The effects of flunixin meglumine on viral shedding in calves

by

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Abstract

Background – The effects of a Food and Drug Administration approved non-steroidal anti-inflammatory (NSAID) on viral shedding in cattle has not been investigated.

Hypothesis/Objective - The hypothesis of this study was that flunixin meglumine would increase the magnitude and duration of viral shedding in calves inoculated with bovine herpesvirus-1 (BHV-1). Another objective was to investigate the consistency of BHV-1 shedding in inoculated calves.

Animals - Twelve Holstein cross-bred steer calves, BHV-1 PCR-negative.

Methods – In this randomized-control study, calves were randomized into treatment (FM) and control (CON) groups. All calves were inoculated intranasally with approximately 4-mLs of $1x10^5$ $TCID_{50}$ of BHV-1. Nasal swabs for BHV-1 PCR testing were collected every 24 hours following inoculation. Calves in the FM group were treated with 2.2mg/kg of flunixin meglumine intravenously after the first BHV-1 PCR positive sample. Magnitude and duration of viral shedding between groups was compared, and a p-value of <0.05 was considered significant.

Results - The least square mean of the BHV-1 total log_{10} AUC of the FM group was 6.11 (95% CI: 4.62-7.60) and CON group was 7.49 (95% CI: 6.00-8.98). The FM group's mean duration from initial to last positive BHV-1 PCR was 7.33 days (Range: 3-12, SD: 4.41), and the CON group's mean was 9.5 days (Range: 1-17, SD: 5.80). There was a treatment-by-time interaction, and there was no difference in BHV-1 shedding by treatment group when comparing mean log_{10} $TDIC_{50}$.

Conclusions and Clinical Importance – Intranasal inoculation with BHV-1 had a wide range of shedding magnitude and duration. Flunixin meglumine administration in BHV-1 challenged calves did not result in an increase in magnitude or duration of viral shedding.

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Chapter 1 - Literature Review

Bovine respiratory disease (BRD) affects cattle across all production systems within the United States (US), causing significant economic loss. Greater than 15% of feedlot cattle show clinical signs of BRD, and BRD is associated with at least 50% of deaths in feedlots.[1,2] This paper reviews the causes and clinical signs of BRD, the pulmonary immune response related to BRD, the mechanism of action of NSAIDs, the history of NSAIDs, and the effects of NSAIDs on BRD and human viral respiratory disease.

BRD results in mild to severe pulmonary inflammation and changes to the respiratory tract microbiome.[3] Pathological agents most associated with BRD are either viral or bacterial. Viral pathogens include Bovine Herpes Virus-1 (BHV-1), Bovine Respiratory Syncytial Virus (BRSV), Parainfluenza-3 (PI-3), Bovine Viral Diarrhea Virus (BVDV), and Bovine Coronavirus (BCoV). Bacterial pathogens include *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Mycoplasma bovis*, and *Bibersteinia trehalosi*. Epithelial or pulmonary cellular destruction by a BRD viral pathogen causes the production of chemokines and cytokines, alters neutrophil recruitment, and initiates the inflammatory cascade.[4,5] The cellular destruction creates a welcoming environment for opportunistic bacteria, likely worsening pulmonary inflammation.[4] Opportunistic bacteria, such as *M. haemolytica*, produce leukotoxins, causing neutrophils and other phagocytes to secrete cytokines, release reactive oxygen species, and initiate cell death.[4] Bacteria increase activation and up-regulation of TLR-2 and TLR-4 causing the production of IL-1B, IL-6, and TNF-a.[5] Tumor necrosis factor-alpha, IL-1, and IL-6 are the main factors inducing inflammation, with IL-1 activating macrophages to develop cyclooxygenase-2, TNF-a which initiates endothelial adhesion molecules for recruitment/adherence of neutrophils, and IL-6 causing production of acute phase proteins (such

as fibrinogen) from the liver.[6,7] Decreases in nuclear factor erythroid 2-related factor 2, a pathway protecting cells from oxidative stress, also contributes to inflammatory-induced pulmonary damage.[5] Initially, the inflammatory response contributes to eliminating the pathogen(s), but as the inflammatory cascade persists, the natural immune response results in considerable pulmonary damage.[8] Potential pathological destructive outcomes are consolidation of lung fields, thickened alveolar walls, fibrosis, pleural fluid, intrapulmonary abscesses, pulmonary edema, and/or hemorrhage.[5] The pathology associated with BRD can cause a myriad of clinical signs: pyrexia, anorexia, thoracic pain, tachypnea, dyspnea, mucopurulent nasal discharge, coughing, bloat, decreased average daily gain, and/or death. To help mitigate the inflammatory response and associated clinical signs, most BRD cases are now being treated with an NSAID.[2]

In 400 B.C., Hippocrates was the first to prescribe a treatment for fever and inflammation, consisting of the leaves and bark of a willow tree.[9] Over 2,000 years later, Bayer, in 1899, started mass-producing aspirin (acetylsalicylic acid).[9] The mechanism of action for NSAIDs, specifically aspirin, was not discovered until the 1970s by John Vane.[9] NSAIDs provide clinical relief by inhibiting the inflammatory cascade. First, cellular damage releases phospholipase A_2, initiating the inflammatory cascade.[10] Phospholipase A_2 releases arachidonic acid (AA).[10] The enzyme cyclooxygenase (COX) then converts AA into prostaglandins and thromboxanes.[10, 11] These inflammatory mediators are signaling molecules causing tissue-specific inflammatory responses.[10, 11] There are two main isotypes of COX, COX-1, and COX-2.[10] COX-1 is produced uniformly throughout the body as a homeostatic agent.[10, 11] COX-2 is produced within the body at targeted sites of inflammation. NSAIDs act by inhibiting COX from converting AA into inflammatory mediators and are classified as either COX-1 specific, COX-2

specific, or non-specific COX inhibitors.[10, 11] NSAIDs are used to control pyrexia, systemic inflammation, and pulmonary inflammation.[12, 13]

With the recent global pandemic in 2020, significant research on the effects of NSAIDs on COVID-19 and viral respiratory disease has been published. An editorial by Dr. Paul Little mentioned that multiple complications ranging from the spreading of pulmonary infection systemically to a longer duration of disease have been associated with NSAID administration in patients with respiratory infections.[14] A study reviewed by Dr. Little revealed that either short or long duration of NSAID therapy was associated with respiratory complications.[14] Robb CT et al. published a review article in 2020 evaluating the pathophysiology of NSAIDs and its effect on patients with COVID-19.[15] Similar to cattle with BRD, patients with COVID-19 had increases in IL-6 and TNF-a, denoting comparable inflammatory responses between species.[15] The review article discussed potential adverse and positive effects of NSAIDs in patients with COVID-19.[15] Adverse effects included worsening inflammation, decreased anti-viral response, hypercoagulation, gastrointestinal ulceration, renal injury, and lengthened illness.[15] NSAIDs worsened inflammation by activating macrophages and further neutrophil extracellular traps.[15] Positive effects included decreases in COVID-19 procurement, inflammatory cytokines (NF-kB and NLRP3), Th1 cells, Th17 cells, and inflammatory contributions of macrophages and neutrophils.[15] Neutrophils and macrophages are necessary for pathogen and inflammatory damage clearance. So, the potential positive consequence of decreased function of macrophages and neutrophils could also be a negative consequence.[15] A systemic review by von Philipsborn et al. comprised 87 studies evaluating outcomes associated with NSAIDs and viral respiratory disease.[16] The highest certainty of evidence within the review, though low, was of one randomized control trial that noted children taking ibuprofen compared to paracetamol had an

increased risk of representing to a physician with unresolved or new respiratory signs.[16] The review concluded that for respiratory viral infections, no additional risk was associated with NSAID use , though the evidence was of low to very low certainty.[16] Another systematic review was conducted in 2021, evaluating NSAIDs and acute viral respiratory disease.[17] The review included the evaluation of 34 studies, with ibuprofen, aspirin, and naproxen being the most investigated NSAIDs.[17] The outcomes assessed were sore throat, respiratory distress, adverse events, pyrexia, cough, and hospitalization duration.[17] Ibuprofen, naproxen, and aspirin decreased pyrexia in pediatric and adult patients with moderate to high evidence.[17] Acute respiratory distress syndrome (ARDS) and mortality associated with ibuprofen treatment in adult patients had low evidence of minimal to no difference.[17] In pediatric patients treated with ibuprofen, moderate evidence noted no significant difference in hospitalization.[17] Naproxen revealed low evidence for a decrease in ARDS and moderate evidence of decreased mortality.[17] Aspirin used in adults did not significantly decrease ARDS, hospitalization, or mortality.[17] Overall, the review concluded that NSAIDs were considered a good treatment course for outpatient care, but more research is required for management recommendations in hospitalized patients.[17] With multiple studies showing mixed effects, human medicine still does not have a clear recommendation regarding NSAID treatment and viral respiratory disease.

As knowledge of NSAIDs in human medicine continues to expand, so does veterinary knowledge of NSAID's effect on cattle. It is important to look at multiple outcomes to give the full scope of the effects of NSAIDs in cattle with BRD. Pyrexia can result in hyporexia/anorexia, lethargy, and/or depression; thus, pyrexia is considered in a negative consequence of BRD. But pyrexia is a natural immune reaction allowing for a quicker immunologic response. Decreasing pyrexia can result in outward positive effects, but there are also potential negative effects.

Multiple research studies note a direct correlation between NSAID administration in the treatment of BRD and a decrease in pyrexia/body temperature.[18-23] Studies such as Bringhenti, et al. and Tomazi, et al. had a major confounding factor, in that the neither the NSAID or the antimicrobial was individually evaluated, but were used in combination, so the decrease in pyrexia could not be confirmed strictly due to the NSAID or antimicrobial.[18, 19] Martin, et al. noted no significant difference in rectal temperature versus the control group when an NSAID was administered in the treatment of BRD in dairy steers.[24] The finding by Martin, et al. contradicts the findings of Word, et al.; both studies inoculated cattle with *M. haemolytica* and used transdermal flunixin meglumine, but Word, et al. also inoculated beef heifers with BHV-1 and evaluated vaginal temperature.[23, 24] Though different populations, these findings potentially indicate that transdermal flunixin meglumine is inconsistent in decreasing body temperature.[23, 24] One of the most common outcomes consistent across multiple research models (induced pulmonary inflammation, inoculated BRD, and natural BRD), and cattle breeds is a decrease in pyrexia with NSAID administration.[18-23] Thus, there is strong evidence that NSAIDs decrease pyrexia.[18-23]

Clinical scores help the producer or veterinarian determine if a bovid is improving, but NSAIDs decreasing clinical scores could result in withholding further treatment due to subjective improvements masking worsening pathology. Bednarek, et al. evaluated effects of meloxicam in natural BRD cases in lowland breed veal calves and investigators were not blinded to treatment.[20] The Walsh, et al. (2016, 2020) studies were blinded, randomized control studies investigating the effects of ibuprofen on Holstein calves inoculated with BRSV. Both studies had similar parameters composing the clinical scores between the studies were body temperature (discussed previously), respiratory rate, mentation, and anorexia. The ibuprofen groups had

better clinical scores compared to the control group.[22,25] Other studies, such as Lockwood, et al. and Guez, et al., evaluated flunixin meglumine as an NSAID therapy and found no significant difference in clinical scores between treatment and control groups.[13, 26] Wilson, et al. and Dudek, et al. both contradict previous studies and found worse improvement in clinical signs in cattle administered flunixin meglumine versus those not given flunixin meglumine.[27, 28] Steers in the Wilson, et al. study receiving a second treatment for BRD had significantly improved clinical scores if they did not receive a NSAID.[27] Dudek, et al. saw inferior improvement of clinical signs with the administration of an NSAID compared to just antimicrobial therapy alone.[28] Of all these studies evaluating clinical scores, there is a major deficit in that only the Walsh, et al. (2016, 2020) studies were blinded. In addition, there is still an inherent bias in clinical scores due to subjective measurements and scores being taken at singular timepoints.[22, 25] The differences in outcomes associated with clinical scores could be different between studies due to multiple factors, including differences in NSAIDs, research models, management systems, and BRD pathogens. Even with the differences in study populations and model (inoculated versus natural), the results provide mild to moderate evidence that NSAIDs have an inconsistent effect on clinical scores.

Clinical or perceived signs of pain associated with BRD, not directly evaluated in most clinical scoring systems, is an important factor for veterinarians to choose an NSAID as indicated by a questionnaire revealing at least 66% of practitioners indicating that BRD was moderately painful, and over 50% of veterinarians within the study choose an NSAID and antimicrobial combination over just an antimicrobial alone.[29] One study evaluated transdermal flunixin meglumine on pain associated with induced BRD in Holstein steers.[24] The only parameter measured with the greatest significance for difference in pain mitigation was right

forelimb force, with the flunixin group placing more force (96.5 kgs) than the control inoculated group (85.5 kgs).[24] The other measurements for pain did not have a significant difference compared to the control group.[24] The study indicated that transdermal flunixin meglumine may help mitigate pain associated with BRD, but with only one parameter having a significant difference it is clear that further work is needed.[24]

Decreased average daily gain and weight loss is a notable negative economic BRD outcome. Only two studies demonstrated positive correlations between body weight and treatment.[22, 30] These studies were vastly different with Filho, et al. evaluating the effect of meloxicam on transported beef cattle performance and Walsh, et al. evaluating the effects of ibuprofen on induced BRSV in dairy calves.[22, 30] Filho, et al. found a statistical difference in that cattle given meloxicam had an increase in ADG compared to the control, but Walsh, et al. noted the ibuprofen group had an increase in body weight compared to the control group at the end of the study, though it was not statically different ($p = 0.08$).[22, 30] The Filho, et al. study is not directly related to cattle with BRD, but looking at the effects of administering an NSAID upon entering a feedlot, temporally near when BRD causes a significant number of deaths, is important.

Nine different studies showed no significant difference in ADG or weight gain in cattle given an NSAID. [18, 19, 27, 31-36] All BRD cases within these studies were from natural infection and treated with an NSAID either at entrance into a commercial setting or onset of acute BRD clinical signs. [18, 19, 27, 31-36] Homerosky, et al., Compiani, et al., and Cook, et al. evaluated the effects of meloxicam or flunixin meglumine on cattle entering the feedlot, as did Filho, et al.[30-33] The study designs were similar, but Homerosky, et al. and Compiani, et al. evaluated meloxicam, and Cook, et al., evaluated flunixin meglumine.[31-33] The Bringhenti, et al. and Tomazi, et al.

studies were performed similarly with randomized untreated controls and calves within the treatment groups were given their respective treatment individually based on clinical signs consistent with BRD, but the NSAID used was flunixin meglumine in combination with florfenicol, resulting in a confounding factor limiting the evaluation of the effect of flunixin meglumine due to no florfenicol or flunixin meglumine-only treatment group.[18, 19] Lisuzzo A et al. looked at meloxicam in combination with florfenicol and had similar ADG outcomes as the Bringhenti L et al. and Tomazi ACCH et al studies, but also had the same major confounding factor.[18, 19, 34] The greatest limiting factor to clinically applying the results from these six studies is that the research conducted fails to directly demonstrate that NSAID administration alone in BRD cases results in no significant difference in ADG, due to either cattle given an NSAID prior to clinical onset of disease or given in combination with an antimicrobial.[18,19, 31-34] Mahendran SA et al. (2017, 2020), and Wilson BK et al., all evaluated NSAIDs on cattle (Holstein calves and beef feedlot steers respectively) with clinical signs consistent with BRD.[27, 35, 36] Mahendran SA et al. (2017, 2020) and Wilson BK et al., randomized studies directly correlated flunixin meglumine administration in cattle with presumed BRD with no significant increase in ADG. [27, 35, 36] Even with the study limitations, there is moderate evidence that NSAIDs in BRD cattle at minimum have do not improve ADG.[18, 19, 27, 31-36]

NSAIDs are used as an ancillary therapy to help decrease pulmonary inflammation, thus evaluating studies that look at pulmonary pathology is important. Wilson, et al., Lockwood, et al., and Tomazi, et al. noted positive effects of an NSAID on pulmonary pathology.[19, 26, 27] The NSAID evaluated in all the studies was flunixin meglumine and the BRD was naturally occuring.[19, 26, 27] In the Lockwood, et al. study, the most significant finding associated with flunixin meglumine was decreased lung consolidation.[26] In contrast, Tomazi, et al. noted less

lung consolidation (9.4%) versus other treatment groups (23.5% and 20.5%), and Wilson, et al. noted decreased lung adhesions in cattle given flunixin meglumine, though that same study did note numerically increased lung consolidation in the NSAID treatment group, but not statistically different.[19, 27] As mentioned previously, Tomazi, et al. evaluated flunixin meglumine in combination with florfenicol, so the confounding effect of florfenicol limits the validity of positive effects of flunixin meglumine on pulmonary pathology.[19.] Lisuzzo, et al., and two other studies, did note that pulmonary pathology was not significantly different between treatment groups.[22, 34, 37] Lisuzzo et al.'s control group had 20.8% lung lesions, and the treatment group had 27.7% lung lesions, with no significant difference between the groups.[34] The same limitations mentioned previously still affect the interpretation of these results.[34] Randomized studies by Walsh, et al. (2016), and Hägglund, et al., evaluated Holstein calves inoculated with BRSV and treated with ibuprofen, or aspirin or meloxicam, respectively. [22, 37] In both studies, no statistical difference in pulmonary pathology between treatment and control groups was demonstrated. In contrast to the first study performed by Walsh,et al. (2016), Carvallo Chaigneau, et al. investigated the lung histopathologic consequences of the Walsh, et al. (2020) study.[22, 25, 38] When ibuprofen was administered later post-inoculation (specifically day 5), though calves clinically looked better, the histological score was worse compared to all other groups. Dudek, et al. also noted worsening pulmonary pathology associated with flunixin in a BRD induction model inoculated with *Mycoplasma bovis*.[28] At the end of the study, calves treated with flunixin meglumine had a higher number of calves with caseous necrosis of the lungs than those not treated with flunixin meglumine.[28] Histopathology of the lungs of calves treated with flunixin meglumine in either group had severe infiltration of leukocytes, particularly macrophages, and

neutrophils, with some necrosis.[28] Walsh, et al. (2020) and Dudek, et al. suggest that NSAID administration during clinical BRD could cause pulmonary damage.[25, 28, 38]

There is a wide range of results when pulmonary pathology is reviewed, from potentially positive to significantly negative. From the current literature there is mixed evidence on the outcome as it is related to pulmonary pathology, thus there needs to be more research into the exact effects of NSAIDs on the pulmonic system in cattle with active BRD.

Looking at retreatment is a pertinent outcome as it relates to initial BRD treatment failure. Manhendran, et al. (2020) and Wilson, et al., did not find a statistical difference in BRD retreatment requirement between groups.[27, 36] In Manhendran, et al. (2020) a large portion of calves only treated for BRD with flunixin meglumine later received an antimicrobial within the study.

Manhedran, et al. (2017) found that calves initially treated with only flunixin meglumine were significantly more likely to be given an antimicrobial in 72 hours with an odds ratio of 5.09.[35] Treatment success of just flunixin meglumine was 25.7%.[35] This study suggests that flunixin meglumine is not a sufficient primary treatment for BRD.[35] The largest limitation to this study was there was no negative control group.

Evaluating carcass quality and mortality is an important outcome, as these outcomes have significant economic effects. Wilson, et al. showed no statistical differences in carcass quality and mortality.[27] In Compiani, et al., mortality was also not significantly different from the control group when meloxicam was administered to cattle entering the feedlot.[32] Lisuzzo et al., corroborated Wilson, et al. findings, with no significant difference in carcass quality, though the Lisuzzo et al. study evaluated meloxicam and did not have a group that was given only florfenicol or only meloxicam, which does hinder further clinical extrapolation related to the

administration of flunixin alone.[27, 34] Overall the evidence for effects on mortality and carcass quality is low, so significantly more research is required to see if there is any true correlation between NSAID administration and carcass quality or mortality.

The immunomodulatory effects of NSAIDs have been noted in multiple studies. In vitro research models evaluating flunixin meglumine on white blood cells in calves revealed flunixin meglumine significantly decreases lymphocyte growth and IFN-g expression compared to the control group.[39] In another study, flunixin meglumine decreased neutrophil migration in all groups of cattle and significantly lowered viability of monocytes and neutrophils in cattle aged 6-9 months compared to the control; calves from 1 week to </= 5 months of age had higher apoptosis than the other age groups.[40] Noting these immune changes in vitro is important, but cannot be completely extrapolated to active BRD in cattle due to the effects being noted in unnatural conditions.

Homerosky, et al., Compiani, et al., Filho, et al., and Cooke, et al. noted conflicting effects of NSAIDs on titers and acute phase proteins in cattle going into feedlots.[30-33] Homerosky, et al. noted BHV-1, BRSV, BCov, and PI-3 titers were not significantly different in seroconversion or titer levels between the meloxicam treatment group and saline control group.[31] They did find a positive effect on serum bactericidal activity at day 30, and decreased haptoglobin concentrations at day 30 in the meloxicam group. In contrast, Compiani, et al. found that meloxicam given upon entry to a feedlot increased BHV-1 antibody production.[32] There is more research needed to determine if NSAIDs, particularly meloxicam, positively affect titer levels. Filho, et al. had similar lower haptoglobin levels as Compiani, et al.[30, 32] Instead of evaluating meloxicam, Cooke RF et al. evaluated the administration of flunixin meglumine and noted plasma acute-phase proteins, haptoglobin, and ceruloplasmin were significantly lower than

the non-treatment group at multiple time points.[33] There is agreement that acute phase proteins decrease with NSAID administration, but flunixin meglumine might cause different effects as cattle that received flunixin meglumine in Cooke, et al. had higher plasma acute-phase proteins than the control group (no treatment and not transported). [30-33] Bednarek, et al. (1999, 2003) in a natural BRD infection model found the NSAID-treated cattle displayed an increase in IFN in blood and bronchoalveolar lavage (BAL) fluid, decreased TNF production, and decreased inflammation by decreasing neutrophil adherence and oxidative burst.[20, 41] However, the NSAID group did not exhibit significant changes to phagocytic cell function or number of cells.[20] Word, et al., in another study with an inoculated BRD model, found similar results with oxidative bursts of neutrophils being significantly decreased in multiple groups given an NSAID at the time of challenge (inoculation with *M. haemolytica*), and significantly lower neutrophil L-selectin levels from baseline in multiple groups post challenge.[23] In models inoculating calves with BRSV, an increased proportion of neutrophils and decreased proportion of macrophages in BAL fluid were noted in groups given meloxicam and aspirin at specific time points.[37] Also, decreases in regulation of B-cells, IFN-g, white blood cell adhesion, and acquired immune response were noted in calves ibuprofen.[42] Lebedev, et al. also noted increases in chemotaxis of multiple white blood cells, chemokine signaling, and virus defense mechanisms in calves given ibuprofen.[42] Other immunological findings, specifically in studies evaluating NSAIDs in calves inoculated with BRSV, affected neutrophil function and response by downregulation of the Liver X Receptor/ Retinoid X Receptor (LXR/RXR) pathway, complement factor C4, and KNG1.[37] In particular calves treated with meloxicam had an abnormal leukotriene B4 response, which contributes to neutrophil inflammatory response.[37] Another study demonstrated decreased COX-dependent products and a narrower range of expression of IFN-g, IL-17, and IL-13, which are

important for neutrophil and goblet cell response, in the ibuprofen group as compared to the placebo group.[22]

There is very strong evidence from multiple studies that immune function is significantly affected by NSAIDs; in vitro, induced, and natural BRD models repeatably show alterations in immune function. [30-33, 37, 39, 40, 42]

NSAIDs notably affecting the immune system raises questions on how do NSAIDs effect viral shedding. Walsh, et al. (2016, 2020) repeatably found increased BRSV shedding in calves given ibuprofen.[22, 25] These are the first studies to correlate an NSAID to increased viral shedding in cattle.[22, 25] Interestingly the ibuprofen groups had decreased IL-8 production.[22] IL-8 is normally elevated with increased viral shedding, but this was not the case for this study. Though, unlike the effects of ibuprofen on BRSV, Hägglund S et al. found that neither meloxicam or aspirin effect viral loads of BRSV in calves.[22, 25, 37] All three studies were performed similarly, this again raises the question of whether the type of COX-inhibitor alters effects on different BRD pathogens. [22, 25, 37] This review demonstrates the paucity of data related to NSAID effects on viral shedding, and the resulting effects on disease occurrence and outcome.

When evaluating the use of NSAIDs in human and bovine medicine, evidence supports that NSAIDs cause a significant immunomodulatory response. The evidence presented within this literature review does not come to a clear conclusion on the use of NSAIDs being significantly positive or negative in BRD cases. However, this review does demonstrate that there is a wide gap in knowledge of the effects of an NSAID on BRD.

Chapter 2 - The Effect of Flunixin Meglumine on Viral Shedding in Calves

Bovine respiratory disease (BRD) is the most significant animal health challenge facing the feedlot industry in the United States (US). BRD-associated treatment, production losses, and deaths account for nearly a billion dollars of lost revenue yearly.[1] Approximately 16 percent of cattle within feedlots exhibit clinical signs of BRD and 50-70 percent of all feedlot deaths are attributed to BRD.[1,2] BRD symptoms range from mild to severe, causing a myriad of clinical signs: pyrexia, inappetence, thoracic pain, tachypnea, dyspnea, mucopurulent nasal discharge, coughing, decreased weight gain, and/or bloat. Ninety-nine percent of feedlot producers use an injectable antimicrobial and 55.9 percent of feedlot producers will administer a non-steroid anti-inflammatory drug (NSAID) for alleviation of clinical signs associated with BRD.[2]

NSAIDs are commonly used in feedlot protocols with the goal of mitigating pyrexia, inflammation, and pain associated with BRD. Recent studies evaluated NSAID immunomodulatory effects within humans and cattle. In humans, NSAIDs decrease the accumulation of neutrophils at acute sites of inflammation and of macrophages at end stages of inflammation.[43] In a study by Word, et al., after administration of transdermal flunixin meglumine the oxidative burst and phagocytic ability of neutrophils were temporally decreased in cattle with BRD.[23] In a randomized placebo-control study evaluating the use of ibuprofen in calves inoculated with bovine respiratory syncytial virus (BRSV), calves given ibuprofen had statistically higher magnitudes of viral shedding than the placebo group.[22] The study noted decreases in cytokines, specifically IL-8, post-ibuprofen administration. IL-8 is important for white blood cell function and increases in IL-8 are associated with higher viral loads.[22, 44] The ibuprofen treatment group had an elevated viral shedding load despite having decreased levels of

IL-8.[22] In these studies, improvement in clinical signs and significant modulation of cytokine response post-treatment with an NSAID were noted by the investigators.[22, 23]

NSAIDs with reported uses in cattle within the U.S. are flunixin meglumine, meloxicam, and ketoprofen.[12] Flunixin meglumine is labeled for use in cattle in the U.S. and is available as an intravenous injection, a topical product, and in combination with florfenicol (Resflor Gold, Merck Animal Health Intervet Inc., Rahway, NJ) and oxytetracycline (Hexasol, Norbrook, Tullamarine, Victoria). Flunixin meglumine is a non-selective COX inhibitor and is a part of the carboxylic acid group of NSAIDS along with ibuprofen and ketoprofen.[10, 45] NSAIDs have not been shown to positively impact treatment outcome.[46-49]

Currently, no study has evaluated the effects of an FDA-approved NSAID on viral shedding in cattle. With the high frequency of NSAID usage in feedlots, and the important role of viral agents in BRD pathogenesis, it is reasonable to investigate the effects of a commonly used NSAID on viral shedding. We hypothesized that calves challenged with BHV-1 and treated with flunixin meglumine would demonstrate higher viral shedding than calves not treated with an NSAID. Our other objective was to evaluate consistency over time and between animals of BHV-1 shedding in inoculated calves.

Materials and Methods: Study procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) of Kansas State University.

Sixteen Holstein cross-bred steer calves, 6-8 weeks of age, were enrolled in the study. All calves were vaccinated at the farm of origin with two autogenous vaccines for *Salmonella* and *Mycoplasma*. Upon arrival to the facility, approximately 5-mL of blood and a superficial nasal swab was collected for BHV-1 serum neuralization titers and PCR, respectively. Calves were

placed in individual outdoor housing units, consisting of one calf hutch and an approximately 100 sq. ft. pen. Pens were placed approximately 3 feet apart and a wire mesh panel was placed on one side of the pen to prevent nose-to-nose contact between individuals. All calves were monitored for 5 days (-4 to 0) for depression, lethargy, increased respiratory rate or effort, open mouth breathing, excessive nasal discharge, consistent coughing, or dyspnea before Bovine Herpes Virus-1 (BHV-1) inoculation. Transthoracic ultrasonography was used to assess for pre-existing and/or concurrent respiratory disease. Calves were provided ad libitum access to water, prairie hay, and fed a concentrate feed (Calf Feed-MKC Custom Calf B68) at 2% of the calves' approximate body weight. Net consumption of concentrate was recorded every 12 hours.

Propagation of the BHV-1 stock was performed utilizing a split confluent flask of clean BT (Bovine nasal turbinate, ATCC CRL-1390) cells, with a cell splitting ratio of 1:2, letting the cells grow to a 70-80% monolayer over approximately 24 hours. After incubation, the flasks were evaluated by a light microscope to determine the degree of confluency reached. The BHV-1 Colorado Strain, NVSL050-BDV stock virus was used for the study.

Titration of a new BHV-1 stock was performed using a 96-well plate seeded with BT cells and incubated for 24 hours. The clarified BHV-1 stock was made into 10 serial 10-fold dilutions in 4-mL serum tubes using growth media as the diluent. Once the dilutions were made, the 96-well BT plate was inoculated and placed back in the incubator for 72 hours after which the median tissue culture infectious dose ($TCID_{50}$/mL) was calculated using the Spearman-Karber method by marking each of the plate wells showing cytopathic effect. The final titer or $TCID_{50}$/mL was expressed in log/mL. The virus was diluted in the media to obtain a final concentration of 10^5 $TCID_{50}$/mL.

On day-0, each calf was inoculated with 4 mLs of $1x10^5$ $TCID_{50}$/mL of the Colorado strain of BHV-1 in the right nostril with a plastic nasal cannula. Study animals were randomly assigned to treatment group with six calves placed into the treatment (FM) group and 6 calves in the control (CON) group. Assignment was conducted using the random number generator in Microsoft Excel. Study design was influenced by a pilot study that informed us of the duration and individual variability in BHV-1 shedding.

Nasal swab sampling started 36 hours following intranasal BHV-1 inoculation. A superficial nasal swab was collected from each calf every 24 hours, alternating nostrils at each sampling time point. The left nostril was the first nostril sampled. The nasal swab was placed into transport medium (Liquid Amies media) and submitted for BHV-1 PCR testing through the Kansas State Veterinary Diagnostic Lab (KSVDL). PCR values were measured in cycle threshold (CT) and transformed to $TCID_{50}$/mL. CT values greater than 39 were negative for BHV-1, values between 38 and 39 were suspect/inconclusive, and values less than or equal to 38 were positive for BHV-1. As each cycle of amplification doubles the PCR product concentration, one CT difference reflects a two-fold difference in concentration. The CT value is negatively correlated to concentration. The relationship between CT and the viral concentration was calculated as Concentration at CT0 / $[2^{-(CT0-CTX)}]$, where CT0 refers to the CT value obtained from the stock solution, and CTX refers to the CT value for a given sample as tested by the PCR assay. As CTX is always larger than CT0 in this type of experiment, the formula can be simplified to Concentration at CT0 / $[2^{(CTX-CT0)}]$. In this particular experiment, the initial concentration of the viral stock is 10^6.8 (6309573.44) $TCID_{50}$/mL, and the corresponding CT is 25.65; thus, the specific formula used in this experiment was: 6309573.44 / $[2^{(CTX-25.65)}]$.[50]

Nasal swabs were collected for a minimum of 7 sample periods following the initial BHV-1 positive sample and until the animal was negative at 2 consecutive sample timepoints. Clinical illness scores (CIS) were determined every 12 hours, including respiratory rate, respiratory effort, rectal temperature, hydration status, appetite, and mentation. The clinical illness score was on a grading system from 1 – 4 as depicted in Supplemental Table 1. The final CIS was assigned for the highest value noted.

The FM group was administered 2.2 mg/kg flunixin meglumine intravenously after the initial BHV-1 positive result, approximately 24 to 64 hours post-inoculation depending on timing of shedding and individual PCR test turnaround time. The CON group was inoculated with BHV-1, but did not receive any treatments throughout the study period.

Once a calf reached the 7-sample minimum and had two consecutive negative PCR samples the animal was considered as having completed the study. Upon completion of the study, all calves were euthanized by first administering 0.2 mg/kg of xylazine, intravenously, for sedation. Calves were then euthanized with a penetrating bolt and administered magnesium sulfate intravenously to effect. Post-euthanasia, the lungs of all the calves were evaluated as described by Fajt et. al.[51]

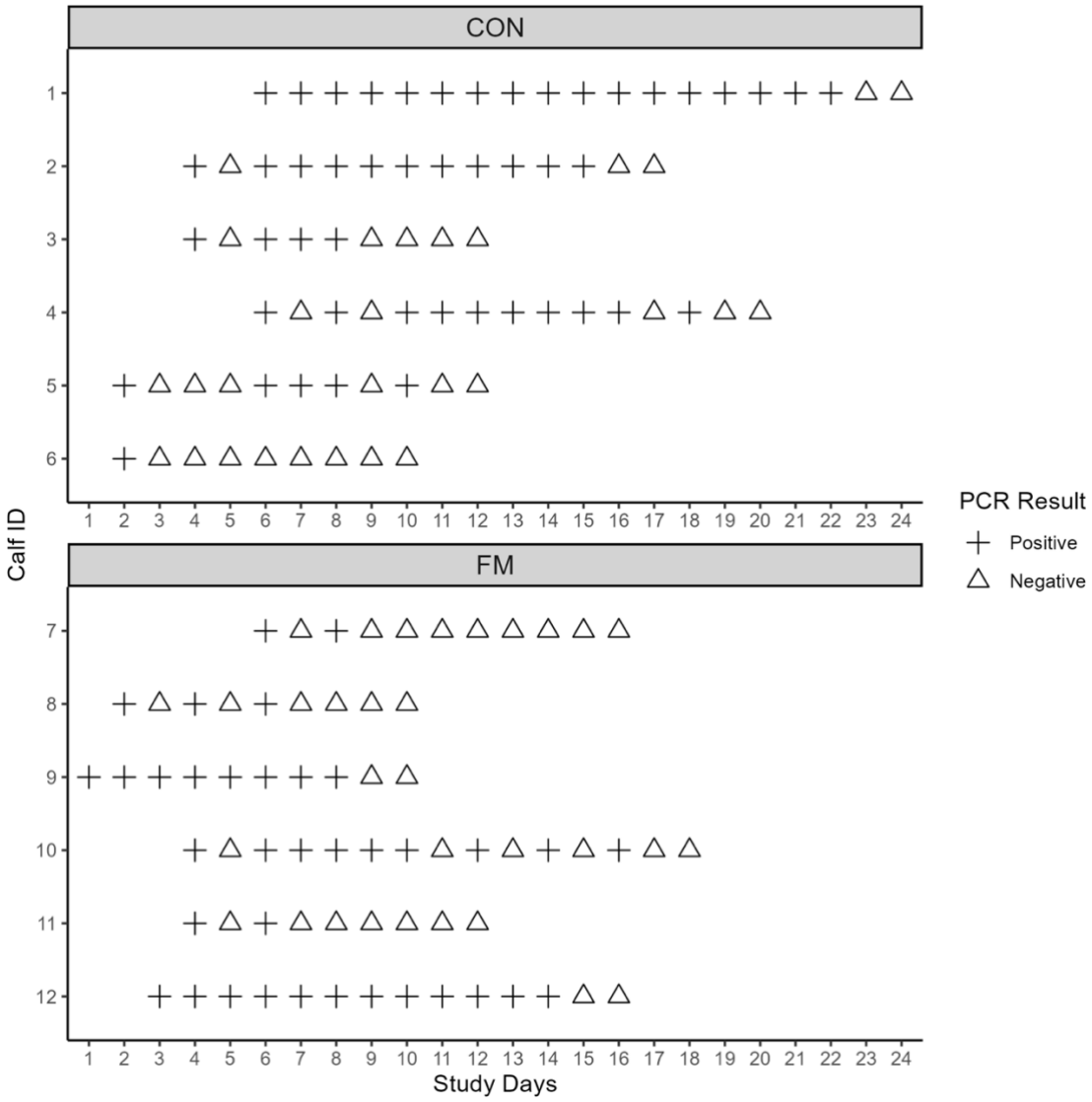
Statistical analysis: The qPCR data expressed as CT values were converted to $TCID_{50}$, using the formula above. Values for Area Under the $TCID_{50}$ Log_{10} curve (AUC), collected (CT values), and computed ($TCID_{50}$ values) were analyzed using a linear mixed model. Fixed effects of the model included treatment, time, and treatment-by-time interaction. Random effect was animal (i.e., the error term vector). The qPCR data using total AUC (on the log_{10} scale) computed at the last study day was analyzed using a one-way ANOVA. Area Under the Curve was formulated by taking the mean of $TCID_{50}$ values over each 24-hour sampling period during

which shedding occurred. Fixed effects of the model included treatment. All tests were two-sided, and significance was defined at the 0.05 level. No multiplicity adjustment was applied. Statistical analyses were performed via Statistical Analysis Software (SAS version 9.4; Cary, NC) using the MIXED procedure.

Descriptive Results: All calves were negative on intake PCR for BHV-1. Four calves were excluded from the study due to evidence of pre-existing respiratory disease on trans-thoracic ultrasonography. Six days post-inoculation, all 12 calves had at least one positive BHV-1 PCR result.

Figure 1 depicts shedding duration per individual calf. Days shed (first positive to last positive) ranged from 1 to 17 days. There could be negative days interspersed between the positive days. Viral shedding for the CON group from initial PCR positive to last positive was a mean of 9.5 days (Range: 1-17, SD: 5.80 days). Viral shedding for the FM group from initial to last PCR positive was a mean of 7.33 days (Range: 3-12, SD: 4.41 days). Number of PCR-positive days within the shedding duration were also determined. Sample days for the CON group had a mean of 8 days that were PCR-positive (Range: 1-17, SD: 5.80 days). PCR-positive sample days for the FM group had a mean of 6 days (Range: 2-12, SD: 4.24 days).

Figure 1: PCR-positive days per individual calf for CON and FM groups post-inoculation. All calves were negative prior to first positive.



Titers on intake in the FM group ranged from <1:2 to 1:32. Titer on intake in the CON group ranged from 1:4 to 1:32. A summary for each calf's BHV-1 titers is shown in Supplemental Table 2. Table 1 summarizes the shedding start-date post-inoculation, shedding period, and total $TCID_{50}$ Log_{10} AUC per individual.

The CIS did not go over a score of 1 after the initial positive BHV-1 PCR in any calf. No calf got over a CIS of 2 and the modal CIS was 1. Total positive samples from the right nostril

from both groups was 54 samples with 24 from the FM group and 30 from the CON group. The total positive samples from the left nostrils from both groups was 28 samples with 12 coming from the FM group and 16 from the CON group. Gross lung lesions were minimal (<5% consolidation) in all study animals, regardless of treatment. The intent was to perform complete necropsies on all animals, however only nine out of 12 calves' tracheas were examined, and no gross lesions were present.

Table 1: Summary of study day first PCR positive, shedding period, and $TCID_{50}$ Log_{10} total AUC per individual.

Group	Calf ID	Study Day First PCR Positive	Shedding Period Days	TCID50 Log10 AUC Total
CON	1	6	17	8.59
CON	2	4	12	8.71
CON	3	4	5	6.25
CON	4	6	13	8.55
CON	5	2	9	8.62
CON	6	2	1	4.24
FM	7	6	3	4.81
FM	8	2	5	5.01
FM	9	1	8	6.58
FM	10	4	13	7.73
FM	11	4	3	4.28
FM	12	3	12	7.65

Inferential Results: Treatment effect was evaluated at each time point. There were no statistical differences in BHV-1 shedding between treatment groups when comparing mean log_{10} $TCID_{50}$ by day (p value = 0.417, CI of 95%). The total log_{10} AUC between CON and FM groups was not significantly different (p value = 0.173). Least square mean of the total log_{10} AUC of the FM group was 6.11 (95% CI: 4.62-7.60) and CON group was 7.49 (95% CI: 6.00-8.98).

Discussion: Neither the magnitude nor duration of BHV-1 shedding in experimentally challenged calves was significantly different in calves that were treated with flunixin meglumine as compared to the control group. Total length of BHV-1 shedding was comparable to previously reported data, with the max length of shedding being 17 days.[52] Incubation periods were quite variable among individuals. Intermittent shedding of BHV-1 during the study period and differences in nostril shedding was unexpected. Infection of calves following BHV-1 inoculation was demonstrated by decreasing CT values, indicating higher positivity over the course of the study. Positivity strictly due to residual BHV-1 from the inoculum would have resulted in increasing CT values. Except for 2 calves, one calf in each group, all calves had decreasing CT values indicating an increasing viral load over time, which these investigators interpret as true nasal shedding of BHV-1.

Intermittent shedding was noted in multiple calves in a study evaluating nasal shedding of BRSV post-intranasal vaccination.[53] The reason for the intermittent shedding of BRSV in calves could not be determined.[53] A postulation for the unique shedding characteristics, was that superficial nasal swab PCR had low sensitivity, but this is unlikely.[53] A recent publication revealed that superficial nasal swab PCRs were positive for a viral agent that infects the respiratory epithelial cells, similar to BHV-1, than the gold-standard trans-tracheal wash.[54, 55] The potential clinical importance of this observation is that serial superficial nasal swab PCR samples for BHV-1 may be needed to determine if an individual is truly negative or positive. As serial samples are not always feasible for a commercial production system, cattle with active BHV-1 may be missed with a one-time superficial nasal swab sample. With no calf going over a CIS of 2, calves with an uncomplicated BHV-1 infection may be shedding and appear clinically healthy.

Recommendations for future BHV-1 challenge studies would be intranasal inoculation with 4-mLs of $1x10^5$ $TCID_{50}$/mL of the Colorado strain of BHV-1 in both nostrils.

Titers were measured on intake for each calf. The calves' titers may have been derived from colostrum and/or exposure; there was no history of BHV-1 vaccination in study animals. Maternal-derived antibodies for BHV-1 in calf serum has been noted to last approximately 122 days in beef calves.[56] Despite the pre-existing titers, although low, viral shedding was demonstrated in this study.

There remains a lack of research into the effects of NSAIDs on shedding of BRD viral pathogens. The study reported here found no effect of flunixin meglumine on the shedding of BHV-1. This finding contrasts with Walsh et al. who demonstrated increased BRSV shedding when calves were given ibuprofen.[31] Further investigations are warranted to evaluate differences in NSAIDs and viral pathogens as well as the effects of different study designs.

The current study did not inoculate calves with a BRD bacterial pathogen, thus not replicating the natural progression of BRD complex. Evaluating flunixin meglumine in calves with a combination BRD viral and bacterial pathogens could result in different effects. Investigating if other flunixin meglumine formulations or other NSAIDs that specifically inhibit COX-1 or COX-2 change viral shedding characteristics is indicated. The evaluation of the effect of NSAID regimen on viral shedding characteristics would also be informative. Calves between 6-8 weeks were used in the study but cattle of varying ages, breeds, and management settings with NSAIDs could be investigated as well. The limitations of the study were the low sample size, lag between time of PCR results to time of treatment, and inability to distinguish PCR positive results being from infection and shedding or residual challenge virus.

Conclusions: There was no significant difference in magnitude or duration of BHV-1 shedding between calves treated with flunixin meglumine following intranasal inoculation with BHV-1. The study demonstrated substantial individual variation in duration and intermittent shedding of BHV-1 post-inoculation.

Supplemental Tables:

Supplemental Table 1: CIS grading system used post-inoculation to determine extent of BRD signs

Clinical Illness Score (CIS)						
CIS	Respiratory Rate	Respiratory Effort	Rectal Temperature	Hydration Status	Appetite	Mentation
1	< 80 breathes per minute (brpm)	Normal	< 104.0^{0}F	Not dehydrated	Normal	Bright, alert, and responsive
2	80-120 brpm	Mild increased effort • Inconsistent coughing • Excessive serous nasal discharge	>104.0^{0}F, < 106.5^{0}F	< 5% dehydrated (mild) • Skin tent 1-2 seconds • 1-2-mm globe recession	Normal	Quiet, alert, and responsive
3	> 120 brpm	Moderate increased effort • Intermittent open mouth breathing	>106.5^{0}F for 24 hours duration	5-8% dehydrated (moderate) • Skin tent 2 seconds • 3-4-mm globe recession	Anorexia > 12 hours	Depressed • Stands with assistance
4	>120 brpm	Marked abdominal effort • Constant open mouth breathing	>107.0^{0}F for 12 hours	8-10% dehydrated (severe) • Skin tent >3 seconds	Anorexia >24 hours	Obtunded • Unable to stand

				• >6-mm globe recession		

Supplemental Table 2: Summary of BHV-1 titer levels of individual calves in the FM group and CON group.

BHV-1 Titers Per Group				
FM Group			CON Group	
Animal ID	BHV-1Titer		Animal ID	BHV-1Titer
Calf 3	1:32		Calf 4	1:32
Calf 7	1:32		Calf 18	1:32
Calf 10	1:8		Calf 6	1:16
Calf 8	1:8		Calf 2	1:8
Calf 21	1:8		Calf 14	1:8
Calf 19	<1:2		Calf 22	1:4

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Chapter 3 - Future Considerations

The multiplicity of pathogens associated with BRD can result in many inflammatory responses. Research and treatment become increasingly difficult with each BRD pathogen causing its own inflammatory response. From this literature review and thesis research project, it is apparent that treatment with NSAIDs can have a range of consequences, either positive or negative. The gold standard to come to a consensus on NSAID therapy for BRD is to perform blinded, randomized, controlled trials with large groups of cattle at each stage of production, evaluating effects associated with every viral and bacterial combination in an experimentally induced and natural state and testing every approved NSAID for bovine use on varying BRD infection combinations. Assessing the effect of NSAIDs with every viral and bacterial combination can lead to a more refined treatment protocol. This extensive investigation is prudent because studies have revealed varying outcomes with different NSAIDs and pathogens, such as flunixin meglumine causing significant lung pathology in calves with *M. bovis*, and ibuprofen increasing BRSV shedding in calves but not increasing BRSV shedding in calves given meloxicam.[22, 25, 28, 37] Obviously, performing these studies would take immense time and finances.

The next step in understanding NSAIDs as an ancillary therapy is to continue evaluating the effects of NSAID therapy on BRSV. Ibuprofen has been consistent in causing increased viral shedding, immunology response, and histopathological changes.[22, 25, 38, 37, 42] These findings were found in the same age and production population of cattle. [22, 25, 38, 37, 42] Instead of investigating ibuprofen, veterinary medicine should investigate the effects of meloxicam, flunixin meglumine, and ketoprofen on BRSV. This would allow for comparison of baseline research and potential variation in different COX inhibitors. Further investigating the effects of NSAIDs on BRSV

could benefit treatment recommendations for human children with RSV. The Carvallo Chaigneau, et al. study found that ibuprofen given later in the disease duration causes a worse histological outcome.[38] As a follow-up study, investigating the effects of NSAID treatment on lung pathology of chronic or late-diagnosis of BRD in feedlot cattle and pre-weaned calves is recommended. The outcomes that should be focused on are ADG, gross lung pathology, lung histopathology, and changes in inflammatory cytokines, especially those affecting neutrophil recruitment, adhesion, pathogen elimination capabilities, and viral shedding. There have been mixed results on viral shedding in calves with BRD given an NSAID. The research in this thesis and Hägglund, et al. did not have a statistical difference in viral shedding between the NSAID treatment group and the control group, but ibuprofen increased BRSV shedding in other studies. [22, 25, 37] Ibuprofen and meloxicam are both COX-2 specific inhibitors, so further evaluation into which specific immunologic response due to ibuprofen results in increased viral shedding is necessary for future understanding of treating BRD patients with an NSAID. If ibuprofen causes a specific immune response that increases viral shedding unique compared to all other NSAIDs, then the next step would be investigating whether ibuprofen-induced viral shedding can cause increased morbidity and mortality in a herd. Also, comparing the effects of flunixin meglumine on BRSV shedding to this thesis research project would be prudent. Though ibuprofen is not an approved NSAID for cattle in the US, it is essential to know its effects for future guidelines in cattle and human medicine.

Further investigating the exact immune response to COX inhibitors is important for future treatment guidelines. The main goal of therapy with COX inhibitors is to decrease inflammation and fever, and to provide analgesia. As discussed, COX inhibitors stop the conversion of AA into inflammatory mediators, resulting in a decreased inflammatory

response.[10, 11] This anti-inflammatory response results in effects that are still not completely understood. One of the most significant components noted in both human and veterinary literature is the effect of COX inhibitors on leukocytes, specifically neutrophil function.[15, 23, 39, 40] As a major contributor to clearing pathogens, neutrophils can cause the most significant pulmonary damage. Refining veterinary knowledge of COX inhibitors on neutrophil function in BRD cases to determine which eicosanoids, interleukins, and/or cytokines are responsible for neutrophil function is important, but more importantly, is knowing which of these pro- or anti-inflammatory neutrophil modulating factors has potential to develop new targeted therapies. Targeted therapies of the inflammatory response would be superior to the blanketed response of COX inhibitors, as this will cause more direct positive effects while hopefully preventing deleterious effects.

In conclusion, in the review of recent literature and the conduction of this thesis research project, NSAIDs have immunomodulatory effects. The significance of these immunomodulatory effects should be a continued area of research to better guide the treatment of BRD cases and human medicine. Reduction of pyrexia is a very consistent response, but there is quality research revealing that NSAIDs can have significant adverse effects on BRD cases. Currently, antibiotics result in the most significant improvement in BRD cases, so consideration of hydration status, severity of fever, pathogens, chronicity of respiratory disease, and potential long-term negative pulmonary consequences should be considered before treating BRD cattle with an NSAID.

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