enzymes and marked liver pathology. Liver pathology correlates with the presence of HAZV.
antigen, which can be detected in hepatocytes, Kupffer cells, endothelial cells, and stellate cells. Consistent with human disease, infected mice also have high levels of inflammatory systemic cytokine activity, including TNF alpha and IL-6 activity. Thus, HAZV shows promise as a BSL-2 mouse model that can be used to provide insight into the pathogenic processes that lead to the liver injury and mortality incurred by CCHFV infection.

In contemplating the appropriateness of withholding analgesics, this study sought to test the hypothesis that discomfort associated with HAZV infection in female mice genetically lacking IFN-I could be treated with sustained release buprenorphine (Bup-SR) administered by subcutaneous injection, or acetaminophen (APAP) administered in drinking water, without significantly altering disease pathogenesis. We sought to minimize the handling stress of sick animals by selecting analgesics that could be given in water or only required a single injection. In an effort to make our experimental use of analgesics more translatable to human medicine, we utilized a “trigger-to-treat” model in our study design in which treatment was administered once clinical signs were observed. Based on earlier work with HAZV in IFNAR mice, we expected our first clinical sign of disease to be weight loss at Day 3 post-infection with most animals succumbing to disease between Day 5 and Day 6. By starting analgesic administration on Day 3, we hoped to simulate health-seeking behavior manifested by humans. In addition, by waiting until disease was established to initiate treatment, we hoped to minimize the immune modulating effects of analgesics on the infectious disease model. For the purposes of our study, significantly altering disease pathogenesis was defined as statistically significant changes in viremia levels, liver enzymes (ALT / AST), TNF alpha and IL-6 cytokine activity, as well as liver histopathology in infected animals receiving analgesics when compared to infected control animals. Differences in weight loss and water consumption were used as potential indicators of improved welfare resulting from use of analgesics.

**MATERIALS AND METHODS**

**Animals, husbandry, and welfare.** All animal research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the AAALAC International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council.8

The study population comprised 56 Female B6.129S2-Ifnar1tm1Agt/Mmjax mice (Mus musculus; age 5 to 12 week; The Jackson Laboratory, Bar Harbor, ME). Animals represented unused inventory from a breeding colony maintained by Jackson Laboratory for institute use. These mice were used opportunistically as a form of animal reduction. All animals were negative for the following pathogens and opportunistic organisms: *Ectromelia* virus, *Theiler* mouse encephalomyelitis virus, *Hanta* virus, *K* virus, lactate dehydrogenase-elevating virus, lymphocytic choriomeningitis virus, murine adenovirus, murine cytomegalovirus, murine hepatitis virus, murine minute virus, murine parvovirus, murine thymic virus, murine norovirus, pneumonia virus of mice, polyoma virus, reovirus 3, rotavirus, Sendai virus, *Bordetella* spp., cilia-associated respiratory bacillus, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Corynebacterium bovis*, *Pasteurella* spp., *Helicobacter* spp., *β*-hemolytic *Streptococcus* spp., *Klebsiella pneumoniae*, *Pneumocystis murina*, *Pseudomonas* spp., *Proteus mirabilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Mycoplasma pulmonis*, *Salmonella* spp., *Streptobacillus moniliformis*, *Encephalitozoon cuniculi*, fleas, fur mites, lice, follicle mites, pinworms, roundworms and other helminths, tapeworms, and opportunistic protozoa (for example, Giardia, *Spirillum*). Mice were housed in groups of 3 to 9 in solid-bottom polycarbonate cages (Allentown Caging, Allentown, NJ; Lab Products, Seaford, DE) on autoclaved cellulose bedding (7070C Teklad Certified Diamond Dry Cellulose Bedding, Envigo, Madison, WI), were fed an autoclaved standard pelleted diet (no. 2018S Teklad Certified Mouse Rodent Diet, Envigo, Madison, WI), and were provided autoclaved municipal water using sipper water bottles without restriction. To minimize water loss from the drinking bottles, the husbandry and veterinary staff were requested to not handle the racks or cages. For enrichment the mice were provided autoclaved compressed cotton squares (Nestlets, Ancare, Bellmore, NY) and paper nesting material (EnviroDri, Fibercore, Cleveland, OH) along with polycarbonate igloo/nestlets (BioServ, Flemington, NJ). The room temperature range was maintained at 68 to 79°F (20.0 to 26.1°C) with a set point of 74.5°F (23.6°C), relative humidity was maintained at 30% to 70%, and the room was on a 12 h light and 12 h dark...
cycle. Mice were uniquely identified using Stoelting small animal ear tags and provided an acclimation period of at least 1 week prior to commencement of experiment.

**Experimental design.** Mice were randomized to 1 of 6 experimental groups (Table 1). Following randomization, weight difference was assessed using the Kruskal-Wallis test which found there was no significant difference between groups (P = 0.9518). Groups 1-3 were infected experimental groups and groups 4-6 served as uninfected controls for hematology and serology assays. Body weight and water consumption were measured and recorded daily beginning on Day -7. On day 0, Groups 1-3 were infected intraperitoneally (IP) with 100 PFUs of HAZV. Following infection, mice were monitored twice daily for signs of disease. At the pre-determined trigger-to-treat time point of Day 3 infected mice were given 1 of 3 treatment options. Group 1 (n=15) was the experimental control group and received a 0.02 mL SC injection of 0.9% NaCl. Group 2 (n=16) was the first analgesic test group and received acetaminophen treated drinking water (1.1mg/mL) and a 0.02 mL SC injection of 0.9% NaCl. Group 3 (n=16) was the second analgesic test group and received a 0.02mL (1.0mg/kg) dose of Bup-SR. Uninfected negative control mice (Groups 4-6) were also given 1 of 3 treatment options on Day 3. Group 4 (n=3) received a 0.02 mL SC injection of 0.9% NaCl. Group 5 (n=3) received acetaminophen treated drinking water (1.1mg/mL) and a 0.02 mL SC injection of 0.9% NaCl. Group 6 (n=3) received a 0.02mL (1.0mg/kg) dose of Bup-SR. On Day 4 a subset of infected mice from Group 1 (n=6), Group 2 (n=7), and Group 3 (n=7) were euthanized subsequent to anesthesia to capture data at disease mid-point. Terminal blood sampling was performed under deep anesthesia to measure viremia, cytokine levels, and liver enzymes (AST, ALT). Liver and spleen samples were collected at necropsy and examined for histopathology and presence of virus (n=3 per group). Mice were chosen for the mid-point serial sac based on severity of scoring and percentage of weight loss with the most clinically affected animals in each group selected. Three mice (Group 1=1, Group 2=2) met early endpoint criteria and were euthanized on Day 4. Two mice succumbed to disease (Group 3=2) and remaining mice (Groups 1-6) were euthanized subsequent to anesthesia on Day 5. Terminal blood sampling was performed under deep anesthesia to measure viremia, cytokine levels, and liver enzymes (AST, ALT). Liver and spleen samples were collected at necropsy and examined for histopathology and presence of virus (n=3 per group).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>PVU</th>
<th>Pain Treatment</th>
<th>Viremia (Day 4)</th>
<th>Cytokines (Day 5)</th>
<th>Terminal Blood (Day 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>300</td>
<td>0.02 mL SC NaCl</td>
<td>200±50</td>
<td>7±2</td>
<td>3±2</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>300</td>
<td>Acetaminophen</td>
<td>400±100</td>
<td>12±3</td>
<td>6±3</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>300</td>
<td>Bup-SR 1.0mg/kg</td>
<td>300±50</td>
<td>8±2</td>
<td>5±2</td>
</tr>
</tbody>
</table>

**Acetaminophen solution.** The recommended dose of oral acetaminophen is 110 to 305mg/kg. At an anticipated total daily consumption of 3 to 6 mL per mouse, the desired concentration of water was approximately 1.1mg/mL to achieve a dose within the recommended range (approximately 200mg/kg). To prepare acetaminophen-treated water, 34.5 mLs of cherry favored dye free acetaminophen liquid (Infants' Pain & Fever, CVS, Woonsocket, RI) was mixed with 1000 mLs of autoclaved municipal water. The acetaminophen concentration was 160 mg per 5 mL, resulting in drinking water containing acetaminophen at 1.1 mg/mL. In order to prevent neophobic avoidance, acetaminophen treated drinking water was provided to Groups 2 and 5 for two days beginning at Day -7 prior to being introduced as a treatment on Day 3. The amount of water consumed (increment, 0.1g) was determined for all groups beginning on Day -7 by weighing the bottles daily in the morning when mice were weighed. The scale (Scout Pro SP601, OHAUS Corp) was calibrated daily before and after data collection using certified weights. A control water bottle was kept in a separate cage containing no animals in the same housing rack and removed from the rack and cage at each time points when cages were manipulated (that is, during each assessment activity). The control water bottle was used to calculate any loss from the bottle that was not associated with consumption in order to provide a more accurate measurement of water consumption. After accounting for control bottle loss, the difference in weights of the water was divided by the number of mice per cage to determine the average amount of water consumed in milliliters per day per mouse beginning on Day -6. The average acetaminophen dose per mouse was calculated by taking the average amount of water ingested (in mL) multiplied by the concentration (1.1mg/mL) of acetaminophen in the water divided by the average weight (in g) of the mice in the cage each day multiplied by 1000 (to get mg/kg).
Buprenorphine-SR. The recommended dose of sustained release buprenorphine (Bup-SR) in mice ranges from 0.6 to 1.5mg/kg. Day 3 treatment for Groups 3 & 6 consisted of a 0.02mL subcutaneous injection (0.5-mL syringe, 31-gauge needle) of Bup-SR (1.0 mg/mL stock, Buprenorphine SR Lab ZooPharm, Windsor, CO). Based on a 15 to 22g weight range this represented a 0.91 to 1.3mg/kg dose. Mice not receiving Bup-SR were given a 0.02mL SC injection of sterile saline using identical procedures to control for the handling stress of the SC injection on Day 3. All injections were administered subcutaneously between the shoulder blades. In hopes of avoiding previously reported injection site skin lesions the Bup-SR was allowed to come to room temperature before it was injected into the mice.

Virus. Huh7 propagated in Dulbecco’s Modified Eagle Medium with Earle’s Salts (DMEM) (Corning) supplemented with 10 % (vol/vol) fetal bovine serum (FBS) (Gibco) 10 U/ml Penicillin/10µg/ml Streptomycin (Gibco), 1% 100mM Sodium Pyruvate (Sigma), and 1% 200 mM L-Glutamine (HyClone) and 1% 1M HEPES (Gibco). Hazara virus (HAZV) strain JC-280 was provided courtesy of the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch. This stock of HAZV was previously passaged 11 times in suckling mouse brains and we further passaged it twice in Huh7 cells. The virus was collected from clarified cell culture supernatants and stored at -80 °C.

RT-qPCR. Mouse serum samples were inactivated using a 3:1 ratio of TRIzol LS Reagent (Thermo Fisher Scientific) and stored at -80° C. Total nucleic acid was purified using the EZ1 Virus Mini Kit v 2.0 (Qiagen) on the EZ1 Advanced XL robot (Qiagen) according to the manufacturer’s recommendations. Viral load was determined in triplicate using a real-time RT-PCR assay specific to HAZV. Challenge stock virus was used to generate the standard curve, allowing calculation of the number of PFU equivalents (PFUe) in the tested samples.

Liver enzymes. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured in serum from HAZV-infected animals using a Piccolo Xpress (Abaxis) and a General Chemistry 13 panel (Abaxis) following a 1:3 dilution with PBS.

Histology. Liver and spleen were collected at necropsy. Tissues were immersed in 10 % neutral buffered formalin for 30 - 50 days. Tissue were then trimmed and processed according to standard protocols. Histology sections were cut at 5 - 6 µM on a rotary microtome, mounted onto glass slides and stained with hematoxylin and eosin (H&E). Examination of the tissue was performed by a board-certified veterinary pathologist.

In Situ Hybridization (ISH). To detect Hazara virus (HAZV) genomic RNA in formalin-fixed paraffin-embedded (FFPE) tissues, in situ hybridization (ISH) was performed using the RNAscope 2.5 HD RED kit (Advanced Cell Diagnostics) according to the manufacturer’s instructions. Briefly, an ISH probe targeting nucleotides 166-2095 of HAZV genome (GenBank access# NC_038710.1) was designed and synthesized by Advanced Cell Diagnostics. FFPE tissue sections underwent deparaffinization with Xyless II (Val Tech Diagnostics, LabChem Inc.) and a series of ethanol washes and peroxidase blocking. The sections were then heated in kit-provided antigen retrieval buffer and digested by kit-provided protease. Sections were exposed to ISH target probe pairs and incubated at 40°C in a hybridization oven for 2 hours. After rinsing with wash buffer, ISH signal was amplified using kit-provided Pre-amplifier and Amplifier conjugated to alkaline phosphatase and incubated with Fast Red substrate solution (Advanced Cell Diagnostics) for 10 minutes at room temperature. Sections were then stained with hematoxylin (Poly Scientific R&D Corp), air-dried, and mounted.

Cytokine and chemokine analysis. Serum cytokine and chemokine analysis was performed using a magnetic bead-based plex mouse panel (Thermo fisher) targeting the indicated molecules. 25 µl of serum per mouse per time point was used. Plates were analyzed on a MAGPIX system (Millipore Sigma) and quantitated against standard curves using MILLIPLEX analyst software.

Statistical methods and data analysis. All analyses were performed using SAS Version 9.4. For weight, descriptive statistics including mean, standard deviation, minimum, and maximum were calculated for each group based on individual observations. Average water consumption of each group was recorded and calculated based on pan observations. Due to reduced blood sample collection, statistical analysis of certain parameters was limited in selected groups. Cytokine levels were limited to four mice per group per time point from groups 1-3. Viremia levels were limited to five mice per group per time point.
from groups 1-3. Analysis of liver enzymes was also limited to five mice per group at the later time point for groups 1 and 3 only. Normality was assessed by Shapiro-Wilks tests. For all groups, an insufficient number of variables were normally distributed, warranting a non-parametrized approach to assessing differences. As day 4 and day 5 samples were taken from different animals, Wilcoxon rank sum tests were used to assess differences between study day 4 and 5 within groups 1, 2, and 3. Kruskal Wallis tests were used to assess differences on Day 5 between groups 1, 2, and 3, as well as groups 4, 5, and 6.

**RESULTS**

**Body weight.** Mice weighed 15-22 g on the day of viral challenge. Compared with baseline measurements, all experimentally infected animals (Groups 1-3) showed an initial drop in body weight at 72 hr after infection ranging from 4% to 15% (Figure 1B). Negative trending body weight continued in experimentally infected mice with final weight loss ranging from 17% to 24% at the time of euthanasia. In comparison, mock infected control animals (Groups 4-6) showed stable or slightly increased body weight (Figure 1A). In the HAZV infected Groups 1-3, there was no statistical difference between group weights. In the HAZV uninfected Groups 4-6 there was also no statistical difference between group weights.

**Water intake.**

Infected mice averaged between 2.8 - 4.0 mL/day water consumption across all treatment groups until Day 3 post-infection when consumption dropped to less than 1.1 mL/day and remained below 2.2 mL/day for all groups for the remainder of the study (Figure 2). Water consumption was slightly higher in the Bup-SR group compared with the other infected treatment groups but was still decreased compared to the pre-infection baseline. The acetaminophen treated water infected group had the lowest water consumption of the three infected groups with an average water consumption of 0.14mL on Day 3, 1.28mL on Day 4, and -0.2mL on Day 5 post-infection. As a result, mice received a sub therapeutic dosing of acetaminophen throughout the experiment. In comparison, uninfected control mice drank 2.6 – 5.1 mL/day with no substantial changes in water consumption post-infection across all treatment groups.

**Viremia.** We examined viral load on days 4 and 5 using five serum samples per infected group at each time point (Figure 3). No significant differences in viremia were detected between infected groups (Kruskal Wallis tests) or within groups between study day 4 and study day 5 (Wilcoxon rank sum tests). These findings indicate that viral replication did not vary significantly between untreated infected animals and those given subcutaneous Bup-SR or subtherapeutic dosing of acetaminophen treated drinking water.

**Liver Enzymes.** Statistical analysis of liver enzymes was limited to infected Groups 1 and 3 on Day 5 due to low serum volumes. Liver enzymes were extremely elevated across all infected groups on days 4 and 5 requiring a 1:3 dilution with PBS in order to fall within the assay range needed for the Piccolo Xpress (Abaxis) to generate results. Wilcoxon rank sum tests showed no statistical significance between groups 1 and 3 for either AST or ALT liver enzyme levels (Figure 4). These results indicate elevated liver enzymes did not vary significantly between untreated infected animals and those given subcutaneous Bup-SR.

**Cytokines.** Cytokine activity was evaluated by multiplex ELISA (Magpix) on days 4 and 5 in infected mice (Groups 1-3) using four serum samples per infected group at each time point (Figure 5). Cytokine activity was evaluated on day 5 in uninfected mice (Groups 4-6) using three serum sample per group. IL-10, IL-1beta, IL-2, IP-10, IL-4, IL-5, IL-6, IL-22, IL-9, IL-13, IL-27, IL-23, IFN-Gamma, IL-12p70, GM-CSF, Gro-alpha/KC, RANTES, TNF-alpha, MIP-1alpha, MCP-3, MCP-1, IL-17, MIP-2, Eotaxin, IL-18, MIP-1beta were all analyzed. According to the Wilcoxon rank sum tests, there were no statistically significant differences between the cytokine levels of study day 4 and study day 5 for groups 1, 2, and 3. Kruskal Wallis tests showed no statistical significance between the cytokines of groups 1, 2, and 3 on study day 5. Kruskal Wallis tests also showed no statistical significance between groups 4, 5, and 6 on study day 5, except for parameter IL_5. This data indicates that cytokine activity did not vary significantly between untreated infected animals and those given subcutaneous Bup-SR or subtherapeutic dosing of acetaminophen treated drinking water.

**Pathology.** Microscopic evaluation of tissues (liver and spleen) included histopathology and in situ hybridization of 27 animals. Animal numbers were limited to three mice per group on Day 4 and Day 5 in Groups 1-3. Mice in Groups 4-6 were euthanized on Day 5 and served as uninfected background controls. Tables 2-3 summarize the
Impact of Analgesics on Disease Pathogenesis in a Murine Model of Crimean-Congo Hemorrhagic Fever Virus

Histopathological findings per treatment group for each infected mouse, separated by day of euthanasia. Table 4 summarizes the in-situ hybridization scoring for all infected groups.

Table 2

<table>
<thead>
<tr>
<th>Organ</th>
<th>1 Saline SC</th>
<th>Acetaminophen H20 (immediate)</th>
<th>2 Acetaminophen H20 (delayed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Spleen</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Histological grading was based on these criteria: 0, no lesions in excess of background levels; 1 (minimal), 10% or less of the cells in the section are affected; 2 (mild), between 11% and 25% of the cells in the section are affected; 3 (moderate), between 26% and 50% of the cells in the section are affected; 4 (marked), between 51% and 79 of the cells in the section are affected; 5 (severe), between 80% or more the cells in the section are affected.

$n = 3$ mice evaluated per group

Table 3

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<tbody>
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<td>4</td>
</tr>
<tr>
<td>Spleen</td>
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<td>2</td>
<td>2</td>
</tr>
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$n = 3$ mice evaluated per group; M annotates missing spleen sample

Table 4

<table>
<thead>
<tr>
<th>Organ</th>
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<th>Acetaminophen H20 (immediate)</th>
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<tr>
<td>Spleen</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Intensity of staining was graded on the following scale: 1 (minimal), 10% or less of the cells in the section are immunoreactive; 2 (mild), between 11% and 25% of the cells in the section are immunoreactive; 3 (moderate) between 26% and 50% of the cells in the section are immunoreactive; 4 (marked) between 51% and 79 of the cells in the section are immunoreactive; 5 (severe), between 80% or more the cells in the section are immunoreactive.

Comparison of Groups 1 (HAZV + Saline) and Group 2 (Acetaminophen H20 + HAZV + Saline):

Within the liver, three of the animals in group 2 had marked lipid degeneration, but this was similar to what was present in group 1 animals euthanized on day 5. There was slight variation in the degree/severity of all types of necrosis between groups 1 and 2; however, no specific pattern emerged. The degree/severity of inflammation and presence of fibrin thrombi was similar between groups.

Within the spleen, lymphocyte depletion was less severe for the early euthanasia time point in group 2 as compared to group 1. However, groups 1 and 2 were similar for the later day 5 euthanasia time point. Additionally, the lymphocyte apoptosis appeared slightly increased in two animals in group 2. This variation between groups was slight and the significance unclear. The extramedullary hematopoiesis (EMH) was less in group 2 as compared to group 1 for two animals (note – one spleen is missing from group 2). The severity of histiocyte accumulation and presence of fibrin thrombi in the spleen were also less severe in group 2 as compared to group 1. These variations between groups were slight and the specific relationship, if any, to acetaminophen administration is unclear.

More animals in group 2 had a marked (compared to moderate) ISH score in the liver as compared to group 1 (5/6 versus 3/6 respectively), but ISH scoring in the spleen was similar between groups.
Comparison of Groups 1 (HAZV + Saline) and Group 3 (Buprenorphine-SR SC + HAZV):

The degree of lipid degeneration in the liver between groups 1 and 3 was similar. There were some slight variations in the degree of necrosis between groups 1 and 3, but a specific pattern did not emerge. Two animals in group 3 had mild coagulative necrosis, compared to all minimal coagulative necrosis in group 1; however, one animal in group 3 had no coagulative necrosis. Severity of inflammation and the presence of fibrin thrombi was similar between groups for the liver.

Lymphocyte depletion was similar between groups 1 and 3, but lymphocyte apoptosis was moderate for three animals in group 3, and only mild for all animals in group 1. EMH was similar between the two groups. Histiocyte accumulation was less severe in group 3 and there were fewer fibrin thrombi as compared to group 1. This difference between groups was slight and the specific relationship, if any, to buprenorphine administration is unclear.

ISH scoring in the liver is identical between groups 1 and 3. In animals euthanized on day 5, more animals had a moderate (versus mild) ISH score in the spleen in group 3 as compared to group 1 (3/3 versus 1/3 respectively).

**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**
The ethical justification of animal research requires a careful balance between the potential benefits of research against the cost to experimental animals in the form of pain or distress experienced as a result of that research. Researchers must strive to maximize animal welfare while minimizing the pain and distress of the animals involved. When laboratory animals are utilized for studies that may cause pain or distress and in which there is a scientific justification for the withholding of relief, then ethically—at least from a utilitarian perspective—the benefits must outweigh the costs. Despite a general acceptance of both the ethical imperative and regulatory requirements intended to maximize animal welfare, the use of effective regimens for mitigating pain remains underutilized in infectious disease research. Factors contributing to the gap between the need for and the actual use of analgesia include lack of sufficient evidence-based data on effective regimens, under-dosing due to labor required to dose analgesics at appropriate intervals, and concerns that the use of analgesics may impact study...
outcomes. Experiments assessing analgesic efficacy are challenging and results vary widely depending on the species, strain, sex, model, and environment. For these reasons we designed a research study to specifically explore the model impact of two low labor analgesics in a newly developed ABSL-2 CCHF model utilizing female IFNAR mice.

Altered disease pathogenesis in this study was defined as statistically significant changes in viremia levels, liver enzymes ALT & AST, TNF alpha and IL-6 cytokine activity, and/or liver pathology in infected animals receiving analgesics when compared to infected control animals. Results showed that viral replication did not vary significantly between untreated infected animals and those given subcutaneous Bup-SR or subtherapeutic doses of acetaminophen treated drinking water. Low serum volumes limited the analysis of elevated liver enzymes to Group 1 and Group 3 on Day 5, but results indicated that elevated liver enzymes AST and ALT did not vary significantly between untreated infected animals and those given subcutaneous Bup-SR. Although our study looked at a multitude of cytokines, the cytokines of greatest interest and relevance to our model were TNF alpha and IL-6. High levels of these two cytokines correlate to more severe disease in humans with CCHFV and are strongly associated with fatal outcomes. Our results showed that cytokine activity did not vary significantly between untreated infected animals and those given subcutaneous Bup-SR or subtherapeutic doses of acetaminophen treated drinking water. Pathology results suggest that model specific lesions (Figure 6) such as lipid degeneration, necrosis, neutrophilic inflammation, and the presence of fibrin thrombi were similar across treatment groups. Histopathological findings did reveal a few minor variations in results between Group 1 and Groups 2 and 3, but the changes were slight and patterns suggesting a specific treatment effect of acetaminophen or buprenorphine, which may have altered the disease pathogenesis, are not evident histologically. However, this absence may be based on the low number of animals involved in the study or the subtherapeutic dose of acetaminophen ingested. According to ISH findings, virus appears to localize to areas of inflammation and lytic (but not coagulative) necrosis (Figure 7). More animals in Group 2 have a higher ISH score as compared to group 1. However, owing to the low number of animals involved in the study, it is unclear if this finding is treatment related. These results support our hypothesis that discomfort associated with acetaminophen’s impact on viremia, cytokine activity, and pathology in these animals. Based on our study results we determined that analgesics delivered via drinking water are unsuitable for this model and moving forward it would be prudent to rely only on parenteral administration of analgesics for relief of pain or discomfort. Additionally, it is worth noting that acetaminophen is well documented to alter disease pathogenesis. Unfortunately, interpretation of acetaminophen treated drinking water on disease pathogenesis is severely limited by the fact that mice received far less than the intended 200mg/kg target dose of acetaminophen. As a result, we were not able gather interpretable data to test our hypothesis that discomfort associated with HAZV infection in IFNAR mice could be treated with acetaminophen administered in drinking water, without significantly altering disease pathogenesis.

A key take away from our study was that use of voluntary ingestion of analgesics should be avoided in this rodent model due to reduced water intake resulting in inadequate dosing. By the time analgesics were initiated on Day 3, water consumption had decreased by 76% to 96% across the infected groups when compared to Day 2. While the intended target dose of oral acetaminophen in Group 2 had been 200mg/kg, the actual dose administered based on water consumption data was 77mg/kg on Day 4 and 0mg/kg on Day 5. Oral self-administration of analgesics has several advantages, but its effectiveness has also been criticized as voluntary ingestion of the drug can be uncertain. Prior to selecting acetaminophen treated drinking water as a study analgesic, we tested our intended water consumption monitoring technique on an earlier leg of HAZV model development that utilized antibody-mediated IFN-1 blockade in C57BL/6J mice. In these earlier studies that relied upon disruption of IFN-1 signaling, water consumption remained adequate in the face of universal weight loss and 50% lethality. Switching to IFNAR mice genetically lacking functional IFN-1 signaling in later model development resulted in increased model lethality and dramatically decreased water consumption. The fact that Group 2 animals received inadequate dosing of acetaminophen significantly limits the value of study data related to acetaminophen’s impact on viremia, cytokine activity, and pathology in these animals. Based on our study results we determined that analgesics delivered via drinking water are unsuitable for this model and moving forward it would be prudent to rely only on parenteral administration of analgesics for relief of pain or discomfort. Additionally, it is worth noting that acetaminophen is well documented to cause liver injury in mice (and humans), including hepatic necrosis and increases in liver enzymes depending on the dose administered. However, differences in the level of hepatic necrosis were not observed between groups that did
and did not receive acetaminophen in this study, likely a reflection of the limited dosing the animals received.

Due to fulminant nature of HAZV disease severity in mice, Bup-SR was an attractive parenteral option for our study because a single subcutaneous dose given in a trigger-to-treat method on Day 3 was expected to provide analgesic coverage for the entire symptomatic period of disease. Unfortunately, during the first 4-8 hours after SC administration of Bup-SR signs of adverse opioid-induced effects were seen in the form of neuroexcitation (mania) in all mice administered the drug. While the neuroexcitation resolved without any resulting loss of study animals, moving forward we would caution against the use of Bup-SR in this specific strain and model due to the significant side effects seen in our study.

Non-pharmacologic pain management is an often overlooked technique that should be further explored in this model. Based on the severity of dehydration seen, administration of fluids either subcutaneously or intraperitoneally may prove extremely beneficial in future studies. We found this mouse strain easy to handle during daily weighing activities but challenging to restrain for injections. Therefore, for future studies we recommend exploring the use of isoflurane anesthesia to minimize handling related stress prior to administering intraperitoneal or subcutaneous injections in IFNAR mice.

In conclusion, both of the low labor analgesics explored in our study were deemed unacceptable for use in this model. Subcutaneously administered Bup-SR did not significantly alter disease pathogenesis in our model, but had unacceptable side effect. Acetaminophen treated drinking water resulted in subtherapeutic dosing due to decreased water consumption. Additional studies are needed to optimize analgesic selection, dosing regimen and efficacy for this model. Future studies exploring non-pharmacologic pain management techniques are highly recommended.

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