Pharmacokinetic properties of transdermal flunixin in cattle and its use in pain models.

by

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A.S., The Ohio State University Agricultural Technical Institute, 2001
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
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Abstract

Flunixin meglumine has been used as an antipyretic and anti-inflammatory since the 1980s. In 2013, a novel formulation was released in the European Union for topical administration and transdermal absorption. Approval for transdermal flunixin in cattle in the United States occurred in 2017, and included a label claim for the control of pain associated with infectious pododermatitis (foot rot). This new formulation allows for needle-less delivery of flunixin with minimal restraint and training required.

In this dissertation, the pharmacokinetics of transdermal flunixin in Holstein calves at 2 months of age and adult lactating cows is described. In these pharmacokinetic studies, plasma flunixin concentrations were determined using high-pressure liquid chromatography coupled with mass spectroscopy. Pharmacokinetic modeling was completed using non-compartmental modeling methods using a commercially available computer program.

Ex vivo prostaglandin E_2 (PGE_2) production using a whole blood model served as a biomarker for the anti-inflammatory effects of flunixin meglumine and suppression of cyclo-oxygenase enzyme-2. The concentrations of PGE_2 were determined using a commercially available enzyme-linked immunosorbent linked assay (ELISA) kit.

The effects of age and pain on the pharmacokinetics of flunixin were investigated. Both influenced the pharmacokinetics and anti-inflammatory effects of flunixin. Cautery dehorning without local anesthetic was used in the calf model to generate pain. The pain associated with dehorning caused lower absorption of the transdermal flunixin and a longer terminal half-life. This longer half-life did result in lower PGE2 concentrations at later time points. The influence of age was determined in the same group of Holstein calves at 2 months and 8 month of age. Age related effects included lower clearance, a longer half-life, and longer suppression of PGE2

following intravenous injection. Following transdermal administration, older animals had a prolonged absorption leading to a longer half-life and apparent 'flip-flop' pharmacokinetics. Additionally, the suppression of PGE2 was not observed in older calves following transdermal flunixin administration.

The analgesic properties of transdermal flunixin were tested using three different pain models. Those pain models include cautery dehorning, surgical castration, and induced lameness. The reduction in plasma cortisol following transdermal administration was the most consistent finding in each model for pain. Infrared thermography (IRT) was used to assess either activation of the autonomic nervous system or local inflammation. Flunixin did not have any effects on substance P concentration in all three pain models. Gait analysis using a floor based pressure mat was used in the assessment of castration and lameness pain. Although there were no observed effects of flunixin in those studies, the use of this technology for pain assessment is promising.

Future studies of transdermal flunixin to determine its utility as part of a multi-modal analgesic plan are still warranted. Specifically, the use a of a local anesthetic block at the time of cautery dehorning, as flunixin has minimal effects on pain, and its pharmacokinetics were altered by the painful stimulus. Timing of the dose relative to the painful procedure is also needed as flunixin is rapidly absorbed. Field studies in lame cattle are needed as there is a deficiency in the literature as only models of lameness induction have been reported.

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Dedication

I would like to dedicate this dissertation to the group of Holstein calves and dairy cows that made much of this work possible; as they made the ultimate sacrifice for the betterment of cattle everywhere. To my parents, Mike and Merrilee Kleinhenz for their support. To my daughters, Harriet and Irene, who make me smile, give me joy beyond measure, and put everything into a better perspective. To my wife Dr. Katie Kleinhenz who has provided me unending support and love. Her assistance in my personnel and professional life is immeasurable. Finally, to the Lord, for it is He who made all of this possible.

Chapter 1 - Literature Review

INTRODUCTION

Flunixin meglumine has been used in cattle as an anti-inflammatory and antipyretic since the 1980s. Flunixin is a nonsteroidal anti-inflammatory drug (NSAID) derived from nicotinic acid with activity on cyclooxygenase enzymes in the arachidonic acid pathway. There are currently two formulations of flunixin approved for cattle in the United States, Canada and European Union. The first approved formulation is for intravenous (IV) use in beef and dairy cattle. It has US Food and Drug Administration (FDA) label approval for the control of pyrexia due to bovine respiratory disease, endotoxemia, and acute mastitis; and control of inflammation due to endotoxemia. The second formulation received FDA approval in July 2017. It is designed for topical administration and transdermal absorption of flunixin in cattle. The FDA granted label approval for the control of pyrexia due to bovine respiratory disease and the control of pain associated with foot rot. It is the first pharmaceutical to receive FDA approval for pain control in food producing animals. There still is no labelled pain control for common husbandry activities such as castration and dehorning.

The lack of objective and repeatable pain measures are the main reason for the lack of approvals. The costs and convenience of administering analgesic drugs has also been seen as a barrier to widespread adaptation. The NSAID meloxicam has a relatively low cost when giving 15 mg pills in a gelatin capsule in the US. However, the oral administration of meloxicam has not been widely adapted in countries with approved meloxicam formulations. The chute-side logistics and time required to administer analgesics has been cited as a barrier to use (Moggy, Pajor et al. 2017). A 2017 survey of veterinarians in the United Kingdom indicated £5.00

(~$6.50) as a maximum acceptable cost of analgesics per calf for castration and disbudding (Remnant, Tremlett et al. 2017). The majority of veterinarians in that survey felt £20.00 (~$26.00) per cow was an acceptable cost for providing analgesia to cows with lameness due to a sole ulcer, but the authors do not mention if that cost includes discarding milk (Remnant, Tremlett et al. 2017).

With the approval of the transdermal formulation, there has been a renewal in the use of flunixin in cattle. To answer questions on the utility of the new formulation, a systematic review of the literature was performed using the online resources PubMed and Web of Sciences in July of 2018. Search terms included species (cattle); NSAID as whole group or flunixin individually; and then conditions/procedures for NSAID use. Conditions and procedures included in the search include endotoxemia, scours, mastitis, BRD, castration, dehorning, and lameness. Studies from Europe and North America were included in the study, and limited to those published in English.

PHARMACOKINETICS

The pharmacokinetics of flunixin following IV injection has been described in multiple studies (**Table 1**). The half-life of flunixin ranges from 3.14 h to 12.9 h (Hardee, Smith et al. 1985, Kissell, Brinson et al. 2016). The age of the animal appears to impact the pharmacokinetics of flunixin when administered by IV injection. Kissell et al. demonstrated a longer half-life in 53 kg calves of 12.9 h (Kissell, Brinson et al. 2016). This compares to a half-life of 6.9 h in 118.9 kg cattle, and 3.4 h in cattle ranging 545 to 676 kg (Landoni and Cunningham 1995, Kissell, Smith et al. 2012). In two studies by Kissell et al., clearance of the younger cattle was lower at 64.4 mL/h/kg compared to the 150.6 mL/h/kg in adult cows (2016). The volume of distribution (Vd)

in these studies were different with younger cattle determined to be 0.634 L/kg and adult cows having a Vd of 0.254 L/kg (Kissell, Smith et al. 2012, Kissell, Brinson et al. 2016).

The presence of lipopolysaccharide (LPS) and mastitis has been shown to alter the pharmacokinetics of flunixin given intravenously (Kissell, Leavens et al. 2015, Smith, Shelver et al. 2015). Cows challenged with LPS had a shorter half-life compared to saline controls. The difference in half-life between groups was attributed to a higher clearance in the LPS group (Smith, Shelver et al. 2015). Cows with clinical mastitis caused by *Klebsiella pneumoniae* or *Escherichia coli* had altered flunixin pharmacokinetics compared to health controls. Cows with mastitis had lower clearance compared to control cows, but the half-lives of the two groups was statistically similar (Kissell, Leavens et al. 2015). Additionally, the area under the curve (AUC) was higher in the mastitis group. The authors question the concurrent use of ceftiofur in the mastitis cows as a source of difference. However, Gorden et al. demonstrated cows administered ceftiofur concurrently with flunixin had similar flunixin pharmacokinetics as flunixin only cows (2018). Thus, the role of inflammation or disease associated with LPS, either as pure LPS or bacteremia, contributes to altered pharmacokinetics and possibly violative residues.

Milk pharmacokinetics

The marker residue for flunixin in milk is 5-hydroxyflunixin (5OHF) which can be measured in both plasma and milk. The half-life of 5OHF in plasma is 1.4 h following IV injection. Following IM and SC injection, both extra-label uses, the half-life of 5OHF is 6.0 and 7.0 h, respectively (Kissell, Smith et al. 2012). In the US, the established milk withdrawal period is 36 h. However, following IM and SC injection 5OHF was detectable in milk samples from healthy cows beyond the 36 h mark. In cows with clinical mastitis, 5OHF was detectable out to 48 h in 3 of 10 cows (Kissell, Leavens et al. 2015).

It should be noted that endotoxemia has been shown to alter the pharmacokinetics of flunixin in the milk of lactating dairy cows. Smith et al. found that cows infused with LPS had higher flunixin: 5-OH flunixin ratios in milk over a 96-h period. Additionally, LPS exposed cows had a shorter half-life, higher clearance, higher volume of distribution, and lower AUC compared to cows receiving flunixin only (Smith, Shelver et al. 2015). Cows with naturally occurring severe coliform mastitis had detectable flunixin in their milk 60 h after IV flunixin administration (Kissell, Leavens et al. 2015). Control cows matched by lactation and enrolled on the same day had milk flunixin levels below the level of quantification (2 ng/mL) by 36 h post-drug administration. Cows with mastitis had lower plasma clearance and larger AUC. However, the terminal half-life in plasma was not different than control cows (Kissell, Leavens et al. 2015). The findings of these two studies indicate that cows treated with flunixin for an endotoxic condition may require an extended withdrawal period for milk.

Anti-inflammatory actions

As an NSAID, flunixin has actions on both isomers of the COX enzyme. Since COX-2 is associated with induced inflammation, its product, prostaglandin E_2 (PGE_2) has been the focus of published analgesic research. Flunixin suppresses *ex vivo* PGE_2 production as soon as 5 min after IV administration at 2.2 mg/kg (Fraccaro, Coetzee et al. 2013). Concentrations of PGE_2 were suppressed out to 12 h following injection. Landoni et al. demonstrated suppression of thromboxane B_2 (TxB_2) for 12 h using an *ex vivo* model after an IV injection at 2.2 mg/kg (1995). Using a tissue cage model, PGE_2 production in exudate was suppressed out to 24 h (Landoni and Cunningham 1995). Transdermal flunixin suppresses the production of PGE2 in a tissue cage model out to 48 h (Thiry, Fournier et al. 2017). This suppression may be longer as the study ended at 48 h with a 90% inhibition of PGE_2 production, so transdermal flunixin has about

quadruple the anti-inflammatory time as when administered by IV injection. However, a true head-to-head study has not been published.

ENDOTOXEMIA

Endotoxemia is the result of the release of endotoxins into the bloodstream from the death and breakdown of Gram negative bacteria cell walls. Lipopolysaccharide (LPS) is the most common endotoxin associated with Gram negative bacteria in cattle. The release of endotoxin into the blood leads to the production of interleukin (IL)-1, IL-6 and TNF-α from the immune system. IL-1, IL-6 and TNF-α stimulate the COX-2 production of PGE_2 in the hypothalamus (Smith 2005). The hypothalamic production of PGE_2 leads to an increase in the set point for body temperature and thus the febrile response.

Endotoxins result in local tissue damage and breakdown of cell membrane phospholipids. The cell membrane phospholipid breakdown, mediated by phospholipase A_2 (PLA_2), leads to the production of arachidonic acid (AA). The AA is then converted into TxA_2, $PGF_{2\alpha}$, PGI, and PGE_2 by the COX enzymes; with TxA_2 and PGE2 having major roles in inflammation. Flunixin has been shown to inhibit both COX-1 and COX-2 isoforms and shows no preference or selection as to which COX isoform it inhibits (Landoni and Cunningham 1995). It does not have effects against the lipoxygenase (LOX)/leukotriene pathway leading to bronchial smooth muscle constriction, vascular permeability and activation of leukocytes (Donalisio, Barbero et al. 2013). Flunixin administration results in decreased production of thromboxane A_2 (TxA_2) via COX-1 and prostaglandin E_2 (PGE_2) via COX-2. Flunixin activity is at the level of the COX enzyme as it does not alter mRNA expression for the COX enzyme (Myers, Scott et al. 2010). Flunixin has also been shown to suppress interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α)

production *in vitro* in whole bovine blood (Donalisio, Barbero et al. 2013). Furthermore, flunixin lowers mRNA synthesis of TNF-α (Myers, Scott et al. 2010). Reductions in prostaglandins, INF-γ, and TNF-α are the reason flunixin has effective antipyretic and anti-inflammatory activity consistently seen in the literature.

CALF SCOURS

Flunixin, administered by IV administration, has been investigated in calves with endotoxemia. When administered at 1.1 mg/kg, flunixin blocked the production of TxB_2 the major metabolite of TxA_2 (Semrad 1993). This resulted in lower lactate levels compared to saline controls with LPS-induced endotoxemia. Flunixin did not have effects on blood glucose concentrations and white blood cell counts (Semrad 1993). When clinically evaluated, calves treated with flunixin had higher clinical scores (Semrad 1993). The clinical scores were based on calves responsiveness and head carriage. All flunixin treated calves ate their milk feeding at 12 h (Semrad 1993).

When calves were administered an *Escherichia coli* enterotoxin to induce diarrhea, flunixin treated cows had lower fecal output compared to control cows (Roussel, Sriranganathan et al. 1988). Additionally, flunixin treated calves had improved hemoconcentration parameters 12 h after diarrhea induction (Roussel, Sriranganathan et al. 1988). In calves with naturally occurring scours, a single dose of flunixin resulted in fewer morbid-days (days of illness) compared to saline treated controls (Barnett, Sischo et al. 2003). Calves receiving two doses of flunixin had numerically lower morbid-days than saline controls, but there was no statistical significance. There were numerical, but not statistical differences in mortality rates between treatments. There were more mortalities in the flunixin treated groups, but there differences were

not statistically significant. Furthermore, calves receiving a single dose of flunixin had fewer antibiotic treatments compared to saline controls (Barnett, Sischo et al. 2003).

MASTITIS

Flunixin is used as a supportive therapy in dairy cows with clinical mastitis. Its use is often limited to moderate or severe cases where the mammary gland is showing local signs of inflammation such as swelling or firmness. Greater than 95% of veterinarians treating cows with acute toxic mastitis administer a NSAID (Fajt, Wagner et al. 2011). Use of supportive treatments, including flunixin, were administered to 18% of all clinical mastitis cases in a survey of 51 Wisconsin dairy farms (Oliveira and Ruegg 2014). The percentage of cows administered supportive treatment increased as clinical severity increased in that survey of Wisconsin farms. The use of flunixin for the treatment of severe mastitis has been a mainstay of veterinary medicine; yet there is little literature with naturally occurring mastitis cases. Much of the literature regarding flunixin and mastitis use *Escherichia coli* or LPS to induce endotoxic mastitis. The dry matter intake of cows following induction of endotoxic mastitis decreases and is a common finding of control cows in these studies. Flunixin has mixed results on the amelioration of dry matter intake reductions. Two studies have found no effects of flunixin on dry matter intake of cows with LPS-induced endotoxic mastitis. The flunixin dose of these studies was either low (Zimov, Botheras et al. 2011) or based on herd average instead of the actual animal body weight (Chapinal, Fitzpatrick et al. 2014). Yeiser et al. used flunixin at the upper end of the dosing range and based dosing on actual study animal body weight on the day of challenge with *E. coli* (2012). They found an increase in dry matter intake in flunixin treated cows on the first day following treatment. The authors also report improved milk production in multiparous-flunixin treated cows. However, this effect of flunixin treatment was not seen in

primiparous cows. Others have reported milk production as an outcome measure, but have not observed any effects (positive or negative) of flunixin on milk production (Wagner and Apley 2004, Zimov, Botheras et al. 2011). A difference in induction model (E. coli versus LPS) may confound comparisons.

Other outcome measures reported in the literature other than milk production and dry matter intake include: udder edema, rumination times, rectal temperatures, rear leg weight shifting, and behavioral variables. Of these, a decreased rectal temperature in flunixin treated cows is consistently reported. This observation is expected as flunixin carries a label approval for control of pyrexia. Wagner and Apley observed flunixin treated cows had lower heart rates compared to control cows (2004). Rumination frequency increased in cows treated with flunixin compared to placebo controls (Chapinal, Fitzpatrick et al. 2014).

A sequelae of endotoxin-induced mastitis is swelling of the mammary gland. Clinically, this swelling is painful to the cow (Fitzpatrick, Chapinal et al. 2013). Veterinarians consider mastitis to be painful, with toxic mastitis having a higher pain score than mild mastitis (Remnant, Tremlett et al. 2017). Additional studies investigating flunixin and/or other NSAIDs are warranted as these drugs are administered by greater than 90% of veterinarians treating toxic mastitis (Fajt, Wagner et al. 2011). Flunixin did not decrease swelling of the gland in endotoxin-induced mastitis cases (Wagner and Apley 2004). In an effort to quantify the pain associated with udder swelling and edema, Chapinal et al. measured rear leg weight shifting of cows with induced endotoxic mastitis (Chapinal, Fitzpatrick et al. 2013). The authors hypothesize that the swelling causes increased friction and thus pain when shifting weight. Weight shifting did not differ between treatment groups.

Behavioral changes investigated include time lying, eating time, and cud chewing. These behavioral changes were correlated with physiological changes following induction of endotoxic mastitis (Zimov, Botheras et al. 2011). The authors found that flunixin treated cows spent more time eating and chewing their cud compared to control cows with LPS-induced mastitis. Despite these improvements in behavior, they found no difference in dry matter intake or milk production between groups.

Despite mastitis having a prominent role in dairy cow health, there are few studies investigating NSAID use and naturally occurring mastitis. A controlled clinical study with 45 cows diagnosed with acute clinical mastitis compared flunixin, phenylbutazone, and saline control (Dascanio, Mechor et al. 1995). The authors found no differences in rectal temperature, milk production, or clinical depression scores at 24 h initial treatment. A three-year, multi-practice study compared flunixin only, flunixin plus IV fluids, and fluids only (Green, Green et al. 1997). The study only had 54 cows enrolled, and no differences between treatments were observed. Additionally, the authors cite variation in the objective measures taken as eight veterinarians were involved in data collection as a major issue with the study.

RESPIRATORY DISEASE

Flunixin has long been used in the treatment of bovine respiratory disease due to its antipyretic and anti-inflammatory properties. Data from the National Animal Health and Monitoring Service (NAHMS) indicates 63% of feedlots use NSAIDs in treatment or prevention measures (USDA 2011). Flunixin is approved as an antipyretic in the US and Canada. However, only 18% of feedlot veterinarians recommend flunixin as an ancillary treatment for bovine respiratory disease (BRD) despite its approval and widespread use (Lee, Terrell et al. 2015).

Reasons for not recommending flunixin in BRD treatment include lack of perceived clinical efficacy and poor economic returns (Lee, Terrell et al. 2015).

A decrease in rectal temperature is the most commonly reported effect of flunixin in cattle with respiratory disease. This effect can be seen as early as 6 h (Thiry and Brianceau 2014) in calves treated with transdermal flunixin meglumine. When administered by IV injection, the anti-pyretic effect is observed at 24 h post-treatment, but no longer significant at 48 h.

When included in the study, the outcomes are mixed in regards to the effect flunixin had on clinical scores. Cattle treated with flunixin and tulathromycin had better mean clinical scores compared to tulathromycin only treated cattle (Guzel, Karakurum et al. 2010). Additionally, calves treated with transdermal flunixin had improved clinical scores when compared to baseline (Thiry and Brianceau 2014). In an early study, calves treated with flunixin at 2.2 mg/kg IV had lower respiratory rates compared to controls following experimental parainfluenza-3 induction. Despite the lower respiratory rates, and authors suggesting the calves appeared clinically better, there was no difference in daily feed intakes between groups (Selman, Allan et al. 1984). Lockwood et al. did not observes differences in clinical scores compared to other treatment groups with and without NSAID treatment (Lockwood, Johnson et al. 2003). Despite this finding, they did observe lower lung consolidation of flunixin treated calves. In calves with experimentally induced acute respiratory distress using 3-methylindole. Flunixin was shown to lower respiratory rates and postmortem lung weights. The authors contribute the lower lung weights to decreased congestion and epithelial hyperplasia in flunixin treated calves (Selman I 1985).

The use of flunixin in the treatment of respiratory disease does not appear to be beneficial to the growth of the cattle as those reporting average daily gains observed no difference (Wilson,

Step et al. 2015, Mahendran, Booth et al. 2017). Mahendran et al. treated calves with flunixin only at the first observation of fever and found they were five times more likely to need a second treatment compared to calves treated with an antibiotic only at first treatments. The authors do acknowledge that 25% of calves treated with just a NSAID at first pull did not require a second treatment; and thus a reduction in overall antibiotic usage. Cattle treated with flunixin and an antibiotic tended to have a higher treatment success rate and lower overall medication costs (Hellwig, Kegley et al. 2000). The authors also observed lower treatment failures and relapse rates in that study. In a similar study, Scott et al. found that 15.5% of cattle treated with flunixin and tilmicosin at first pull required a second treatment. Of the calves treated with tilmicosin only, 27.9% required a second treatment, but these differences were not statistically significant (Scott 1994).

PAIN MANAGEMENT OF FLUNIXIN

Cattle of all ages can feel pain as this fact has been well documented and established among animal caretakers, animal scientist, and veterinarians. Commonly the pain cattle experience is a result of an elective procedure or a disease condition such as lameness. It is imperative that the pain be mitigated using management techniques and pharmacological interventions. The relief of pain and/or pyrexia is important for the health and wellbeing of the animal, as well as positive public perception. In the US, non-steroidal anti-inflammatory drugs (NSAID) are commonly used for fever reduction and analgesia in cattle (Fajt, Wagner et al. 2011, USDA 2011). This is due to their lack of sedation, longer duration of action, and lower regulatory oversight compared to other classes of drugs.

Flunixin meglumine is the only approved NSAID for cattle in the US, and is the only drug with an approved label claim for pain control in cattle. There has been interest in researching the role flunixin may have as an analgesic due to its known anti-inflammatory properties. Castration, dehorning, and lameness are three commonly encountered painful conditions in which there are multiple research reports regarding the analgesic actions of flunixin.

Castration

Castration is a common husbandry practice performed on male cattle worldwide. In 2017, there were over 14.5 million castration procedures in the US (NASS, 2018). In separate surveys of producers and veterinarians, the use of analgesia is as high as 50% to as low as 20% (Coetzee, Nutsch et al. 2010, Fajt, Wagner et al. 2011). Veterinarians consider surgical castration to be a painful procedure, but less than 40% administer a NSAID for analgesic (Remnant, Tremlett et al. 2017). In contrast only 10% of western Canada beef producers utilized pain mitigation for castration despite a country wide code of practices and approved analgesic drug available such as meloxicam.

The most consistent observation in the literature is the reduction of cortisol in flunixin treated calves at the time of castration. Webster et al. found that flunixin treated calves had lower cortisol levels when compared to placebo treated calves (2013). Calves in this study were also administered a local anesthetic block of lidocaine prior to the procedure. Flunixin administered by the transdermal route significantly lowered cortisol levels for 4 hours after surgical castration (Kleinhenz, Van Engen et al. 2018). This was without the use of a local anesthetic block in larger Holstein bulls. Lower salivary cortisol was seen in calves treated with flunixin at the time of band castration (Gonzalez, Schwartzkopf-Genswein et al. 2010).

The evidence is mixed in regard to the effects of flunixin on behavior following castration. Flunixin does not appear to improve average daily gains (Webster, Morin et al. 2013, Mintline, Varga et al. 2014). Its relative short half-life when administered by IV injection may be a factor as the majority of flunixin is eliminated from the body in the first 24 h. There is evidence that flunixin does provide some pain relief based on the lying behavior of calves following castration. Treatment of flunixin increased the lying time of castrated calves following either band or surgical castration compared to non-medicated calves (Gonzalez, Schwartzkopf-Genswein et al. 2010). Mintline et al. found no difference in lying behavior based on body position between flunixin and sham calves (Mintline, Varga et al. 2014). These two observations indicate that flunixin treated calves are more comfortable lying down; and stay lying longer in a more natural posture. Additionally, Currah et al. demonstrated lower pain score in flunixin treated calves at 4 h post-castration and longer, more normal stride lengths as well (Currah, Hendrick et al. 2009).

Dehorning

Dehorning, like castration, is a common husbandry procedure that causes pain and distress. Dehorning is performed on over 90% of dairy farms (USDA and 2014). A Canadian survey found that 48% of veterinarians and 24% of producers utilize NSAID therapy at dehorning (Winder, LeBlanc et al. 2016). Furthermore dehorning is conducted on over 94% of dairy farms in the US, but only 28% of these operations provide analgesia and/or anesthesia at the time of the procedure (USDA and 2014). A survey of veterinarians revealed over half use analgesia of some type (Fajt, Wagner et al. 2011).Calves treated at dehorning with transdermal flunixin had lower maximum concentrations, but a longer half-life compared to sham dehorn calves (Kleinhenz,

Van Engen et al. 2018). This also resulted in lower PGE2 concentrations at 48 and 72 h in the dehorn calves.

Cortisol levels are suppressed by flunixin administration at the time of dehorning (Kleinhenz, Van Engen et al. 2017). The use of a local anesthetic block does not fully attenuate the cortisol response (Stafford and Mellor 2005). Attenuation of the cortisol response is also linked to preventing stress associated leukocytosis and neutropenia (Ballou, Sutherland et al. 2013). Increased temperatures of the medial canthus of the eye taken by infrared thermography has been correlated to increased stress response. Kleinhenz et al. found no difference in ocular temperatures following dehorning or sham dehorning (2017). Changes in substance P levels have not been observed following IV and transdermal flunixin administration (Glynn, Coetzee et al. 2013, Kleinhenz, Van Engen et al. 2017).

The use of pressure algometry to test pain tolerance has been reported. The use of flunixin does not improve pressure algometry scores following dehorning (Glynn, Coetzee et al. 2013, Kleinhenz, Van Engen et al. 2017). Flunixin has been shown to decrease central sensitization when pressure algometry is measured at a location away from the horn tissue (Kleinhenz, Van Engen et al. 2017). Flunixin treated calves had numerically fewer head shakes and head rubs compared to calves receiving saline at the time of dehorning (Huber, Arnholdt et al. 2013). Glynn et al. observed improved average daily gains in flunixin treated calves compared to saline controls and gabapentin only treated calves (Glynn, Coetzee et al. 2013).

Lameness

Lameness is a painful condition that impacts the health and well-being of the animal. Prevalence of lameness cited in the literature ranges from 6.9% to 27.9% (Espejo, Endres et al. 2006, Adams, Lombard et al. 2017). Additionally, there are many causes of lameness in the

bovine. Due to the complexity of pathologies and treatments; the readers are referred to Coetzee et al. in Veterinary Clinics of North America – Food Animal for a more robust discussion (Coetzee, Shearer et al. 2017). Despite acknowledging the associated pain, 76% did not recommend the use of analgesia as part of the treatment of sole ulcers (Kleinhenz, Plummer et al. 2014).

Research regarding the use of flunixin as an analgesic for lameness in the literature is sparse. Chapinal et al. followed cows after seeing the hoof trimmer and found no effect of intravenous flunixin on the improvement of weight shifting (2010). The study did include non-lame cows and their inclusion may have diluted any treatment effect. The group then followed only lame cows following a hoof trim and found that flunixin treated cows had decreased amounts of weight shifting of the rear legs (Wagner, Young et al. 2017). The authors attribute this change to pain alleviation of lame cows by flunixin.

Transdermal flunixin is approved for the control of pain associated with foot rot. The drug manufacturer was able to show improved step force using a floor based pressure mat system following flunixin administration (USDA, 2017). Schulz et al. used a similar pressure mat system to evaluate flunixin administered intravenously to cattle with induced lameness (2011). They found that flunixin treated cattle had increased force and contact pressures of the lame foot. Additionally, flunixin treated cows had lower total lying time than saline treated lame cattle.

TOPICAL DRUG DELIVERY

As the newer formulation of flunixin meglumine is for topical application and transdermal absorption, a review of topical drug delivery and absorption is prudent. The physical characteristics of mammalian skin varies between species and anatomical location of the body.

These differences have been shown to cause drastic differences in the uptake of drugs across the skin (Mills, Magnusson et al. 2005). The skin of livestock species (and humans) is comprised of three distinct layers. These three layers are the outermost epidermis; the dermis, and the inner hypodermis. There are differences in the thickness, presence of appendages (i.e. hair follicles), and vascularity (Riviere and Papich 2001). The chemical properties of the drug and vehicles in which it is contained must also be taken into account when discussing transdermal drug absorption.

The epidermis is avascular and comprised of numerous distinct layers of keratinocytes in various degrees of differentiation. The keratinocytes are surrounded by a continuous bilayer of lipids. This gives rise to the epidermis to be described as a "brick and mortar" structure. This mixing of cell and lipid layer is key to the diffusion of drugs from the skin surface to the blood. The major barrier to drug absorption is the stratum corneum as it is the outermost layer of the skin. The stratum corneum serves as an effective waterproof barrier of the skin.

The dermis is a vascular layer of the skin located between the outer epidermis and inner hypodermis. The dermis has a role in thermoregulation of the body using its network of blood vessels and nerves. Blood flow to the dermis is adjusted based on the environmental, health and stress of the animal. The dermis also contains sebaceous glands, apocrine sweat glands, and hair follicles. The concentration of these structures varies between species and anatomical location on the body.

The hypodermis is the inner most layer of skin. It is comprised of adipocytes, fibroblasts, endothelial cells and macrophages. It connects the skin to the underlying cutaneous muscle layers. Its major function is to carry vessels and nerves to the skin (Mills and Cross 2006).

There are three major mechanisms for the transport of drugs across the epidermis (Mills and Cross 2006). The three mechanisms for trans-epidermal drug movement are: intercellular, transcellular, and trans-appendageal. Intercellular transport is the movement of a drug using the lipid layer between the keratinocytes. For a drug to be efficiently absorbed, it would have to be lipid soluble and small size. The transcellular movement of drugs is the least likely path of absorption as the drug compound would have to diffuse into and out of each keratinocytes. Transport using appendages is a likely route as these structures bypass the epidermis due to their openings to the surface.

The chemical properties of the drug molecules intended for transdermal absorption must also be taken into consideration. The polarity, relative lipophilicity, and particle size are molecular properties that dictate the absorption of drugs transdermally. Non-polar drugs with higher lipophilicity are more readily absorbed through the lipid layer around keratinocytes. Additionally, chemical sizes less than 40,000 Daltons are better suited for transdermal drug delivery (Riviere and Papich 2001).

In addition to the chemical properties of the drug intended for transdermal delivery, the vehicle used to deliver the drug aids it absorption and ability to cross the epidermis. Vehicles used to deliver topical pharmaceuticals are used to enhance the delivery of the drug and alter the stratum corneum to allow the drug to cross (Mills and Cross 2006). The chemical properties of the vehicle can alter the absorption of drugs across the skin. Baynes demonstrated enhanced avermectin absorption with alcohol-based vehicles compared to oil-based vehicles (Baynes 2004). The solubility of the drug in the vehicle impacts the partition coefficient of the drug. The partition coefficient describes the release of the drug from the vehicle making it available for absorption.

CONCLUSIONS

Flunixin has repeatedly been shown as an effective antipyretic and anti-inflammatory. The literature is mixed on the utility of flunixin in treating cattle with mastitis or respiratory disease. For respiratory disease, flunixin lowers fevers and respiratory rates; but it does not appear to improve daily gains and feed intakes. A similar pattern is seen when cows are administered flunixin for the treatment of mastitis. Flunixin improves rumen function, dry matter intake, and decreases leg shifting associated with swelling. These changes do not appear to translate into improved milk production or lower culling rates. When treating for mastitis or respiratory disease, the short-term improvements in animal health do not appear to correlate to a positive long-term outcome. However, much of this work has yet to be repeated using transdermal flunixin which appears to have a longer anti-inflammatory effect.

The utility of flunixin as an analgesic is dependent on the cause of pain and use of other included drugs as part of a multi-modal analgesic plan. There is supporting evidence for the use of flunixin in lame cattle that do not have foot rot. The use of flunixin in cases of lameness not associated with foot rot appear to be positive. Further work into the utility of transdermal flunixin for treating lameness not caused by foot rot is needed. For castration and dehorning, the evidence is not as supportive, as flunixin does appear to alter nociception threshold tests and substance P. It does lower cortisol and reduce head shaking behavior.

Future work is needed to investigate the utility of transdermal flunixin in different age of cattle, various painful situations, and time relative to the pain stimulus. Since the transdermal formulation is easy to administer and requires relatively less restraint; its adaptation may be considered by those who felt the IV formulation was difficult to administer. Differences in the analgesic and anti-inflammatory components of the IV and transdermal formulations will be due

to differences in the pharmacokinetics of the two formulations. It appears the transdermal formulation will provide a longer anti-inflammatory effect and possibly a more pronounce analgesic effect. Situations that alter the absorption of flunixin from the skin will ultimately alter the effects of the drug.

Table 1-1 Selective pharmacokinetic parameters for cattle receiving flunixin meglumine by intravenous injection.

	Dose	$T_{1/2}$	Vd	Cl	Weight range	Animals class
Reference	mg/kg	h	L/kg	mL/kg/min	kg	
Hardee, Smith et al. (1985)	1.1	8.12	1.05	1.5	346-506	Immature Cows
Anderson, Neffdavis et al. (1990)	1.1	3.14	0.397	2.51	NR	Mature Cows
Landoni and Cunninham (1995)	2.2	6.87	2.11	3.33	118.9±1.5	Calves
Odensvik (1995)	2.2	5.2	0.782	1.92	407-562	Mature Cows
Kissell, Smith et al. (2012)	1.1	3.4	0.254	2.51	545-676	Mature Cows
Kissell, Brinson et al. (2016)	2.2	12.9	0.634	1.08	53.3±9.5	Calves
Gorden, Kleinhenz, et al. (2018)	2.2	4.14	0.538	3.54	573-722	Mature Cows

NR = Not reported

Table 1-2 Summary of outcomes of published literature investigating the use of flunixin meglumine in the treatment of bovine respiratory disease.

Reference	Dose (mg/kg)	Route of Administration	Number per group	Key outcomes
Guzel, Karakurman et al. (2010)	2.2	IV	30	Improved clinical scores 1st 48 hours Lower rectal temps. and respiratory rates
Hellwig, Kegley et al. (2000)	2.2	IV	26	Lower treatment failure and relapse rates Tendency to have lower average medicine costs
Lockwood, Johnson et al. (2003)	2.2	IV	17	No difference in depression scores, dyspnea or cough Lower lung consolidation at slaughter
Mahendran, Booth et al. (2017)	2	IM	42	No effects on average daily gain Flunixin only treated calves 5x more likely to have 2nd pull for treatment
Scott (1994)	2.2	IV	60	Non-significant reduction in percent of calves requiring a 2nd treatment No difference in treatment interval compared to antibiotic only
Selman, Allan et al. (1984)	2.2	IV	5	Lower respiratory rates compared to controls No difference in feed intake between groups
Selman (1985)	2.2	IV	5	Lower respiratory rates and better clinical appearance Lower post-mortem lung weights due to decreased congestion and epithelial hyperplasia
Thiry and Brianceau (2014)	3.3	TD	23	Improved clinical depression scores compared to placebo Larger decrease in rectal temperature at 6 hr post-treatment
Wilson, Step et al. (2015)	2.2	IV	80	No difference in rectal temperature, mortalities or removal No difference in daily gains or feed conversion

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Chapter 2 - Short Communication: The pharmacokinetics of transdermal flunixin meglumine in Holstein calves

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ABSTRACT

This study describes the pharmacokinetics of topical and intravenous (IV) flunixin meglumine in Holstein calves. Eight male Holsteins calves, age 6 to 8 weeks were administered flunixin at a dose of 2.2 mg/kg intravenously. Following a 10 day washout period, calves were

dosed with flunixin at 3.33mg/kg topically (transdermal). Blood samples were collected at predetermined times from 0 to 48 hours for the intravenous portions and 0 to 72 hours following topical dosing. Plasma drug concentrations were determined using liquid chromatography with mass spectroscopy. Pharmacokinetic analysis was completed using noncompartmental methods. The mean bioavailability of topical flunixin was calculated to be 48%. The mean AUC for flunixin was determined to be 13.9 h×ug/mL for IV administration and 10.1 h×ug/mL for topical administration. The mean half-life for topical flunixin was 6.42 h and 4.99 h for the intravenous route. The C_{max} following topical application of flunixin was 1.17 μg/mL. The time to maximum concentration was 2.14 h. Mean residence time (MRT) following IV injection was 4.38 h and 8.36 h after topical administration. In conclusion, flunixin when administered as a topical preparation is rapidly absorbed and has longer half-life compared to IV administration.

Keywords: Flunixin, Topical Pharmacokinetics, Cattle, Pharmacology, Mass Spectrometry

Flunixin meglumine is the only nonsteroidal anti-inflammatory drug (NSAID) in the United States that is approved for use in cattle. Flunixin is specifically labeled for intravenous administration to treat pyrexia associated with bovine respiratory disease and endotoxic mastitis as well as the control of inflammation in endotoxemia. Intramuscular administration of the intravenous formulation of flunixin causes profound tissue blemishes and carcass trim (Pyorala, Laurila et al., 1999). Recently a novel formulation of flunixin meglumine intended as a pour-on application for transdermal absorption has been approved in the European Union. Like the injectable product, it is only labelled to treat pyrexia associated with bovine respiratory disease

and acute mastitis. The aim of this study was to describe the pharmacokinetics of flunixin meglumine when administered as a transdermal topical preparation.

Eight weaned male Holstein calves were enrolled into the study. The calves were 6 to 8 weeks of age, weighted 60.2 ± 7.3 kg, and were procured from a single source farm. The study was approved by the Institution Animal Care and Use Committee (IACUC log # 6-15-8039-B) at Iowa State University. The calves were acclimated for 14 days after arrival where they were trained to be restrained by halter and lead rope. During the acclimation period, all calves were administered amprolium (Corid, Duluth, GA) at 10mg/kg per os (PO) for five days.

The study was completed in two phases with a 10 day washout period between the two phases. The first phase of the study was to determine pharmacokinetics of flunixin when administered by the intravenous route to facilitate determination of transdermal bioavailability. Calves were administered flunixin meglumine (Banamine®, Merck Animal Health, Madison, NJ) at 2.2 mg/kg into the left jugular vein via catheter. Blood collections, for plasma concentrations, were obtained from the right jugular catheter before drug administration and at 3, 6, 10, 15, 30, 45, 60 minutes and 2, 4, 6, 8, 12, 24, 36, and 48 hours.

In the second phase of the study, the transdermal route of administration was tested for the pharmacokinetics of flunixin. Calves were administered the label dose of flunixin meglumine (Finadyne Pour-On, MSD Animal Health, Dublin, Ireland) at 3.33 mg/kg via transdermal route. The drug was applied to the calf on the top-line starting at the shoulders and ending at the tail head using a single use syringe, following label directions. Blood collections, for plasma drug concentrations, were obtained from the jugular catheter before drug administration and at 10, 20, 30, 40, 50, 60, and 90 min and at 2, 4, 6, 8, 12, 24, 36, 48, 56 and 72 hours post drug administration.

At each time point, blood was collected from the catheter using a syringe and placed into sodium heparin tubes (BD Vacutainer, Franklin Lakes, NJ). The samples were centrifuged at 1,500 g for 10 minutes. The plasma was pipetted off and placed into cryovials. Plasma samples were stored at -80°C until analyzed.

Plasma concentrations of flunixin were determined using high-pressure liquid chromatography coupled with mass spectroscopy. Briefly, plasma samples were thawed and vortexed and 100 µL aliquots were transferred into a vial to which 400 µL of 50 ng/mL internal standard, flunixin D-3, in acetonitrile with 0.1% formic acid was added. Samples were vortexed and centrifuged at 2500 g for 20 minutes. Supernatant was transferred, and samples were dried down, reconstituted in 200 µL of 25% acetonitrile in water, vortexed and transferred to an autosampler vial (with glass insert), centrifuged for 20 min at 2500 g and analyzed via LC-MS/MS.

Concentrations of flunixin in plasma samples were determined utilizing an ABSciex QTRAP 4500 mass spectrometer coupled to a 1260 Infinity pump and autosampler (ABSciex, Framingham, MA, USA). The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. The flow rate was 0.5 mL/min. The mobile phase began at 2% B with a linear gradient to 80% B at 7.27 min and 99% B at 7.37 min. It was re-equilibrated to 2% B at 11 min. Separation was achieved with a C18 column (Gemini C18, 50mm x 2mm 3µm particles Phenomenex, Torrance, CA, USA). The three ions measured for flunixin in negative ion mode were (*m/z*) 295 → 251, 210, and 197 at retention time of 5.60 minutes, however only 251 was used for quantification. The standard curve was quadratic in bovine plasma from 0.005 to 10 µg/ml. Samples with flunixin concentrations greater than 10 µg/ml were diluted with blank bovine plasma. The curve was accepted when the correlation coefficient exceeded 0.99 and the

measured values were within 20% of their expected value. The accuracy and coefficient of variation for flunixin was 108% and 7.3% respectively. The limit of quantification was 0.005 μg/ml and the limit of detection was 0.003 μg/ml.

Pharmacokinetic analysis was completed using computer software (Phoenix 64, Certara, Princeton, NJ, USA) and noncompartmental methods. The individual animal pharmacokinetic values were calculated and the descriptive statistics (geometric mean, minimum, median and maximum values) were reported (Julious & Debarnot, 2000). The bioavailability (F) was calculated after topical administration with the following equation:

$$\frac{Topical\ AUC / Topical\ Dose}{IV\ AUC / IV\ Dose}$$

Table 1 summarizes the pharmacokinetic parameters for flunixin when administered by the IV route. Table 2 summarizes the pharmacokinetic parameters for flunixin when administered by the transdermal route. The mean plasma concentrations for flunixin for both intravenous and transdermal routes are shown in Figure 1.

To our knowledge this is the first report describing the systemic pharmacokinetics of flunixin meglumine after transdermal administration. A randomized cross-over design with the IV formulation was not conducted in this study since the pharmacokinetics of the topical formulation was not known, and an appropriate washout period could not be estimated. Since the pharmacokinetics of the IV formulation have been well described, the study was designed to administer the IV formulation first, followed by the topical formulation after the observed washout period. No drug carryover was present in time 0 samples for the transdermal route. Although the lack of randomization could bias the results of the transdermal portion of the study,

the short time period of the study and lack of repeated dose effects on flunixin pharmacokinetics would be expected to have minimal effects on the transdermal pharmacokinetics.

In this study, cattle were restrained to prevent self-grooming and allo-grooming for the first four hours post-dosing. It has been suggested that the grooming and licking behaviors should be considered when evaluating bioavailability of topical and transdermal drugs (Toutain, Modric et al., 2012). However, data presented at the 2014 World Buiatrics Congress reported no difference in absorption of transdermal flunixin if calves were allowed to groom versus not allowed to groom (Crouch, Brianceau et al., 2014).

In the United States, there is currently only one veterinary approved topical NSAID. Surpass®, 1% diclofenac sodium (Boehringer-Ingelheim Vetmedica, Inc., St. Joseph, MO, USA) is approved for the control of pain and inflammation associated with osteoarthritis in equine. However, there is no published literature on its use and pharmacokinetics in cattle to compare data.

Other investigators have described the pharmacokinetics of flunixin given by the oral, intramuscular and subcutaneous routes (Odensvik, 1995; Kissell, Smith et al., 2012). In our study, topical flunixin had a lower mean bioavailability (48%) compared to the oral route (60%); intramuscular (85%) or subcutaneous (104%) (Odensvik, 1995; Kissell, Smith et al., 2012). The half-life ($t_{1/2}$) was 6.42 h for the topical route and 4.99 h of the IV formula. This is compared to the half-life of 6.20 h, 4.48 h, and 5.39 h for the oral, intramuscular and subcutaneous routes respectively (Odensvik, 1995; Kissell, Smith et al., 2012). However until a direct comparison of the different routes of administration using a randomized crossover design, comparisons should be cautious.

The mean maximum concentration (C_{max}) of flunixin for the topical route was 1.17µg/mL, which was similar to the other routes. The reported C_{max} for the intramuscular, subcutaneous and oral routes was 2.2 µg/mL, 1.33 µg/mL, and 0.9 µg/mL respectively (Odensvik, 1995; Kissell, Smith et al., 2012). The mean absorption time (MAT) of 3.82 h was between the MAT for the intramuscular route (2.71 h) and subcutaneous route (4.78 h), but was slower than the 6.3 h MAT for oral administration (Odensvik, 1995; Kissell, Smith et al., 2012).

One clinical study on the use of topical flunixin as an adjunct therapy in bovine respiratory disease has been published (Thiry & Brianceau, 2014). The authors were able to show a decrease in rectal temperature and fever reduction of 1.68°C.An improvement in respiratory signs and clinical depression scores was seen 6 hours after administration.

In conclusion, when flunixin is administered as a topical transdermal preparation, it is rapidly absorbed, has longer half-life, compared to IV administration. Additional work needs to be completed to investigate the suppression of prostaglandin synthesis by topical flunixin administration, which is a proxy of its pharmacodynamics. Additionally, more work needs to be done to evaluate how pain or diseased states can alter the pharmacokinetics and/or pharmacodynamics of the topical preparation of flunixin.

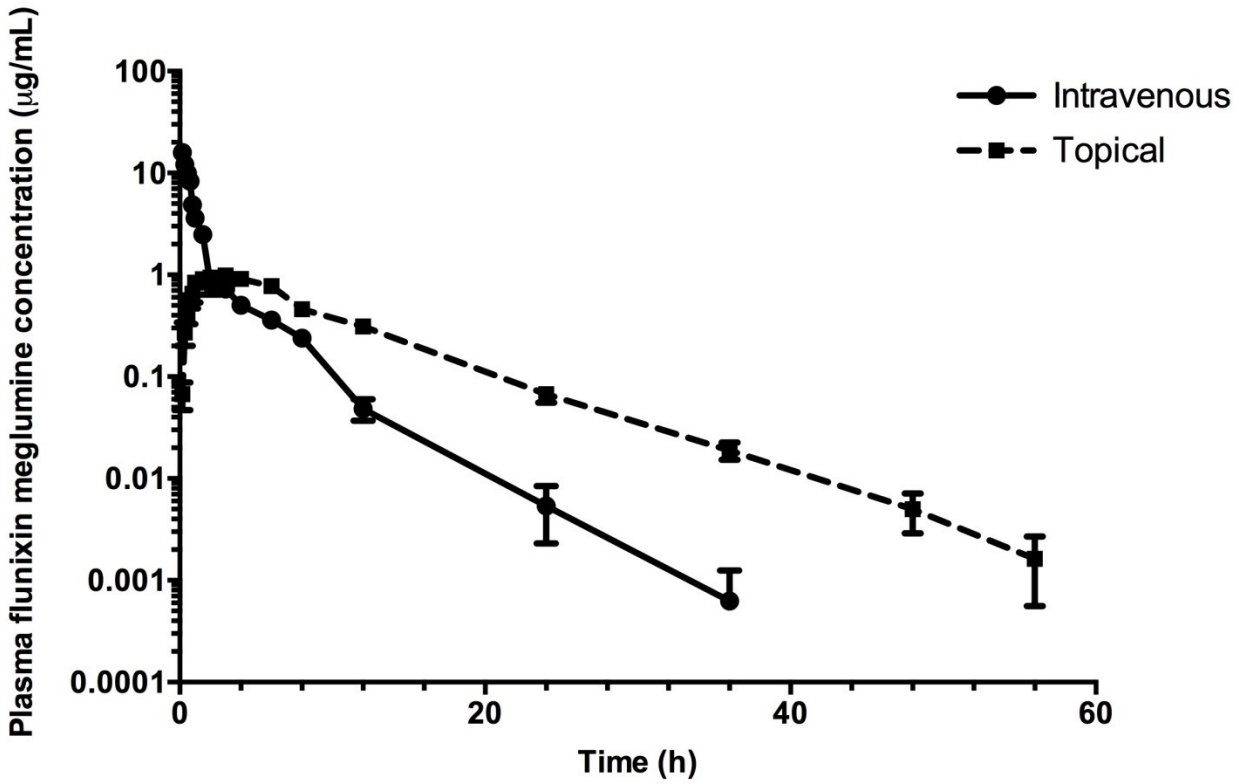

Figure 2-1 Plasma concentrations (Mean ± SEM) of flunixin over time by the intravenous and topical routes.

Table 2-1 Pharmacokinetics of IV flunixin at a dose of 2.2 mg/kg. AUC Extrap = percent of the AUC extrapolated to infinity; AUC = area under the curve extrapolated to infinity; Cl = plasma clearance; C0 = plasma concentration extrapolated to time 0; T ½ = terminal half-life; λz = terminal rate constant; MRT = mean residence time extrapolated to infinity; Vss = volume of distribution at steady state; Vz = volume of distribution (area method). [1]

Parameter	Units	Individual Animal ID								Geometric Mean	Min	Median	Max
		1460	1466	1468	1469	1470	1471	1475	1493				
AUC Extrap	%	0.7	1.4	2.6	1.6	1.2	1.5	0.3	0.2	0.9	0.2	1.3	2.6
AUC	h×ug/mL	13.0	12.4	9.58	10.2	13.3	15.6	18.5	23.0	13.9	9.58	13.1	23.0
C0	ug/mL	15.8	26.5	14.1	19.6	23.7	22.4	18.0	18.0	19.4	14.13	18.8	26.5
Cl	mL/min/kg	2.82	2.95	3.83	3.61	2.77	2.36	1.99	1.59	2.64	1.59	2.79	3.83
T ½	h	5.82	4.67	5.69	4.72	4.37	4.52	4.79	5.55	4.99	4.37	4.76	5.82
λz	1/h	0.12	0.15	0.12	0.15	0.16	0.15	0.15	0.13	0.14	0.12	0.15	0.16
MRT	h	4.81	3.79	4.32	3.88	3.67	4.12	5.34	5.45	4.38	3.67	4.22	5.45
Vss	L/kg	0.81	0.67	0.99	0.84	0.61	0.58	0.64	0.52	0.69	0.52	0.65	0.99
Vz	L/kg	1.42	1.19	1.88	1.47	1.05	0.92	0.82	0.77	1.14	0.77	1.12	1.88

[1] The following parameters were calculated for IV administration: AUC = area under the curve extrapolated to infinity, using the linear trapezoidal method; AUC Extrap = percent of the AUC extrapolated to infinity; Cl = plasma clearance; C0 = plasma concentration back extrapolated to time 0 using log linear regression of the first two time points; T ½ = terminal half-life; λz = terminal rate constant; MRT = mean residence time extrapolated to infinity; Vss = volume of distribution at steady state; Vz = volume of distribution (area method).

Table 2-2 Pharmacokinetics of topical flunixin at a dose of 3.33 mg/kg. AUC Extrap = percent of the AUC extrapolated to infinity; AUC = area under the curve extrapolated to infinity; Cl/F = clearance per fraction of the dose absorbed; C_{MAX} = maximum plasma concentrations; T ½ = terminal half-life; λz = terminal rate constant; MRT = mean residence time extrapolated to infinity; MAT = mean absorption time; T_{max} = time of C_{max}; Vz/F = volume of distribution (area method) per fraction of the dose absorbed; F = fraction of the dose absorbed. [2]

Parameter	Units	Individual Animal ID								Geometric Mean	Min	Median	Max
		1460	1466	1468	1469	1470	1471	1475	1493				
AUC Extrap	%	0.9	0.8	0.6	1.0	1.1	0.9	0.9	0.4	0.8	0.4	0.9	1.1
AUC	h×ug/mL	8.34	10.1	8.31	9.41	10.0	11.05	9.45	15.8	10.1	8.31	9.71	15.8
Cl/F	mL/min/kg	6.65	5.49	6.68	5.90	5.54	5.02	5.89	3.51	5.49	3.51	5.72	6.68
C_{max}	ug/mL	0.68	1.27	1.38	1.23	0.86	1.32	1.38	1.56	1.17	0.68	1.30	1.56
T ½	h	7.05	5.37	6.88	5.35	9.76	5.73	5.22	7.06	6.42	5.22	6.30	9.76
λz	1/h	0.10	0.13	0.10	0.13	0.07	0.12	0.13	0.10	0.11	0.07	0.11	0.13
MRT	h	11.2	7.64	7.64	7.37	9.13	7.31	7.23	10.3	8.36	7.23	7.64	11.2
MAT	h	6.39	3.85	3.32	3.49	5.46	3.19	1.89	4.82	3.82	1.89	3.67	6.39
T_{MAX}	h	4	2	2	1.5	2	1.5	1.5	4	2.14	1.5	2	4
Vz/F	L/kg	4.06	2.55	3.98	2.73	4.68	2.49	2.66	2.14	3.05	2.14	2.70	4.68
F		0.42	0.54	0.57	0.61	0.50	0.47	0.34	0.46	0.48	0.34	0.48	0.61

[2] For topical administration the Cl/F = Cl per fraction of the dose absorbed and Vz/F = Vz per fraction of the dose absorbed were also calculated. The C_{max} (maximum plasma concentrations) and T_{max} (time of C_{max}) were determined directly from the data. The MAT (mean absorption time) was calculated by subtracting the IV MRT from the topical MRT.

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Chapter 3 - Short Communication: Pharmacokinetics of multiple doses of transdermal flunixin meglumine in adult Holstein dairy cows

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ABSTRACT

A transdermal formulation of the nonsteroidal anti-inflammatory drug, flunixin meglumine has been approved in the United Stated and Canada for single dose administration. Transdermal flunixin meglumine was administered to 10 adult Holstein cows in their second or third lactation at the label dose of 3.33 mg/kg every 24 hours for 3 total treatments. Plasma flunixin concentrations were determined using high-pressure liquid chromatography with mass spectroscopy (HPLC-MS). Pharmacokinetic analysis was completed on each individual animal with noncompartmental methods using computer software. The time to maximum drug concentration (Tmax) was 2.81 h and the maximum drug concentration was 1.08 µg/ml. The mean terminal half-life (T ½) was determined to be 5.20 h. Clearance per fraction absorbed (Cl/F) was calculated to be 0.294 L/h/kg and volume of distribution of fraction (Vz/F) absorbed was 2.20 L/kg. The mean accumulation factor was 1.10 after 3 doses. This indicates changes in dosing may not be required when giving multiple doses of flunixin transdermal. Further work is required to investigate the clinical efficacy of transdermal flunixin after multiple daily doses.

Keywords: Dairy cattle, Flunixin, NSAID, Pour-on, Topical

Flunixin meglumine is a nicotinic acid derivative, nonsteroidal anti-inflammatory drug (NSAID) that has been approved as an injectable formulation for intravenous use in cattle for the treatment of pyrexia associated with bovine respiratory disease (BRD) and endotoxic mastitis and the treatment of endotoxemia associate with coliform mastitis (2017). A new transdermal pour-on formulation was approved by the US Food and Drug Administration (FDA) in 2017, and previously approved in the European Union in 2014, for single dose administration. In the

United States, the new label approval is for the control of pyrexia from bovine respiratory disease (BRD) and the control of pain associated with foot rot (US FDA, 2017). Due to the label indication for control of pain associated with foot-rot, this formulation of flunixin has the potential to be an important tool as an extra-label drug use (ELDU) for the control of pain in other disease processes. The primary objective of the present study was to describe the pharmacokinetics of topical flunixin following multiple dose administration in lactating dairy cattle.

This study was approved by the Institutional Animal Care and Use Committee at Iowa State University (Log # 7-16-8314-B). Ten Holstein dairy cows averaging 86 days in milk and in their second or third lactation were enrolled in the study. Cows were administered flunixin meglumine (Finadyne Pour-On, MSD Animal Health, Dublin, Ireland) at the label dose of 3.33mg/kg every 24 hours for a total of 3 doses via the transdermal route. The flunixin product used in this study was the European Union approved product with the trade name Finadyne Transdermal. It is manufactured by the same parent company as the transdermal flunixin product approved in the United States and Canada. The drug was administered by applying the product to the top-line, starting at the shoulders and ending at the tail head, using a syringe in accordance to the manufacturer's directions. Blood samples were obtained before initial drug application and at 30, 60, and 90 min and 2, 3, 4, 6, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 96, and 120 hours post-drug application. Blood samples at 24 and 48 h were collected prior to the second and third dosing of the transdermal flunixin, respectively.

At each collection time point, up to 20 ml of blood was collected via jugular venipuncture using a syringe and needle. Collected blood was placed into two 10ml blood collection tubes with sodium heparin (BD Vacutainer, Franklin Lakes, NJ) and inverted to mix contents.

Samples were immediately placed on ice and transported to the lab within 1 hour. The samples were centrifuged at 3,500 rpm for 10 minutes. The plasma was pipetted off and placed into cryovials. Plasma samples were immediately placed onto dry ice and further stored at -80°C until analyzed.

Concentrations of flunixin in plasma samples were determined utilizing high-pressure liquid chromatography (Agilent 1100 Pump, Column Compartment, and Autosampler, Santa Clara, CA) with mass spectrometry detection (LTQ, Thermo Scientific, San Jose, CA). Calibration standards were made by spiking blank bovine plasma with flunixin to concentrations of 0.2; 1; 2; 5; 10; 20; 50; 100; 200; 500; 1,000; 2,000; and 5,000 ng/ml. Quality control (QC) samples were prepared by spiking blank bovine plasma with flunixin to a concentration of 15, 150, and 1,500 ng/ml. Samples underwent an extraction via protein precipitation by adding a 100 µl aliquot to a vial followed by 400 µl of acetonitrile with 0.1% formic acid containing 50 ng/ml of internal standard, flunixin D-3. Samples were centrifuged at 2,400 rpm for 20 minutes. Supernatant was transferred to glass tubes, samples were dried down under a stream of nitrogen. Samples were then reconstituted in 200 µL of 25% acetonitrile in water, vortexed and transferred to an autosampler vial (with glass insert). Autossampler vials were then centrifuged for 20 minutes at 2,400 rpm and analyzed via LC-MS/MS.

The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. The flow rate was 0.275 mL/min. The mobile phase began at 25% B with a linear gradient to 95% B at 5.0 min, which was maintained for 1.5 min at 0.35 ml/min, followed by re-equilibration to 25% B at 0.35 ml/min for 3.5 min. Separation was achieved with a C_{18} column 100mm x 2.1mm 3µm particles (HypersilGold C_{18}, Thermo Scientific, San Jose, CA, USA) maintained at 50°C. Flunixin and flunixin D-3 eluted at 4.87±0.05 min. Full scan wideband MS

of the parent ions of flunixin (m/z 297) and flunixin D-3 (m/x 300) was used for analyte detection. Plasma concentrations of flunixin were calculated by computer software (Xcalibur Software, Thermo Scientific, San Jose, CA, USA) based on the calibration curve. The standard curve was linear with an $R^2 \geq 0.9970$ and coefficient of variation for flunixin was 2.5% for each concentration of 15, 150 and 1,500 ng/ml. The limit of quantification (LOQ) was 0.5 ng/ml with a limit of detection (LOD) of 0.2 ng/ml.

Plasma flunixin concentrations versus time data for each individual cow were subjected to non-compartment analysis methods using a commercially available software program (Phoenix® Win-Nonlin® 7.0, Certara, Inc. Princeton, NJ, USA). The area under the concentration time curve (AUC) and the area under the first moment of the concentration-time curve (AUMC) were calculated using the linear/logarithmic trapezoidal method. For the calculation of AUC_{last} and $AUMC_{last}$, time range from the first measurement to the last measurable drug concentration, as well as the extrapolation to infinity (AUC_{∞}, $AUMC_{\infty}$) was used. The rate constant associated with the terminal elimination phase (λ) was estimated by means of linear regression of the terminal phase of the log plasma concentration vs. time curve, and the half-life (T1/2) and volume of distribution of fraction absorbed (V/F) associated with (Vd_{λ}) was calculated. Overall clearance of fraction absorbed (CL/F), mean residence time (MRT_{last}), and Accumulation factor (R) were calculated from AUC and AUMC. The values of peak plasma concentration (Cmax), time to obtain peak concentration (T_{max}) were reported as observed values.

The pharmacokinetic parameters for multiple dose transdermal flunixin are summarized in Table 1 and the individual animal pharmacokinetics are presented in Table 2. The time course of mean plasma concentrations for flunixin is shown in Figure 1.

Following multiple doses, there was no accumulation of flunixin in the plasma, indicating dose adjustments may not be required to avoid toxicities if given at 24-hour frequencies. However, further work investigating the anti-inflammatory effects (specifically, PGE_2 suppression) is needed to evaluate potential clinical efficacy after multiple administrations. Additionally, there is a need to explore any potential tissue residues to evaluate if the label meat withdrawal time is adequate following multiple doses. This information is critical as this product is relatively easy to dose with minimal animal restraint required making the potential for inappropriate use high.

The half-life reported here(5.20h) is shorter in these cattle following multiple doses compared to those (6.42h) published following a single dose in younger Holstein calves (Kleinhenz, 2016). The time to maximum flunixin concentration (T_{max}), maximum flunixin concentration (C_{max}), and mean resident time (MRT) are similar to those in younger calves. This is noteworthy because the C_{max} and MRT of transdermal flunixin have been shown to be influenced by age (Kleinhenz, In Press). However, the Cl/F = 0.294 L/h/kg is slightly lower in adult cattle compared to those (0.321L/h/kg) previously published and is possibly influenced by age, sex and lactation status (Igarza, 2004; Modric & Martinez, 2011).

The pharmacokinetics of flunixin following multiple intravenous (IV) or intramuscular (IM) injections have been published (Smith, 2015). The half-life of the transdermal formulation (5.2 h) is shorter compared to 3 doses given either IV or IM (11.6 h and 15.5 h respectively) to adult dairy cows (Smith, 2015). The MRT for the multiple doses by IV (36.7h) or IM (44.3h) routes were greater than the transdermal routes. Furthermore, the Cmax of the transdermal formulation was lower than the C_0 following IV or C_{max} following the IM routes. The C_{max} was 1.09 µg/ml for the transdermal route in the present study. Following IV administration the C_0

was determined to be 11.9 µg/ml and the Cmax was 5.50 µg/ml after IM administration in adult Holsteins (Smith, 2015).

The housing in the current study was intended to mimic standard industry practices. Cows were group housed and only restrained from grooming for the first 4 hours of the study. Cows were allowed to practice normal grooming practices thereafter. Flunixin has an oral bioavailability of 60% (Odensvik, 1995). The test product was shown to have true transdermal absorption in calves that were prevented from grooming following drug application (Kleinhenz, 2016). Furthermore, there is supporting data showing no difference between cattle allowed to groom and those not allowed to groom (US FDA, 2017).

In conclusion, multiple doses of transdermal flunixin given at 24-hour intervals appear to have similar pharmacokinetics as a one-time dose. Further work is needed to address clinical effectiveness and to what degree, if any, tissue accumulation occurs.

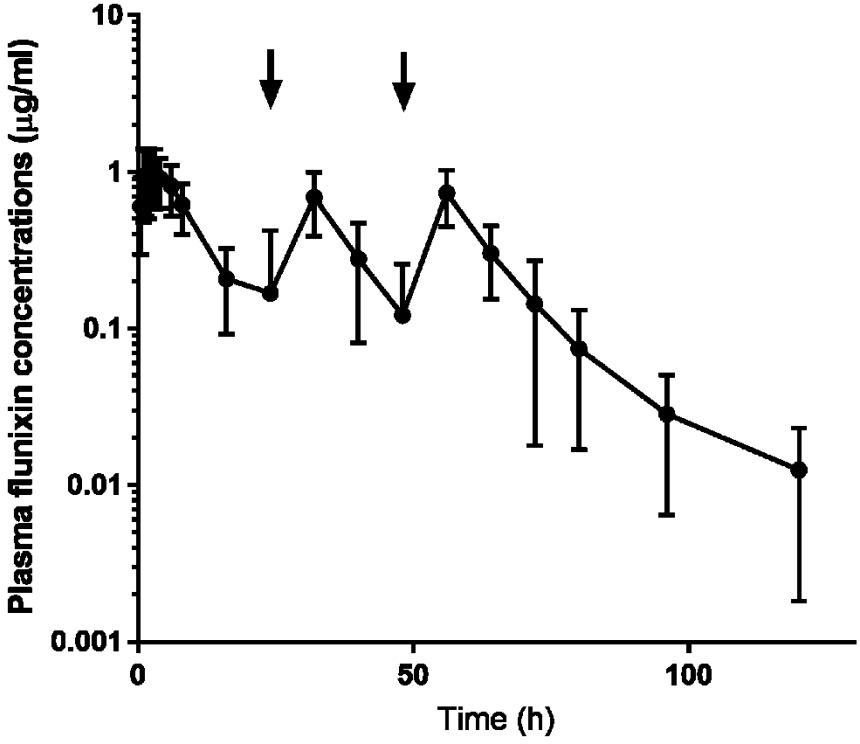

Figure 3-1 Mean (±SEM) plasma flunixin concentrations (ng/ml) following multiple doses of flunixin meglumine by transdermal route in adult Holstein cows. The ↓ symbols indicate drug administration at 24 and 48 hours after the first dose.

Table 3-1 Summary of pharmacokinetic parameters for adult Holstein cows (n=10) following transdermal administration of flunixin meglumine every 24 hours for 3 total doses at 3.33 mg/kg.

Parameter	Unit	Geometric Mean	SD	Min	Median	Max
$T_{1/2}$	h	5.20	1.34	3.43	4.90	7.82
Vz/F	L/kg	2.20	1.41	1.43	1.95	6.20
T_{max}	h	2.81	2.41	1.00	3.00	8.00
C_{max}	µg/ml	1.08	0.39	0.46	1.21	1.74
CL/F	L/h/kg	0.29	0.11	0.20	0.26	0.55
AUC_{tau}	µg*h/ml	10.68	3.13	5.35	11.73	15.60
$AUMC_{tau}$	µg*h2/ml	76.97	34.92	42.65	79.83	143.24
MRT_{last}	h	7.20	1.74	4.72	6.95	9.94
R=accumulation factor		1.10	0.11	1.03	1.07	1.30

Table 3-2 Individual animal pharmacokinetic parameters for adult Holstein cows following transdermal administration of flunixin meglumine every 24 hours for 3 total doses at 3.33 mg/kg.

		Cow ID									
Parameter	Unit	1	2	3	4	5	6	7	8	9	10
$T_{1/2}$	h	4.14	5.61	6.97	3.43	6.29	4.62	4.75	7.82	5.00	4.79
Vz/F	L/kg	1.43	1.62	2.49	1.74	2.26	2.02	1.88	6.20	1.64	3.06
T_{max}	h	3.00	6.00	8.00	1.00	6.00	1.50	4.00	2.00	1.00	3.00
C_{max}	µg/ml	1.74	1.33	0.83	1.64	0.99	1.22	1.20	0.46	1.35	0.79
CL/F	L/h/kg	0.24	0.20	0.25	0.35	0.25	0.30	0.27	0.55	0.23	0.44
AUC_{tau}	h*µg/ml	13.69	15.60	11.74	9.39	12.13	10.64	11.72	5.35	14.04	7.25
$AUC_{48h-72h}$	h*µg/ml	11.24	14.60	17.60	5.61	11.32	7.01	7.35	6.54	7.22	5.23
$AUMC_{tau}$	µgh2/ml	80.20	143.24	113.21	44.34	120.50	66.95	79.46	42.65	99.90	46.35
MRT_{last}	h	5.86	9.18	9.64	4.72	9.94	6.29	6.78	7.97	7.12	6.39
R=accumulation factor		1.06	1.03	1.30	1.03	1.30	1.03	1.08	1.12	1.03	1.08

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Chapter 4 - Effect of age on the pharmacokinetics and pharmacodynamics of flunixin meglumine following intravenous and transdermal administration to Holstein calves

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ABSTRACT

OBJECTIVE

To determine the effect of age on the pharmacokinetics and pharmacodynamics of flunixin meglumine (flunixin) following IV and transdermal administration to calves.

ANIMALS

Eight healthy weaned Holstein bull calves.

PROCEDURES

At 2 months of age, all calves received an injectable solution of flunixin (2.2 mg/kg, IV); then, after a 10-day washout period, calves received a topical formulation of flunixin (3.33 mg/kg, transdermally). Blood samples were collected at predetermined times before and for 48 and 72 hours afterward. At 8 months, calves received flunixin by the transdermal route first. Other factors remained constant. Plasma flunixin concentrations were determined by liquid chromatography-tandem mass spectroscopy. For each administration route, pharmacokinetic parameters were determined by noncompartmental methods and compared between the 2 ages. Plasma prostaglandin E_2 (PGE_2) concentration was determined with an ELISA. The effect of age on the percentage change in PGE_2 concentration was assessed with repeated measures analysis. The half maximal inhibitory concentration of flunixin on PGE_2 concentration was determined by nonlinear regression.

RESULTS

Following IV administration, the mean half-life, area under the plasma concentration time curve, and residence time were lower and the mean clearance was greater for calves at 8 months. Following transdermal administration, the mean maximum plasma drug concentration was lower and the mean absorption time and residence time were greater for calves at 8 months. The half maximal inhibitory concentration of flunixin on PGE_2 concentration at 8 months was significantly greater. Age was not associated with the percentage change in PGE_2 concentration following IV or transdermal flunixin administration.

CONCLUSIONS AND CLINICAL RELEVANCE

In calves, the clearance of flunixin at 2 months was slower than that at 8 months following IV administration. Flunixin administration to calves may require age-related adjustments to the dose and dosing interval and an extended withdrawal interval.

ABBREVIATIONS

AUC	Are under the plasma concentration-time curve
Cl	Plasma clearance rate
C_{max}	Maximum plasma concentration
COX	Cyclooxygenase
IC_{50}	Half maximal inhibitory concentration
MRT	Mean residence time
MAT	Mean absorption time
PG	Prostaglandin
$t_{1/2}$	Terminal half-life
V_{ss}	Volume of distribution at steady state after IV administration

Scientific literature regarding the effects of age on the pharmacokinetics of drugs in cattle and other veterinary species is sparse. Cattle, in particular, undergo changes in body structure and composition as they mature from preruminant calves to adult ruminants. Those changes include alterations in total water composition, body surface area, and adipose tissue development, and, for females, changes associated with gestation and lactation.[1] Changes in drug absorption, metabolism, and renal excretion as individuals mature are the basis for age-dependent differences in the pharmacokinetics of various drugs.[2] In cattle, the effect of age on the pharmacokinetics of some NSAIDs[3–5] have been investigated. However, there is a lack of information regarding the effect of age on the pharmacokinetics of flunixin meglumine (flunixin) in cattle.

Flunixin is a nicotinic acid derivative and is the only NSAID approved by the FDA for the use in cattle in the United States. Injectable formulations of flunixin are labelled for the treatment of pyrexia associated with bovine respiratory disease and endotoxic mastitis and inflammation associated with endotoxemia in adult cattle. Flunixin has also been reported to be useful as an adjunctive treatment for neonatal calves with diarrhea.[6]

Because flunixin in an NSAID, it has direct effects on COX enzymes associated with inflammation. The COX pathway is responsible for the conversion of arachidonic acid to PGE_2, PGI, $PGF_{2\alpha}$, and thromboxane. Cyclooxygenase has 2 isoforms, COX-1 and COX-2, and each isoform has distinct functions. The COX-1 isoform is constitutively produced in tissues and is associated with homeostasis. The COX-2 isoform is constitutively expressed in some tissues, and can be markedly upregulated subsequent to inflammatory insults, tissue injury, or localized hypotension. Expression of COX-2 products such as PGE_2 and PGI_2 correlates best with the anti-inflammatory function of flunixin and other NSAIDs. For example, inhibition of PGE_2 and PGI_2

is positively correlated with anti-inflammatory activity and an increase in PGE_2 and PGI_2 concentrations is associated with poor anti-inflammatory activity.

The objective of the study reported here was to investigate effect of age on the pharmacokinetics and pharmacodynamics of flunixin following intravenous and transdermal administration to Holstein calves. We hypothesized that the pharmacokinetics and associated pharmacodynamics of flunixin would differ as calves age owing to changes in physiology, body composition, and growth.

MATERIALS AND METHODS

Animals and study design

All study procedures were reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC Nos. 6-15-8039-B and 5-15-8016-B). The study was conducted in 2 periods with a 6-month interval between the 2 periods, and the same calves were used for both periods. Eight 6-week-old Holstein bull calves were obtained from a single source before initiation of period 1. The calves were weaned and had a mean ± SD weight of 60.2 ± 7.3 kg. The calves were acclimated for 14 days prior to initiation of period 1, during which they were accustomed to being restrained with a halter and lead rope and were drenched with amprolium[a] (10 mg/kg, PO) once daily for 5 consecutive day for coccidiosis control. At the beginning of period 2, the calves were 8 months old and had a mean ± SD weight of 222 ± 7.3 kg.

Each period consisted of IV and transdermal phases and had a crossover design, with a 10-day washout period between the 2 phases. For the first phase of period 1, all calves received flunixin meglumine[b] (2.2 mg/kg), IV, to facilitate determination of the bioavailability of flunixin

following transdermal administration. For each calf prior to IV flunixin administration, a catheter was aseptically placed in each jugular vein. The catheter in the left jugular vein was used for flunixin administration, after which it was flushed with heparinized saline solution (0.9% NaCl solution with 1% sodium heparin) and immediately removed. The catheter in the right jugular vein was used for collection of serial blood samples. From each calf, a blood sample (approx 20 mL) was collected immediately before and at 3, 6, 10, 15, 30, 45, and 60 minutes and 2, 4, 6, 8, 12, 24, 36, and 48 hours after flunixin administration. Each blood sample was collected into a syringe and then immediately placed into two 10-mL blood collection tubes[c] that contained sodium heparin as an anticoagulant. The tubes were gently inverted several times to ensure that the blood was adequately mixed with the anticoagulant. The catheter was flushed with heparinized saline solution after collection of each blood sample, and was removed immediately after collection of the last blood sample for phase 1. All blood samples were placed on ice immediately after collection and transported to the laboratory for processing within 1 hour after collection. At the laboratory, all blood samples were centrifuged at 1,500 X *g* for 10 minutes. The plasma was harvested from each blood sample, placed in a cryovial, and stored frozen at –80°C until analyzed.

During phase 2 of period 1, each calf was administered flunixin meglumine[d] (3.33 mg/kg) transdermally. The flunixin formulation used was approved for transdermal administration to cattle in the European Union and Canada and was administered with a single-use syringe in accordance with the label directions. For each calf, the drug was applied to the skin of the dorsal top line beginning between the scapulas and extending to the tail head. Prior to drug administration, a catheter was aseptically placed in a jugular vein of each calf for collection of serial blood samples. From each calf, a blood sample was collected via the jugular catheter as

described for phase 1 immediately before and at 10, 20, 30, 40, 50, 60, and 90 minutes and 2, 4, 6, 8, 12, 24, 36, 48, 56, and 72 hours after flunixin administration. The catheter was removed immediately after collection of the last blood sample for phase 2.

Period 2 was initiated 6 months after completion of phase 2 of period 1, and the 8 calves used for period 1 were used for period 2. The experimental protocol for period 2 was identical to that described for period 1, except the administration route order was reversed. During phase 1 of period 2, all calves received flunixin meglumine[d] (3.33 mg/kg) transdermally, and blood samples were collected immediately before and at 30, 60, and 90 minutes and 2, 4, 6, 8, 12, 24, 48, and 72 hours after drug administration. During phase 2 of period 2, all calves received flunixin meglumine[b] (2 mg/kg), IV, and blood samples were collected immediately before and at 3, 6, 10, 15, 30, 45, and 60 minutes and 2, 4, 6, 8, 12, 24, 36, and 48 hours after drug administration.

Plasma flunixin meglumine determination

The flunixin concentration in each plasma sample was determined by use of high-performance liquid chromatography–tandem mass spectroscopy as described.[7] Briefly, the mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Separation was achieved with a C18 column.[e] Three ions for flunixin were measured in negative ion mode at m/z 295 → 251, 210, and 197, with a retention time of 5.60 minutes. Only the 251 ion was used for quantification.

The standard curve for flunixin in bovine plasma had a quadratic shape and ranged from 0.005 to 10 µg/mL. Samples with flunixin concentrations > 10 µg/mL were diluted with blank bovine plasma (ie, plasma from cattle that were not treated with flunixin). The curve was accepted when the correlation coefficient exceeded 0.99 and the measured values were within

15% of the expected values. The accuracy and coefficient of variation for flunixin were 108% and 7.3%, respectively. The limit of quantification for flunixin was 0.005 µg/mL, and the limit of detection was 0.003 µg/mL.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed by use of noncompartmental methods with computer software.[f] Individual animal pharmacokinetic values were calculated, and descriptive statistics (geometric mean and median [range]) were reported as described.[8] For each period, the bioavailability of flunixin following transdermal administration was calculated as follows: ($AUC_{transdermal}$/transdermal dose)/(AUC_{IV}/IV dose).

Determination of plasma PGE_2 concentration

Ex vivo plasma PGE_2 concentrations were determined by use of methods adapted from Fraccaro et al[9] and Stock et al.[10] Prior to centrifugation, 2-mL aliquots of anticoagulated blood were reserved from blood samples collected before and at 1, 2, 4, 12, 24, and 48 hours after IV flunixin administration and from blood samples collected before and at 1, 2, 4, 12, 24, 48, 72 hours after drug administration during the transdermal phase of each period for determination of plasma PGE_2 concentration. With the exception of aliquots reserved from blood samples collected before flunixin meglumine administration (ie, baseline samples), each 2-mL aliquot was spiked with lipopolysaccharide from *Escherichia coli* 0111:B4[g] (10 µg/mL). Baseline samples were used as controls and were spiked with a volume of PBS solution equal to the volume of lipopolysaccharide added to each of the other 2-mL aliquots. All samples were incubated for 24 hours at 37°C, after which they were centrifuged at 400 X *g* for 10 minutes.

Plasma was harvested from each sample and pipetted into individual cryovials. The cryovials were placed on dry ice to freeze the plasma and then stored at –80°C until analyzed.

To determine PGE_2 concentration, plasma proteins were precipitated with methanol at a 1:5 dilution. Then, samples were centrifuged at 3,000 X *g* for 10 minutes, and the supernatant was decanted and used for analysis. The PGE_2 concentration in each supernatant sample was determined by use of a commercially available ELISA.[h] The coefficient of variation for intra-assay variability was 8.2% and interassay variability was 11.2%. For each calf during each phase of each period, the IC_{50} of flunixin on PGE_2 was determined by nonlinear regression performed by use of computer software.[i] For statistical analyses, the percentage change in PGE_2 concentration for each sample relative to that for the baseline sample for the phase and period being evaluated was calculated as follows: (sample PGE_2 – baseline PGE_2)/baseline PGE_2 X 100.

Statistical analysis

The respective data distributions for all pharmacokinetic parameters were assessed for normality by means of Shapiro-Wilk Test. Comparisons between the 2 age groups were performed with unpaired *t* tests for parametric parameters and Wilcoxon Rank sum tests for nonparametric parameters. One calf had a negative MAT during the transdermal phase (phase 1) of period 2, and MAT data from that calf were excluded from that comparison between the 2 ages.

For each route of flunixin administration, a mixed linear regression model was used to assess the effect of age (2 vs 8 months), duration between flunixin meglumine administration and blood sample collection (time), and the interaction between age and time on the percentage change in plasma PGE_2 concentration; a random effect for calf was included in the model to

control for repeated measures within individual calves. The IC_{50} of flunixin on plasma PGE_2 concentration was compared between the 2 age groups by use of a Wilcoxon Rank sum test, and the difference in the IC_{50} between the 2 age groups at each time was evaluated with a paired *t* test. All analyses were performed with statistical software,[j] and values of $P \leq 0.05$ were considered significant.

RESULTS

Flunixin pharmacokinetics

No adverse effects associated with flunixin administration were observed in any of the calves during periods 1 and 2. The plasma flunixin concentrations over time after IV and transdermal administration for calves at both ages were summarized **(Figure 1)**. The pharmacokinetic parameters for flunixin following IV and transdermal administration for both ages were also summarized **(Table 1)**. When flunixin was administered IV, the median AUC ($P = 0.002$) and $t_{1/2}$ ($P < 0.001$) and geometric mean MRT ($P < 0.001$) were significantly lower, whereas the median Cl ($P = 0.002$) and terminal rate constant ($P < 0.001$) were significantly greater for calves at 8 months old, compared with the corresponding values at 2 months old. When flunixin was administered transdermally, the geometric mean C_{max} was significantly ($P = 0.021$) lower, whereas the geometric mean MAT ($P = 0.004$) and MRT ($P = 0.006$) were significantly greater for calves at 8 months old, compared with the corresponding values at 2 months old.

Percentage change in PGE_2 concentration

The percentage change in PGE_2 concentration over time for blood samples collected after IV and transdermal flunixin administration to calves at both ages was plotted **(Figure 2)**. When flunixin was administered by the IV route, the percentage change in PGE_2 concentration was significantly ($P < 0.001$) associated with the duration between drug administration and blood sample collection (time) but was not associated with age ($P = 0.172$) or the interaction between age and time ($P = 0.228$). Likewise, when flunixin was administered by the transdermal route, the percentage change in PGE_2 concentration was significantly ($P = 0.001$) associated with time but was not significantly associated with age ($P = 0.090$) or the interaction between age and time (0.706). The mean ± SEM IC_{50} of flunixin on plasma PGE_2 concentration for calves at 2 months old (10.8 ± 5.9 ng/mL) was significantly ($P = 0.026$) lower than that at 8 months old (37.7 ± 11.7 ng/mL).

DISCUSSION

Results of the present study indicated that the pharmacokinetics of flunixin following both IV and transdermal administration to calves varied with age. Interestingly, more pharmacokinetic parameters had significant differences between calves at 2 months and 8 months when flunixin was administered by the IV route than when it was administered by the transdermal route. Following IV administration, the plasma Cl of flunixin was slower for calves at 2 months old than at 8 months old, which resulted in the AUC, $t_{1/2}$, and MRT of flunixin being greater for calves at 2 months of age than at 8 months of age. Those differences were not observed when flunixin was administered by the transdermal route.

The pharmacokinetics of flunixin following IV and transdermal administration to calves at 2 months of age were similar to those reported for similarly aged calves in another study[7] conducted by our research group. The MRT and MAT for flunixin following transdermal administration to the 2-month-old calves of that study[7] were similar to those for the 2-month-old calves and substantially lower than those for the 8-month-old calves of the present study. Thus, the findings of the present study appear to be supported by the results of that study.[7]

In another study[11] in which the pharmacokinetics and tissue depletion of flunixin following IV administration to 3- to 6-week-old calves were described, the V_{ss} (0.63 L/kg) was similar to the V_{ss} (0.68 L/kg) for calves at 2 months old in the present study, but the MRT (12.54 h) and $t_{1/2}$ (12.88 h) were greater than twice the mean MRT (4.56 h) and $t_{1/2}$ (5.44 h) for the 2-month-old calves of this study. The authors of that report[11] attributed the fairly long MRT and $t_{1/2}$ to the fact that the Cl of drugs in calves is generally slower than that in adult cattle. Following IV administration of flunixin in the present study, the mean Cl (4.08 mL/min/kg) of the drug for calves at 8 months old was 1.6 times the mean Cl (2.48 mL/min/kg) of the drug at 2 months old, whereas the mean MRT (2.53 h) and $t_{1/2}$ (3.45 h) of flunixin for calves at 8 months old were significantly shorter than the corresponding values for calves at 2 months old. Collectively, these findings support an age-dependent effect on the pharmacokinetics of flunixin following IV administration to cattle and provide compelling evidence of the need to extend the slaughter withdrawal interval from the labeled slaughter withdrawal time when the drug is administered in an extralabel manner to calves, especially those intended for veal production.

The effect of age on the pharmacokinetics of other NSAIDs has been described in other studies.[3–5] However, unlike the present study, those studies[3–5] did not use the same animals for all experimental phases. When phenylbutazone was administered IV, the Cl was slower and the

MRT and $t_{1/2}$ were longer for young calves relative to older calves.[5] Following IV administration of either the R-(-) or S-(+) enantiomer of ketoprofen, the AUC and MRT of both enantiomers were greater and the Cl of the S-(+) enantiomer was slower for calves, compared with the corresponding values for adult cattle.[3] Similar age-dependent pharmacokinetic changes have been described following IV administration of carprofen to calves.[12] Following IV administration of the stereoselective drug flurbiprofen, the Cl was slower and the AUC was lower for calves than for adult cattle[4]; however, the MRT and $t_{1/2}$ of flurbiprofen did not differ significantly between the calves and adult cattle of that study. Thus, the effect of age on the pharmacokinetics of NSAIDs following administration to cattle appears to vary, and a clear generalization of age-dependent effects on the pharmacokinetics of NSAIDs as a drug class cannot be provided.

Results of a study[13] on the effect of age on the pharmacokinetics of flunixin following IV administration to horses suggest that age is negatively correlated with Cl and positively correlated with $t_{1/2}$. Those findings are opposite those observed for the calves of the present study following IV administration of flunixin. However, as with other NSAIDs, age did not appear to have a significant effect on the V_{ss} of flunixin for the horses of that other study[13] or the calves of this study.

The effect of age on the pharmacokinetics of various antimicrobials following administration to various mammals has been reviewed.[2] For calves specifically, the author of that review[2] reported that the plasma elimination half-life of antimicrobials tended to decrease as age increased. In another study,[14] the Cl of ceftiofur, an antimicrobial that, like flunixin, is highly protein bound, was significantly slower and the AUC was significantly greater for 1-day-old calves than for 6-month-old calves.

In the present study, age was not significantly associated with the percentage change in PGE_2 concentration following either route of flunixin administration, despite the fact that the mean ± SEM IC_{50} of flunixin on plasma PGE2 concentration for calves at 2 months old (10.8 ± 5.9 ng/mL) was significantly ($P = 0.026$) lower than that at 8 months old (37.7 ± 11.7 ng/mL). Notably, transdermal administration of flunixin failed to induce a negative percentage change in PGE_2 in 8-month-old calves, and IV administration of the drug resulted in a smaller negative percentage change in PGE_2 in calves at 8 months old than that at 2 months. This suggested that suppression of PGE_2 synthesis was reduced when flunixin was administered to the calves at 8 months old relative to that when the drug was administered to the calves at 2 months. The percentage change in PGE_2 concentration mirrored the plasma flunixin concentration and associated pharmacokinetic parameters in the present study. The differences in the percentage change in PGE_2 concentration between the 2 ages was attributed to the slower Cl and longer $t_{1/2}$ of flunixin and lower IC_{50} of flunixin on PGE_2 concentration for calves at 2 months old relative to those at 8 months. On the basis of the ex-vivo PGE_2 suppression results, flunixin administered IV at the high end of the label dose (2.2 mg/kg) should produce anti-inflammatory effects for 24 hours in calves ≤ 2 months old, and a dosing interval of 24 hours should be sufficient for such calves.

The differences between the 2 age groups in the percentage change in PGE_2 concentration following transdermal administration of flunixin likely reflected age-related effects on pharmacokinetic and pharmacodynamic parameters. The significant differences in the percentage change in PGE_2 concentration (ie, flunixin-induced anti-inflammatory effects) between the 2 ages over time were attributed to the lower C_{max}, longer MRT, and greater IC_{50} for 8-month-old calves relative to the corresponding values for 2-month-old calves. Consequently, the clinically

perceived anti-inflammatory effects of flunixin for 8-month-old calves may be less than those for 2-month-old calves, but further research in which specific clinically relevant endpoints are evaluated is warranted to validate that supposition.

The fact that most of the pharmacokinetic parameters for flunixin did not differ significantly following transdermal administration to calves at 2 months old and again at 8 months old might be attributable to skin thickness. The skin of cattle thickens as animals age; thus, the skin of 8-month-old calves is thicker than the skin of 2-month-old calves. Thicker skin typically results in slower drug absorption (ie, longer MAT) and a lower C_{max}, and longer $t_{1/2}$ following transdermal drug administration. Those pharmacokinetic changes are indicative of flip-flop kinetics, where drug absorption becomes the rate-limiting step in drug elimination. However, for the calves of the present study, the skin thickness difference between calves at 2 months and 8 months was not sufficient to significantly affect the Cl of flunixin following transdermal administration.

Other significant differences in pharmacokinetic parameters observed between the 2 ages evaluated in the present study might be attributed to age-related differences in body composition and development. As calves mature, the amount of body water decreases as muscle mass and adipose tissue increase.[1] Results of multiple studies[2–5,14] indicate that age-related changes in body composition affect the MRT, $t_{1/2}$, and Cl of various drugs. Additionally, hepatic metabolism changes as cattle mature, and the hepatic clearance of drugs in young calves is generally slower than that in older cattle.[2] Those principles held true for the calves of the present study, as evidenced by the fact that the Cl increased and $t_{1/2}$ decreased following IV administration of flunixin to calves at 8 months old relative to those at 2 months.

Published literature regarding the IC_{50} of flunixin on the plasma PGE_2 concentration of cattle is sparse, and evaluation of the effect of age on the IC_{50} of flunixin on PGE_2 concentration is lacking. The primary goal of the studies[15,16] that have evaluated IC_{50} data for cattle were conducted to determine the COX-1 or COX-2 preference of and compare different NSAID formulations. On the basis of the IC_{50} results of the present study, further research is necessary to elucidate the anti-inflammatory mechanism of flunixin.

The design of the present study was unique in that the same calves were used for both experimental periods. The phases in each period were blocked such that there was only 1 treatment in each block to avoid a period effect in the final analysis. Moreover, by blocking the treatments, the washout period could be standardized to reduce the possibility of carry-over flunixin between phases. Although we recognize that not having both treatments represented in all study phases might be a source of bias, we feel that such bias was unlikely given the short time (10 days) between the 2 phases of each period.

When the present study was conducted, the transdermal formulation of flunixin meglumine was approved for use in cattle in the European Union and Canada. After the study was completed, the FDA approved that formulation for the treatment of fever associated with bovine respiratory disease and the control of pain associated with foot rot in US cattle.

In the present study, multiple significant age-related differences were identified for the pharmacokinetic parameters of flunixin following IV and transdermal administration to calves at 2 months and 8 months of age. The differences in pharmacokinetics following IV administration of flunixin reflected changes in the Cl of the drug from the body as calves mature. However, age was not associated with flunixin-induced PGE_2 inhibition, which suggested that age-related changes in the pharmacokinetics of a drug might not be reflected as alterations in clinically

evident outcomes. Following transdermal flunixin administration, significant age-related differences were observed only for MAT and MRT. Age was not significantly associated with the overall percentage change in PGE_2 concentration, but the percentage change in PGE_2 concentration was greater for 2-month-old calves than for 8-month old calves during the first 48 hours after transdermal flunixin administration. Thus, results of the present study suggested that flunixin administration to young calves may require age-related dose adjustments and an extended withdrawal interval. Additionally, age-related differences in the affinity of flunixin for COX enzymes requires further investigation.

Footnotes

a. Corid, Merial LLC, Duluth, Ga.

b. Banamine injectable solution, Merck Animal Health, Madison, NJ.

c. BD Vacutainer, Franklin Lakes, NJ.

d. Finadyne Transdermal Pour-On, MSD Animal Health, Dublin, Ireland.

e. Phenomenex, Torrance, Calif.

f. Phoenix 64, Certara, Princeton, NJ.

g. Sigma-Aldrich, St Louis, Mo.

h. Cayman Chemicals, Ann Arbor, Mich.

i. Prism7, GraphPad Software Inc, La Jolla, Calif.

j. JMP Pro, version 12.0, SAS Institute Inc, Cary, NC.

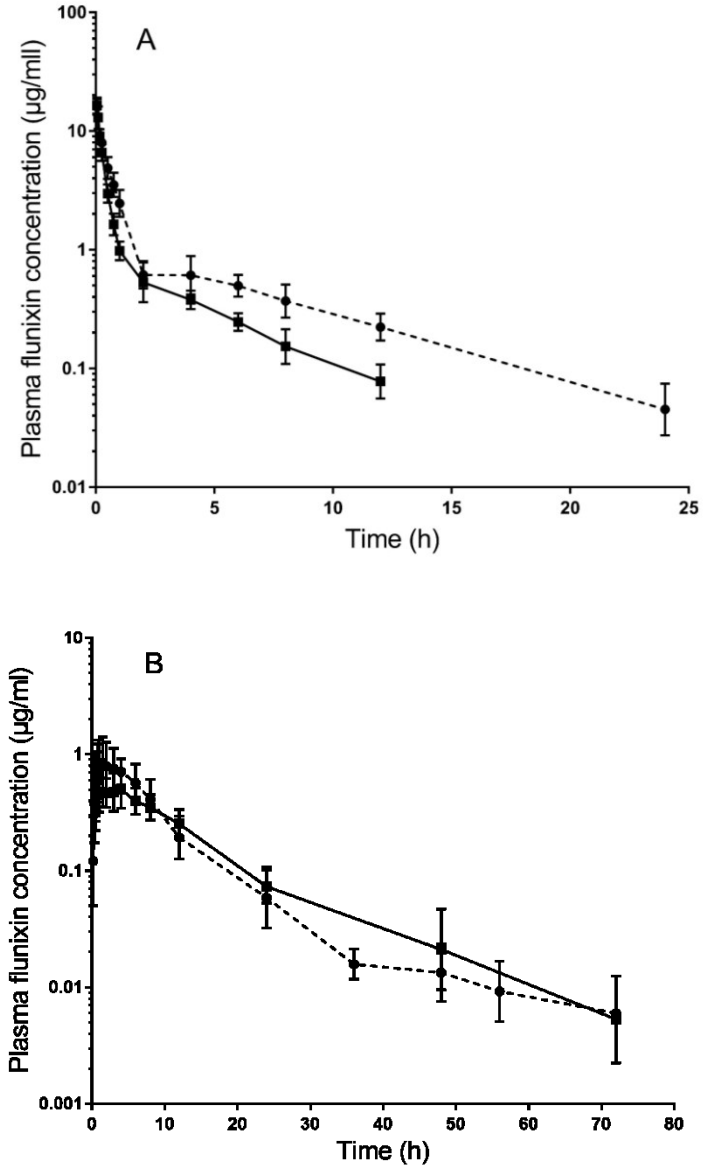

Figure 4-1 **Mean ± SEM plasma flunixin concentrations over time following administration of an injectable solution of the drug at a dose of 2.2 mg/kg, IV (A) or a topical formulation of the drug at a dose of 3.33 mg/kg, transdermally (B), to 8 healthy weaned Holstein bull calves at 2 months (dashed line) and 8 months (solid line) of age. At 2 months of age, all calves received the injectable solution of flunixin; then, after a 10-day washout period, calves received the topical formulation of flunixin. Blood samples were collected at predetermined times before and for 48 and 72 hours, respectively, after IV and transdermal drug administration. At 8 months of age, the experimental protocol was repeated except all calves received flunixin by the transdermal route first.**

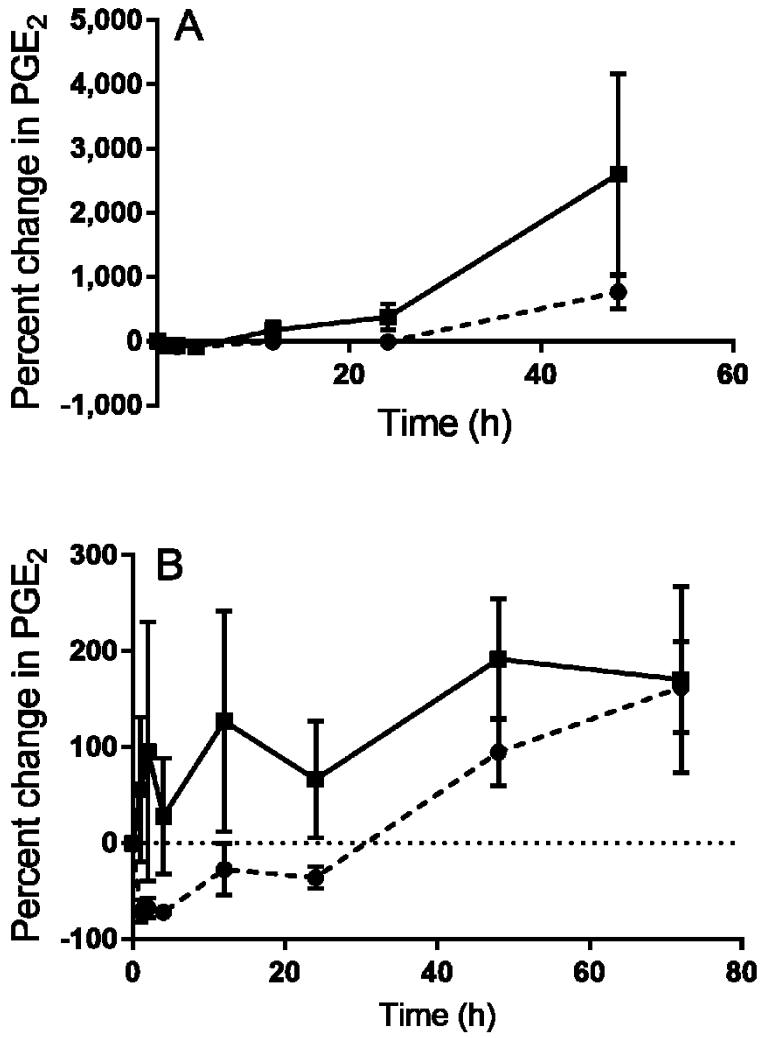

Figure 4-2 **Mean ± SEM percentage change in PGE_2 concentration over time following IV (A) and transdermal (B) administration of flunixin meglumine to 8 calves at 2 months (dashed line) and 8 months (solid line) of age as described in Figure 1. For each blood sample collected following flunixin meglumine administration, the percentage change in plasma PGE2 concentration was calculated as follows: (sample PGE_2 – baseline PGE_2)/baseline PGE_2 X 100, where baseline PGE_2 was the concentration of PGE_2 in the plasma of the blood sample collected immediately before drug administration. Notice the scale of the y axis differs between the 2 panels.** *See* **Figure 1 for remainder of key.**

Table 4-1 Pharmacokinetic parameters determined for flunixin meglumine following IV and transdermal administration to 8 healthy weaned Holstein bull calves at 2 months and 8 months of age.

Route of administration	Parameter	2 months old		8 months old		*P* value*
		Geometric mean	Median (range)	Geometric mean	Median (range)	
IV	$AUC_{0-\infty}$ (h•µg/mL)	14.76	14.63 (10.17–23.04)	9.00	9.33 (6.52–10.39)	0.002
	C0 (µg/mL)	21.24	22.09 (15.80–30.87)	20.72	19.60 (16.50–32.05)	0.875
	Cl (mL/min/kg)	2.48	2.51 (1.59–3.61)	4.08	3.95 (3.53–5.62)	0.002
	$t_{1/2}$ (h)	5.44	5.21 (4.37–7.61)	3.45	3.61 (2.68–4.00)	< 0.001
	λ_z (1/h)	0.13	0.13 (0.09–0.16)	0.20	0.19 (0.17–0.26)	< 0.001
	$MRT_{0-\infty}$ (h)	4.56	4.34 (3.67–7.24)	2.53	2.50 (1.88–3.52)	< 0.001
	V_{ss} (L/kg)	0.68	0.69 (0.56–0.84)	0.62	0.60 (0.50–0.89)	0.277
	V_z (L/kg)	1.17	1.13 (0.92–1.58)	1.22	1.22 (1.05–1.38)	0.801
Transdermal	$AUC_{0-\infty}$ (h•µg/mL)	8.99	9.67 (4.60–14.04)	8.52	9.47 (4.03–11.5)	0.683
	C_{max} (µg/mL)	0.96	1.16 (0.48–1.68)	0.54	0.48 (0.33–1.20)	0.021
	Cl/f (mL/min/kg)	6.18	5.79 (3.96–12.07)	6.52	5.87 (4.83–13.76)	0.820
	$t_{1/2}$ (h)	9.31	8.16 (5.59–25.31)	13.24	15.33 (6.10–28.80)	0.189
	λ_z (1/h)	0.07	0.09 (0.03–0.12)	0.05	0.05 (0.02–0.11)	0.188
	t_{max} (h)	1.89	1.75 (1.00–4.00)	2.14	2.50 (1.00–4.00)	0.513
	MAT (h)	3.07	2.81 (–0.48–14.98)†	12.94	13.97 (6.11–21.53)	0.004

	$MRT_{0-\infty}$ (h)	9.10	8.06 (6.76–14.90)	15.74	16.29 (9.05–24.36)	0.006
	V_z/f (L/kg)	4.98	5.68 (2.55–13.84)	7.47	7.37 (2.77–19.89)	0.243
	F	0.40	0.43 (0.23–0.61)	0.63	0.69 (0.31–1.17)	0.199

The study was conducted in 2 periods with a 6-month interval between the 2 periods, and the same calves were used for both periods. Each period consisted of 2 phases, with a 10-day washout period between the 2 phases. At 2 months of age (period 1), all calves received flunixin meglumine[b] (2.2 mg/kg, IV) during the first phase and flunixin meglumine[d] (3.33 mg/kg, transdermally) during the second phase. Blood samples were collected at predetermined times before and for 48 and 72 hours, respectively, after IV and transdermal drug administration. At 8 months of age (period 2), the experimental protocol was repeated except all calves received flunixin by the transdermal route first. Data were not normally distributed for $AUC_{0-\infty}$, Cl, $t_{1/2}$, and λ_z when the drug was administered by the IV route or for $t_{1/2}$ when the drug was administered by the transdermal route. *Comparisons between the 2 age groups were performed with unpaired *t* tests for parametric parameters and Wilcoxon Rank sum tests for nonparametric parameters. †One calf had a negative MAT and was excluded from the analysis for that parameter. λ_z = Terminal rate constant. $AUC_{0-\infty}$ = Area under the plasma concentration–time curve from time 0 extrapolated to infinity. C0 = Plasma concentration extrapolated to time 0. Cl/f = Clearance per fraction of the dose absorbed. F = Bioavailability. $MRT_{0-\infty}$ = Mean residence time from time 0 extrapolated to infinity. t_{max} = Time of maximum plasma concentration. V_z = Volume of distribution during terminal phase after IV administration. V_z/f = Volume of distribution per fraction of the transdermal dose absorbed.

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Chapter 5 - The impact of pain on the pharmacokinetics of transdermal flunixin meglumine administered at the time of cautery dehorning in Holstein calves

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ABSTRACT

Objective To study the influence of pain on the pharmacokinetics and anti-inflammatory actions of transdermal flunixin administered at dehorning.

Study design Prospective, crossover, clinical study.

Animals A total of 16 male Holstein calves, aged 6-8 weeks weighing 61.3 ± 6.6 kg.

Methods Calves were randomly assigned to one of two treatments; transdermal flunixin and dehorning (PAIN) or ~~a~~ transdermal flunixin and sham dehorning (NO PAIN). Flunixin meglumine (3.33 mg kg^{-1}) was administered topically as a pour-on concurrently with hot iron dehorning or sham dehorning. The calves were subjected to the alternative treatment 14 days later. Blood samples were collected at predetermined time points out to 72 hours for measurement of plasma flunixin concentrations. Pharmacokinetics parameters were determined using non-compartmental analysis. Prostaglandin E_2 (PGE_2) concentration was determined using a commercial ELISA assay. The 80% inhibition concentration (IC_{80}) of PGE_2 was determined using nonlinear regression. Pharmacokinetic data were statistically analyzed using paired *t* tests and Wilcoxon rank sums for nonparametric data. Flunixin and PGE_2 concentrations were log transformed and analyzed using repeated measures.

Results A total of 15 calves completed the study. Plasma half-life of flunixin was significantly longer in PAIN (10.09 hours) than NO PAIN (7.16 hours) ($p = 0.0202$). Bioavailability of transdermal flunixin was 30% and 37% in PAIN and NO PAIN, respectively ($p = 0.097$). Maximum plasma concentrations of flunixin were 0.95 μ*g* mL^{-1} and 1.16 μ*g* mL^{-1} in PAIN and NO PAIN, respectively ($p = 0.089$). However, there was a treatment (PAIN *versus* NO PAIN) by time interaction ($p = 0.0353$). PGE_2 concentrations were significantly lower at in the PAIN

treatment at 48 and 72 hours ($p = 0.0092$ and $p = 0.0287$ respectively). The IC_{80} of PGE_2 by flunixin was similar in both treatments ($p = 0.88$).

Conclusion and clinical relevance Pain alters the pharmacokinetics and anti-inflammatory effects of transdermally administered flunixin.

Keywords cattle, NSAID, pain, pharmacokinetics.

INTRODUCTION

Dehorning is a procedure performed on >90% of dairy farms in the United States with the goal of preventing animal injury, human injury and carcass bruising (USDA 2007). Of the various methods described, cautery dehorning is the most widely practiced (USDA 2007). The pain associated with dehorning has been documented to cause acute changes in physiology, behavior and neuroendocrine system function of the animal (Stafford & Mellor 2011). One change at the time of dehorning is the activation of the sympathetic nervous system, which redirects the blood flow from the skin to other vital organs (Allen et al. 2013; Stock et al. 2015).

Administration of flunixin meglumine by the transdermal route is approved in cattle for the control of pain associated with foot rot. Transdermal flunixin is the only drug in its class with a Food and Drug Administration (FDA) label for pain control in cattle. Studies have shown that flunixin exerts analgesic effects when administered intravenously (IV) at the time of dehorning (Huber et al. 2013). By contrast, transdermal flunixin does not provide substantial analgesia when used as the sole analgesic drug at dehorning (Kleinhenz et al. 2017). Calves treated with transdermal flunixin at the time of dehorning had elevated cortisol concentrations and decreased

mechanical nociception threshold measures compared with sham controls (Kleinhenz et al. 2017). The changes in cortisol and mechanical nociception threshold measures are consistent with dehorning studies investigating other non-steroidal anti-inflammatory drugs (Glynn et al. 2013; Stock et al. 2016). Flunixin is also known to be a highly effective inhibitor of cyclo-oxygenase-2 (COX-2) and its product PGE_2 (Myers et al. 2010). PGE_2 production serves as a marker for the anti-inflammatory effects of flunixin (Fraccaro et al. 2013).

The objective of this study was to determine if the pain associated with cautery dehorning in calves alters the pharmacokinetics of topically applied flunixin meglumine and its suppression of PGE_2. It is our hypothesis that pain alters the pharmacokinetics of topically administered flunixin meglumine.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (no. 6-15-8039-B). As part of the IACUC approval, a rescue analgesia protocol was in place if a calf was determined to show signs of distress including changes in attitude, changes in behavior, not eating or drinking and excessive discharge from the dehorning site.

Study animals

Sixteen weaned male Holstein calves that were 6–8 weeks of age were enrolled into the study. Mean calf weight (± standard deviation) was 61.3 ± 6.6 kg. Upon arrival, calves underwent a 2-week acclimation period. Calves were conditioned to tolerate restraint using the lead of their rope halter; and to interact with study personnel. Calves were group housed in an

open faced building with indoor/outdoor access. There was eight calves per pen with each treatment equally distributed between pens. Pen space and housing conditions met or exceeded recommendations set forth in the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS 2010). Calves were fed a diet consisting of grass hay, cracked corn and a commercial protein/mineral supplement containing a coccidiostat. The diet was formulated to meet or exceed the nutrient requirements for growth and development. Calves had *ad libitum* access to water.

Experimental design

PGE_2 was considered the outcome with the most expected variability and, therefore, was used to determine the number needed per group. The study was designed to have statistical power of 0.8 assuming an effect size (delta) in differences of 1 pg mL^{-1} PGE_2, a standard error (sigma) of 0.1 and a significance level (alpha) of 0.05 as previously reported by (Stock et al. 2016). A sample size of six calves per group was determined.

This study was completed in 2 phases (Fig. 1). A crossover block design was used for Phase 2 (dehorning and sham dehorning) with each calf subjected to both treatments. Calves were randomly assigned to 1 of 2 initial treatments: 1) topical flunixin and dehorning (PAIN) or 2) topical flunixin and sham dehorning (NO PAIN) with eight calves in each treatment. Calves were placed into treatments using a random number generator (Excel; Microsoft Corp., WA, USA) blocked by body weight prior to the start of the study to ensure equal weight distribution among treatments.

In Phase 1, all 16 calves were administered a single IV dose of flunixin meglumine (2.2 mg kg^{-1}; Banamine; Merck Animal Health, NJ, USA). Pharmacokinetic analysis of IV dosing

was used to determine bioavailability of transdermally administered flunixin. Phase 1 also served as an additional training and acclimation period for Phase 2. A 10 day washout period was observed after Phase 1. Calves were administered the assigned treatment at the start of Phase 2. A 14 day washout period was observed between replicates and calves were subsequently subjected to the alternate treatment. Thus calves in NO PAIN in replicate 1 were enrolled in PAIN for replicate 2.

Preparation

A 16 gauge, 6.35 cm catheter (Surflo, Terumo Medical Co., NJ, USA) was aseptically placed in the right jugular vein 24 hours before the start of each phase for collecting blood samples. A second catheter was placed in the in the left jugular vein in Phase 1 for flunixin administration. The catheters were capped with an injection port (Hospira Inc., IL, USA) and sutured into place with #0 nylon suture (Ethilon; Ethicon US LLC, NJ, USA). Just prior to each blood collection time point, the injection port was cleansed with an alcohol-soaked gauze.

Phase 1. IV flunixin administration

In Phase 1 all calves were administered flunixin via the left jugular catheter. Following flunixin administration, the catheter was flushed with 3 mL heparinized saline and immediately removed to prevent accidental use during blood sample collection. Blood samples were collected from the right jugular catheter to determine plasma flunixin concentrations. In Phase 1 blood samples were collected prior to IV flunixin meglumine administration (time 0) and at 3, 6, 10, 15, 30, 45 and 60 minutes as well as 2, 4, 6, 8, 12, 24, 36 and 48 hours post-administration.

Phases 2. Transdermal flunixin administration

Transdermal flunixin administration and dehorning methods were adapted from those described by Kleinhenz et al. (2017). Transdermal application of flunixin and dehorning procedure occurred simultaneously. Treatment application was used to mark the start of the experiment (time 0). Calves were tightly restrained with a rope halter and lead rope for treatment application. Flunixin meglumine (Finadyne Pour-On; MSD Animal Health, Ireland) was applied to the skin along the top-line starting at the withers and ending at the tail head at the labeled dose of 3.33 mg kg^{-1} using a single-use syringe. Drug application was completed in accordance of the manufacturer's product directions.

An electrocautery unit (Rhinehart Development Corp., IN, USA) was used to dehorn the calves. The unit was applied for 10 seconds on each horn to achieve 'a copper ring around the horn tissue' in the PAIN treatment. An identical inactive cautery unit was applied to each horn for 10 seconds in the NO PAIN (sham) treatment. In Phase 2 blood samples were collected prior to topical flunixin meglumine administration (time 0) and at 10, 20, 30, 40, 50, 60 and 90 minutes as well as 2, 4, 6, 8, 12, 24, 36, 48, 56 and 72 hours post-administration. Dehorning sites were monitored for signs of infection for 7 days after the dehorning procedure in each replicate.

Plasma flunixin concentration determination

Blood (≤ 20 mL) was collected into a syringe and transferred to two 10 mL blood collection tubes with sodium heparin (BD Vacutainer; Becton Dickinson & Co., NJ, USA) and inverted to mix the contents. The catheters was flushed with heparinized saline after each collection. Samples were immediately placed on ice and transported to the laboratory within an hour. The samples were centrifuged at 1,500 ***g*** for 10 minutes. The plasma was separated and

placed into cryovials which were immediately frozen using dry ice and then stored at −80 °C until analyzed.

Plasma concentrations of flunixin were determined using high-pressure liquid chromatography (HPLC) coupled with mass spectroscopy (Kleinhenz et al. 2016). Plasma samples were extracted using acetonitrile combined with an internal standard, flunixin D-3, to verify sample accuracy through the machine. Samples were passed through the HPLC column in two phases using 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. The standard curve for flunixin determination was created by spiking blank bovine plasma with known concentrations of flunixin 0.005–10 μg mL^{-1}. Blank bovine plasma was used to dilute samples with flunixin concentrations >10 μg mL^{-1}. The curve was accepted when the measured values were within 15% of their expected value and the correlation coefficient exceeded 0.99. The accuracy and coefficient of variation for flunixin was 108% and 7.3%, respectively. The lower limit of quantification was 0.005 μg mL^{-1} and the lower limit of detection was 0.003 μg mL^{-1}.

Pharmacokinetic analysis

Pharmacokinetic analysis was accomplished using noncompartmental methods performed on computer software (Phoenix 64; Certara, NJ, USA). The individual animal pharmacokinetic values were calculated and the descriptive statistics (geometric mean, minimum, median and maximum values) were reported (Julious & Debarnot 2000). The bioavailability (F) was calculated after topical administration in each phase using the following equation: F = (Topical AUC/Topical Dose)/(IV AUC/IV Dose).

Plasma PGE_2 determinations

Ex vivo prostaglandin concentrations were determined by modifying methods described by Stock et al. (2015). Blood samples were collected from the right jugular catheter prior to flunixin administration and at 1, 2, 4, 12, 24, 48 and 72 hours post-administration. Whole blood (2 mL) from each of the post-administration samples was spiked with 20 µg lipopolysaccharide from *Escherichia coli* 0111:B4 (Sigma-Aldrich, MO, USA). Samples were incubated for 24 hours at 37 °C. The samples were then centrifuged at 400 ***g*** for 10 minutes and the plasma was pipetted into individual cryovials, which were immediately frozen using dry ice and then stored at −80 °C until analyzed. After thawing, plasma proteins were precipitated using methanol (5:1 ration of methanol to plasma). Samples were then centrifuged at 3,000 ***g*** for 10 minutes and the PGE_2 concentration of the supernatant was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemicals, MI, USA). The coefficient of variation for intra-assay variability was 8.2% and interassay variability was calculated as 11.2%.

Inhibition 80 determination

The 80% inhibition concentration of PGE_2 was determined using computer software (GraphPad Prism7; GraphPad Software, Inc., CA, USA). The IC_{80} was calculated using nonlinear regression for each calf in each phase by comparing the log plasma flunixin concentration to the PGE_2 concentration. The means of the individual calf IC_{80} for each study phase were used in the statistical model.

Statistical analysis

Statistical analysis was performed using analytical software (JMP Pro 12.0; SAS Institute, NC, USA). Plasma flunixin and PGE_2 curves were log transformed for normality. Plasma flunixin and PGE_2 was analyzed using linear mixed effect model. The fixed effects were treatment (PAIN or NO PAIN), time, Phase 2 replicate and treatment by time interaction. The calf and first assigned treatment were random effects. In order to test the significance of main effects and interactions, *F*-tests were used. If significant overall differences were identified, pairwise comparisons were performed using Tukey's *t* tests based on the least square means (lsmeans) statement with Bonferroni correction adjustment. Pharmacokinetic parameters and IC_{80} data were compared using nonparametric tests (Wilcoxon Rank Sums) as the flunixin concentrations used to determine the parameter were previously log transformed (Powers 1990). Statistical significance was set at $p \leq 0.05$.

RESULTS

One calf was removed from the study and further statistical analysis owing to an unrelated pneumonia and subsequent death after replicate 1 in Phase 2. Thus, there were 15 animals that completed the study with seven calves in NO PAIN for replicate 1 and PAIN for replicate 2 (Fig. 1).

Flunixin concentrations

There was no impact of treatment (PAIN *versus* NO PAIN; $p = 0.6846$) or replicate ($p = 0.4040$) on flunixin concentrations (Fig. 2). There was an effect of time ($p < 0.0001$) and treatment by time interaction ($p = 0.0125$). Plasma flunixin concentrations were higher for the

PAIN calves at 48 (0.012 µg mL^{-1} *versus* 0.006 µg mL^{-1}; $p = 0.0320$) and 56 hours (0.006 µg mL^{-1} *versus* 0.002 µg mL^{-1}; $p = 0.0052$) post-procedure and drug application.

Flunixin pharmacokinetics

Calves in the PAIN treatment had a longer plasma flunixin half-life than NO PAIN (10.09 hours *versus* 7.16 hours; $p = 0.0202$; Table 1). Maximum flunixin concentration was 0.95 µg mL^{-1} for PAIN compared with NO PAIN 1.16 µg mL^{-1} ($p = 0.089$). Volume of distribution per fraction ($Vz\ F^{-1}$) absorbed was 5.43 L kg^{-1} (PAIN) *versus* 3.73 L kg^{-1} (NO PAIN; $p = 0.0564$). Bioavailability was 0.30 (PAIN) *versus* 0.37 (NO PAIN; $p = 0.097$). Mean absorption time (MAT) was numerically lower for the PAIN treatment (3.79 hours *versus* 5.27 hours), but this difference was not significant ($p = 0.2802$). Clearance, mean resident time (MRT) and AUC_{inf} were not significantly different between phases of PAIN *versus* NO PAIN.

Prostaglandin E_2

There was no effect of procedure ($p = 0.1883$) or replicate ($p = 0.7166$) on PGE_2 concentrations (Fig. 3). There was an effect of time ($p < 0.0001$) and a treatment by time interaction ($p = 0.02$). PGE_2 concentrations were lower for the PAIN treatment at 48 hours (217.6 pg mL^{-1} *versus* 424.5 pg mL^{-1}; $p = 0.0092$) and 72 hours (422.2 pg mL^{-1} *versus* 828.4 pg mL^{-1}; $p = 0.0287$) post-procedure and drug application.

IC_{80}

The IC_{80} of flunixin on PGE_2 was 0.039 µg mL^{-1} (NO PAIN) *versus* 0.026 µg mL^{-1} (PAIN; $p = 0.88$).

DISCUSSION

Dehorning is a painful procedure that has been shown to activate the sympathetic nervous system (Stewart et al. 2008). Activation of the sympathetic nervous system causes blood to be redirected towards vital organs such as the heart and skeletal muscle. This redirection of blood flow lowers the perfusion to the skin and should slow uptake of a transdermally applied drug from the skin, which is the basis of the authors' hypothesis. A decrease in ocular temperature using infrared thermography has been observed in calves post-dehorning (Stewart et al. 2008; Kleinhenz et al. 2017). The decrease in temperature has been linked to reduction of blood flow to the medial canthus associated with sympathetic nervous system activation (Stewart et al. 2010).

The results of this study demonstrate that the pain associated with dehorning has an effect on the pharmacokinetics and pharmacodynamics of transdermally applied flunixin. The most plausible explanation for the pharmacokinetic changes observed is a result of diversion of blood from the skin to other tissues. After the painful event, calves tended to have lower bioavailability, most likely due to decreased skin blood flow resulting in slower absorption from the administration site. Calves in the study were restrained to prevent grooming of themselves and reduce potential drug uptake by routes other than transdermal absorption. Calves were restrained for four hours after flunixin administration. Thus, plasma flunixin concentrations are those from transdermal absorption only (i.e. the transdermal drugs were not ingested). Maximum plasma flunixin concentrations (Cmax) tended to be lower following dehorning (PAIN) as a result of lower bioavailability.

The Vz F^{-1} tended to be larger following dehorning (PAIN). The significantly longer half-life in the PAIN treatment can be attributed to the higher Vz F^{-1} as there was no difference

in clearance (Cl F^{-1}) between PAIN and NO PAIN. Additionally, the change in Vz F^{-1} was proportionally much larger than the change in F. The differences in Vz F^{-1} further support activation of the sympathetic nervous system and resulting redistribution of blood flow away from the skin.

A potential confounding issue is the activation of the sympathetic nervous system as a result of study procedures and animal handling. In order to minimize these effects, calves were acclimated to being restrained with a halter, having human interactions and finally intense blood collection in Phase 1 (IV administration). An exaggerated sham response can mask changes when using a crossover design. Treatments that produce greater tissue injury and/or require longer healing time are more likely to produce an exaggerated sham response. Cortisol concentrations spike following both dehorning and sham dehorning (control). Dehorned calves have a significantly higher spike in cortisol, but concentrations return to baseline and that of control calves by 2 hours post-procedure (Stock et al. 2015; Kleinhenz et al. 2017). Kleinhenz et al. (2017) found no differences in ocular temperatures using infrared thermography after cautery dehorning and administration of transdermal flunixin. This is consistent with data observed following cautery dehorning and treatment with the drugs carprofen and firocoxib (Stock et al. 2015, 2016).

PGE_2 concentrations differed over time, with animals in NO PAIN having higher concentrations at 48 and 72 hours post flunixin administration. At these time points the plasma concentration of flunixin was approaching the level of detection for both PAIN and NO PAIN treatments. In tissue cage models, transdermal flunixin has been shown to suppress PGE_2 production for 48 hours, but tissue cage models may not be predictive of drug concentrations at the surgical site due to disproportionate partitioning of drug into the tissue cage (Thiry et al.

2017). However, it is still expected that flunixin suppression of COX-2 in tissues occurs beyond when flunixin is measurable in plasma. This may result from increased persistence of flunixin in inflammatory exudate (Landoni et al. 1995). Furthermore, COX-2 mRNA is not influenced by flunixin in whole blood *in vitro* models (Myers et al. 2010). The IC_{80} of PGE_2 is a more appropriate measure of NSAID clinical efficacy (Lees et al. 2004). The IC_{80} was not significantly different between treatments. This indicates flunixin should have similar clinical efficacy, PAIN *versus* NO PAIN. Further work is needed to investigate the mechanism(s) responsible for the lower PGE_2 concentrations at 48 and 72 hours in the PAIN treatment, specifically the interaction of flunixin and the COX-2 enzyme.

Local anesthetic blockade was not utilized in the current study. This allowed the full impact of pain on transdermally administered flunixin pharmacokinetics to be evaluated. This is especially important as the exact pharmacokinetics of transdermally applied flunixin in Holstein calves of this age were not fully known at the time of the study. A Canadian survey revealed there were still a large number of producers (38%) that are dehorning without the use of local anesthetic blockade despite a country wide code of conduct (Winder et al. 2016). Similar data has been published for veterinarians (Fajt et al. 2011). The ease of administration and FDA label for control of pain may sway producers to solely use transdermal flunixin as a 'needle-less' method of analgesic.

In conclusion, pain impacts the plasma concentrations, pharmacokinetics and anti-inflammatory effects of transdermally administered flunixin. Further studies are needed to determine the exact mechanisms responsible as well as the influence of local anesthetics and drug application time relative to the painful procedure. Additionally, the data supports the utility of the calf as a model for future pain studies.

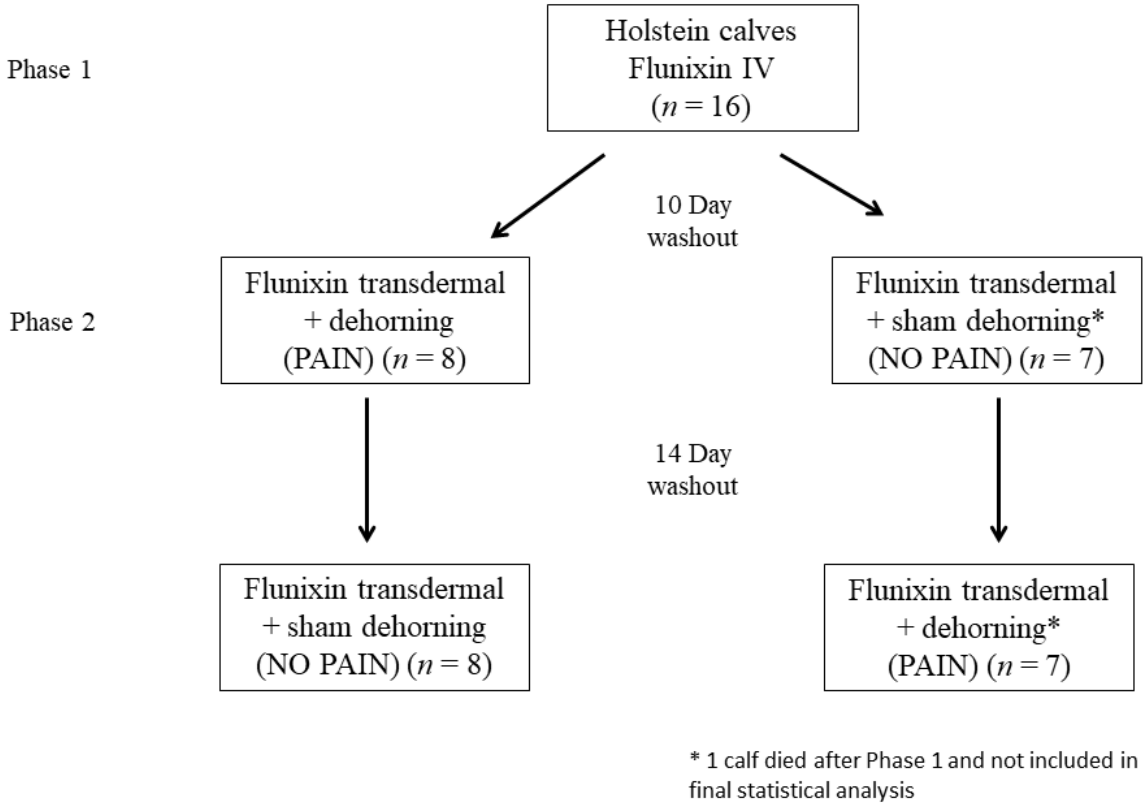

Figure 5-1 Study design to determine the pharmacokinetics of transdermal (topical) flunixin in calves subjected to cautery dehorning (PAIN) or sham dehorning (NO PAIN).

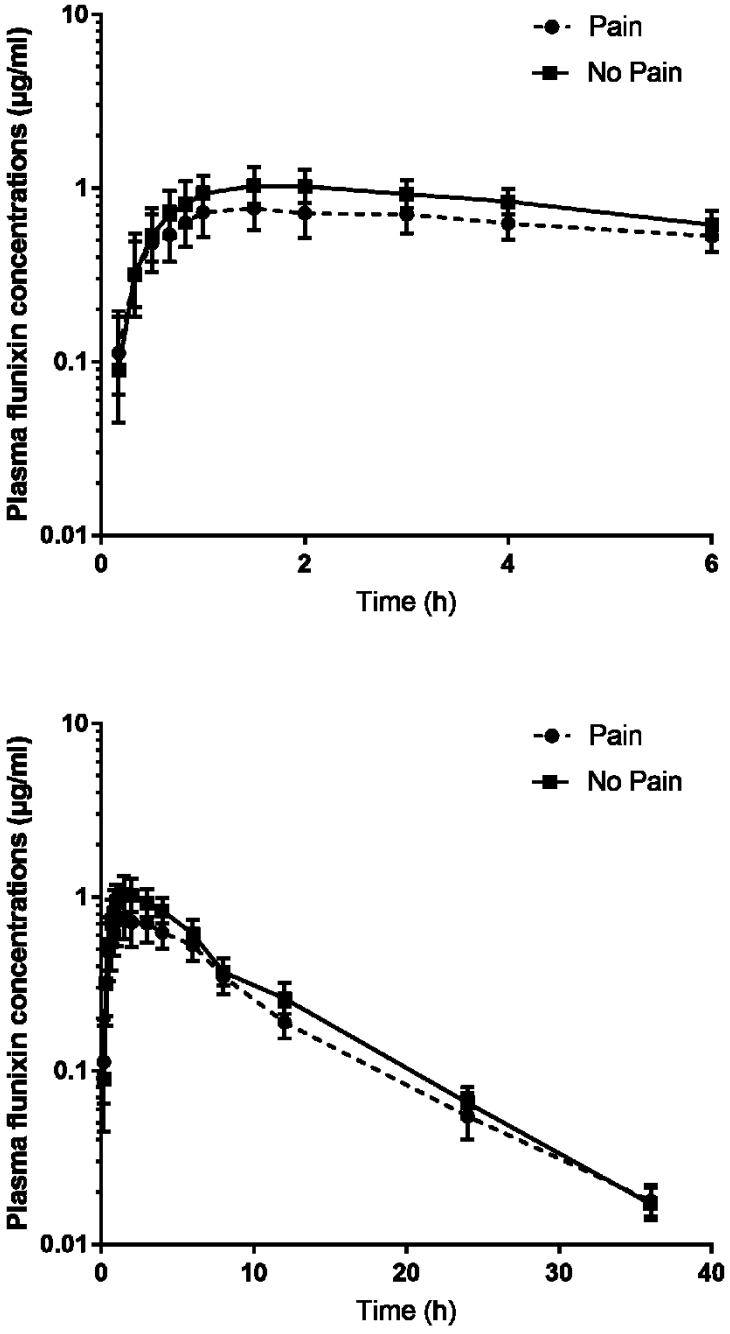

Figure 5-2 Plasma flunixin concentrations (mean ± standard error of the mean) (a) over 6 hours and (b) 60 hours in calves subjected to cautery dehorning (PAIN) or sham dehorning (NO PAIN) with concurrent administration of transdermal flunixin (3.33 mg kg^{-1}). * Significant difference between treatments ($p < 0.05$).

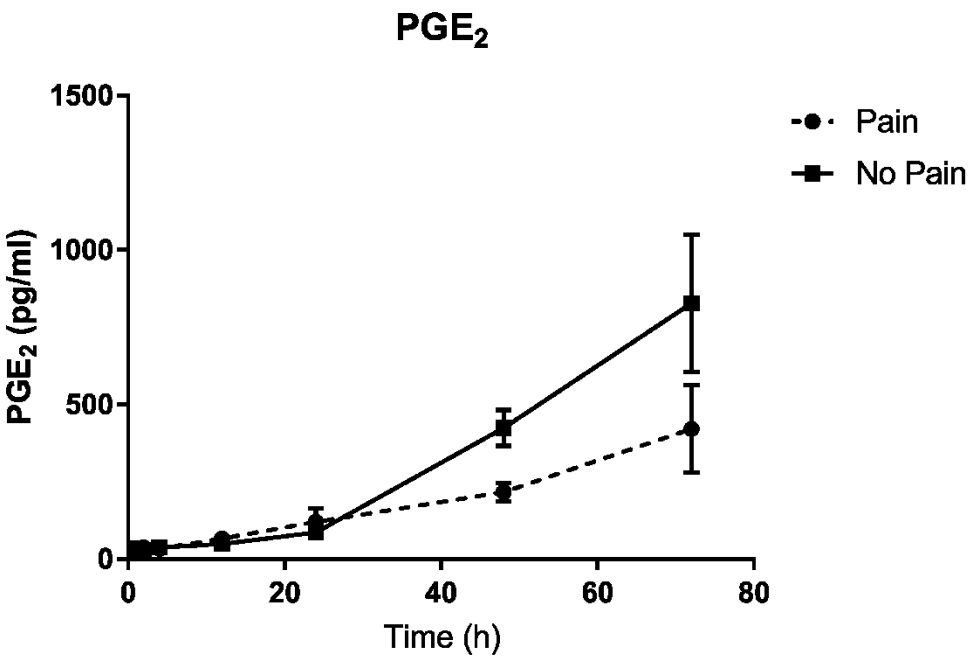

Figure 5-3 Plasma prostaglandin E_2 concentrations (mean ± standard error of the mean) in calves subjected to cautery dehorning (PAIN) or sham dehorning (NO PAIN) with concurrent administration of transdermal flunixin (3.33 mg kg^{-1}). * Significant difference between treatments ($p < 0.05$).

Table 5-1Summary table of the pharmacokinetic parameters for calves subjected to cautery dehorning (treatment PAIN) or sham dehorning (treatment NO PAIN) with concurrent administration of transdermal flunixin at 3.33mg kg^{-1}.

Parameter	NO PAIN Geometric Mean	NO PAIN Median (Range)	PAIN Geometric Mean	PAIN Median (Range)	*p*-value
λz ($hour^{-1}$)	0.10	0.10 (0.05 - 0.13)	0.07	0.08 (0.03 - 0.12)	0.0191
T 1/2 (hour)	7.16	6.88 (5.27 - 14.57)	10.09	8.42 (5.59 - 25.31)	0.0202
Tmax (hour)	1.66	1.50 (0.67 - 4.00)	1.84	2.00 (0.83 - 4.00)	0.6232
Cmax (μg mL^{-1})	1.16	1.27 (0.37 - 2.26)	0.95	0.96 (0.48 - 1.68)	0.089
AUC all (hour*μg mL^{-1})	9.12	9.31 (4.73 - 18.16)	8.76	9.10 (4.52 - 13.94)	0.7716
Vz/F (L kg^{-1})	3.73	3.84 (1.42 - 10.84)	5.43	5.50 (2.55 - 13.84)	0.0564
CL/F (mL $minute^{-1}$ kg^{-1})	6.02	5.90 (3.05 - 11.51)	6.21	6.04 (3.96 - 12.07)	1
MRT inf (hour)	9.20	8.49 (6.87 - 14.57)	9.89	9.24 (6.76 - 20.33)	0.6482
MAT (hour)	5.27	5.42 (2.06 - 11.27)	3.88	3.88 (0.60 - 14.98)	0.1711
F	0.37	0.40 (0.17 - 0.58)	0.30	0.29 (0.18 - 0.56)	0.0971

λz, terminal rate constant; T ½, terminal half-life; T_{MAX}, time of C_{MAX}; C_{MAX}, maximum plasma concentrations; AUC all, area under the curve extrapolated to infinity; Vz/F, volume of distribution (area method) per fraction of the dose absorbed; CL/F, clearance per fraction of the dose absorbed; MRT, mean residence time extrapolated to infinity; MAT, mean absorption time; F, fraction of the dose absorbed (bioavailability)

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Chapter 6 - Effects of transdermal flunixin meglumine on pain biomarkers at dehorning in calves

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ABSTRACT

The objective of this study was to evaluate the analgesic properties of transdermal flunixin meglumine when given at the time of dehorning on pain biomarkers. Twenty-four weaned male Holstein calves, 6 to 8 weeks of age were enrolled into the study. The calves were randomly assigned to one of three treatment groups: 1) transdermal flunixin and dehorn (DH-FLU); 2) transdermal flunixin and sham dehorn (SHAM-FLU); and 3) placebo and dehorn (DH-PLBO). Transdermal flunixin at a label dose of 3.33 mg/kg (or placebo at an equivalent volume) was administered as a pour-on along the top-line of the calves in each treatment group concurrently with electrocautery dehorning, or sham dehorning. Biomarker parameters collected and analyzed included: infrared thermography (IRT), mechanical nociception threshold (MNT), plasma cortisol, and Substance P (SP). There were no differences in maximal temperatures detected for the IRT measurements of the medial canthus of the eye for the DH groups. Mean control point MNT measurements at 48 hours were 3.14 kgF, 3.46 kgF and 1.43 kgF for the DH-FLU, Sham-FLU and DH-PLBO groups respectively (P = 0.0001). No other differences of MNT were detected between the dehorned groups for the other test sites and time points. Plasma cortisol reached peak concentration at 20 minutes post dehorning for the DH-FLU and DH-PLBO groups and 10 minutes for SHAM-FLU group. Peak plasma cortisol concentrations were 32.0 ng/ml, 12.7 ng/ml, and 28.8 ng/ml for the DH-FLU, SHAM-FLU and DH-PLBO groups respectively. Cortisol concentrations were lower for the DH-FLU group at 90 minutes post dehorning compared to the SHAM-FLU and DH-PLBO group ($P = 0.04$). Area under the effect curve (AUEC) were similar for all groups ($P = 0.93$). No statistical differences in SP concentrations between groups were detected for any of the time points. In conclusion, transdermal flunixin meglumine given at the time of dehorning did not provide substantial analgesia based on the pain

biomarkers investigated. Further investigation into its role as part of a multi-modal analgesic plan is warranted.

Key words: NSAID, cattle, nociception, analgesia, topical

INTRODUCTION

Dehorning is a common practice in cattle production systems to decrease human and animal injury, and carcass bruising. According to the 2007 National Animal Health Monitoring Survey, dehorning was performed on 94% of dairy farms according to the USDA National Animal Health Monitoring Survey (2007). There has been an increase in the welfare concerns of cattle undergoing dehorning and interest in ways to mitigate the pain associated with dehorning (Stafford and Mellor, 2015). Many veterinarians feel it is important to provide analgesia to calves at the time of dehorning, but many still do not provide it (Fajt et al., 2011).

Flunixin meglumine is the only non-steroidal anti-inflammatory drug (NSAID) in the United States that is approved for use in cattle. It is only labeled for intravenous administration for pyrexia associated with bovine respiratory disease and endotoxic mastitis as well as the control of inflammation in endotoxemia (2017). Giving the intravenous formulation via an intramuscular route of administration causes profound tissue blemishes and carcass trim (Pyorala et al., 1999; Smith et al., 2008). Recently, a novel formulation of flunixin meglumine has been approved in the European Union. This formulation of flunixin has been developed for topical administration and transdermal absorption. This formulation also provides for convenient and non-invasive administration that requires little to no training. Like the injectable product, it is only labelled for the treatment of pyrexia associated with bovine respiratory disease. Flunixin

meglumine has been studied for pain mitigation in the past and has been shown to be effective in the reduction of cortisol and behavioral traits (Huber et al., 2013).

Our hypothesis was that flunixin meglumine, when given by transdermal route, would provide analgesia at dehorning. The objective of this study was to investigate the analgesic effects of transdermal flunixin meglumine on nociception and stress at the time of dehorning.

MATERIAL AND METHODS

This study was approved by the Institutional Animal Care and Use Committee at Iowa State University (Log # 6-15-8039-B).

Animals and Housing

Twenty-four weaned male Holstein calves, 6 to 8 weeks of age, were obtained from a single dairy farm in South Dakota. The calves weight between 43.2 kg and 87.3 kg with an average of 68.6 kg. Each calf was identified with a plastic ear tag in each ear and a RFID tag in the right ear by the source farm. Calves were acclimated upon arrival for 2 weeks prior to the start of the trial. During the acclimation period, calves were trained to be restrained with a rope halter. Animal caretakers also moved about the calves to mimic the activities that would occur during the experiment. Additionally, all calves were drenched with amprolium (Corid, Duluth, GA) at 10mg/kg PO for five days during the acclimation period for the treatment of coccidiosis.

Calves were grouped housed in an open faced building with indoor/outdoor access. Calves were fed a diet of cracked corn, grass hay and a commercial protein supplement containing a coccidiostat (Bovatec, Zoetis Animal Health, Kalamazoo, MI). The diet was formulated to meet or exceed NRC recommendations for growth and development. Calves were fed in a portable feed bunk twice daily so all animals could access feed at the same time. Calves had access to water at all times in water troughs.

Experimental Design

A randomized block design was used for this study with 8 calves in each treatment group. Calves were randomly assigned to one of three treatment groups: 1) topical flunixin and dehorn (DH-FLU); 2) topical flunixin and sham dehorn (SHAM-FLU), and 3) placebo and dehorn (DH-PLBO). Calves were randomized based on body weight using a random number generator (Excel, Microsoft Corp. Redmond, WA) and interspersed throughout all pens.

Twenty-four hours prior to the start of the study, calves were restrained and an intravenous catheter was aseptically placed. For catheter placement, the hair over the jugular vein was clipped using a #40 blade and electric clippers (Oster, Boca Raton, FL). The skin was surgically scrubbed for 5 minutes using 4% Chlorohexidine surgical scrub (First Priority Inc., Elgin, IL) and 70% isopropyl alcohol (First Priority Inc., Elgin, IL). The calf was restrained by study personnel and a 16 gauge catheter (Surflo, Terumo Medical Co., Somerset, NJ) was placed in the jugular vein. The catheter was capped with an injection port (Hospra Inc., Lake Forest, IL) and sutured in place with #0 nylon suture (Ethilon, Ethicon US LLC, Somerville, NJ). Prior to each blood draw, the injection port was disinfected with an alcohol soaked gauze.

The hair immediately adjacent to the horn buds was clipped prior to the start of the study. Baseline mechanical nociception threshold and infrared thermographic images (described later) were collected prior to the time of intravenous catheter placement. Blood samples for baseline cortisol and substance P samples were collected 20 minutes prior to dehorning and drug application.

Drug application and dehorning.

Drug application and dehorning occurred concurrently, but drug application was considered the start of the experiment (time 0). For the drug application, calves were tightly restrained with a rope halter. Flunixin meglumine (Finadyne Pour-On, MSD Animal Health,

Dublin, Ireland) or placebo was applied to the skin along the top-line, starting at the shoulders and ending at the tail head, at a dose of 3.33 mg/kg using a single use syringe. The placebo consisted of propylene glycol, isopropyl alcohol, and a red dye to mimic the test product in color, viscosity, and odor. Calves were dehorned by applying electrocautery (Rhinehart Development Corp., Spencerville, IN) to each horn for 10 seconds to achieve a copper ring around the horn tissue. Calves allocated to the sham dehorn group had a cold electric dehorner applied to each horn for 10 seconds to mimic dehorning restraint. Dehorning sites were monitored for signs of infection for 7 days after the dehorning procedure.

Mechanical Nociception Threshold (MNT).

Mechanical nociception threshold, as defined by a maximum force which induces a withdrawal response(Tapper et al., 2013), was determined 24 hours prior to dehorning and 6, 24 and 48 h post-dehorning using methods described by Stock *et. al* (2016). Briefly, calves were restrained using a halter for mechanical nociception threshold determination. Using a hand held pressure algometer (Wagner Instruments, CT, USA), a force was applied perpendicularly at a rate of approximately 1 kg of force per second at 2 locations (lateral and caudal) adjacent to the horn bud. The 1 cm^2 rubber tip of the algometer was placed immediately adjacent to cauterized skin. Additionally, a third control location on the frontal bone between the eyes was used to evaluate MNT of an area that was not adjacent to cauterized skin. A withdrawal response was indicated by an overt movement away from the applied pressure algometer. The MNT values were recorded by a second investigator to prevent bias by the investigator performing the MNT collection. A maximum value of 5 kgf was determined a priori. To avoid provoking a withdrawal from a startle response, the investigator placed a hand on the posterior aspect of the poll and removed it immediately prior to placing the algometer. Calves were blindfolded prior to MNT to avoid a response associated with visual cues. Both the order of locations tested and the

side of the calf the investigator stood were randomized for each calf. Locations were tested three times in sequential order and the value was averaged for statistical analysis.

Infrared Thermography Imaging (IRT).

Infrared thermography images were obtained 24 hours prior to dehorning and 1, 2, 4, 6, 12, 24, 48 h post-treatment application using a research grade infrared camera (FLIR SC 660, FLIR Systems AB, Danderyd, Sweden). The camera was calibrated prior to obtaining images with the ambient temperature and relative humidity. Changes in ambient temperature and relative humidity were automatically obtained by the camera throughout the imaging session. Calves were restrained with a halter and an image of the lateral aspect of the head was obtained so that the image contained the medial canthus of the eye and the dehorning site. The image was obtained by pointing the camera at the calf's head at a 45° angle and distance of 0.5 meters. Both sides of the calf's head were imaged. Images were saved and the image file number was recorded for analysis at a later time. The average ambient temperature and relative humidity was 21.5ºC and 67% respectively over the course of the study.

Infrared images were analyzed using research grade computer software (FLIR ExaminIR, Inc., North Billerica, MA). The maximum temperatures of the medial canthus of the eye were recorded and analyzed. The maximum temperature of the medial canthus was determined by centering a 2 centimeter circular window over the target area.

Plasma Cortisol Determination.

Blood samples for cortisol levels were collected at time 0 for a baseline, 10, 20, 30, 40, 50, 60, and 90 minutes, as well as 2, 3, 4, 6, 8, 12, 24, 36, 48, 56, and 72 hours, post treatment application. Baseline samples were collected first thing in the morning of the experiment to account for the normal circadian rhythm of cortisol levels. Briefly, 15 ml of blood was collected at the predetermined time points via jugular catheterization. The blood was immediately

transferred to blood tubes containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ). The tubes were inverted 3 times and then placed on ice until transported to the lab. Once in the lab, the blood was centrifuged at 3000g for 10 minutes. The plasma was pipetted from the tube and placed into cryovials. The plasma samples were placed on dry ice and then stored at -80°C until analysis.

Cortisol levels were determined using a commercially available radioimmunoassay (MP Biomedicals, Santa Ana, CA). Samples were run in duplicates and repeated if a large difference in cortisol levels between the samples were determined. The assay had a detection range of 0.64 to 150 ng/ml. The coefficient of variation for the intra-assay variability was 11.72% and the inter-assay variability was calculated to be 13.6%. The area under the effect curve was calculated by the linear trapezoidal rule as described by Glynn et al.(2013).

Substance P Determination.

Substance P concentrations were determined as a baseline on the morning of the experiment and at 1, 2, 4, 12, 24, and 48 hours post treatment application. For substance P determination, 200µg benzamidine was added to EDTA blood tubes 48 hours prior to the start of the study. To collect the sample, 6 ml of blood was added to the spiked EDTA tube and inverted 3 times to mix thoroughly. The samples were immediately placed on ice and transported to the lab. The samples were centrifuged within 30 minutes of collection and the plasma was placed into cryovials. The cryovials were placed on dry ice to freeze immediately and then stored at -80°C until analysis.

Substance P levels were determined using the methods described by Van Engen *et.al*. (Engen et al., 2014) using non-extracted plasma. The coefficient of variation for the intra-assay variability was 10.3% and the inter-assay variability was calculated to be 32%. The average R^2

for the calibration curve was 0.981. The area under the effect curve was calculated by the linear trapezoidal rule.

Statistical Analysis

Statistical analysis was completed using repeated measure analysis with compound symmetric structure performed using the Mixed Procedure in SAS (version 9.4, SAS Inst. Inc., Cary, NC). In the model statement, cortisol, substance P, IRT, and MNT were each response variables. The fixed effects were treatment (flunixin, placebo, dehorn, sham), time point, and treatment by time interaction. In order to test the significance of main effects and interactions, *F*-tests were used. If significant overall differences were identified, pairwise comparisons were performed using Tukey's *t* tests based on the lsmeans statement with Bonferroni correction adjustment. Statistical analysis for AUEC was run using the model as described above. Statistical significance was set at $P < 0.05$ and a tendency for significance at $P = 0.05$ - 0.1.

RESULTS AND DISCUSSION

The main objective of this study was to examine the analgesic efficacy of transdermal flunixin meglumine at the time of dehorning. Lidocaine was not used as it would have interfered with the direct pharmacologic effect of flunixin on pain biomarkers. It is also important to note that investigators performing MNT and IRT were not blinded to dehorning procedure (dehorn or sham), but they were blinded to treatment (dehorn or placebo).

Mechanical Nociception Threshold (MNT)

There was a treatment ($P = 0.0002$), time ($P < 0.0001$), and treatment by time interaction ($P < 0.0001$) for MNT at the dehorn sites (Figure 1) for the 6 h, 24 h and 48 h timepoints.

MNT has been used to evaluate the level of pain associated with dehorning in previous studies (Heinrich et al., 2010; Allen et al., 2013; Glynn et al., 2013; Stock et al., 2015).

Intravenous flunixin, given at the time of dehorning, has been evaluated and no effect of flunixin was seen for MNT values (Glynn et al., 2013). MNT values for the dehorning sites in this study did not return to baseline by the end of the study period (48 h). These finding are similar to other studies that examined MNT. Stock et al. (Stock et al., 2015) reported MNT values for dehorned calves below baseline and sham controls for 4 days post dehorning. Additionally, Summer et al. (Summer et al., 2007) reported that hyperalgesia can be present for 4 weeks in partial epidermal burns in rat models. Based on the finding of the present study and other studies, further research is needed to follow pain sensitivity in calves following dehorning.

Additionally, there was a treatment ($P = 0.0364$), time ($P < 0.0001$), and treatment by time interaction ($P = 0.0060$) for MNT at the control site (Figure 2). At 48 hours post dehorning, there was a significant difference in the MNT at the control site between the DH-FLU and DH-PLBO groups (DH-FLU 3.14 Kgf vs. DH-PLBO 1.59 Kgf; P = 0.0023). This could be attributed to a reduction in centralized pain and/or an increase in pain tolerance. These findings warrant further investigation especially in light of the lack of difference in other pain biomarkers.

Infrared Thermography Imaging

There was no effect of treatment on ocular IRT ($P = 0.5421$). However, there was a time effect ($P <0.0001$) but no treatment by time interaction ($P = 0.9831$) for ocular IRT (figure 3).

In this study, the maximum temperature decreases from baseline to 2 hours post procedure and then increases at 6 hours post procedure. The decrease over the first 2 hours does not indicate activation of a stress response as expected and reported by others (Mellor et al., 2000; Stewart et al., 2010). The activation of the autonomic nervous system may not have been seen due to the 1 hour window from the dehorning procedure to the first IRT timepoint. Glynn et al. (Glynn et al., 2013) noted a stress response post procedure using a 5 minute time interval

instead of 60 minutes as in the current study. The ambient temperature may have had a larger role in the ocular temperature pattern seen in these calves. Calves were kept under shade and out of direct sunlight to minimize environmental factors that have been shown to influence IRT temperature measurements (Church et al., 2014). Despite control measures, temperatures in the barn ranged from a low of 15°C in the morning to a high of 30°C during the middle of the day.

Plasma Cortisol

There was a treatment ($P = 0.0014$), time ($P < 0.0001$), and treatment by time interaction ($P < 0.001$) for cortisol (figure 4). Peak plasma cortisol concentrations were reached at 20 minutes post dehorning for the DH-FLU and DH-PLBO groups and 10 minutes for SHAM-FLU group. Maximum plasma cortisol concentrations were 32.0 ng/ml, 12.7 ng/ml, and 28.8 ng/ml for the DH-FLU, SHAM-FLU and DH-PLBO groups respectively. Cortisol concentrations tended to be lower for the DH-FLU group at 90 minutes post dehorning compared to the DH-PLBO group ($P = 0.0819$). There was a rise in cortisol for the SHAM-FLU group at 4 hours post dehorning procedure. This spike in cortisol only tended to have significance ($P = 0.0854$) ~~and~~ but coincided with the timing of feed delivery to the pens.

When the area under the effect curve (AUEC) was examined, there were no differences in the total area under the effect curve ($P = 0.93$). When broken down into distinct time periods, differences in AUEC were found for the time frames of 0 to 2 h and 2 to 4 h (Table 2). The SHAM-FLU group had a lower AUEC at 0 to 2h ($P <0.0001$) and is explained by the lack of spike of cortisol associated with the dehorning procedure. At 2 to 4 h, the DH-FLU group had lower AUEC compared to the SHAM-FLU and DH-PLBO groups ($P = 0.05$). This difference can be explained by the lower cortisol concentrations for the DH-FLU group compared to the DH-PLBO group, and the rise in cortisol concentrations of the SHAM-FLU group. The total proportion of the AUEC is represented in figure 5. The DH-PLBO group had a high proportion

of the AUEC occur in the first 12 hours compared to the other groups. The DH-FLU had a significantly lower proportion of the AUEC occur at 2 to 4 hours compared to the DH-PLBO group. This difference at 2 to 4 hours was seen compared to the SHAM-FLU group likely due to the rise of cortisol levels seen in that group. Although the two dehorn groups (DH-PLBO and DH-FLU) had elevated cortisol over the first 2 hours, this accounted for a small portion of the overall AUEC.

Cortisol concentrations in calves given intravenous flunixin meglumine have been previously reported (Glynn et al., 2013; Huber et al., 2013). In both studies, flunixin was shown to reduce cortisol when given at the time of dehorning. In the study by Huber et al. (Huber et al., 2013), there was a significant difference in cortisol levels in calves given two doses of flunixin compared to placebo calves. The second dose of flunixin was given 3 hours after the dehorning procedure. However, calves given a single dose of flunixin 20 minutes prior to dehorning only tended to have lower cortisol levels. A difference between the two aforementioned studies and the current study is the route of administration. The studies by Glynn (2013) and Huber (2013) utilized flunixin given by the intravenous route. Differences in the pharmacokinetics of intravenous and transdermal routes need to be addressed. The time to maximum (Tmax) concentration has been shown to be 2 h for calves treated with transdermal flunixin resulting in a delay between drug application and potential full effect (Kleinhenz et al., 2016). At the time of Tmax, the calves in the dehorn groups (DH- FLU and DH- PLBO) did not have a difference in cortisol levels (6.12 ng/ml vs. 9.68 ng/ml; $P = 0.3513$). These finds suggest an alternative dosing time, such as 2 hours prior to dehorning may be more appropriate.

Others have investigated cortisol at the time of dehorning using different NSAIDs. Allen et al. found differences in cortisol levels of calves administered meloxicam compared to placebo

controls (2013). Calves administered carprofen and firocoxib did not have significant differences in cortisol levels (Stock et al., 2015; Stock et al., 2016).

Substance P

There was no effect of treatment ($P = 0.9473$) or treatment by time interaction ($P = 0.1073$) for SP concentrations (table 1). However, there tended to be an effect of time ($P = 0.0699$). When the AUEC was examined, there were no differences between groups ($P = 0.98$; see Table 2)

These SP findings are similar to other reports of calves treated with flunixin at dehorning (Glynn et al., 2013). Substance P levels were not different from calves given firocoxib or carprofen (Stock et al., 2015; Stock et al., 2016). Calves treated with meloxicam did have lower SP level than their placebo counterparts (Allen et al., 2013). The lack of difference between the dehorn and sham groups is interesting given the fact local anesthetics were not used in the dehorning procedure.

CONCLUSION

Cautery dehorning in dairy calves causes an acute increase in cortisol and subsequent increase in nociception. The treatment of calves with transdermal flunixin meglumine as a stand-alone analgesic does not provide adequate analgesia when given at the time of dehorning. Further research is needed to determine the utility of transdermal flunixin meglumine as part of a multimodal analgesic protocol that includes the use of a local anesthetic block, alternative dosing strategies, and/or comparison to other NSAIDs. Additional work investigating the effects of pain on the pharmacokinetics of transdermal flunixin meglumine is warranted.

Figure 6-1 Mean (±SEM) mechanical nociception threshold (MNT) at the dehorn sites over 48 h as measured with pressure algometry for flunixin and placebo treated groups following cautery dehorning. DH-FLU = dehorned and flunixin treated (n = 8); DH-PLBO = dehorned and placebo treated (n = 8); SHAM-FLU = sham dehorned and flunixin treated (n = 8).

* Differences ($P \leq 0.05$)

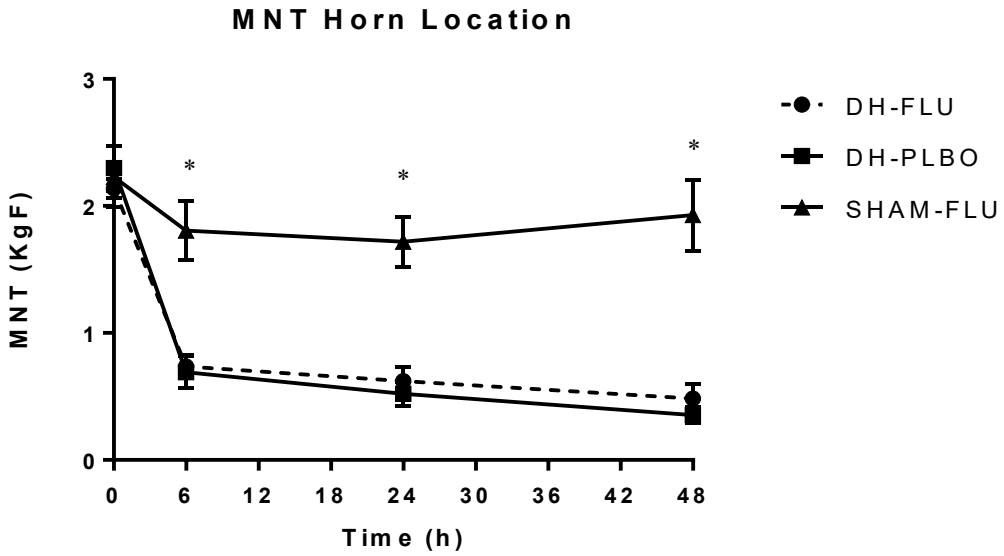


Figure 6-2 Mean (±SEM) control location mechanical nociception threshold (MNT) over 48 h as measured with pressure algometry for flunixin and placebo treated groups following cautery dehorning. DH-FLU = dehorned and flunixin treated (n = 8); DH-PLBO = dehorned and placebo treated (n = 8); SHAM-FLU = sham dehorned and flunixin treated (n = 8).

* Differences ($P \leq 0.05$)

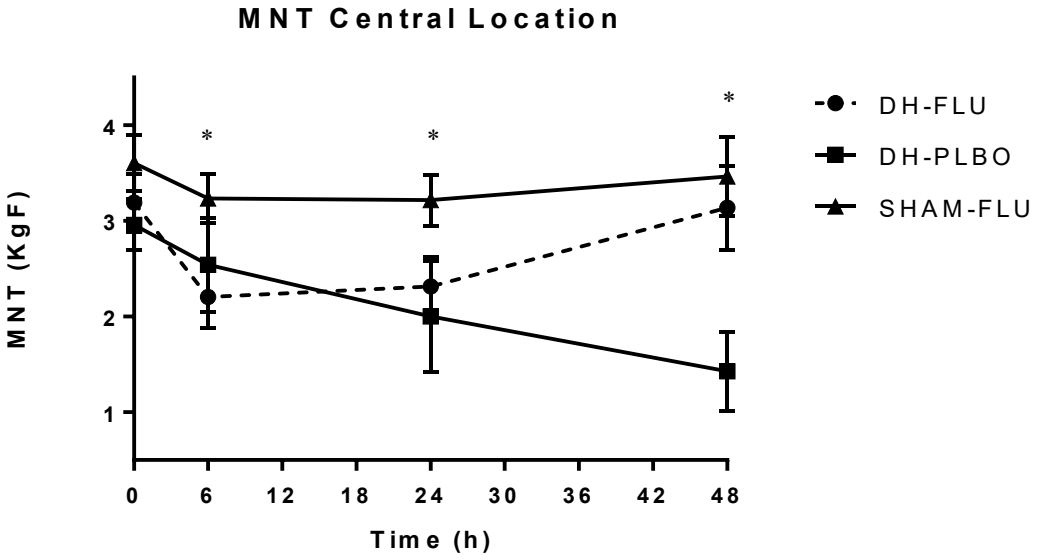


Figure 6-3 Mean ocular temperature (±SEM) for 48 h for flunixin and placebo treated groups following cautery dehorning. DH-FLU = dehorned and flunixin treated (n = 8); DH-PLBO = dehorned and placebo treated (n = 8); SHAM-FLU = sham dehorned and flunixin treated (n = 8).

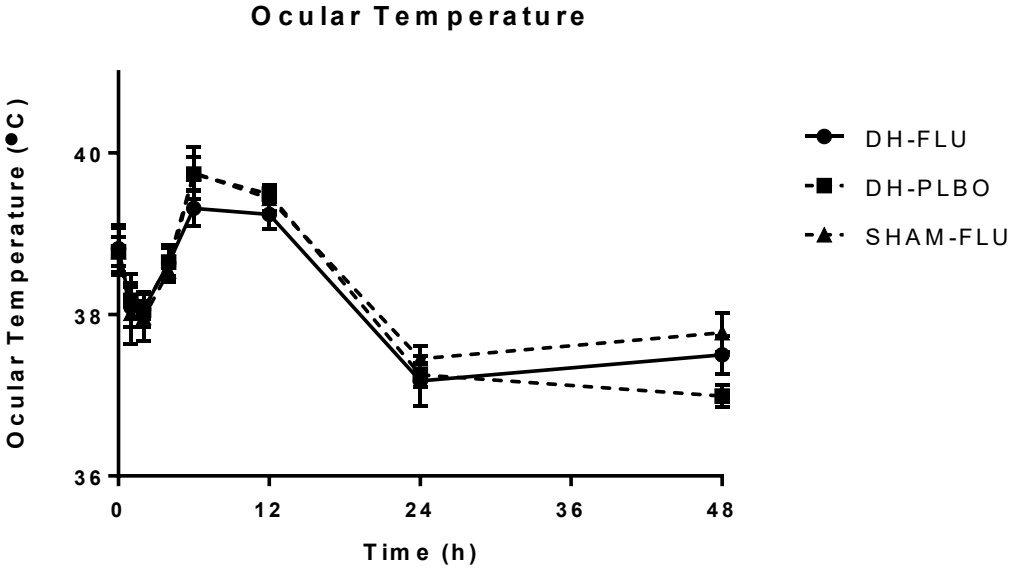
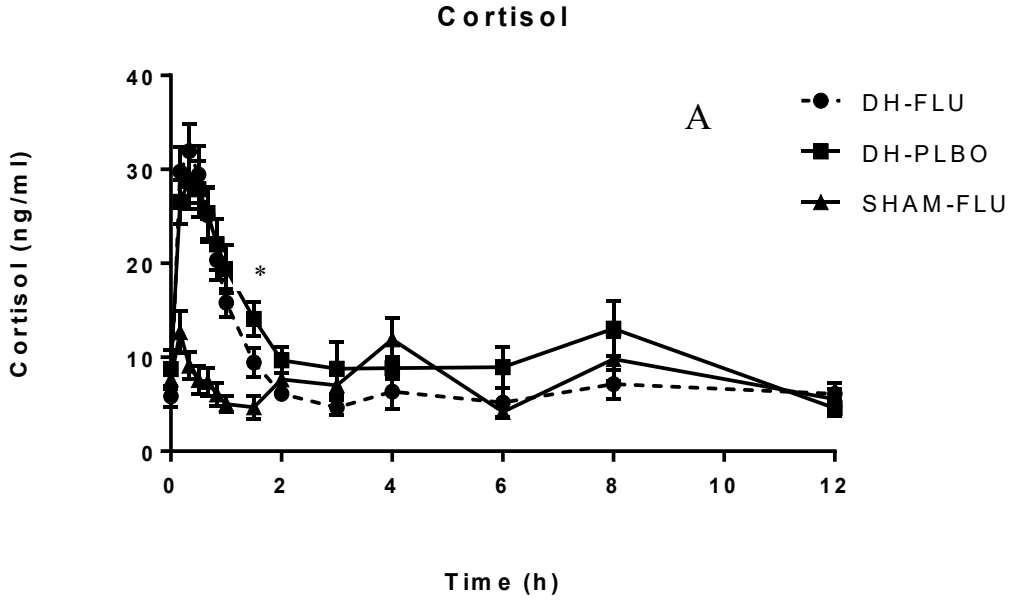


Figure 6-4 Mean cortisol concentrations (±SEM) over 12 (A) and 72 h (B) for flunixin and placebo treated groups following cautery dehorning. DH-FLU = dehorned and flunixin treated (n = 8); DH-PLBO = dehorned and placebo treated (n = 8); SHAM-FLU = sham dehorned and flunixin treated (n = 8). * Differences ($P \leq 0.05$)



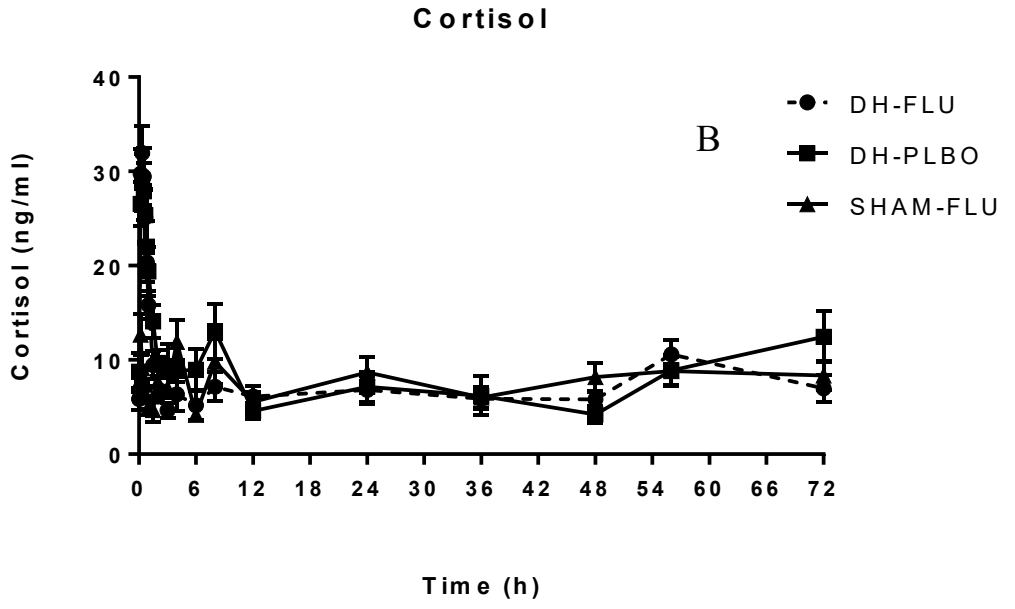
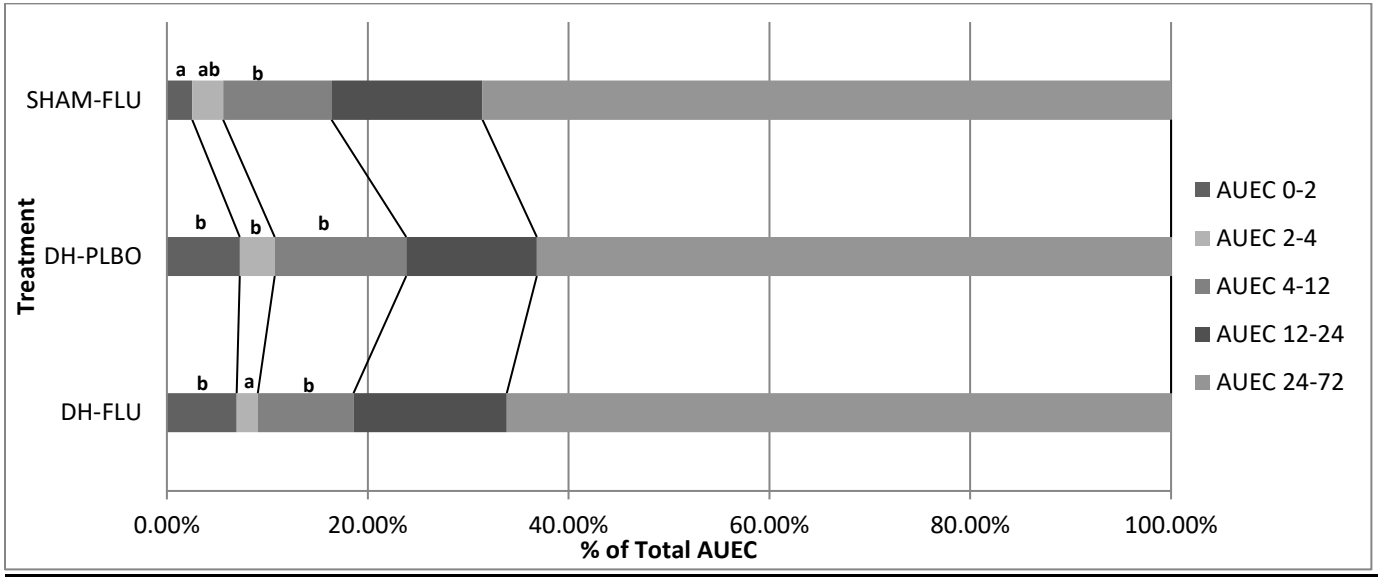


Figure 6-5 Proportion of the total area under the effect curve (AUEC) for cortisol by successive time periods for DH-FLU = dehorned and flunixin treated (n = 8); DH-PLBO = dehorned and placebo treated (n = 8); and SHAM-FLU = sham dehorned and flunixin treated groups (n = 8).



[a,b] Different superscript letters within time period differ (P ≤0.05).

$AUEC_{0-2}$ = area under the effect curve from time 0 to 2 h; $AUEC_{2-4}$ = area under the effect curve from time 2 to 4 h; $AUEC_{4-12}$ = area under the effect curve from time 4 to 12 h; $AUEC_{12-24}$ = area under the effect curve from time 12 to 24 h; $AUEC_{24-72}$ = area under the effect curve from time 24 to 72 h

Table 6-1 Summary table of overall mean response variables for flunixin and placebo treated calves after dehorning or sham dehorning (n = 8) at 48 h

	DH-FLU[1]	DH-PLBO	SHAM-FLU	Treatment, *P*-value	Time, *P*-value	Treatment x Time, *P*-value
MNT (horn),[2] kgf	1.2[a] (1.0 to 1.3)	1.3[a] (1.2 to 1.4)	2.0[b] (1.9 to 2.1)	0.0002	< 0.0001	< 0.0001
MNT (control),[3] kgf	2.8[a] (2.6 to 3.1)	2.4[b] (2.1 to 2.6)	3.4[c] (3.1 to 3.6)	0.04	< 0.0001	0.006
Cortisol, ng/ml	12.6[a] (11.2 to 14.0)	14.0[a] (12.6 to 15.4)	7.7[b] (6.3 to 9.1)	0.0014	< 0.0001	< 0.0001
Substance P, pg/ml	103.5[a] (92.7 to 114.4)	104.6[a] (96.2 to 113.1)	99.7[a] (94.3 to 105.2)	0.95	0.09	0.09
Ocular temperature, ºC	38.4[a] (38.2 to 38.6)	38.4[a] (38.2 to 38.6)	38.5[a] (38.3 to 38.7)	0.54	< 0.0001	0.98

[a,b] Different superscript letters within a row, means differ ($P \leq 0.05$).

[1]DH-FLU = topical flunixin and dehorn; SHAM-FLU = topical flunixin and sham dehorn; DH-PLBO = placebo and dehorn

[2]MNT (horn) = Mechanical nociception threshold at the horn bud

[3]MNT (control) = Mechanical nociception threshold at the control point or middle of the head

Table 6-2 Area under the effect curve (AUEC) and maximum concentration for cortisol and substance P concentrations for flunixin and placebo treated calves after dehorning or sham dehorning (n = 8).

Response[1]	DH-FLU[2]	DH-PLBO	SHAM-FLU	*P* – value
		Mean (95% confidence interval)		
Cortisol, ng x h/ml				
$AUEC_{0-72}$	532.1[a] (431.8 to 632.5)	555.6[a] (351.1 to 760.0)	563.7[a] (449.1 to 678.2)	0.93
$AUEC_{0-2}$	35.2[a] (27.6 to 43.1)	38.3[a] (29.6 to 46.9)	13.9[b] (9.6 to 18.3)	<0.0001
$AUEC_{2-4}$	11.0[a] (7.5 to 14.5)	18.0[b] (12.0 to 24.1)	17.1[b] (12.3 to 21.9)	0.05
$AUEC_{4-12}$	51.0[a] (29.5 to 72.6)	73.8[a] (41.9 to 105.8)	61.8[a] (41.0 to 82.7)	0.34
$AUEC_{12-24}$	80.2[a] (48.2 to 112.1)	71.1[a] (44.0 to 98.2)	85.1[a] (57.5 to 112.8)	0.72
$AUEC_{24-72}$	354.8[a] (273.5 to 436.0)	354.3[a] (205.8 to 502.8)	385.6[a] (302.3 to 469.0)	0.86
Cmax, ng/ml	32.0[a] (25.2 to 38.8)	28.8[a] (21.6 to 36.0)	12.7[a] (7.5 to 17.9)	<0.0001
Substance P, pg x h/ml				
$AUEC_{0-48}$	7638.1[a] 5955.2 to 9320.9	7626.3[a] (5943.4 to 9309.1)	7411.4[a] (5728.5 to 9094.2)	0.98
$AUEC_{0-12}$	1191.5[a] (918.4 to 1464.6)	1259.2[a] (986.1 to 1532.3)	1209.0[a] (935.9 to 1482.1)	0.93
$AUEC_{12-48}$	6446.6[a] (5026.6 to 7866.7)	6367.1[a] (4947.1 to 7787.1)	6202.4[a] (4782.3 to 7622.4)	0.97
Cmax, pg/ml	134.5[a] (106.2 to 162.7)	123.6[a] (95.3 to 151.8)	120.3[a] (92.4 to 148.5)	0.74

[a,b] Different superscript letters within a row, means differ ($P \leq 0.05$).

[1] $AUEC_{0-72}$ = area under the effect curve from time 0 to last sample collected; $AUEC_{0-2}$ = area under the effect curve from time 0 to 2 h; $AUEC_{2-4}$ = area under the effect curve from time 2 to 4 h; $AUEC_{4-12}$ = area under the effect curve from time 4 to 12 h; $AUEC_{12-24}$ =

area under the effect curve from time 12 to 24 h; $AUEC_{24-72}$ = area under the effect curve from time 24 to 72 h; $AUEC_{0-48}$ = area under the effect curve from time 0 to last sample collected; $AUEC_{0-12}$ = area under the effect curve from time 0 to 12 h; $AUEC_{12-48}$ = area under the effect curve from time 12 to 48 h; Cmax = maximum plasma concentration

[2]DH-FLU = topical flunixin and dehorn; SHAM-FLU = topical flunixin and sham dehorn; DH-PLBO = placebo and dehorn

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Chapter 7 - The impact of transdermal flunixin meglumine on biomarkers of pain in calves when administered at the time of surgical castration without local anesthesia.

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ABSTRACT

Castration is a common husbandry practice performed on cattle worldwide. Although non-steroidal anti-inflammatory drugs provide analgesia at the time of castration, labor and medicine costs have been cited as reasons for not including analgesic into castration protocols. This study was conducted to assess the impact of transdermal flunixin meglumine on biomarkers of pain in calves when administered at the time of surgical castration without local anesthesia Twenty three Holstein calves were randomly assigned to (1) a flunixin castrated group (CAST+FLU) (n=8); (2) a placebo castrated group (CAST+PLBO) (n=8) or (3) a previously castrated, negative control group (SHAM+PLBO) (n=7). Treated calves received topical flunixin meglumine applied to their dorsal midline at the label dose of 3.33 mg/kg during the surgical castration procedure. Outcomes collected and analyzed included: plasma cortisol, substance P, ocular infrared thermography (IRT), and gait analysis (step force, foot contact area, foot contact pressure, impulse). Biomarkers were statistically analyzed using repeat measures analysis. Plasma cortisol concentrations were higher ($P = 0.0016$) and the area under the effect curve tended to be higher ($P = 0.0979$) in the CAST+PLBO. Specifically, the CAST+FLU group had significantly lower cortisol levels compared to CAST+PLBO groups at 2, 3, 4, and 12 hours. There were no differences between treatment groups for substance P levels. Mean IRT values tended to be higher for CAST+FLU calves (35.4ºC) compared to CAST+PLBO (34.5ºC) and SHAM+PLBO (34.3ºC) calves ($P = 0.06$). The total step force applied was similar for all treatment groups. The calves undergoing surgical castration placed more force onto their fore limbs ($P = 0.02$) indicating a shift in their weight distribution to the front limbs. There were no measured differences in total step contact area and step contact pressure. SHAM+PLBO calves has lower total impulses compared to CAST+FLU and CAST+PLBO ($P = 0.004$). Transdermal

flunixin reduced plasma cortisol concentrations and mitigated the stress response for 8 hours when given at the time of castration. Transdermal flunixin provided negligible analgesic effects on the pain biomarkers of substance P, IRT and gait analysis when given at the time of castration. Further research is needed to assessment the impact of transdermal flunixin when administered as part of a multimodal analgesic protocol that includes local anesthesia.

Key Words: Cattle, Gait analysis, Substance P, Analgesia, NSAID

INTRODUCTION

Castration is a common husbandry practice performed on cattle worldwide and has been documented to be a source of stress and pain (Stafford and Mellor, 2005; Coetzee et al., 2010). Veterinarians and farm personnel consider the use of analgesics important in mitigating pain associated with castration, but less than 20% routinely implement such practices (Coetzee et al., 2010; Fajt et al., 2011). Logistics, medication costs, and labor costs have been cited as reasons for not providing analgesic strategies when performing painful procedures like castration (Moggy et al., 2017).

Transdermal flunixin (Banamine Transdermal, Merck Inc, New Jersey) is the only drug with approval from the United States Food and Drug Administration (US FDA) for the control of pain in cattle. Specifically, transdermal flunixin is approved for the control of pain associated with foot rot (US FDA, 2017). There have been published reports demonstrating the analgesic benefit of non-steroidal anti-inflammatory drugs (NSAIDs) when administered at the time of

surgical castration (Earley and Crowe, 2002; Webster et al., 2013; Roberts et al., 2015). Flunixin meglumine is a common NSAID administered at the time of castration (Coetzee et al., 2010).

The topical formulation of flunixin for transdermal delivery has been shown to decrease PGE_2 concentrations in a tissue cage model (Thiry et al., 2017). Based on the pharmacokinetic profile of transdermal flunixin, these recognized anti-inflammatory properties, and FDA approval for pain control; this novel formulation shows potential for inclusion into an analgesic plan for castration (Kleinhenz et al., 2016).

The objective of this study was to investigate the analgesic effects of transdermal flunixin meglumine when administered at the time of surgical castration, without local anesthesia, by measuring pain associated biomarkers. We hypothesize that transdermal flunixin, when administered at the time of castration, would provide some analgesic benefit by lowering cortisol, substance P concentrations, ocular temperatures taken by infrared thermotherapy, and improving stride length.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee at Iowa State University (Log # 5-15-8016-B).

Animals and Housing

A group of 23 Holsteins calves, 9 months of age and weighting 350 +/- 50 kg, obtained from a previous vaccine study were utilized for this study. The group consisted of 16 intact males and 7 previously castrated males. Each calf was identified with a plastic ear tag in each ear. Calves had been previously halter trained and were restrained with halter and lead rope.

The study was conducted in an enclosed barn to control environmental conditions. The barn had pens along the walls with a center access alley in the middle. The barn was maintained

at 16±3°C for the duration of the study. Calves were housed in groups of 7 or 8 calves per pen for the study. Pen space per calf exceeded the requirements in the Guide of the Care and Use of Agricultural Animals in Research and Teaching. Calves were fed a diet consisting of cracked corn, dried distillers grains, a custom protein and mineral mix, and dry hay. The diet was formulated to meet or exceed nutrient requirements set forth by the National Research Council guidelines for beef cattle. Calves had access to water at all times.

Study Design

This was a prospective clinical study where the calves were divided into 3 treatment groups with the intact males being randomly assigned to either a flunixin castrated group (CAST +FLU) (n=8) or a placebo castrated group (CAST+PLBO) (n=8) using computer software (Excel, Microsoft Corp. Redmond, WA). The third group of steers served a negative control (SHAM+PLBO) group (n=7) for pain associated outcome measures. Calves in the SHAM+PLBO group were surgically castrated at 3 months of age as part of another research experiment. Numerical order of ear tags (lowest to highest) was used to determine the order in which calves underwent their castration procedure, treatment application, and subsequent data collection for the entire study. Study personnel were blinded to treatment groups for castrated calves. Pressure mat analysis was considered to be the most variable outcome assessed in this trial. For this reason, the study was designed to have statistical Power of 0.8 assuming an effect size (delta) in stride length of 8.5 cm, a standard error (sigma) of 5 and a significance level (alpha) of 0.05 as previously reported by Currah et al. (2009).

Blood Sample Collection

Twenty-four hours before study commencement, calves were restrained and an intravenous catheter was aseptically placed. For catheter placement, the hair over the jugular vein was clipped using a #40 blade and electric clippers (Oster, Boca Raton, FL). The skin was

surgically scrubbed using 4% chlorhexidine surgical scrub (First Priority Inc., Elgin, IL) and 70% isopropyl alcohol (First Priority Inc., Elgin, IL). The calf was restrained by study personnel and a 14 gauge catheter (Jorgensen Labs, Loveland, CO) was placed in the left jugular vein. The catheter was capped with an injection port (Hospra Inc., Lake Forest, IL) and sutured in place with #0 nylon suture (Ethilon, Ethicon US LLC, Somerville, NJ). Before each blood draw, the injection port was wiped with an alcohol-soaked gauze.

Castration Procedure and Drug Application

Drug application and castration occurred concurrently, but drug application was considered the start of the experiment (time 0). For drug application, calves were restrained in a stanchion and squeezed with a gate. Flunixin meglumine (Finadyne® Pour-On, MSD Animal Health, Dublin, Ireland) or placebo was applied to the skin of the top-line, starting at the shoulders and ending at the tail head, at a dose of 3.33 mg/kg using a single-use syringe. Drug dosage and administration were done following the manufacturer's written instructions accompanying the product. The placebo consisted of propylene glycol, isopropyl alcohol and a red dye to mimic the test product in color, viscosity and odor.

Calves were surgically castrated without local anesthesia, which is currently considered standard industry practice in the United States (Coetzee et al., 2010; Fajt et al., 2011). Calves were restrained and the scrotum was cleaned with water and disinfectant. The distal third of the scrotum was removed using a sterile scalpel to expose the testicles, the fascia was bluntly dissected and the testicles were removed by applying a twisting and pulling action. Calves assigned to the SHAM+PLBO group underwent a sham castration procedure, where the remnant of their scrotum was disinfected and manipulated to mimic the handling that occurs up to the cutting portion of surgical castration procedure.

Plasma Cortisol Determination

Blood samples for cortisol concentrations were collected at T0 (baseline), 30, 60, and 90 minutes as well as 2, 3, 4, 6, 8, 12, 24, 48, and 72 hours after drug application. Baseline samples were collected first thing in the morning of the experiment to account for the normal circadian rhythm of cortisol concentrations. Briefly, calves were loosely restrained with a halter a lead rope and 15 ml of blood was collected at the predetermined time points via the jugular catheter. The blood was immediately transferred to a blood tube containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ). The tubes were inverted 3 times and then placed on ice until transported to the ~~lab~~ laboratory. Once in the lab, the blood was centrifuged at 3,000g for 10 minutes. The plasma was pipetted from the tube and placed into cryovials. The plasma samples were placed on dry ice and then stored at -80°C until analysis. Samples were analyzed within 30 days of collection.

Cortisol concentrations were determined using a commercially available radioimmunoassay (MP Biomedicals, Santa Ana, CA). Samples were ~~run~~ analyzed in duplicate and repeated if large differences (inter-assay coefficient of variation > 25%) in cortisol concentrations among the duplicate samples were determined. The assay had a detection range of 0.64 to 150 ng/ml. The coefficient of variation for the intra-assay variability was 16.11% and the inter-assay variability was calculated to be 9.0%.

Plasma Substance P Determination

Substance P (SP) concentrations were determined as a baseline in the morning of the experiment and at 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours after drug application. For SP determination, 200µg benzamidine was added to EDTA blood tubes (BD Vacutainer, Franklin Lakes, NJ) 48 hours before the start of the study. To collect the sample, 6 ml of whole blood was added to the spiked EDTA tube and inverted 3 times to mix thoroughly. The samples were

immediately placed on ice and transported to the ~~lab~~ laboratory. The samples were centrifuged within 30 minutes of collection and the plasma was placed into cryovials. The cryovials were placed onto dry ice to freeze immediately and then stored at -80°C until analysis. Samples were analyzed within 60 days of collection.

Substance P concentrations were determined using radioimmunoassay methods described by Van Engen et al. (Van Engen et al., 2014) using nonextracted plasma. The coefficient of variation for the intra-assay variability was 14.38% and the inter-assay variability was calculated to be 17%.

Infrared Thermography

Infrared thermography images were obtained 24 hours before castration and 2, 4, 6, 8, 12, 24, 48, and 72 hours post-drug application using a research grade infrared camera (FLIR SC 660, FLIR Systems AB, Danderyd, Sweden). The camera was calibrated before obtaining images with the ambient temperature and relative humidity. Changes in ambient temperature and relative humidity were automatically obtained by the camera throughout the imaging session. Calves were restrained with a halter and an image of the lateral aspect of the head was obtained so that the image contained the medial canthus of the eye by directing the camera lens at the calf's head at a 45° angle and distance of 0.5 meters. Images were saved and the image file number was recorded for analysis at a later time.

Infrared images were analyzed using research grade computer software (FLIR ExaminIR, Inc., North Billerica, MA). The maximum, minimum and mean temperatures of the medial canthus of the eye were recorded and analyzed. Additionally, a temperature data logger (HOBO®, OnSet Computer Co., Cape Cod, MA) was employed to retrospectively analyze environmental temperature.

Gait Analysis

A commercially available floor mat-based pressure/force measurement system (MatScan, Tekscan, Inc., South Boston, MA) was used to record and analyze gait, step force, step pressure, and stride length of each calf. Calves were walked across the pressure mat 24 hours before castration and 2, 4, 6, 8, 12, 24, 48, and 72 hours after drug application. The pressure mat was calibrated daily and each time the computer software was engaged using a known mass to ensure accuracy of the measurements at each time point. Video synchronization was used to ensure consistent gait between and within calves for each time point. Using research-grade software, (HUGEMAT Research 5.83, Tekscan, Inc., South Boston, MA) force, contact pressure, contact area, impulse and stride length were assessed using the methods as described by Coetzee et al. (Coetzee et al., 2014). Definitions for each outcome are shown in **Table 1**.

Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Inst. Inc., Cary, NC), via the GLIMMIX procedure using repeated measures. Data for cortisol and substance P outcomes were transformed to natural logarithms for normality. The fixed effects were treatment (CAST+FLU, CAST+PLBO, SHAM+PLBO), time and treatment by time interaction. In order to test the significance of main effects and interactions, *F*-tests were used. If significant overall differences were identified, pairwise comparisons were performed using Tukey's *t* tests based on the lsmeans statement. Statistical significance was set at p-value ≤ 0.05. P-values between 0.05 and 0.10 were considered to be approaching significance and are reported as tendencies toward significance.

RESULTS AND DISCUSSION

Calves in this study were castrated using standard practices in the United States (Coetzee et al., 2010). A rescue analgesia protocol was in place, per IACUC protocol, for calves deemed to be experiencing severe pain. Severe pain was described as excessive lying, reluctance to rise, in appetence, reluctance to move, and/or excessive swelling. No calves met the criteria to require rescue analgesia.

Plasma Cortisol

Plasma cortisol concentrations are presented in **Figure 1**. There was a treatment (P = 0.003), time ($P < 0.0001$) and treatment by time interaction ($P < 0.0001$) for plasma cortisol. Plasma cortisol concentrations spiked following surgical castration and started to decline over the next hour. Cortisol concentrations in the CAST+PLBO group increased at 2 hours after castration and were significantly higher than the other treatment groups ($P < 0.0001$). Additionally, calves in the CAST+FLU group had lower mean cortisol concentrations at the 2 h time point (6.50 ng/ml vs 32.14 ng/ml; $P < 0.0001$), suggesting the absorbed flunixin was effective at controlling the spike in cortisol level associated with castration pain. This correlates with the pharmacokinetics of the transdermal flunixin, which achieved a maximum plasma concentration (Cmax) around 2 hours (Kleinhenz et al., 2016). There were significant differences in cortisol concentrations between the CAST+FLU and CAST+PLBO groups at 3 hours (4.79 ng/ml vs. 24.35 ng/ml; $P = 0.0025$) and 4 hours (6.75 ng/ml vs. 20.30 ng/ml; P = 0.0012) after drug administration that can be explained by the flunixin treatment.

Cortisol area under the effect curve (AUEC) data was presented in **Table 2**. There tended to be a treatment difference for cortisol $AUEC_{0\text{-}72}$ ($P = 0.097$). When the AUEC is further broken into distinct time periods, the CAST+PLBO group had higher $AUCE_{0\text{-}8}$ compared to the

CAST+FLU and SHAM+PLBO groups ($P < 0.0001$). The difference in $AUEC_{0-8}$ between the CAST groups suggests that the flunixin treatment helped in reducing the cortisol effects associated with castration pain. Despite the CAST+FLU group having a numerically lower $AUEC_{8-72}$, there were no statistically significant differences between the CAST+PLBO and SHAM+PLBO treatment groups.

The cortisol results of the present study are similar to other studies investigating NSAID effects on surgical castration. Webster et al. (2013) used flunixin in Holstein calves before castration and reported a similar spike in cortisol concentrations followed by a decrease in concentrations for the remainder of the study. This study utilized flunixin at 1.1 mg/kg administered intravenously. These authors concluded that flunixin provided analgesia following surgical castration. Calves given ketoprofen following surgical castration also had a similar plasma-cortisol-over-time profile as the current study, where they had a cortisol spike after castration followed by lower concentrations (Early and Crowe, 2002). Furthermore, calves given ketoprofen, either on its own or with local anesthetic using lidocaine, had lower cortisol AUC concentrations compared to calves given lidocaine local anesthetic only or no analgesia (Early and Crowe, 2002). Due to the number of animals available for this study, including a local anesthetic group was not possible in order to achieve the appropriate statistical rigor. Future research should investigate the benefits of adding a local anesthetic to the pain management protocol at the time of castration. Not using a local anesthetic at the time of castration is consistent with current industry practice (Coetzee et al., 2010).

A study using meloxicam in surgically castrated calves reported a similar secondary cortisol spike in untreated castrated animals (Roberts et al., 2015). The authors of the aforementioned attributed this second spike to pain associated with moving the calves into chutes

for data collection. Like the present study, meloxicam-treated calves did not have a second spike in cortisol indicating a potential analgesic effect.

Substance P

There was no effect of flunixin on SP concentrations following castration ($P = 0.67$). Furthermore, there was no time effect ($P = 0.82$) or treatment by time interaction ($P = 0.79$) between groups. The lack of effect of flunixin on SP concentrations was reported by Mintline et al.(2014), where flunixin was given intravenously to calves at the time of castration. In that study, an effect of day was seen where SP concentrations dropped following castration in both treated and untreated calves, but there were no differences between treatment groups. In calves undergoing band castration or a sham procedure, there were no differences in SP concentrations at 24 hours post-castration compared to baseline samples taken 24 hours prior to castration (Repenning et al., 2013). In that same study, calves given meloxicam had similar SP concentrations as untreated castrated calves and SHAM controls.

Infrared Thermography

The maximum, mean, and minimum ocular temperatures in the CAST+FLU group were higher than the CAST+PLBO and SHAM+PLBO groups after castration and drug application (**Table ~~2~~ 3**). There tended to be a treatment effect for the maximum IRT ($P = 0.05$), but no treatment by time interaction ($P = 0.38$). For the mean ocular temperature, there was a treatment effect ($P = 0.007$) and tended to be a treatment by time interaction ($P = 0.06$). There was a significant treatment effect and treatment by time interaction for the minimum ocular temperature ($P = 0.004$ and $P = 0.01$ respectively).

Environmental conditions have been documented to influence IRT measurements (Church et al., 2014). The current study was performed in an enclosed barn with environmental controls to minimize the influence of environment as well as for the logistics of the pressure mat

equipment. The barn was maintained at 16±3°C for the duration of the study and was monitored via digital thermometers used to calibrate the IRT camera.

The outcomes for IRT were expected as the SHAM+PLBO group had the lowest temperatures. Due to activation of the sympathetic nervous system and subsequent peripheral vasodilation of the eye, we expected the SHAM+PLBO group to have the lowest IRT temperatures. The decrease in ocular temperature in the CAST+PLBO and lack of change in the CAST+FLU group are opposite those seen by Stewart et al. (Stewart et al., 2010). In that study, calves undergoing surgical castration with and without local anesthetics had an increase in the maximum ocular temperature recorded via IRT. However, IRT data were only collected over a 45 minute period with the last reading occurring at 20 minutes after castration. In the present study, the first IRT reading was taken 2 hours post-procedure and treatment application. Further investigation into ocular thermography before and after castration is warranted to evaluate a potential physiological rebound effects that could take place following acute pain and subsequent analgesia.

Gait Analysis

Outcome variables for the gait analysis are shown in **Table 4**. The total force applied was similar for all treatment groups. However, when the amount of force was partitioned onto the front or rear limbs, the calves undergoing castration placed more force onto their fore limbs ($P = 0.02$). This result indicated castrated cattle shifted weight forward onto the front limbs after castration. This was also seen in the contact pressure applied to the pressure mat. Total contact area and contact area for front or rear feet were not different among groups. The shift of weight forward suggests the castrated cattle were attempting to modify their gait in response to castration pain. This shows step force analysis has potential as an outcome test for surgical pain just as it has been shown for lameness pain (Kotschwar et al., 2009; Schulz et al., 2011)

The SHAM+PLBO calves had a lower total impulse compared to the CAST+FLU and CAST+PLBO groups ($P = 0.004$). Impulse is a function of force (kg) and time (s). Since there were no differences in total force among the groups, the differences seen are likely due to the speed and step duration of which the SHAM+PLBO calves crossed the pressure mat. Thus the contact time for each step was lower for SHAM+PLBO calves for each step. This indicates the SHAM+PLBO calves moved across the pressure mat at a higher velocity than the two castration groups; suggesting they were less painful than the castration groups.

There was no difference in stride length between treatment groups ($P = 0.65$) or effect of time on stride length ($P = 0.78$). All treatment groups had similar stride length both before and after the castration procedures. The use of a pressure mat to analyze steps after castration has not been described in the literature. A pitfall of the equipment is the fixed length of the pressure mat itself. The calves in the present study may have been too large since some calves did not have complete strides recorded. A study using video recording to evaluate stride length following surgical castration has been published (Currah et al., 2009). In that study, the authors found castrated calves had shorter stride lengths post-castration compared to pre-castration baselines. Additionally, calves receiving flunixin as part of their treatment regimen had decreased stride lengths post-castration, but these calves had significantly longer stride lengths at 4 and 8 hours post-castration compared to non-flunixin calves (Currah et al., 2009).

Results of the present study should be interpreted in the context of several potential trial limitations. Firstly, the study duration of 72 h did not fully encompass the full healing time frame of the castrated calves. However, based on the known pharmacokinetics of transdermal flunixin, 72 hours was beyond the time when flunixin could be detected in the plasma and it was therefore hypothesized that the impact of the drug on biomarkers of pain would not be anticipated beyond

72 h (Kleinhenz et al., 2016). Secondly, due to the size of the calves in the experiment, the length of the pressure mat did not allow for each calf to have two foot-falls per time-point to fully evaluate stride length. Additional acclimation to the pressure mat is warranted along with the use of a longer mat for this category of animal. In spite of this, the use of the pressure mat has been shown to be a useful tool in validating pain assessment; as it was used to gain FDA approval of transdermal flunixin the control of pain associated with foot rot in cattle (US FDA, 2017).

In conclusion, transdermal flunixin mitigated plasma cortisol concentrations and the stress response in calves when administered at the time of surgical castration without local anesthesia. However, transdermal flunixin alone did not have a significant impact on substance P, IRT and gait analysis when given at the time of castration in older calves. From the results of the study, we can conclude that castration is a painful event based on cortisol response and the shift of weight from the hind limbs to front limbs using the pressure mat system. Further research is needed to investigate the analgesic properties of transdermal flunixin meglumine using additional measurements that can be appropriately validated for pain assessment. Furthermore, additional work is needed to investigate the impact of transdermal flunixin meglumine combined with local anesthesia as part of a multimodal analgesic protocol following surgical castration in calves.

Figure 7-1 Mean cortisol concentrations (±SEM) over 8 (Top) and 72 h (bottom) for calves castrated given transdermal flunixin (CAST+FLU; n =8), castrated and given placebo (CAST+PLBO; n = 8) and calves sham castrated and given placebo (SHAM+PLBO; n = 7).

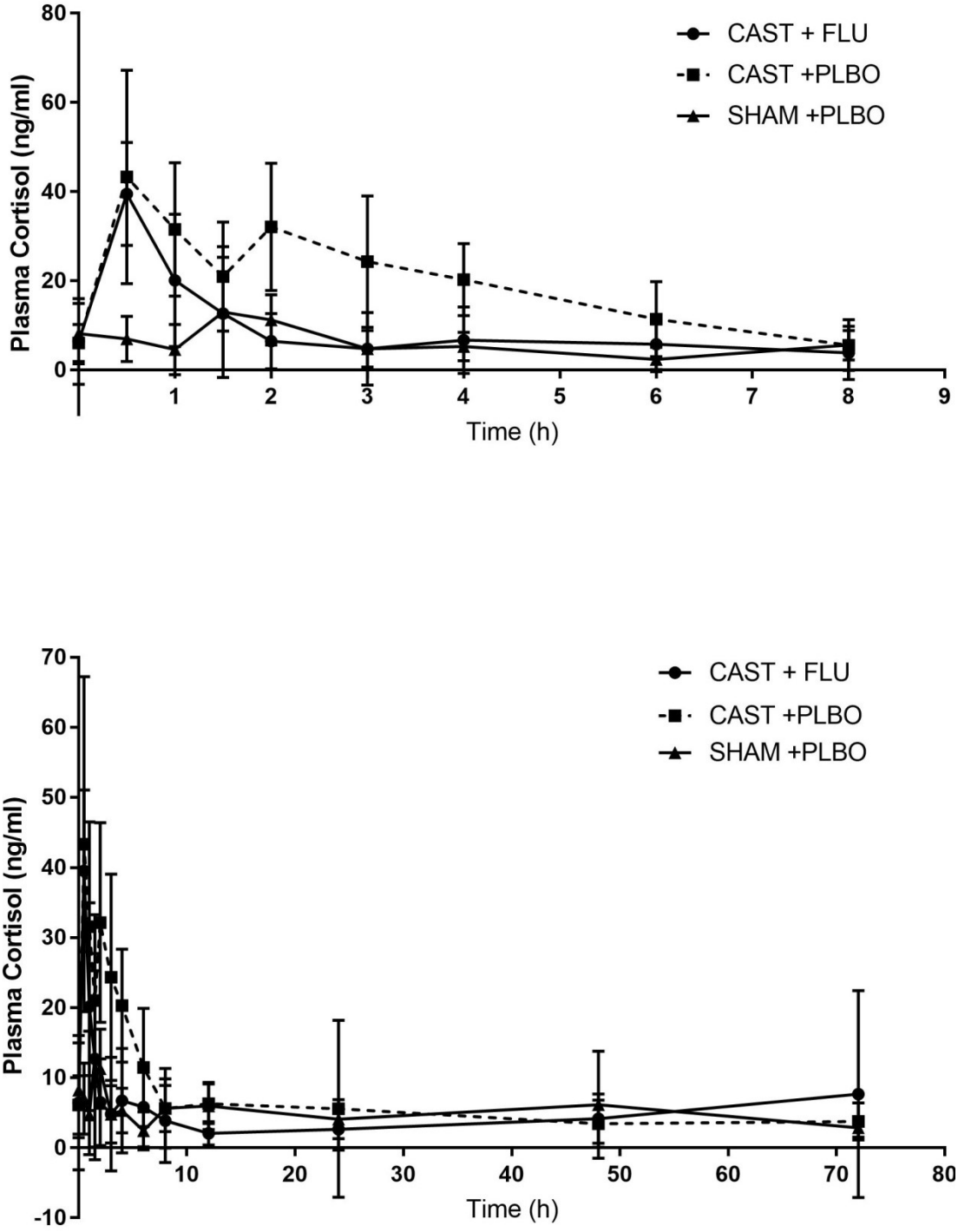

Table 7-1 Definitions of outcome variables for pressure mat analysis

Term	Unit of measure	Definition
Force	kg	Maximum force applied for each step
Contact pressure	kg/cm^2	Maximum peak pressure applied for each step
Contact Area	cm^2	Total area of each step
Impulse	kg x s	Maximum force per unit of time
Stride length	cm	Distance measured parallel to line of progression between the posterior of the heel for consecutive foot falls.

Table 7-2 Mean and 95% confidence interval for area under the effect curve (AUEC) and maximum concentration for cortisol concentrations for calves receiving transdermal flunixin at castration (CAST+FLU; n = 8), a placebo at castration (CAST+PLBO; n = 8) or a placebo and a sham castration (SHAM+PLBO; n = 7).

Cortisol, ng x h/ml	CAST + FLU	CAST + PLBO	SHAM + PLBO	*P* – value
	Mean (95% confidence interval)			
$AUEC_{0-72}$	195[a] (88 to 303)	360[a] (253 to 468)	253[a] (138 to 368)	0.098
$AUEC_{0-8}$	73[b] (45 to 102)	157[a] (128 to 186)	46[b] (16 to 77)	<0.0001
$AUEC_{8-72}$	122[a] (13 to 231)	202[a] (94 to 312)	207[a] (90 to 324)	0.46
Cmax, ng/ml	39.5[a] (27.8 to 51.3)	43.3[a] (31.6 to 55.1)	11.3[b] (3.7 to 18.9)	0.0005

[a,b] Different superscript letters within a row, means differ ($P \leq 0.05$).

[1] $AUEC_{0-72}$ = area under the effect curve from time 0 to last sample collected; $AUEC_{0-8}$ = area under the effect curve from time 0 to 8 h; $AUEC_{8-72}$ = area under the effect curve from time 8 to 72 h; Cmax = maximum plasma concentration

Table 7-3 Summary of the Least Square Means (±SEM) for the Maximum, Mean and minimum ocular temperature (°C) of the medial canthus using Infrared Thermography for calves receiving transdermal flunixin at castration (CAST+FLU; n = 8), a placebo at castration (CAST+PLBO; n = 8) or a placebo and a sham castration (SHAM+PLBO; n = 7)

	LS means (± SE)			*P*-value		
Parameter	CAST + FLU	CAST + PLBO	SHAM + PLBO	Treatment	Time	Treatment x time
Maximum IRT, °C	38.18 (0.16)	37.89 (0.16)	37.53 (0.17)	0.05	< 0.0001	0.38
Mean IRT, °C	35.42 (0.24)	34.54 (0.29)	34.29 (0.25)	0.007	< 0.0001	0.06
Minimum IRT, °C	29.41 (0.50)	28.95 (0.50)	26.71 (0.53)	0.004	< 0.0001	0.01

Table 7-4 Gait analysis and stride length outcome measures, as least square means ± SEM, for calves castrated given transdermal flunixin (CAST+FLU; n =8), castrated and given placebo (CAST+PLBO; n = 8) and calves sham castrated and given placebo (SHAM+PLBO; n = 7).

Parameter	LS means (± SEM)			*P*-values		
	CAST + FLU	CAST + PLBO	SHAM + PLBO	Treatment	Time	Treatment x time
Total force, kg force	245.1 (9.3)	251.1 (9.3)	252.8 (10.0)	0.82	0.71	0.71
Force (front limbs), kg force	265.9 (11.1)	272.1 (11.1)	257.9 (11.9)	0.69	0.78	0.27
Force (rear limbs), kg force	242.7 (9.0)	232.0 (9.1)	247.7 (9.7)	0.23	0.68	0.95
Force Diff. Font -Rear. kg force	-23.2 (7.6)	-40.1 (7.6)	-10.2 (8.2)	0.02	0.49	0.30
Total contact area, cm^2	79.4 (2.5)	79.1 (2.5)	81.6 (2.7)	0.77	0.0007	0.42
Contact area (front limbs), cm^2	81.4 (2.7)	80.1 (2.7)	83.2 (2.9)	0.75	0.03	0.71
Contact area (rear limbs), cm^2	77.4 (2.5)	78.0 (2.5)	79.9 (2.7)	0.78	0.17	0.76
Contact area Diff. Front-Rear, cm^2	-4.0 (1.6)	-2.1 (1.6)	-3.3 (1.7)	0.61	0.90	0.76
Total contact pressure, kg/ cm^2	153.0 (5.2)	160.1 (5.2)	156.7 (5.5)	0.63	0.4	0.81
Contact pressure (front limbs), kg/ cm^2	163.7 (5.9)	170.5 (6.0)	157.6 (6.4)	0.36	0.73	0.51
Contact pressure (rear limbs), kg/ cm^2	142.7 (5.5)	150.0 (5.6)	156.1 (6.0)	0.27	0.52	0.84
Contact pressure Diff. Front-Rear, kg/cm^2	-21.0 (5.3)	-20.6 (5.3)	-1.5 (5.7)	0.04	0.79	0.32
Total impulse, kg x s	133.6 (12.4)	140.5 (12.4)	109.1 (13.2)	0.22	0.08	0.004
Impulse (front limbs), kg x s	137.6 (11.7)	149.8 (11.7)	109.7 (12.5)	0.08	0.07	0.007

Impulse (rear limbs), kg x s	129.3 (14.7)	131.8 (14.7)	108.7 (15.7)	0.52	0.68	0.54
Impulse Diff. Front-Rear, kg x s	-8.3 (9.2)	-18.0 (9.3)	1.0 (9.9)	0.43	0.72	0.83
Stride length, cm	127.2 (1.7)	123.9 (1.4)	126.0 (1.7)	0.49	0.78	0.65

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Chapter 8 - Effects of transdermal flunixin meglumine on experimentally induced lameness in adult dairy cattle.

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ABSTRACT

Lameness is a common animal health condition with significant production and welfare implications. The transdermal formulation of flunixin meglumine is the only approved drug for pain control in cattle in the United States. Thirty (30) adult dairy cows were enrolled in a study to determine the effect of transdermal flunixin on cattle with induced lameness. Cows were allocated to one of three treatment groups with 10 cows per group: lameness and flunixin (**LAME + FLU**); lameness and placebo (**LAME + PLBO**); or sham induction and placebo (**SHAM + PLBO**). An arthritis-synovitis was induced in the distal interphalangeal joint of the left hind lateral digit using 20 mg of amphotericin B 6 h prior to the application of treatment. Cows enrolled into the sham induction group had 4 mL of isotonic saline injected into the joint. Cows were dosed with transdermal flunixin at 3.33 mg/kg (1 mL/15 kg) every 24 h for 3 days or a placebo at 1 mL/15 kg every 24 h for 3 days. The first treatment of flunixin or placebo was

considered the start of the study identified as time 0 h. Data was collected from all cows for 120 h following the initial treatment application. Outcome measures obtained included plasma cortisol; substance P (SP); visual lameness assessment; mechanical nociception threshold (**MNT**) presented as difference between left and right feet; infrared thermography (**IRT**) presented as difference between left and right feet; and gait analysis using a pressure mat. Cortisol concentrations were lower for the LAME + FLU group starting at 1.5 h after drug administration. Substance P levels were not different among treatment groups. Differences between the left hind MNT and right hind MNT were detected with the SHAM + PLBO having the lowest difference at -0.04 kgf (95% CI: -1.86 – 1.78 kgf), and the LAME + PLBO having the highest at -2.96 kgf (95% CI: 1.55 – 4.36 kgf). The LAME + FLU group was intermediate at -2.08 kgf (95% CI: 0.89 – 3.27 kgf). Similarly, when the difference between the maximum temperatures of the coronary band via IRT were examined; the LAME + PLBO group had the highest difference at 1.64 °C (95% CI: 1.02 – 2.26 °C) with the LAME + FLU and SHAM + PLBO groups measuring 0.57 °C (95% CI: 0.06 – 1.08 °C) and 0.53 °C (95% CI: -0.2 – 1.25 °C) respectively. There was an absence of evidence for treatment groups when analyzing force, contact pressure, step impulse, and stride length. Based on differences in MNT, IRT, and cortisol, transdermal flunixin is an effective analgesic agent for induced lameness. Multiple doses of transdermal flunixin may be required to be clinically effective based on MNT and IRT data. Further investigation of transdermal flunixin and its analgesic effects are warranted in naturally occurring lameness.

Key Words: Dairy cattle, non-steroidal anti-inflammatory drug, pour-on, lameness

INTRODUCTION

Lameness has a direct impact on the welfare of cows (Whay and Shearer, 2017). This has resulted in lameness being included as a component of third-party animal care audits (Coetzee et al., 2017). The prevalence of lameness on dairy farms in the US varies from 6.9% to as high as 54.8% (von Keyserlingk et al., 2012, Adams et al., 2017). While regarded as a painful condition, only 53.1% of veterinarians administer analgesics to acutely lame dairy cattle; with nonsteroidal anti-inflammatory drugs (**NSAID**) being the preferred drug for analgesia (Fajt et al., 2011).

Currently, flunixin meglumine is the only NSAID approved for use in cattle in the USA. It has been shown to be effective in treating pain associated with induced and natural lameness (Schulz et al., 2011, Wagner et al., 2017). Furthermore, flunixin meglumine, as a transdermal formulation, is the first NSAID to be approved by the US Food and Drug Administration (**FDA**) specifically for the control of pain in cattle (US, 2017). This approval is limited to the pain associated with foot rot in beef and non-lactating cattle. Despite FDA approval, this formulation of flunixin would be considered extra-label drug use (**ELDU**) in a mature dairy cow.

Amphotericin B induced arthritis and synovitis has been shown to be a reliable method to produce transient lameness in cattle (Kotschwar et al., 2009, Schulz et al., 2011, Coetzee et al., 2014). It has been utilized in cattle, horses and swine as a lameness model to evaluate analgesic drugs and lameness detection methods (McIlwraith et al., 1979, Kotschwar et al., 2009, Pairis-Garcia et al., 2014). In cattle, the amphotericin B lameness induction model has been used to test a variety of pharmaceuticals including meloxicam, meloxicam combined with gabapentin, sodium salicylic acid, and flunixin (Kotschwar et al., 2009, Schulz et al., 2011, Coetzee et al., 2014). Schulz et al. (2011) tested flunixin meglumine at a dose of 2.2 mg/kg via intravenous injection for the amelioration of lameness. In that study, flunixin was shown to improve

lameness based on visual lameness scores and pressure gait analysis. In a similar study in swine, the authors were able to show an improvement of lameness scores in sows given flunixin meglumine (Pairis-Garcia et al., 2014).

The objective of this research was to examine the analgesic efficacy of transdermal flunixin meglumine in adult dairy cows with experimentally induced lameness. Our hypothesis was that multiple treatments of transdermal flunixin would be required to provide sufficient analgesia to lame dairy cows as evident by lower lameness scores and higher mechanical nociception thresholds.

MATERIALS AND METHODS

This project was reviewed and approved by the Institutional Animal Care and Use Committee at Iowa State University (Protocol #7-16-8314-B).

Study animals

Thirty (30) adult Holsteins at 60-90 days in milk, and in their 2nd or 3rd lactation, were enrolled in a study to determine the effect of transdermal flunixin on animals with induced lameness. Cows were eligible for enrollment if they had no record of lameness in the current lactation and were free of clinical signs of lameness (VLS = 0). Cows were also visually examined for clinical signs of foot rot and digital dermatitis at the time of lameness induction. Cows were randomly allocated to one of three treatment groups using the RAND function in a spreadsheet (Excel, Microsoft Co., Redmond, WA) based on the cow's identification number.

- **LAME + FLU**: lameness induction + transdermal flunixin
- **LAME + PLBO**: lameness induction + placebo

- **SHAM + PLBO**: sham lameness induction + placebo

Cows in the LAME + FLU group were dosed with transdermal flunixin at 3.33 mg/kg (1 mL/15 kg; Finadyne Transdermal; MDS Animal Health, Dublin, Ireland) every 24 h for 3 doses as a topical pour-on following label directions. Cows in the LAME + PLBO and SHAM + PLBO groups were given a placebo at the dose of 1mL/15 kg. The placebo was composed of propylene glycol and red-dye to mimic the test product. The first dose was considered time 0 for the study and it was administered 6 hours after lameness induction. For the second and third doses (24 and 48 h), the treatment application occurred after the outcome parameters were obtained.

Animal housing

Cows were group housed in the same pen within the barn with freestalls for resting with one freestall per cow. Only study cows were housed in the pen. Freestalls were bedded with fresh recycled manure solids which was standard practice for the dairy farm. Cows were milked three times per day at 4 a.m., 12 p.m. and 8 p.m. by study personnel. Housing met the requirements of the guide for the care and use of agricultural animals in research and teaching (FASS, 2010). A TMR was provided once a day in the morning, with feed push-ups occurring every 6 h after feeding. The ration was formulated to meet or exceed the National Research Council requirements for lactating dairy cattle. The TMR was provided at a feedbunk with self-locking headlocks, and the number of headlocks exceeded the number of cows in the pen. Cows had access to water at all times, except when away from the pen for milking and outcome parameter collection.

Lameness induction

Lameness was induced using methods described by Kotschwar et al. (2009). Briefly, the cow was restrained in a custom chute designed for hoof trimming. Using the hydrolytic tilt function of the chute, the cow was placed into left lateral recumbency, and the feet were restrained. The chute was used for lameness induction only, including sham injections. Lameness was induced via chemical synovitis and arthritis by injecting 20 mg (4 mL) of amphotericin B (X-Gen Pharmaceuticals Inc., Big Flats, NY) into the left hind lateral distal interphalangeal joint. Cows in the SHAM + PLBO group had 4 mL of sterile saline injected into the left hind lateral distal interphalangeal joint. For induction, the skin of the lateral aspect of the lateral claw just proximal to the coronary band was clipped and aseptically prepared using alternating chlorhexidine scrub and 70% alcohol. The distal interphalangeal joint was identified and a needle was placed in the joint for amphotericin B or placebo injection. Correct placement of the needle into the joint was confirmed by anatomical landmarks, obtaining joint fluid prior to injection and ease of injection. All lameness inductions were performed by the same veterinarian (JAS) to avoid inter-operator variation.

Lameness Scoring

Lameness scores were measured using two methods to document presence of lameness and to visually score severity of lameness. First, lameness was scored using a 0 to 4 scale adapted from Sprecher et al. (1997) (Table 1). Secondly, a visual analog score (VAS) was adapted from Flower and Weary (2006) whereby the degree of lameness was placed along a 100 mm line ranging from not lame (0 mm) to severely lame (100 mm). Lameness scores were taken prior to lameness induction (-6 h), just prior to the first application of treatment, and at 8, 16, 24, 48, 72, 96, and 120 h after initial drug administration. All lameness scores were assigned by a

investigator blinded to treatments trained in both systems prior to commencing the study. All lameness examinations were performed on dry even, non-sloped concrete floors free of obstructions and debris. To determine the lameness score the observer watched the cow walk a minimum of 20 m in a straight line, turn, and walk 20 m back to the starting point. Cows were allowed to walk naturally with an investigator at the turn-around point..

Cortisol

Blood samples for plasma cortisol concentration were collected prior to lameness induction (-6 h) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 16, 24, 32,40, 48, 56, 64, 72, 80, 96, and 120 h after the first treatment administration. Cows were restrained in headlocks for blood collection at 0.5 h to 6 h, but only continually restrained for 0.5h to 3 h. The remaining blood collections occurred in treatment chutes which consisted of a self-locking headgate and side gates. Cows had their head restrained by rope halter for sample collection. Baseline samples were collected prior to lameness induction the morning of the experiment. Briefly, up to 20 mL of blood was collected at the predetermined time points via a jugular venipuncture using a 20 mL syringe (Monoject, Mansfield, MA) and 16 gauge 3.8 cm needle (Monoject, Mansfield, MA). The blood was immediately transferred to a blood tube containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ). The tubes were inverted 3 times and then placed on ice until transported to the lab. Once in the lab, the blood was centrifuged at 3,000 g for 10 min. The plasma was pipetted from the tube and placed into cryovials. The plasma samples were placed on dry ice and then stored at -80°C until analysis.

Cortisol concentrations were determined using a commercially available radioimmunoassay (MP Biomedicals, Santa Ana, CA). Samples were run in duplicate and repeated if large differences in cortisol concentrations among the duplicate samples were

determined. The assay had a detection range of 0.64 to 150 ng/mL. The coefficient of variation for the intra-assay variability was 16.7% and the inter-assay variability was calculated to be 16.0%.

Substance P

Blood samples for substance P determination were collected prior to lameness induction (-6 h) and at 1, 2, 8, 24, 48, 72, 96, and 120 h after induction. At each collection, an additional 6 mL of whole blood was obtained from the jugular venipuncture for cortisol determination. The sample was placed into a 6 mL blood tube (BD Vacutainer, Franklin Lakes, NJ) containing EDTA and 200µg benzamidine. Samples were placed on ice until centrifugation within 1 h. Samples were centrifuged at 3500 g for 10 min. Plasma was pipetted off and placed into pre-labeled cryovials. Once in cryovials, samples were frozen at a temperature of -80°C until analyzed.

Substance P concentrations were determined using an in-house radioimmunoassay as described by Van Engen et al. (2014). Each sample was run in duplicate and the average reported. The coefficient of variation for the intra-assay variability was 14.4% and the inter-assay variability was calculated to be 18%.

Mechanical Nociception Threshold (MNT)

The MNT was determined using a handheld algometer (FPX 100, Wagner Instruments, Greenwich, CT) prior to lameness induction (-6 h) and at 8, 16, 24, 48, 72, 96,and 120 h after initial treatment administration. Cows were restrained in a treatment chute and allowed to stand for all MNT measurements. The MNT was measured using the lateral digit at the level of the coronary band at a point half way between midline and the heel bulb by applying slow steady

pressure until the cow responded. MNT measures were taken on both the left (lame) and right (non-lame) hind feet. The average of three readings for each foot at each time point was used for analysis. The difference between left and right hind feet MNT measures (left hind minus right hind) were determined for each time point. These differences were used for statistical analysis. The investigator determining the MNT was blinded to treatment and the reading of the algometer to prevent testing bias. A second investigator recorded algometer readings to prevent testing bias.

Infrared Thermography (IRT)

Thermographic images of the dorso-lateral aspect of the rear feet were taken prior to lameness induction (-6 h) and at 8, 16, 24, 48, 72, 96, and 120 h following initial treatment administration using a research grade infrared camera (FLIR SC 660, FLIR Systems AB, Danderyd, Sweden). Cows were restrained in the chute used for MNT measurement with full access to the feet. Images were obtained at a 45° angle and 1 m from the coronary band. Infrared images were analyzed using research specific computer software (FLIR ExaminIR, Inc., North Billerica, MA) to determine maximum and minimum temperatures. The results of three images at each time-point were averaged for analysis; and the difference between the temperatures of the left and right hind feet (left hind minus right hind) were determined for each time point. These differences were used for statistical analysis.

Pressure Mat Analysis

A commercially available pressure/force measurement system (MatScan, Tekscan, Inc., South Boston, MA) was used to record and analyze gait and biomechanical parameters of the affected foot of each cow. The sensing area of the mat was 244 cm in length and 45 cm wide. The Data was collected prior to lameness induction (-6 h) and at 8, 16, 24, 48, 72, 96, and 120 h after

initial treatment administration. The pressure mat was calibrated daily and prior to each time the computer software was engaged using a known mass to ensure accuracy of the measurements at each time point. Video synchronization was used to ensure consistent gait between and within cows for each time point. Cows were allowed to move across the mat at their own pace. Using research specific software (HUGEMAT Research 5.83, Tekscan, Inc., South Boston, MA), force, contact pressure, and impulse in the affected feet and non-affected feet was assessed using the methods as described by Coetzee et al. (2014).

Statistical Analysis

Contact force was used to determine the number of cows needed per treatment group. The study was designed to have a statistical power of 0.80 assuming an effect size in differences of 122 kg force, as standard error of 36.8 and statistical Power of 0.80 as reported (Coetzee et al. 2014). A sample size of 10 animals per treatment group was determined.

Plasma cortisol and substance P values were log transformed for normality prior to statistical analysis. Responses were analyzed using mixed linear models with the cow as the experimental unit using AR-1 as the covariance structure. Cows nested in a treatment group were designated as a random effect, with treatment, time, treatment by time interaction, and visual lameness score (VLS) at initial treatment designated as fixed effects. Responses measured include plasma cortisol, substance P, MNT differences, IRT data, visual analog scores (VAS), and gait analysis. Pair-wise comparisons were done using Tukey HDS test. Visual lameness scores were treated as categorical data and Fisher's exact test was used to compare scores. All statistics were performed using statistical software (JMP Pro 13.0, SAS Institute, Cary, NC). Statistical significance was set *a priori* at $P \leq 0.05$. Data are presented as least square means.

RESULTS

Lameness Scoring

The LAME + FLU and LAME + PLBO groups had a higher proportion of observations (0.37 and 0.44 respectively) with a VLS of 1 or greater compared to the SHAM + PLBO group (0.08) ($P < 0.0001$) over the entire study period. At the time of the first flunixin treatment (time 0), all cows in the LAME+ FLU group had a VLS of 2 or above. In contrast, 0.8 of the LAME + PLBO and 0.1 of the SHAM + PLBO groups had VLS of 1 or above at the time if initial treatment (time 0) ($P = 0.007$). At 16 h post initial treatment, there was a higher proportion of lameness (VLS > 1) in the LAME + FLU group (0.7) and LAME + PLBO group (0.6) compared to the SHAM +PLBO group (0.1) ($P = 0.04$). At 48 hours post initial treatment and prior to the final 3rd treatment, 0.7 of the LAME + FLU cows were non-lame (VLS = 0) compared to 0.4 in the LAME + PLBO and 0.9 in SHAM + PLBO group ($P = 0.08$). At 72 hours post initial treatment, all of the LAME + FLU cows were non-lame (VLS = 0) compared to 0.6 in the LAME + PLBO and all of in SHAM + PLBO group ($P = 0.09$).

At the initial treatment post-lameness induction, the LAME + FLU group had higher mean VAS measures [6.7 cm (95% CI: 6.1 – 7.3 cm)] compared to the LAME +PLBO [2.4 cm (95% CI: 1.9 – 3.0 cm)] and SHAM + PLBO [0.2 cm (95% CI: -0.4 – 0.8 cm)] groups ($P < 0.0001$; Table 2; Fig 1). At 8 h post-initial treatment, the mean VAS decreased for the LAME +FLU and LAME + PLBO groups to 2.1 cm (95% CI: 1.6 – 2.7 cm) and 1.9 cm (95% CI: 1.4 – 2.4 cm) where the SHAM + PLBO group increased to 0.6 cm (95% CI: 0.1 – 1.1 cm) ($P < 0.001$).

Cortisol

Mean cortisol concentrations were lower for the LAME + FLU (5.77 ng/mL; 95% CI: 1.88 – 9.66 ng/mL) group compared to the LAME + PLBO (13.68 ng/mL; 95% CI: 9.10 – 18.27 ng/mL) and SHAM + PLBO (14.65 ng/mL; 95% CI: 8.70 – 20.59 ng/mL) groups ($P = 0.0019$; Table 2). There was no evidence of treatment differences prior to lameness induction [LAME + FLU 8.16 ng/mL (95% CI: 1.87 – 14.44 ng/mL) LAME + PLBO 14.52 ng/mL (95% CI: 7.80 – 21.24 ng/mL) SHAM + PLBO 18.28 ng/mL (95% CI: 10.60 – 25.96 ng/mL)] ($P = 0.13$).

Starting at 1.5 h after initial treatment application, the LAME + FLU group maintained lower cortisol concentrations compared to the LAME + PLBO and SHAM + PLBO groups (Fig 2). At 8 h, man cortisol levels were 4.45 ng/mL (95% CI: -1.84 – 10.73 ng/mL) in the LAME + FLU group compared to 16.35 ng/mL (95% CI: 9.63-23.07 ng/mL) and 17.82 ng/mL (95% CI: 10.13 – 25.50 ng/mL) in the LAME + PLBO and SHAM +PLBO groups respectively ($P < 0.0001$). The LAME + FLU had lower cortisol levels at 16 h compared to the LAME + PLBO and SHAM +PLBO groups [4.07 ng/mL (95% CI: -2.22 – 10.35 ng/mL); 18.84 ng/mL (95% CI: 12.12 – 25.56 ng/mL); 26.42 ng/mL (95% CI: 18.74 – 34.10); $P = 0.007$]. Cortisol levels in the LAME + FLU cows were lower than the LAME + PLBO cows at 24 hours [4.12 ng/mL (95% CI: -2.17 – 10.41 ng/mL) *vs*. 17.58 ng/mL (95% CI: 10.87 – 24.30 ng/mL); $P = 0.0052$].

Substance P

No evidence of treatment differences were appreciated. (Table 2) The LAME + PLBO group had mean SP concentrations (84.59 pg/mL; 95% CI: 73.12 – 96.05 pg/mL) similar to the LAME + FLU (81.89 pg/mL; 95% CI: 72.16 – 91.62 pg/mL) and SHAM + PLBO groups (70.59 pg/mL; 95% CI: 55.72 – 85.46 pg/mL) ($P = 0.15$).

Mechanical Nociception Threshold

For the left hind foot, there was evidence of treatment differences between groups. The overall mean MNT for the LH was 8.46 kgf (95% CI: 6.93 – 9.99 kgf), 7.98 (95% CI: 6.17 – 9.79 kgf), and 12.34 kgf (95% CI: 10.00 – 14.69 kgf) for the LAME + FLU, LAME + PLBO, and SHAM +PLBO respectively ($P = 0.0026$). There was a time effect observed in MNT measures with the LAME + FLU [6.29 kgf (95% CI: 4.40 – 8.19 kgf)] and LAME + PLBO [8.52 kgf (95% CI: 6.43 – 10.63 kgf)] decreasing at 8 hours initial treatment. At 48 h post-initial treatment the LAME + FLU MNT measure in the LH increased to 9.31 kgf (95% CI: 7.41 – 11.20 kgf) where the LAME + PLBO group was 6.57 kgf (95% CI: 4.45 – 8.69 kgf) and the SHAM + PLBO group was 13.41 kgf (95% CI: 10.83 – 16.00 kgf) ($P < 0.0001$).

There were no evidence of treatment differences in the right hind MNT measures. The overall mean MNT for the RH was 10.54 kgf (95% CI: 9.33 – 11.75 kgf), 10.93 kgf (95% CI: 9.51 – 12.37 kgf), and 12.30 kgf (95% CI: 10.45 – 14.16 kgf) for the LAME + FLU, LAME + PLBO, and SHAM +PLBO respectively ($P = 0.23$).

When the differences between the left hind (lame) and right hind (control) feet were compared, the LAME + PLBO group had a larger mean differences in MNT -2.96 kgf (95% CI: -4.36 – -1.55 kgf) after initial treatment compared to the LAME + FLU [-2.08 (95% CI: -3.27 – 0.89 kgf)] and SHAM + PLBO [0.04 kgf (95% CI: -1.78 – 1.86 kgf)] groups ($P = 0.007$). Specifically, there was evidence of treatment differences with LAME + PLBO group having larger MNT differences at 48 and 72 h compared to the SHAM + PLBO groups (Fig 2) The mean MNT difference for the LAME + PLBO at 48 h was -4.53 kgf (95% CI: -2.53 - -6.53 kgf) compared to 0.77 kgf (95% CI: -1.52 – 3.08 kgf) in the SHAM + PLBO group ($P = 0.025$).

Infrared Thermography

There was evidence of treatment effects ($P = 0.001$) observed between mean differences of the left and right foot maximum temperatures from IRT (Table 2; Fig 4a). The LAME + PLBO group had higher temperature differences (1.64 °C; 95% CI: 1.01 – 2.26 °C) throughout the entire 120-hour study period compared to the LAME + FLU (0.57 °C; 95% CI: 0.06 – 1.08 °C) and SHAM + PLBO (0.52 °C; 95% CI: -0.20 – 1.25 °C) groups with exception of the baseline time point. Differences of the baseline time point were -0.12 °C (95% CI: -0.71 – 0.47 °C) for LAME + FLU; 0.96 °C (95% CI: 0.27 – 1.65 °C) for LAME + PLBO; and 0.40 °C (95% CI: -0.38 – 1.18 °C) for SHAM + PLBO ($P = 0.12$).

There was no evidence of treatment differences ($P = 0.59$) in the mean minimum temperature differences observed (Table 2; Fig 4b). There was a treatment by time interaction observed. The LAME + FLU group [1.62 °C (95% CI: 0.38 – 2.86 °C)] had higher mean minimum temperature differences at 8 h compared to the SHAM + PLBO group [-0.21 °C (95% CI: -1.73 - 1.31 °C); $P = 0.01$]. The SHAM + PLBO group [0.54 °C (95% CI: -0.98 - 2.06 °C)] had lower mean minimum temperature differences at 24 h compared to the LAME + FLU [1.89 °C (95% CI: 0.66 – 3.12 °C); $P = 0.006$] and LAME+ PLBO [1.83 °C (95% CI: 0.41 – 3.25°C); $P = 0.04$] groups. At 96h, the LAME +PLBO [2.51 °C (95% CI: 1.10 – 3.91 °C)] had higher mean minimum temperature differences compared to the LAME + FLU [-0.03 °C (95% CI: -1.27 – 1.21 °C); $P = 0.01$] and SHAM + PLBO [0.49 °C (95% CI: -1.04 – 2.01 °C); $P = 0.02$] groups.

Pressure Mat Analysis

Data for the pressure mat gait analysis is summarized in Table 3.

Stride length. There were no observed differences in mean stride lengths between treatment groups ($P = 0.81$). Mean stride lengths for the LAME + FLU, LAME + PLBO and SHAM + PLBO groups were 77.4 cm (95% CI: 74.0 – 80.7 cm), 79.5 cm (95% CI: 75.6 – 83.4 cm), and 78.0 cm (95% CI: 72.9 – 83.1 cm), respectively.

Force. There was no evidence for a difference in the amount of force on the left hind limb between the LAME + FLU cows [182.2 kg (95% CI: 153.2 – 211.1 kg)] compared to the LAME + PLBO [174.7 kg (95% CI: 140.6 – 208.8 kg)] and SHAM +PLBO groups [171.4 kf (95% CI: 127.2 – 215.6 kg)] ($P = 0.91$). There was evidence of a time effect for all groups in maximum force of the LH foot being 194.5 kg (95% CI: 167.3 – 221.7 kg) at 96 h; and minimum LH force of 161.2 kg (95% CI: 133.9 – 188.3 kg) at 72h ($P = 0.03$). When examining the non-lame feet, there was no evidence of treatment effects. There was evidence of a time effect in the non-lame feet with the mean force of all groups peaking at 24 h of 142.9 kg (95% CI: 125.8 – 160.1 kg) and had a trough at 72h of 116.8 kg (95% CI: 99.6 – 134.0 kg) ($P = 0.04$).

Impulse. Cows in the SHAM + PLBO group had a mean impulse of 136.3 kg x sec (95% CI: 94.0 – 178.6 kg x sec.) compared to the LAME + FLU and LAME + PLBO cows [125.9 kg x sec (95% CI: 98.1 – 153.6 kg x sec.); 113.8 kg x sec (95% CI: 81.2 – 146.4), respectively] for the left hind limb ($P = 0.51$). When evaluating the non-lame limbs, no treatment effects were observed ($P = 0.46$). Cows in the LAME + FLU had higher impulse of 139.8 kg x sec (95% CI: 122.0 – 157.5 kg x sec) versus 124.1 kg x sec (95% CI: 103.2 – 145.0 kg x sec) and 124.5 kg x sec (95% CI: 97.4 – 151.6 kg x sec) for the LAME +PLBO and SHAM +PLBO groups respectively. There was evidence of a time effect in the non-lame limb impulse with a peak impulse of 191.2 kg x sec (95% CI: 167.1 – 215.3 kg x sec) at 24 h and low of 173.1 kg x sec (95% CI: 149.1 – 197.2 kg x sec) at 72 h ($P = 0.02$).

Contact pressure. Contact pressure was similar for all treatment groups for the left hind limbs ($P = 0.85$) and non-lame limbs ($P = 0.87$) (Table 3). There was a significant time effect ($P < 0.0001$) as contact pressures for all groups decreased over time. Baseline contact pressure was 3.30 kg/cm^2 (95% CI: 2.88 – 3.71 kg/cm^2) with a trough at 72 h [2.88 kg/cm^2 (95% CI: 2.46 – 3.29 kg/cm^2)] after initial drug administration in all limbs. At 96 h post initial drug administration, contact pressures for the left hind feet of all groups peaked at 3.55 kg/cm^2 (95% CI: 3.14 – 3.97 kg/cm^2).

DISCUSSION

The model used to induce lameness in this study has been previously validated using 4 to 6 month old cattle and has been shown to provide a predictable time frame to the onset of lameness (Kotschwar et al., 2009, Schulz et al., 2011, Coetzee et al., 2014). In the current study, amphotericin B was successful in creating lameness, but the degree of lameness in the LAME + PLBO cows was not as severe compared to the LAME + FLU as shown by the VAS measures. The changes in mechanical nociception thresholds and elevations of infrared thermography temperatures in the LAME + FLU and LAME + PLBO groups, but not the SHAM +PLBO group, are indicative of hyperesthesia and inflammation of the affected foot caused by amphotericin B injection into the joint.

Transdermal flunixin meglumine is the only drug with a pain control approval in cattle in the United States. It has a label approval for the control of pain associated with foot rot, and any use beyond that disease condition is considered extra-label drug use. This approval was granted after the study described in this manuscript was conducted. To gain approval by the FDA, the

drug sponsor used a foot rot model to induce disease in calves. That model used the causative agent *Fusobacterium necrophorum* to cause lameness in the desired limb (US, 2017).

Since transdermal flunixin has a pain control label for foot rot in cattle, this study is important because it evaluated pain caused by a different mechanism. Amphotericin B, when placed intra-articular, causes a local arthritis and synovitis (McIlwraith et al., 1979). Injection in the lateral distal interphalangeal joint was used as the majority of lameness is found in the lateral hind digit (Murray et al., 1996). There are no models for induction of sole ulcers or white line disease, two common causes of lameness in dairy cattle (Shearer and van Amstel, 2017). The causative event leading to sole ulcers typically occurs week to months prior to a sole ulcer is clinically noted (Shearer and van Amstel, 2017). These changes cause disruption in growth and development of corium leading to a lesion that cause inflammation to the corium. The inflammation of the corium is different than the arthritis/synovitis caused by amphotericin B. The use of amphotericin B allows for induction of lameness without confounding of lesions in other limbs or multiple lesions in the same foot. Cows with naturally occurring lameness may have either lesions in other feet or not than one lameness causing etiology in the same foot (i.e. sole ulcer and digital dermatitis occurring concurrently). A downfall of the model is the acute lameness it creates; when other lameness lesions are typically more chronic in mature.

Multiple doses of transdermal flunixin were evaluated in the present study and were warranted as cows in the two lame groups had signs of lameness and decreased MNT thresholds beyond 24 h. The pharmacokinetics of transdermal flunixin in adult Holsteins with three doses at 24-hour intervals are described; the plasma half-life was 5.2 h, time to maximum concentration was 2.8 h, and the accumulation factor was 1.1 indicating little to no accumulation in plasma (Kleinhenz et al., 2018). These pharmacokinetic parameters support the dosing regimen of 3.33

mg/kg at 24 h intervals. As the flunixin formulation was not approved for use in the US at the time of the study, cattle in the LAME + FLU group did not enter the food chain. Currently transdermal flunixin is not approved for lactating dairy cattle in the US, use in this production class of animals constitutes an extra-label drug use and requires a valid veterinary-client-patient relationship. Additionally, milk from all cattle was diverted from the food chain to prevent potential amphotericin B residues.

Lameness was evaluated using two methods. The visual lameness score (VLS) has been previously used in lameness research and clinical applications. It describes the grade of lameness with data needing to be compared using categorical methods. A second method for evaluating lameness was a visual analog scale (VAS). The VAS was set on a 10 cm line with 0 cm indicating no lameness and 10 cm as severe lameness. A VAS of 1.5 cm would be equivalent to a VLS of 1 to 2. The VAS measures allows for increased differentiation of lameness severity within VLS classifications. Thus two cows with similar VLS measures could receive different VAS measures. Additionally, VAS measures can be treated as continuous data and incorporated in as such in the statistical model.

Lameness scores, evaluated using either method, were low in this study meaning the cows were only mildly lame. Peak VAS scores were seen at the time of initial treatment. Cows in the LAME + FLU group had VAS score double that of the LAME + PLBO group, but these differences were no longer observed at 8 h post treatment. The lameness, as judged by mean VAS scores persisted longer in the LAME + PLBO group throughout the study. However, it is noted that there were cows in the LAME + PLBO group with no visual lameness. Since there were differences in VLS and VAS measures at the time of initial drug administration; VLS was

included as a covariate in the statistical model. The inclusion of VLS in the statistical model did not change the statistical outcome when it was not included.

Mechanical nociception threshold (MNT) testing has been shown to be an effective noninvasive test for hyperalgesia in dairy cows (Tadich et al., 2013) and sows (Tapper et al., 2013). Mechanical nociception threshold in this study showed increased sensitivity following lameness induction, when the left (affected) foot is compared to the right foot (control) as an inter-animal control and when raw MNT values between groups are compared. The right hind served as a control, but a there was evidence of a time effect with the mean MNT decreasing at 8 and 16 h post initial treatment in all groups. This is likely due to the amount of standing the cows did for sampling prior to the 16 h time point. Additionally, changes in MNT reflect the changes in visual lameness scores. The data supports a second dose of flunixin may be warranted as cows in the LAME + FLU group had similar MNT levels as the LAME + PLBO group at 24 h, but not 48 h (Figure 3).

The MNT data are further supported by the differences in maximum temperatures of the left and right feet taken by IRT. The differences in the LAME + FLU and LAME + PLBO maximum temperature increased from baseline and peaked at 16 h. There are differences between the two LAME groups at 48, 72, 96, and 120 h. These differences were observed after the second and third dose of flunixin were administered at 24 h and 48 h respectively. At 48 h, the LAME + FLU mean temperature difference approached that of the SHAM + PLBO group indicating flunixin had decreased the amount of inflammation in the foot. The elevation of maximum temperature differences by IRT are indicative of inflammation in the area (Rekant et al., 2016). The difference in the left hind temperature and right hind temperature were used to account for environmental factors such as ambient temperature and humidity which play a role in

IRT measures (Church et al., 2014). IRT has been shown to be sensitive in the detection of lameness and inflammation, but specificity lacked (Stokes et al., 2012, Alsaaod et al., 2014). The temperature differences found in the current study correlate to those found in cows with lesions found at routine hoof trimming (Alsaaod et al., 2014).

The blood biomarkers tested in this study provide marginal evidence to support the effectiveness of transdermal flunixin in this pain model. The cortisol concentrations for the LAME + FLU cows were significantly lower than the LAME + PLBO and SHAM + PLBO groups for all time points except the baseline samples; and at 0.5 and 1 h after drug application. It has been suggested that cortisol is not a specific biomarker for pain, but more of a stress response indicator (Anil et al., 2002). In other lameness induction studies, cortisol did not statistically differ between treatment groups (Schulz et al., 2011, Coetzee et al., 2014).

In the current study, all cows were handled in the same system and were housed together in a single pen, thus decreasing study procedures as a source of differences. Although not statistically significant, cows enrolled into the SHAM + PLBO group had higher baseline cortisol level compared to LAME + FLU and LAME + PLBO groups. Baseline cortisol values were examined as a covariate in the model due to the differences observed. Their inclusion in the statistical model did not change the coefficients and interpretation of outcomes; and were not included in the final model. A limitation to the cortisol data is not obtaining a cortisol sample at the time of initial treatment (T0). Furthermore, the location of cortisol sample collection were different for 0.5 h to 6 h compared to baseline and samples collected after 8 h. Cows were restrained in headlock for the 0.5 h to 6 h time points due to farm logistics. Methods to collect the blood sample (restraint via halter and venipuncture) were consistent regardless of sampling location, but still need to be considered when evaluating the cortisol concentrations over time.

There is little published data regarding substance P in either naturally occurring lameness or following induction in lameness models. In an oligofructose overload model inducing laminitis, the authors were able to show a difference in substance P levels between laminitis and non-laminitis groups (Bustamante et al., 2015). The Bustamante et al. research did not include an analgesic component in the study design. The range of treatment differences in the data presented here, although inconclusive, indicate further investigation is warranted into the correlation of lameness and neuropeptide concentrations, especially in naturally occurring lameness. Substance P is a neuropeptide that has been shown to be associated with nociception in cattle (Coetzee, 2008). In horses, synovial concentrations of substance P were elevated in joints with osteoarthritis compared to non-diseased joints. Substance P levels have also been correlated to prostaglandin E_2 concentrations in arthritic joints (Kirker-Head et al., 2000).

Gait analysis using a floor based pressure mat system was used to gain approval for the control of pain due to foot rot (US, 2017). Other studies using the amphotericin B induction model used a similar gait analysis method (Kotschwar et al., 2009, Schulz et al., 2011, Coetzee et al., 2014). Schultz et al. (2011) found significant increases in force, contact area, and impulse between calves treated with flunixin following lameness induction. Similarly, Coetzee et al. (2014) saw significant increases in step impulse between calves treated with meloxicam and gabapentin following lameness induction. There were also increases in total force and contact area for calves treated with meloxicam and gabapentin compared to placebo controls, but these differences were not statistically significant. Using the pressure mat technology in the study presented here; there was no evidence of treatment differences despite differences in lameness at the time of initial treatment. In the previously published studies and freedom of information summary data, the cattle used in those studies were of a younger age than the current study.

Adjustments were made to the mat to allow for the increased weight of mature cows to walk across it. These adjustments include placing a thicker rubber mat over the pressure sensors and decreasing the sensitivity of the system. The pressure mat system was calibrated using a known weight daily. The uneven distribution of lameness in LAME groups may have contributed to this observation and lack of treatment differences. Both LAME groups had cows with VLS of 0 especially at the later time points. Visual lameness scores were tested in the statistical model, but they were not significant and not included in the final model. The time effects seen in the non-lame limbs are interrelated since impulse and contact pressure have a function of force embedded in them.

Flunixin, given by intravenous injection, has been evaluated in cattle lameness previously (Chapinal et al., 2010, Schulz et al., 2011, Wagner et al., 2017). Schulz et al. (2011) used amphotericin B to induce lameness in calves and found flunixin was successful in providing analgesia. Flunixin treated calves spent less time lying after lameness induction; and had increased force and contact area walking across a gait analysis system similar to the one used in the current study. The studies by Chapinal et al. (2010) and Wagner et al. (2017), followed dairy cows and used weight-shifting of the rear limbs as the main endpoint. Chapinal et al. (2010) investigated cows following hoof-trimming and found no evidence for treatment differences between flunixin treated cows and saline controls. However, the inclusion of non-lame cows, may have diluted the differences seen between treatments. Wagner et al. (2017) followed only lame cows before being trimmed by a hoof trimmer. The authors observed a difference in weight shifting between treatment groups with the flunixin treated cows having decreased amount of weight shifting. Changes in weight distribution were evident at 6, 12, and 24 h post-treatment. The authors attributed this decrease in weight shifting to alleviation of pain by flunixin (Wagner

et al., 2017). Based on the Schulz et al. and Wagner et al. studies, flunixin provides analgesia to cattle with lameness. This supports our findings of improved MNT and VAS measures in the LAME + FLU cows following transdermal flunixin administration.

When all outcome variables are taken into account, there appears to be a benefit in providing transdermal flunixin to cows with induced lameness. Lameness scores improved in both the LAME + FLU and LAME + PLBO groups based on lower VAS measures by 24 h. There is evidence of differences in MNT and IRT measures at 24 h in the lame groups. These observed differences became evident following the second administered dose at 24 h and measures taken prior to the third dose at 48 h. The increased MNT scores in the LAME + FLU, but not the LAME + PLBO group, provide evidence of improved tolerance to painful stimuli. The increases in MNT measures are also supported by decreased maximum temperatures taken by IRT and the associated inflammation of foot.

CONCLUSIONS

Transdermal flunixin is an effective analgesic agent when treating induced mild lameness. Multiple doses of transdermal flunixin may be required ~~to be clinically effective~~ as MNT and IRT differences were first noted after a second dose was administered. Further investigation of transdermal flunixin and its analgesic effects in severe lameness cases and naturally occurring lameness are warranted.

Figure 8-1 Mean visual analog scores for lameness adapted from Flower and Weary (2006) for cows with induced lameness using amphotericin B and treated with transdermal flunixin at 3.33 mg/kg (1 mL/15 kg) (LAME + FLU; n = 10); induced lameness using amphotericin B and treated with a topical placebo at 1 mL/15 kg (LAME + PLBO; n = 10); or sham lameness induction and treated with a topical placebo at 1 mL/15 kg (SHAM + PLBO; n = 10). ↓ Indicates dosing times

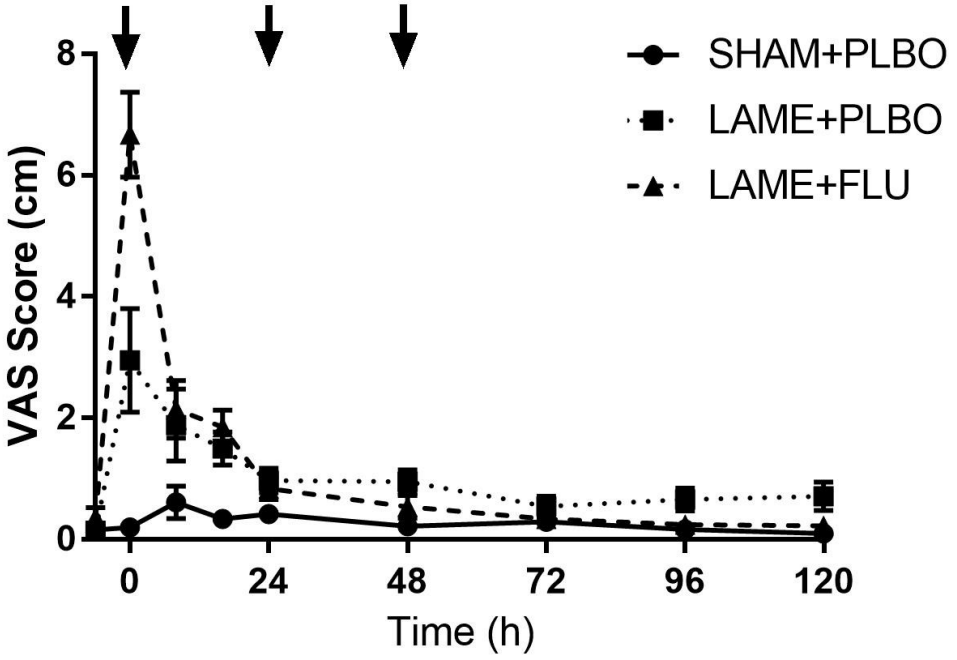

Figure 8-2 Mean cortisol concentrations (ng/mL) of cows with induced lameness using amphotericin B and treated with transdermal flunixin at 3.33 mg/kg (1 mL/15 kg) (LAME + FLU; n = 10); induced lameness using amphotericin B and treated with a topical placebo at 1 mL/15 kg (LAME + PLBO; n = 10); or sham lameness induction and treated with a topical placebo at 1 mL/15 kg (SHAM + PLBO; n = 10)) at -6 h to 6 h (a, top) and -6 h to 120 h (b, bottom). ↓ Indicates dosing times.

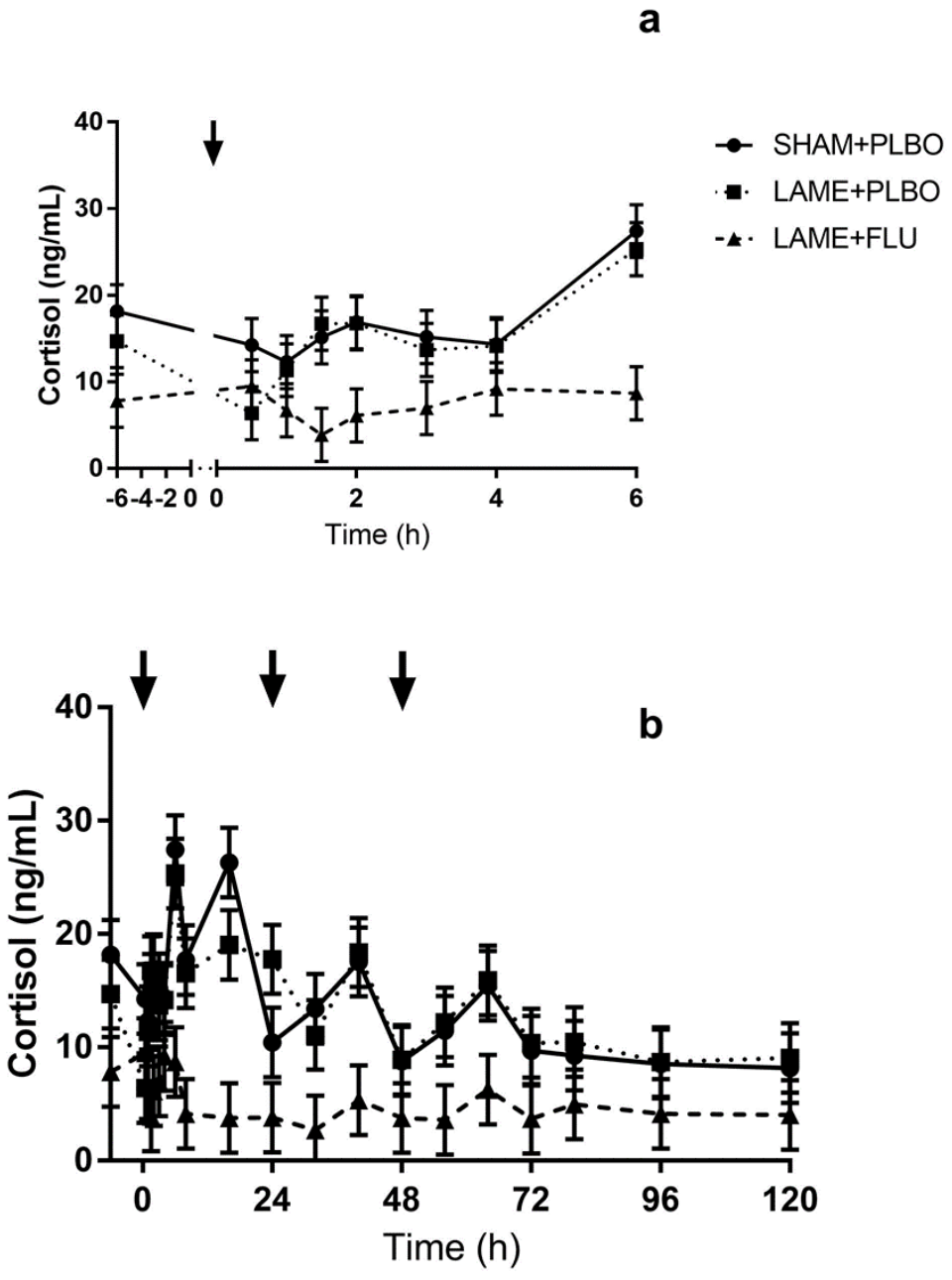

Figure 8-3 Mechanical nociception threshold (MNT) difference between the left hind (lame) and right hind (non-lame) foot of cows with induced lameness using amphotericin B and treated with transdermal flunixin at 3.33 mg/kg (1 mL/15 kg) (LAME + FLU; n = 10); induc induced lameness using amphotericin B and treated with a topical placebo at 1 mL/15 kg (LAME + PLBO; n = 10); or sham lameness induction and treated with a topical placebo at 1 mL/15 kg (SHAM + PLBO; n = 10). ↓ Indicates dosing times.

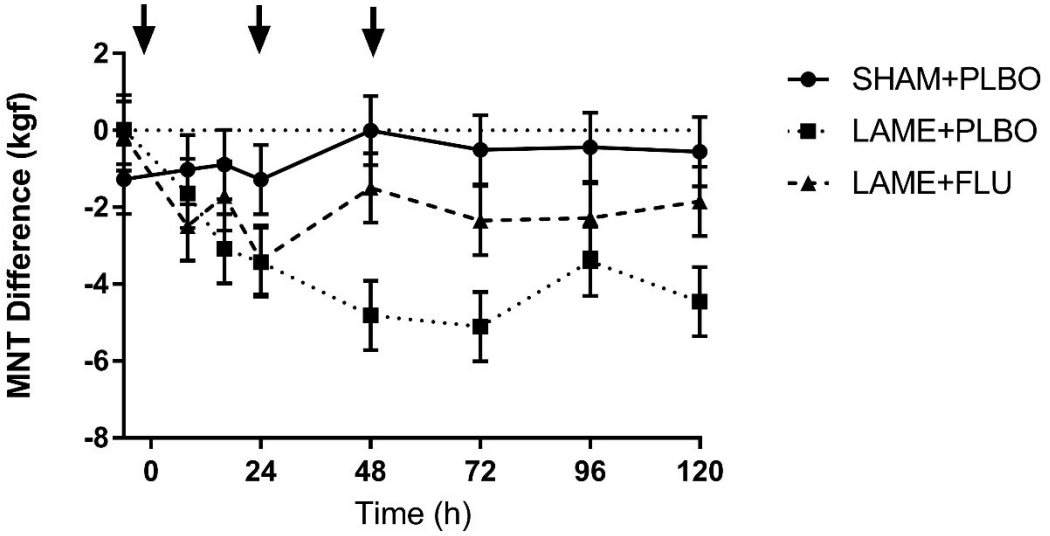

Figure 8-4 Infrared thermography (IRT). Difference between the maximum (a, top) and minimum (b, bottom) temperatures of the left hind (lame) and right hind (non-lame) foot of cows with induced lameness using amphotericin B and treated with transdermal flunixin at3.33 mg/kg (1 mL/15 kg) (LAME + FLU; n = 10) every 24 h for 3 doses; induced lameness using amphotericin B and treated with a topical placebo at 1 mL/15 kg (LAME + PLBO; n = 10) every 24 h for 3 doses; or sham lameness induction and treated with a topical placebo at 1 mL/15 kg (SHAM + PLBO; n = 10) every 24 h for 3 doses. ↓ Indicates dosing times.

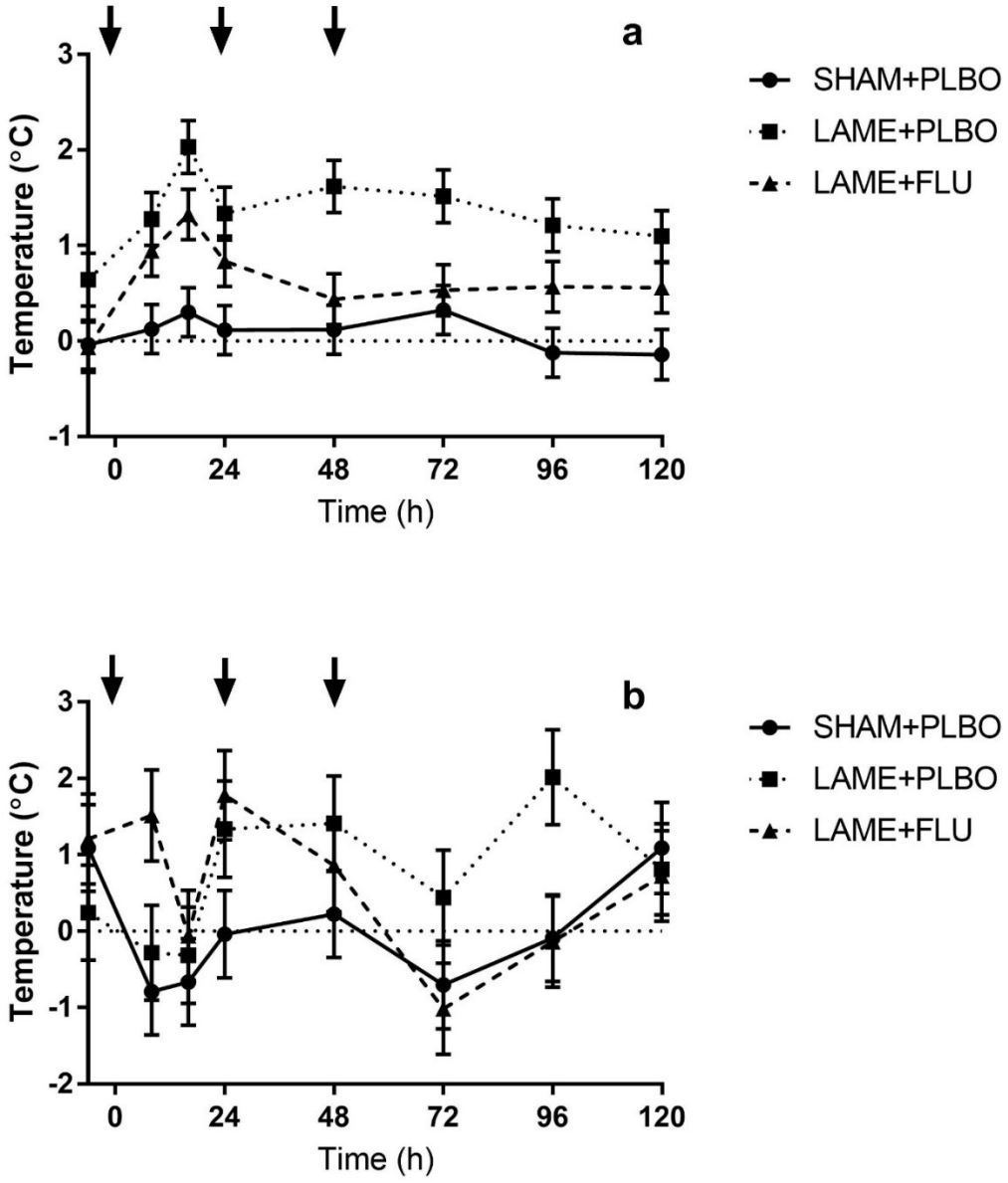

Table 8-1. Visual Lameness Score. Visual lameness score to assess lameness adapted from Sprecher et al. (1997).

Lameness Score	Clinical Description
0	Normal-Stands and walks normally, with all feet placed with purpose.
1	Mildly lame – Stands with flat back, but arches when walks, gait is abnormal
2	Moderately lame – Stands and walks with an arched back, and short strides on one or more legs
3	Lame - Arched back standing and walking, with one or more limbs favored but at least partially weight bearing
4	Severely lame - Arched back, refuses to bear weight on one limb, may refuse or have great difficulty moving from lying position

Table 8-2 Mean (± SE) outcome measures in cows with induced lameness using amphotericin B and treated with transdermal flunixin at 3.33 mg/kg (1 mL/15 kg) (LAME + FLU; n = 10); induced lameness using amphotericin B and treated with a topical placebo at 1 mL/15 kg (LAME + PLBO; n = 10); or sham lameness induction and treated with a topical placebo at 1 mL/15 kg (SHAM + PLBO; n = 10).

Parameter	LS[1] means (±SE)			*P* -values		
	LAME + FLU[2]	LAME + PLBO[3]	SHAM + PLBO	Treatment	Time	Treatment x time
Visual Assessment Score, 0-10 cm	1.47[a] (0.1)	1.11[a] (0.1)	0.28[b] (0.1)	< 0.0001	< 0.0001	< 0.0001
Cortisol, ng/mL	5.77[a] (1.9)	13.68[b] (2.2)	14.64[b] (2.9)	0.0006	< 0.0001	0.0019
Substance P, pg/mL	81.9 (4.7)	84.6 (5.5)	70.6 (7.2)	0.15	0.66	0.92
MNT[4] LH	8.46[a] (0.7)	7.98[a] (0.9)	12.34[b] (1.1)	0.003	< 0.0001	< 0.0001
MNT RH	10.54 (0.6)	10.93 (0.7)	12.34 (0.9)	0.23	< 0.0001	0.73
MNT LH-RH difference	-2.08[a,b] (0.6)	-2.96[a] (0.7)	-0.04[b] (0.9)	0.008	0.03	0.02
Max Temp IRT[5], LH-RH difference, °C	0.57[b] (0.2)	1.64[a] (0.3)	0.53[b] (0.4)	0.0043	< 0.0001	0.052
Min Temp IRT, LH-RH difference, °C	0.71 (0.4)	1.20 (0.5)	0.61 (0.6)	0.59	< 0.0001	0.0007

[a,b] Different superscript indicate significant differences between treatment groups ($P \leq 0.05$)
[1] Least square means
[2] FLU = flunixin
[3] PLBO = placebo
[4] MNT = mechanical nociception threshold
[5] IRT = infrared thermography

Table 8-3 Mean (± SE) outcome measures from pressure mat gait analysis in cows with induced lameness using amphotericin B in the left hind lateral distal interphalangeal joint and treated with transdermal flunixin at 3.33 mg/kg (1 mL/15 kg) (LAME + FLU; n = 10); induced lameness using amphotericin B in the left hind lateral distal interphalangeal joint and treated with a topical placebo at 1 mL/15 kg (LAME + PLBO; n = 10); or sham lameness induction in the left hind lateral distal interphalangeal joint and treated with a topical placebo at 1 mL/15 kg (SHAM + PLBO; n = 10).

Parameter	LS[1] means (±SE)			*P* -values		
	LAME + FLU[2]	LAME + PLBO[3]	SHAM + PLBO	Treatment	Time	Treatment x time
Stride length, cm	77.4 (1.6)	79.5 (1.9)	78.0 (2.4)	0.61	0.11	0.09
Left Hind Foot						
Force, kg	182.2 (14.0)	174.7 (16.5)	171.4 (21.4)	0.91	0.03	0.76
Impulse, kg x sec	125.9 (13.4)	113.8 (15.8)	136.3 (20.5)	0.51	0.22	0.50
Contact Pressure kg/cm^2	3.36 (0.2)	3.18 (0.3)	3.27 (0.3)	0.85	< 0.0001	0.99
Non-lame feet						
Force, kg	193.8 (13.2)	187.7 (15.5)	172.0 (20.2)	0.65	0.02	0.27
Impulse, kg x sec	139.8 (8.6)	124.1 (10.1)	124.5 (13.1)	0.46	0.04	0.06
Contact Pressure kg/cm^2	3.48 (0.2)	3.31 (0.3)	3.37 (0.3)	0.87	< 0.0001	0.92

[1] Least square means
[2] FLU = flunixin
[3] PLBO = placebo

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Chapter 9 - Future directions and conclusions

The use of transdermal flunixin as the only analgesic provided at castration or dehorning is not supported by the studies presented here. Future studies are needed to investigate transdermal flunixin as part of a multimodal analgesic plan that includes local anesthetic blocks and other analgesic drugs such as butorphanol or xylazine. Transdermal flunixin has been shown to provide analgesia in lameness models of foot rot or induced arthritis/synovitis. Clinical field trials are needed to determine if transdermal flunixin's efficacy when the time of lameness onset is not known and multiple lesions may be present.

The use of infrared thermography and pressure mat gait analysis as techniques for the objective measure of pain and inflammation. In our lameness research, temperature differences of the inflamed joint were observed. The use of thermography in studies of local inflammation such as arthritis show promise. Further description of gait changes following castration or parturition are needed. This will allow the differentiation of normal from abnormal and influences of analgesic interventions.

Additional work on the interaction of flunixin and cyclo-oxygenase (COX) enzyme are needed to further characterize the age related inhibition of prostaglandin E_2 seen. Additionally, investigating any age related effects of flunixin on thromboxane production is needed and may allow for further understanding of COX-1:COX-2 partitioning in cattle of various ages. This data will allow for optimizing our understanding and clinical use of transdermal flunixin in cattle.

Finally, information is needed for the use of transdermal flunixin meglumine in lactating dairy cows and other food production animal classes is needed. This includes data on the excretion of flunixin in milk following administration of transdermal flunixin; tissue residue

profiles in lactating dairy cattle; and tissue residues in other food production animals such as goats. Supporting pharmacokinetic data to guide clinical use in species not on the label are also needed.

Conclusions

Transdermal flunixin is rapidly absorbed and achieves maximum plasma concentrations around 2 hours post administration. Transdermal flunixin provides anti-inflammatory effects, as measured by prostaglandin E_2 production, beyond 24 hours in our testing. Following multiple doses at 24 h intervals, no plasma accumulation is observed and no adverse effects were noted.

The use of analgesic at the time of painful procedures has become a norm for some, but is still resisted by others. Having an analgesia that is easy to administer, economical, and efficacious is key to adaptation. Transdermal flunixin is easy to administer, rapidly absorbed, and has anti-inflammatory actions for 24 hours. Its efficacy in castration and dehorning models are not appreciated, but it appears to provide analgesia in lameness models.