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The effects of anti-inflammatory drugs on clinical signs, milk production, and mammary epithelial cells in cows with endotoxin-induced mastitis

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**The effects of anti-inflammatory drugs on clinical signs, milk production, and
mammary epithelial cells in cows with endotoxin-induced mastitis**

by

Sarah Anderson Wagner

**A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY**

Major: Physiology (Pharmacology)

**Program of Study Committee:
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2003

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For The Major Program

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ABSTRACT

Three studies are described in this report. First, the effect of intravenous administration of the steroidal drug isoflupredone acetate on healthy lactating dairy cows and lactating dairy cows with mastitis induced using gram-negative bacterial endotoxin was investigated. Cows were randomly assigned to one of four treatment groups: untreated controls, isoflupredone acetate only, mastitis only, and mastitis plus isoflupredone acetate. Isoflupredone acetate was given to treated groups at a dose of 20 mg intravenously, once. Mastitic cows receiving treatment were given isoflupredone acetate after the development of mammary swelling and an increase in rectal temperature of at least 1° Celsius. When compared with untreated mastitic controls, cows with endotoxin-induced mastitis treated with isoflupredone acetate did not exhibit measurable differences in heart rate, rectal temperature, rumen motility, or changes in mammary gland surface area in the 14 hours following the administration of intramammary endotoxin, nor did they have significantly different milk production following the mastitic episode. Healthy cows treated with isoflupredone acetate had a higher heart rate over the 14 hours following drug administration than did untreated healthy controls, but this was likely caused by repeated blood sampling of the drug-treated cows for another study.

In the second study, somatic cell counts and flow cytometric analysis of cytokeratin-positive epithelial cells in the milk of lactating Holstein cows were performed before and 12 hours after the induction of mastitis using purified bacterial endotoxin. Prior to the induction of mastitis, the mean percentage of cells

that were cytokeratin-positive was $4.18\% \pm 1.05$, and the mean total number of cytokeratin-positive cells per milliliter of milk was $7,711 \pm 2,575$. Twelve hours after the induction of mastitis, cytokeratin-positive cell percentages and numbers in bovine milk were significantly increased; the mean percentage of cytokeratin-positive cells was $40.63\% \pm 10.23$, and the mean number of cytokeratin-positive cells per milliliter of milk was $2,954,554 \pm 877,719$. Treatment with flunixin meglumine or isoflupredone acetate after the development of clinical signs of mastitis did not mitigate the increase in somatic cell count or cytokeratin-positive cells in milk in the first 12 hours following endotoxin administration.

Finally, the effects of flunixin meglumine and isoflupredone acetate were compared in lactating Holstein dairy cows with mastitis induced by the intramammary administration of purified bacterial endotoxin. Drugs were administered to cows following the development of mammary swelling in the affected gland and a rectal temperature of $40\text{ }^{\circ}\text{C}$ or greater. Heart rate, rectal temperature, and rumen motility were recorded hourly for 14 hours following the intramammary administration of endotoxin. Milk production was evaluated for five days before and ten days following the day of mastitis induction and drug treatment. Neither drug decreased milk production loss nor mammary surface swelling when compared to untreated mastitic controls. Both drugs reduced the mean heart rate in treated groups over the 14 hours following endotoxin administration, when compared to untreated mastitic controls. Animals treated with flunixin meglumine also had increased rumen motility and decreased rectal temperature over the same time period, when compared to untreated mastitic controls or mastitic cows treated with isoflupredone acetate.

Taken together, these studies suggest that neither the NSAID flunixin meglumine nor the corticosteroid drug isoflupredone acetate ameliorate milk production losses in cows with endotoxin-induced mastitis when they are administered following the onset of clinical signs, nor do they decrease the magnitude of sloughing of mammary epithelial cells into the milk in the acute phase of endotoxin-induced mastitis. Both drugs may, however, provide some relief from the pain of mastitis, as evidenced by the decrease in heart rate in cows treated with either drug following the development of endotoxin-induced mastitis. Only flunixin meglumine was found to alleviate fever associated with endotoxin-induced mastitis. Until it is known whether relief of fever is beneficial or detrimental in naturally occurring cases of infectious mastitis, it is not possible to infer that one agent would be superior to the other in the treatment of such cases.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is presented as an introductory chapter, three chapters corresponding to papers submitted or accepted for publication, and a chapter of general conclusions. All of the papers included address the central research question of this dissertation: What are the effects of steroidal and non-steroidal anti-inflammatory drugs on endotoxin-induced mastitis in lactating dairy cows? The second and fourth papers describe the physiologic effects of a corticosteroid drug and a non-steroidal inflammatory drug in cows with endotoxin-induced mastitis. The study presented in the third chapter describes the effects of both drugs on epithelial cells in cows' milk. An Appendix describes data analysis for the flow cytometry method used in conducting the research presented in Chapter 3.

The coauthors of the submitted manuscripts contributed in the following ways: Michael D. Apley was the Major Professor and involved in all aspects of the studies including study design, data collection and analysis. Doug Jones was integral in flow cytometry method development, the performance of flow cytometry and the analysis of flow cytometry data.

Literature Review

Part One: Models of Endotoxic Mastitis

Mastitis due to infection of the mammary gland or glands remains a costly disease to the dairy industry and an ongoing topic of investigative research. There are numerous pathogens implicated in infectious bovine mastitis; this literature review and the research described herein focus on endotoxic mastitis caused by coliform bacteria, the most common causative agents in severe clinical cases of mastitis. (Hogan, Smith et al. 1989) The term "coliform" is used to describe gram negative bacteria including *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species. (Rebhun 1995)

Mammary gland infection with gram-negative coliform organisms may result in a mild, moderate or severe case of mastitis. Mild or moderate disease is characterized by localized mammary swelling and abnormal milk, with or without the presence of systemic signs such as depression and fever. These mild or moderate cases may be resolved without any treatment of the cow. (Erskine, Tyler et al. 1991) Coliform mastitis may also be quite severe in its manifestation, particularly in cows suffering immunosuppression, which is commonly observed in dairy cows around the time of calving. (Cai, Weston et al. 1994) Many of the detrimental effects of coliform infection are due to the endotoxin produced by coliform bacteria. (Lohuis, Verheijden et al. 1988) Cows affected with severe coliform mastitis are frequently recumbent, with a hot, swollen mammary gland,

watery abnormal milk in the affected gland, fever or hypothermia, diarrhea, rumen stasis, anorexia, and dehydration. More than 30 percent of severely affected cows may become bacteremic, (Wenz, Barrington et al. 2001) and concurrent hypocalcemia is common. (Rebhun 1995) Severe endotoxic mastitis may be fatal to the cow. Cows that do survive commonly suffer sequelae such as chronic active or subclinical infection, agalactia in one or more quarters, or musculoskeletal damage as a result of prolonged recumbency. (Rebhun 1995)

Because coliform mastitis can be so severe in its manifestation and consequences, much research has been done to investigate how to prevent and treat it. The goal of therapy is to preserve the life of the cow and minimize harmful sequelae. In addition to antimicrobial therapy to eliminate the causative pathogen from the mammary gland and systemic circulation, anti-inflammatory drugs have been investigated in the hope that they may reduce mortality, preserve milk production, and alleviate the clinical signs associated with coliform mastitis.

Research into the therapy of endotoxic mastitis has been conducted using various models, including: 1) naturally occurring cases of endotoxic mastitis due to spontaneous infection, 2) endotoxic mastitis artificially induced by introducing live bacteria, usually *Escherichia coli*, into the mammary gland, and 3) endotoxic mastitis induced by the administration of purified bacterial endotoxin.

Mastitis due to natural infection would be expected to provide the most reliable data about the effects of various therapies for endotoxic mastitis; after all,

it is for natural infection that we are hoping to develop better treatment through research. Unfortunately, difficulties are encountered in using natural infection to research the therapy of endotoxic mastitis. Naturally occurring endotoxic mastitis is progressive over time, but some cases will ultimately become more severe than others. As it is impossible to know the time or size of microbial inoculation of a naturally occurring case of mastitis, it is quite difficult to determine where in the progression of disease each cow is when first examined. For example, a moderately affected cow may have already attained the greatest severity of disease she will suffer during the mastitic episode, or she may be in the process of developing severe, even life-threatening disease. One way to avoid this variation would be to treat only cases of endotoxic mastitis defined as severe. This can certainly be done, but severe naturally occurring endotoxic mastitis is sporadic in nature, (Sargeant, Scott et al. 1998) and it would take either a long duration of time or a large number of participating farms to accumulate enough naturally occurring cases to provide the statistical power necessary to demonstrate the effect of a treatment that is moderately beneficial.

Because of the difficulties associated with using natural infection to study the therapy of coliform mastitis, induced mastitis models have frequently been used instead. One approach has been to induce infection using a known number of colony forming units of coliform bacteria, most commonly *Escherichia coli*. The advantage of this approach is clear; it is an infectious model of an infectious disease. Unfortunately, the variability one encounters in clinical disease due to natural infection is also present in induced infection. Intramammary

administration of the same number of colony forming units of bacteria to a number of cows can result in a “great variation in duration and severity of clinical signs”. (Lohuis, Schukken et al. 1990) This is likely due to variations in immunocompetence between cows and resulting variations in the speed and magnitude of bacterial killing. When cows had approximately 10^4 colony forming units of *Escherichia coli* (of a strain isolated from a clinical case) infused into their left rear quarter, some animals were mildly affected with mastitis as a result; these animals had mean peak bacterial concentrations of 3.3×10^1 Colony Forming Units (CFU) per ml of milk at 12 hours post administration, and they all had bacteriologically negative milk at 29 hours post administration. (Lohuis, Schukken et al. 1990) Other cows in the same study developed severe illness following administration of the same strain and number of bacteria; these animals had mean peak bacterial numbers of 1.2×10^9 CFU per ml of milk at 18 hours post infection and continued to have more than 1000 CFU per ml of milk through the last sampling time at 125 hours post-infection. There were also large variations in the magnitude of milk production loss between cows. In addition to the large amount of variability in clinical disease produced by induction of mastitis using live bacteria, use of live bacteria also compels the investigator to decide whether or not to give antibiotics, as has usually been done, and if so when in the course of disease to administer the drug, which type of antimicrobial drug to administer, and what dosing regimen to use.

Some of the drawbacks encountered when using an induced infection model may be avoided by using a non-infectious model of endotoxic mastitis in which

purified Lipopolysaccharide endotoxin from *Escherichia coli* is administered into one or more mammary glands. There are several advantages to this approach. Unlike the induced infection model, the response to intramammary endotoxin challenge is generally quite consistent between cows, (Tyler, Welles et al. 1994) as the amount of endotoxin to which each cow is exposed is not dependent on the quality of her immune response. Furthermore, the issue of whether and when to administer antimicrobial therapy becomes moot as no infectious agent is administered. Additionally, the course of the clinical response is brief; although typically used doses of endotoxin result in markedly increased rectal temperature, depression, and decreased rumen motility, these clinical signs are short-lived and resolve within 12 to 24 hours. (Lohius, Leeuwen et al. 1989) Milk production losses generally resolve within a few days, (Hoeben, Burvenich et al. 2000) but in one high-dose study mean milk production never returned to pre-challenge levels. (Oostveldt, Tomita et al. 2002) Fatalities using the endotoxin-induced mastitis model were not reported in any of the many such studies reviewed.

The endotoxin-induced mastitis model does have its limitations, however. One limitation is the same as an advantage it provides- the illness is generally short-lived and there have been no documented fatalities. Although this feature is beneficial to the preservation of research animals and their milk production, it is not an accurate reflection of the severe potential consequences of natural infection. Furthermore, qualitative differences between the physiological response of cows with mastitis due to coliform infection and mastitis due to intramammary endotoxin administration have been demonstrated. For example, in one study

blood was taken from cows during the acute phase of mastitis induced using either *Escherichia coli* bacteria or endotoxin. (Oostveldt, Tomita et al. 2002) After incubating the blood samples for three hours, the percentage of apoptotic neutrophils in the blood was increased in cows with *E.coli* mastitis when compared to non-mastitic cows, but this effect was not observed in cows with endotoxin-induced mastitis. There were also many similarities between the two models in the study cited above; *E. coli* and endotoxin both caused milk somatic cell count, rectal temperature, and percentage of immature neutrophils in the blood to increase, and both models caused milk production and total leukocyte count to decrease. These changes were more rapid, more severe and of shorter duration in cows with endotoxin-induced mastitis. Both models have also been demonstrated to raise levels of Tumor Necrosis Factor alpha in plasma and milk, and levels of nitric oxide in milk. (Blum, Dosogne et al. 2000) Again, these effects tend to peak earlier in endotoxin-infused cows than in cows infused with *E. coli* bacteria.

Hoeben et al also found that endotoxin-induced mastitis produced physiological changes more quickly than mastitis induced using *E coli* bacteria. (Hoeben, Burvenich et al. 2000) Overall milk production loss was greater in infected cows, which suffered “substantial” losses in both inflamed and non-inflamed quarters, but it was more acute in endotoxin infused cows, which suffered only “negligible” losses in the non-inflamed quarters. As in other studies, both models increased plasma levels of Tumor Necrosis Factor alpha in blood plasma. Hoeben hypothesized that there existed “differences in kinetics, composition and amounts of different cytokines released in the mammary gland and subsequently

absorbed into the circulation^p between the infectious and endotoxin models. This hypothesis is consistent with the literature, at least in terms of amounts and kinetics of cytokine release in the two models, if not the composition of those cytokines, which has thus far not been demonstrated to be different. One unusual aspect of Hoeben's study was that cows with endotoxin-induced mastitis did not have decreased reticulorumen motility during the resulting episode of mastitis; this finding is inconsistent with the work reported in this dissertation.

Finally, work at the cellular level has demonstrated different effects of endotoxin and infection with *Escherichia coli* bacteria on the epithelium of the mammary gland. Studies in which endotoxin or *E. coli* was administered into the mammary gland, followed by slaughter of animals and microscopic examination of the affected gland, revealed that endotoxin caused inflammation and edema of the mammary epithelium, while bacterial infection resulted in necrosis and sloughing of epithelial cells from the basement membrane. The effects of mastitis on the mammary epithelium are discussed in more detail in a later section of this review.

Overall, administration of purified bacterial endotoxin has produced a consistent result: clinical mastitis of short duration but high intensity, with moderate local and systemic clinical signs and acute but short-lived losses in milk production. This uniformity of response is useful in a research setting, as are the limited milk production loss and low risk of fatality, but these qualities of the model also make it dissimilar to the severe naturally acquired infections that are the target of therapies under investigation. For this reason, endotoxin-induced

mastitis should be seen as a useful tool for preliminary investigation, but not a foolproof replication of “the real thing”. It would be justified to expand investigation of therapies that appear promising using the endotoxin-induced mastitis model into research trials involving naturally occurring cases of mastitis. Still, the utility of the endotoxin model as a less expensive, consistent and useful tool for preliminary investigation has been established.

Part Two: Anti-Inflammatory Drugs and Endotoxic Mastitis

Anti-Inflammatory drugs can be divided into two major categories; corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs). Both NSAIDS and glucocorticoids have been investigated as therapeutic agents in the treatment of endotoxic mastitis. The two types of anti-inflammatory drugs have some pharmacologic similarities and some differences.

Corticosteroid drugs are described as being either primarily mineralocorticoid (regulating electrolyte metabolism) or glucocorticoid (regulating carbohydrate metabolism) in their activity. Corticosteroid drugs used for anti-inflammatory therapy have predominantly or entirely glucocorticoid activity. (Schimmer and Parker 2001) In addition to their anti-inflammatory activity, glucocorticoids affect the cardiovascular system, the central nervous system, the digestive system, the hypothalamic-pituitary-adrenal axis, and protein, fat and bone metabolism. (Ferguson and Hoenig 2001)

Glucocorticoids are among the most commonly used drugs in veterinary medicine. (Ferguson and Hoenig 2001) Their 21-carbon molecular structure includes three 6-carbon rings and one 5-carbon ring. (Schimmer and Parker 2001) Glucocorticoid drugs are lipid-soluble and therefore are able to penetrate cell membranes to access glucocorticoid receptors, which are present in the cytosol. The unbound glucocorticoid receptor is complexed with heat shock protein 90, which is released upon binding to the glucocorticoid and may play a role in glucocorticoid anti-inflammatory activity. (Ferguson and Hoenig 2001) The hormone-receptor complex then travels into the nucleus of the cell, where it can directly or indirectly alter gene expression. Production of some inflammatory mediators, such as chemokines, cytokines, and adhesion molecules, is decreased. Production of anti-inflammatory molecules called lipocortins (also called annexins) is increased. Lipocortins act by inhibiting the enzyme Phospholipase A₂, which catalyzes the conversion of cell membrane phospholipids into arachidonic acid (5,8,11,14-Eicosatetraenoic acid). Consequently, there is decreased production of the inflammatory products of arachidonic acid metabolism: prostaglandins, leukotrienes, and platelet activating factor.

Glucocorticoid drugs also inhibit the production of the enzyme cyclooxygenase 2 (COX2), found at sites of inflammation, which functions in the production of the inflammatory prostaglandin PGG₂ from arachidonic acid. (Schimmer and Parker 2001) The unstable PGG₂ is quickly converted to PGH₂, which is also an unstable intermediate. PGG₂ and PGH₂ are called endoperoxides, have a half life in humans of 4 to 5 minutes, and are able to cause aggregation of

platelets and contraction of smooth muscle. PGG₂ and PGH₂ may ultimately be converted to PGE₂, PGI₂ PGF_{2α}, or thromboxanes. (Ninneman 1988) Prostaglandin E compounds increase vascular permeability, induce vasodilation, are leukotactic, may cause pain, stimulate the release of lysosomal enzymes, and elevate body temperature. (Ramwell 1973) Although they inhibit the inflammation-induced production of COX2, glucocorticoids have not been observed to inhibit production of cyclo-oxygenase 1 (COX1), a constitutive enzyme which catalyzes the production of prostaglandins such as the PGI₂ in the kidneys, which serves an anti-hypotensive function, and PGE₂ in the gastric mucosa, which regulates acid secretion. (Boothe and Mealey 2001; Morrow and Roberts 2001) The wide variety of prostaglandin effects may be explained by the wide variety of prostaglandin receptors; PGE₂, for example, has at least five known receptor types. (Morrow and Roberts 2001) It is important to note that the current understanding of the functions of the cyclo-oxygenase enzymes is incomplete; recent work has described homeostatic functions of COX2 and a role for COX1 in the resolution of the inflammatory process. (Morita 2002) Membrane-associated glucocorticoid receptors have also been identified, and may be responsible for the rapid effects of glucocorticoids, such as changes in cellular membranes in the treatment of shock. (Ferguson and Hoenig 2001)

The glucocorticoid drug Isoflupredone, also called 9-alpha fluoroprednisolone, is a fluorinated prednisolone molecule, with the fluorine molecule at carbon number nine. Dexamethasone is also fluorinated at carbon number 9, but cortisone, hydrocortisone, prednisone, and prednisolone are not.

Isoflupredone is described as having glucocorticoid potency 10 times greater than hydrocortisone and about one third that of dexamethasone, but these relative potencies have not been confirmed in the treatment of inflammation associated with mastitis. (McDonald 1988) Isoflupredone is unique among these compounds because it does not cause abortion in pregnant cattle at any stage of gestation. (Mohammedsadeh 1994) The drug is commonly used in the United States to treat dairy cattle for ketosis and inflammatory conditions, and it has FDA approval for such use [Package Insert, Pharmacia Animal Health, Kalamazoo, MI]. The drug does have some mineralocorticoid activity; repeated administration of high doses has been associated with electrolyte imbalances in lactating dairy cows, specifically hypokalemia. (Sielman, Sweeney et al. 1997)

In contrast to the glucocorticoids, which exert their anti-inflammatory actions at several points along the inflammatory cascade, the anti-inflammatory activity of NSAID drugs is more focused. They also have a markedly different structure: all of the commonly used NSAIDs are organic acids incorporating one, two or three benzene rings. (Roberts and Morrow 2001) Acetaminophen has a number of properties that make it different from the other NSAIDs; as it is not used to treat cattle, acetaminophen will not be included in this review of the actions of NSAIDs.

The mechanism of NSAID activity is the inhibition of prostaglandin production by the cyclo-oxygenase enzymes. Unlike glucocorticoids, which inhibit the production of COX2 only, NSAIDs commonly used in food animal medicine are

active on both COX1 and COX2 enzymes. Some new NSAID drugs used in humans and small animals are selective for action primarily on the COX2 enzyme, but none of the COX2 selective drugs has yet been approved or adopted for use in cattle.

Unlike glucocorticoids, NSAIDs inhibit the activity, not the production, of the cyclo-oxygenase enzymes.

Aspirin covalently and permanently modifies both COX1 and COX2, resulting in a duration of clinical effect that is related to the speed with which new cyclo-oxygenase molecules are generated. The modification of the COX2 enzyme by binding of aspirin not only inhibits the production of pro-inflammatory prostaglandins, it also causes the enzyme to produce the anti-inflammatory product 15-epilipoxin A₄. (Hardman, Limbird et al. 2001) The other NSAIDs are competitive reversible inhibitors of cyclo-oxygenase; their duration of action is related to the rate of clearance of the drug from the body. Although some NSAID drugs have been found to exert other anti-inflammatory activities, inhibition of COX enzymes is still considered to be the most important mechanism of action of the NSAIDs. (Roberts and Morrow 2001)

Flunixin meglumine is an NSAID that has previously been investigated as a therapy for endotoxic mastitis, as described below. Its chemical name is 3-pyridine-carboxylic acid, and its structure contains 2 benzene rings. It has FDA approval for the indications of fever, inflammation, and endotoxemia in cattle [Package insert, Banamine®, Schering-Plough].

As NSAIDs and glucocorticoids have different structures and mechanisms of action, they might be expected to also have different effects when used as anti-inflammatory agents in the treatment of endotoxic mastitis, and this has proven to be the case. The comparative effects of NSAIDs and steroids in the treatment of mastitis are discussed in some detail in the following chapter and will therefore only be summarized here.

Both glucocorticoid and NSAID drugs have been shown to reduce local inflammation of the mammary gland (evaluated externally) when administered systemically prior to the onset of clinical signs of mastitis induced using bacteria or bacterial endotoxin. (Carroll, Schalm et al. 1965; Anderson, Smith et al. 1986; Lohuis, Werven et al. 1991) Studies evaluating NSAIDs in the treatment of induced mastitis have demonstrated a reduction in rectal temperature following the systemic administration of these agents. (Anderson, Smith et al. 1986; Anderson and Hunt 1989; Lohuis, Werven et al. 1991; Morkoc, Hurley et al. 1993) The effects of systemic glucocorticoids on fever have been inconsistent, with studies demonstrating either increased or decreased febrile response in treated animals when compared to untreated mastitic controls. The dose of dexamethasone that decreased fever, 220 mg for a 500 kg cow, was more than seven times higher than the 30 mg per cow dose that increased fever (Lohuis, Leeuwen et al. 1988; Anderson and Hunt 1989).

Research investigating the question of whether anti-inflammatory drugs improve the likelihood of recovery of cows from endotoxic mastitis due to coliform

infection has been uncommon and inconclusive. A study done in goats did not indicate that treatment with the NSAID flunixin meglumine or the glucocorticoid dexamethasone was detrimental to the clearance of infectious *E coli* bacteria from experimentally challenged mammary glands. (Anderson, Hunt et al. 1991) One study did claim an increased odds ratio of recovery in cows with naturally occurring *E.coli* mastitis treated repeatedly with systemic administration of the NSAID drug ketoprofen. (Shpigel, Chen et al. 1994) This study may not be relevant to severe endotoxic mastitis, however, as eligibility criteria for admission to the study were a 25% drop in milk production and “typical clinicopathological changes in milk and the udder”. “Typical” was undefined, and a complete lack of systemic clinical signs did not exclude cows from the study. Recovery was defined as cows that returned to at least 75% of pre-mastitis milk production. These same parameters were used in another study by the same author, which concluded that the NSAIDs phenylbutazone and dipyrone also increase the odds of recovery from acute mastitis. (Shpigel, Winkler et al. 1996) Both of these studies compared cows receiving anti-inflammatory treatment to the same historical control group, so both could be confounded with the same time effects.

To this author’s knowledge, no other published research has claimed improved recovery in cows treated with non-steroidal anti-inflammatory drugs. Completely prospective clinical trials, using contemporary controls, have shown no benefit to treatment with the NSAIDs flunixin or phenylbutazone in naturally occurring cases of clinical endotoxic mastitis. One study compared therapy with flunixin to therapy with intravenous fluids alone or intravenous fluids plus

flunixin; the odds of survival were the same in all three groups, with survival defined as neither dying nor being culled due to the mastitic episode. (Green, Green et al. 1997) All cows admitted to the study also received standardized therapy with antimicrobial drugs and calcium borogluconate. Another study compared therapy with saline solution to therapy with flunixin or phenylbutazone, and did not find any significant effects of drug treatment on the likelihood of survival or the speed and magnitude of milk production recovery in cows that did survive. (Dascanio, Mecher et al. 1995) Both studies randomly allocated cows to treatment groups (drugs, saline solution, or both) as they were enrolled, and both studies had admissions criteria that included both mammary and systemic clinical signs such as weakness, signs of shock, fever or depression; cows without systemic manifestations of disease were not admitted to either study.

After survival of the cow, the parameter that is likely to be most important to the dairy producer with a cow affected by toxic mastitis is subsequent milk production. Aside from the work described above using a historical control group, studies done using naturally occurring cases or mastitis models have not shown systemic use of NSAIDs to be of benefit to subsequent milk production in cows with endotoxic mastitis. (Anderson, Smith et al. 1986; Dascanio, Mecher et al. 1995) It has been reported that intramammary administration of the NSAID drug ibuprofen significantly decreased milk loss in cows with endotoxin-induced mastitis, however, this was reported in abstract form as a preliminary result and no detailed follow-up report has been published to date, so the methods used in the study cannot be evaluated. (Hatfield, Harmon et al. 1984)

The effect of the systemic administration of the glucocorticoid drug dexamethasone on milk production has been evaluated in cows with mastitis induced using *E. coli* bacteria and found to reduce losses in milk production. (Lohuis, Leeuwen et al. 1988) The same drug, in combination with the antimicrobial drugs ampicillin and polymixin E, was also shown to enhance recovery of milk production in cows with endotoxin-induced mastitis. (Ziv, Shem Tov et al. 1998) The inclusion of antimicrobial drugs in the treatment of endotoxin-induced mastitis in this study is unusual, as there is no infectious process at work in endotoxin-induced mastitis. Incubation of polymorphonuclear leukocytes from bovine mammary glands with drugs from the same classes Ziv et al used (the beta-lactam drug dicloxacillin and the polymixin drug polymixin B) did not enhance viability of the cells or phagocytic activity when compared to control cells incubated with phosphate buffered saline. (Nickerson, Paape et al. 1985) In fact, alterations in normal cell morphology were noted in the cells incubated with polymixin B. Any benefit to mammary epithelial cell function or survival from these antimicrobial drugs that did not benefit other cells from the mammary gland, and may have damaged them, seems unlikely. It would be more reasonable to assume that the beneficial effect on milk production in the study by Ziv et al was due to the dexamethasone, as it had also demonstrated such benefit in an infectious mastitis model. It is important to note that in both of the studies in which dexamethasone had a beneficial effect on milk production in mastitic cows, the drug was not administered in response to the development of clinical signs; rather it was given either immediately after intramammary challenge (both studies) or at a set time

afterward (Ziv et al). This would be impossible to do in the case of a natural infection, as one cannot determine which cows have been infected until they manifest clinical signs. No experimental studies were found in which drugs were administered as they would be in practice, in response to the development of clinical signs. Several studies have been done using intramammary administration of glucocorticoid drugs to treat mastitis; however no studies located by this author reported measurement of milk production following such administration. (Hatfield, Harmon et al. 1984; Merminod and Schaellibaum 1985; Lohius, Leeuwen et al. 1989)

Only one previous study has investigated the effect of the steroidal drug isoflupredone acetate in cows with endotoxic mastitis. (Carroll, Schalm et al. 1965) This study, done more than 35 years ago, evaluated isoflupredone acetate as a treatment for mastitis induced by intramammary administration of the coliform bacteria *Aerobacter aerogenes*. The drug was given prior to the onset of clinical signs of mastitis by the intramuscular or intramammary route, with the intramammary dose totaling 500 mg and the intramuscular dose totaling 600 mg per cow. The current label dose is 10 to 20 mg per cow. This high dose was found to have depressant effects on milk production, just as a supra-therapeutic dose of dexamethasone did in a later study. (Anderson and Hunt 1989) Other clinical effects of treatment with isoflupredone were equivocal; there was “some possibility” that local edematous swelling was reduced in treated cows. Prior to the research reported herein, there had not been any published studies of isoflupredone acetate at the label dose as a therapy for endotoxic mastitis.

Part Three: Examination of Epithelial Cells in Milk

Much of the research investigating cells in cows' milk during and after endotoxic mastitis has been concerned with immune cells. It has been determined, for example, that the primary factor affecting the likelihood of recovery of a cow from coliform mastitis is the speed and magnitude of neutrophil recruitment to the infected mammary gland. (Hill 1981) The magnitude of the leukocyte response to endotoxic mastitis is reflected in a marked increase in the number and percentage of neutrophils in the mammary gland and milk during the acute phase of mastitis. (Paape, Wergin et al. 1981)

The percentage and total numbers of epithelial cells in milk have generally been considered to drop during the acute phase of mastitis as numbers of leukocytes, particularly neutrophils, increase dramatically. (Paape, Wergin et al. 1981) However, studies have also demonstrated epithelial cell sloughing into the milk as early as one hour following the administration of a very large number (10^9 colony forming units) of *Escherichia coli* into the mammary gland. (Frost, Hill et al. 1980) Microscopic examination of the mammary glands of cows administered this high dose of bacteria revealed epithelial necrosis and sloughing one hour following the administration of the bacteria, which worsened by two hours post-infection, and was followed by an influx of neutrophils. This work suggests that the observed increase in the neutrophil population in milk may be preceded by an increase in the population of epithelial cells in the milk as they are sloughed early in the onset of clinical mastitis. In later work by the same author, using more moderate doses

of *E. coli* (50 or 200 CFU), epithelial cell necrosis and sloughing took a longer time to develop, and was again followed by an "intense inflammatory response". (Frost, Hill et al. 1982) The lesions caused by epithelial necrosis and sloughing are a short-lived phenomenon; they may be repaired in as few as five hours by migration of remaining epithelial cells across the undamaged basement membrane. (Brooker, Hill et al. 1981)

As described earlier, dexamethasone has been observed to protect milk production in cows with endotoxic mastitis; such an effect has not been convincingly demonstrated for flunixin meglumine. No explanation for the protective effect of dexamethasone on milk production has been established. The anti-inflammatory or gluconeogenic functions of glucocorticoid drugs would be two obvious candidates for explaining how dexamethasone exerts its protective function on milk production during mastitis; however, there must also be some cause for the inhibition of milk production observed when dexamethasone is administered to cows without mastitis. (Hartmann and Kronfeld 1973; Andersson and Olsson 1984) Anti-inflammatory or gluconeogenic effects would seem extremely unlikely to cause decreased milk production in any circumstance. An hypothesis was conceived that would explain both the reported protective effects on milk production by glucocorticoids in mastitic cows and their detrimental effects on milk production in healthy cows. Glucocorticoid drugs inhibit Phospholipase A₂ and promote lipocortin production, both of which inhibit breakdown of the cell membrane into arachidonic acid. Glucocorticoid drugs also interfere with dissolution of connective tissue and cells, and they may stabilize lysosomal

membranes. (Ferguson and Hoenig 2001) If glucocorticoid drugs have these effects on the membranes of the secretory cells of the mammary epithelium, they would be expected to promote the milk production effects observed for dexamethasone in both healthy and mastitic cows. In healthy cows, stabilization of cell membranes would decrease milk secretion, as the apical membrane of the cell must be able to separate and form micelles during the milk secretion process. Glucose uptake might also be depressed by decreased function of the cell membrane. During mastitis, however, these effects of glucocorticoid drugs would be expected to be beneficial, as stabilization of lysosomal membranes and cell membranes might help secretory epithelial cells in the mammary gland survive the endotoxic insult encountered in mastitis. With the resolution of the mastitic episode and elimination of the drug, cows treated with dexamethasone, if this hypothesis is correct, would be expected to have more functional, undamaged secretory cells remaining in their mammary glands than untreated controls, and they would consequently have more robust milk production following the episode of mastitis.

In order to test the hypothesis that a glucocorticoid drug would increase mammary epithelial cell survival through an episode of mastitis, without slaughtering or performing biopsies on any of the cows to directly observe mammary epithelium, it was decided to observe numbers of epithelial cells in the milk before and after the administration of purified bacterial endotoxin. Cows had either no drug treatment, treatment with the NSAID flunixin meglumine, or treatment with the glucocorticoid drug isoflupredone acetate. If the glucocorticoid drug did increase survival of epithelial cells in endotoxin-induced mastitis, it could

reasonably be expected that cows treated with isoflupredone acetate would shed fewer epithelial cells in their milk during the acute phase of mastitis.

Epithelial cells in milk have been identified using a number of methods including light microscopy with Wright's stain or monoclonal antibody-linked stain, and transmission electron microscopy. (Paape and Miller 1988; Leitner, Shoshani et al. 2000) Paape's study indicated a good agreement between Wright's stain and anti-cytokeratin antibody linked stain in the number of cells identified as epithelial cells using light microscopy combined with staining. The study described in this dissertation identified mammary epithelial cells using a combination of anti-cytokeratin antibody linked fluorescent staining and flow cytometry, using a variation on previously described techniques for analysis of epithelial cells in cows' and goats' milk. (Barrington, Besser et al. 1997; Boutinaud, Rulquin et al. 2002) The technique used to preserve and stain milk samples is described in detail in Chapter 3. Following preservation and staining, milk cell samples were analyzed using a FACScan Flow Cytometer (Becton-Dickinson, Union, NJ).

Flow cytometry is a tool for analyzing the characteristics of cells in suspension as they flow past a point of observation. The cells to be analyzed are suspended in solution, which is introduced into the center of a wider diameter flow of sheath solution. This results in laminar flow of the solution past the observation point, with the cells passing by one at a time but very quickly. At the observation point, a laser beam is focused upon each cell. Optic lenses and computer software are used to measure and describe the forward scatter of the laser off the cells, or

the light that is scattered between approximately 0.5 and 10 degrees from the original direction of the laser beam. The intensity of this forward scatter light is measured and translated into an approximation of the diameter of the cell under examination. Another lens is placed perpendicularly to the light source and used to measure the intensity of perpendicular light scatter intensity, which is used to measure the quantity of granular structures within the cell. Fluorescence intensities may also be measured at several different wavelengths, to quantify the uptake of various fluorescent stains by the cell. Light scatter and fluorescence are measured simultaneously as each cell passes through the laser light beam. (Shapiro 1988) Examples of the forward scatter, side scatter, and fluorescence properties of the cells examined in the research described in this dissertation are given in Appendix 1.

The objectives of the studies reported in the following chapters were:

- 1) To describe the physiologic and milk production effects of the steroidal drug isoflupredone acetate on cows with endotoxin-induced mastitis, when the drug was given systemically at the label dose after the appearance of clinical signs;
- 2) To measure the changes in epithelial cell populations in the milk of cows with endotoxin-induced mastitis, and to evaluate whether such changes were affected by systemic treatment of the cows with anti-inflammatory drugs;

- 3) To compare the physiologic and milk production effects of the NSAID drug flunixin meglumine to the effects of the corticosteroid drug isoflupredone acetate in cows with endotoxin-induced mastitis.

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**CHAPTER 2. PHARMACODYNAMICS OF ISOFLUPREDONE ACETATE IN AN
ENDOTOXIN-INDUCED MASTITIS MODEL**

A Paper Accepted for Publication by the Journal of Dairy Science

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Abstract

The effect of intravenous administration of the steroidal drug isoflupredone acetate on lactating dairy cows with mastitis induced using gram-negative bacterial endotoxin was investigated. Cows were randomly assigned to one of four treatment groups: untreated controls, isoflupredone acetate only, mastitis only, and mastitis plus isoflupredone acetate. Isoflupredone acetate was given to treated groups at a dose of 20 mg intravenously, once. Mastitic cows receiving treatment were given isoflupredone acetate after the development of clinical signs. When compared with untreated mastitic controls, cows with endotoxin-induced mastitis treated with isoflupredone acetate did not exhibit measurable differences in heart rate, rectal temperature, rumen motility, or changes in mammary gland surface area in the 14 hours following the administration of intramammary endotoxin. Healthy cows treated with isoflupredone acetate had a higher heart rate over the 14 hours following drug administration than did untreated healthy controls. When compared with untreated mastitic controls, cows treated with isoflupredone acetate did not exhibit statistically significant differences in milk production following endotoxin-induced mastitis.

Introduction

Mastitis is a common affliction of modern dairy cows and a major cause of lost income in the dairy industry. Temporary or permanent decreases in milk production may result from inflammatory damage to the mammary gland caused by mastitis. Antimicrobial drugs can inhibit or destroy mastitis-causing bacteria, but antimicrobial drugs do not generally have any anti-inflammatory activity, thus they do not act directly to limit inflammatory damage to the mammary gland (Nickerson et al, 1986).

Researchers in the past have investigated whether therapy of gram-negative endotoxic mastitis with steroidal or non-steroidal anti-inflammatory drugs is beneficial. Typically, investigators have administered a variety of anti-inflammatory drugs concurrently with or shortly after the induction of mastitis using bacteria or purified bacterial endotoxin, but before the development of systemic clinical signs.

Unfortunately, in a clinical setting it is not possible to identify and treat mastitic cows prior to the development of clinical signs of mastitis. The goal of this project was to test the hypothesis that the steroidal drug isoflupredone acetate may limit milk production loss and reduce clinical signs in cows with endotoxin-induced mastitis when the drug is given parenterally after the development of clinical signs. In the United States, the steroidal drug isoflupredone acetate is labeled for the treatment of inflammatory conditions in dairy cows.

Materials and Methods

This protocol was approved by the Committee on Animal Care at Iowa State University.

Study Population

Thirty-two Holstein cows from the Iowa State University Dairy in Ankeny, Iowa, were used in this study. They were selected based on the following criteria: 30 to 70 days into second or greater lactation, no clinical mastitis in the current lactation, no drug treatment in the previous 30 days, and no abnormalities on physical examination.

Group Assignment and Randomization Procedures

Cows were randomly assigned to one of four treatment groups: untreated controls, drug administration only, mastitis only, and mastitis plus drug administration. Randomization was achieved by assigning a two digit random number from a random number list, read in sequence from a random starting point, to each treatment group on each day. Cows were then assigned to a treatment group by ranking each cow from lowest to highest farm identification number, then assigning them to the group with the randomly assigned number of the same rank. In order to minimize day effects on clinical outcomes, one cow from each of the four treatment groups was enrolled in the trial on each of eight trial days. Two of the 32 selected cows were removed from the study due to lameness on the day they were scheduled to participate, leaving a total of 30 cows enrolled in the study. Ultimately there were eight cows in each treated group and six cows in the control group, as both cows removed due to lameness had been assigned to the untreated control group. All clinical work for this project was conducted between September 2000 and April 2001.

Housing and Husbandry

On trial days, cows were kept in a small barn where they were accustomed to spending time while undergoing treatments and routine examinations such as pregnancy diagnosis. They had unlimited access to water and a total mixed ration composed primarily of corn silage and dry hay, formulated for lactating dairy cows. Cows were caught in headlocks for the hourly recording of clinical observations, but were allowed to roam freely in the barn at other times. The cows were milked at their normal 12-hour interval.

Induction of Mastitis

Cows assigned to treatment groups with mastitis had a negative bacterial culture 72 hours before the induction of mastitis and a negative California Mastitis Test in the treated quarter on the day of their participation the study. The California Mastitis Test on the other three quarters had either negative or trace reaction immediately before the induction of mastitis.

Mastitis was induced in the left front quarter of selected cows unless there was a trace positive California Mastitis Test in that quarter on the day of the study, in which case the right front quarter was used. Mastitis was induced in selected quarters by intramammary administration of 0.1 mg purified Lipopolysaccharide (LPS) from *Escherichia coli* B4:0111 (Sigma-Aldrich, St.Louis, MO) in 20 mL sterile isotonic saline solution.

Clinical mastitis was defined as measurable swelling of the affected mammary gland, defined as an increase above baseline measurements of mammary surface area, and a rise in rectal temperature of at least 1 °C above the baseline measurement taken prior to the administration of LPS. Cows that did not

meet these criteria for development of clinical mastitis following the infusion of LPS into a mammary gland were removed from the study.

Eighteen groups of cows were cultured in order to qualify the nine groups of cows enrolled in the study. One group of four cows was removed from the study on the day of participation because the cows in the groups given intramammary endotoxin did not develop clinical mastitis as defined in this study.

Clinical Variables

Clinical variables recorded hourly for all treatment groups included rectal temperature by electronic thermometer, heart rate by auscultation for 15 seconds with a stethoscope, and number of rumen contractions per two minutes by auscultation for two minutes with a stethoscope. Cows in groups with mastitis also had mammary skin surface area measured hourly. This was done using a modification of the technique of making two indelible marks on the udder skin and measuring the distance between the marks using calipers, as described by Bywater et al (1988). In this study, four indelible marks were made on the udder skin using a rectangular plastic template measuring 100 mm by 120 mm, and the distance between the marks for each side of the rectangle and both diagonals were measured using a flexible plastic ruler. Total area of the rectangle at each time point was calculated as follows: The rectangular area being measured was divided by the two diagonals into four triangles, and the area of each triangle was calculated based on the length of its three sides using Heron's Formula, which states that the area of a triangle with sides of lengths a, b, and c is equal to

$\sqrt{s(s-a)(s-b)(s-c)}$, where s equals $\frac{a+b+c}{2}$. The areas of the four triangles were

summed to total the overall rectangular surface area. This method allowed surface

area measurements to accurately reflect expansion that occurred outward from the initial plane of measurement, in addition to expansion within the two dimensions of the created rectangle.

Study Design

One cow from each treatment group was enrolled in the study on each trial day, in order to minimize day effects on outcome variables.

Beginning immediately after morning milking, each cow was examined and had baseline clinical observation values recorded. Cows in groups with endotoxin-induced mastitis were prepared for udder surface area measurement by clipping the hair of the mammary gland quarter to be treated and placing four black marks on that quarter using a plastic template and a permanent marker. The four experimental groups were then treated as follows (Figure 1):

Cows in the untreated control group had clinical variables recorded for 14 more hourly observations (15 total observations including baseline).

Cows in the group receiving only drug treatment were given 20 mg isoflupredone acetate (Predef 2X, Pharmacia Animal Health, Kalamazoo, MI) one time by jugular venipuncture soon after the recording of baseline observations. Clinical variables were recorded for 14 more hourly observations. Cows in this group also had nine blood samples taken by jugular venipuncture over the next 24 hours for a concurrent pharmacokinetic study.

Cows in the group with endotoxin-induced mastitis but no drug treatment had LPS infused into one quarter shortly after the recording of baseline variables. Mammary surface area measurements and other clinical variables were recorded for 14 more hourly observations.

Cows in the group receiving drug treatment following the development of clinical mastitis had LPS infused into one quarter shortly after the recording of baseline variables. Cows that subsequently developed clinical signs of mastitis as defined above received 20 mg of isoflupredone acetate one time by jugular venipuncture as soon as possible after the hourly observation at which they were noted to have developed clinical mastitis. Observations were continued for at least 12 hours following the establishment of clinical mastitis. This group also had nine blood samples collected by jugular venipuncture over a 24-hour period for a concurrent pharmacokinetic study.

Milk Production

Milk weight data used in this study were collected electronically. Each cow in the study wore a computer chip bearing her farm identification number on a neck strap. This identification number was read by a scanner as each cow entered the milking parlor and used to record the cow's milk production at each milking. Cows enrolled in this study had twice-daily milk weights recorded for the week prior and three weeks following the day of participation in the study.

Statistics

All statistical analyses for this study were performed using the JMP statistical software program (SAS Institute, Cary, North Carolina). The study design provided a power of greater than 0.80 to detect a one-day difference of 10 pounds in daily milk weight between groups. Analysis of baseline clinical variables was performed using analysis of variance. Subsequent clinical variables and four-day baseline milk production data were analyzed using multivariate analysis of variance, which accounts for repeated measurements over time. Statistical

significance was designated a priori as a *P* value less than or equal to 0.05.

Baseline clinical observations were considered covariates for statistical analysis of clinical variables following treatment, in order to compensate for variation in mean baseline measurements between groups.

Initial analysis of milk production during the recovery period using multivariate analysis of variance for repeated measures revealed an interaction between time and treatment. Further statistical analysis was therefore done using analysis of variance separately for each day of the recovery period. Dunnett's method was used to correct the tendency toward type 1 error when comparing treatment groups individually to the control group.

Due to failure of the scanner at the milking parlor entrance to read the cow's identification number, milk weight data were not recorded for every cow at each time point used in the data analysis. Missing milk weights were replaced with an average of the previous and subsequent milk weights. For this herd, the bias introduced by using morning weights to estimate evening weights and vice versa is minimal, as mean morning and evening milking weights for all cows in this study, calculated using only observed weights, varied by less than one percent. One cow from the untreated mastitis group was missing all but one milk weight measurement, and one cow from the same group was missing more than half of the milk weights to be used for analysis. These two cows were removed from statistical analysis of milk production, but their clinical observation data were complete and were used in analysis of treatment effects. Among cows with milk weight data used for statistical analysis, one cow from the control group (no mastitis or drug treatment) was missing 12 of the 24 observations used. Five cows were missing

four to six data points, three cows were missing three data points, seven cows were missing two data points, four cows were missing one data point, and eight cows had milk weights for all time points used in the statistical analysis. The replacement of missing milk weights with the average of previous and subsequent weights may reduce the “within-cow” variation in the data, which may increase the likelihood of differences between treatment groups being found to be statistically significant.

Results

Rumen contractions (Figure 2)

For all groups, baseline observations were not significantly different ($P = 0.8556$). Both drug treated ($P = 0.0413$) and untreated ($P < 0.01$) cows with mastitis had significantly decreased rumen motility over time as compared to the control group. Drug treatment did not significantly effect the magnitude of rumen motility depression in mastitic cows ($P = 0.27$). The group of cows receiving drug treatment alone did not have decreased rumen motility as compared to the untreated control group ($P = 0.4105$).

Heart rate (Figure 3)

Baseline heart rate for the four groups was not different ($P = 0.8611$). Cows receiving only drug treatment had a higher heart rate over time ($P < 0.01$) than untreated controls. Groups with mastitis had the same mean heart rate over time regardless of whether they were treated with isoflupredone acetate or not ($P = 0.4524$). Both mastitic groups had higher mean heart rates than the group receiving only the drug (mastitis plus drug treatment, $P = 0.0245$, mastitis only $P =$

0.0112) and higher mean heart rates than the untreated control group ($P < 0.01$ for both mastitic groups).

Rectal Temperature (Figure 4)

Mean baseline temperatures for all groups were not significantly different ($P = 0.6674$). Temperature changes over time were the same for all cows with mastitis ($P = 0.5307$) and for all cows without mastitis ($P = 0.7663$), regardless of drug treatment status. Mastitic cows with or without drug treatment had higher mean temperatures over time than did control cows or cows receiving only drug treatment ($P < 0.01$ for all groups).

Mammary Surface Area (Figure 5)

Changes in mammary surface area over the 14 hours following the introduction of LPS into the mammary gland were not different for drug treated and untreated mastitic cows ($P = 0.097$).

Milk Production (Figure 6)

Mean baseline milk production for the four days preceding the initiation of the trial was not different between treatment groups ($P = 0.5544$).

There were no statistically significant differences in milk weights between any groups for the day of endotoxin challenge and drug administration ($P = 0.0858$). On the following day, both treatment groups with mastitis had lower milk production than the control group ($P < 0.05$). By the second day following the induction of mastitis, there were once again no statistically significant differences in milk production between the groups ($P > 0.05$). For the drug treated group without mastitis, there was no significant difference in milk production when compared to the control group at any time point ($P > 0.05$).

Discussion

Previous work investigating the effect of anti-inflammatory steroid drugs in cows with endotoxic mastitis has largely been focused on the effect of drug administration prior to or concurrent with the induction of mastitis. Carroll et al (1965) gave 9-alpha fluoroprednisolone acetate (also called isoflupredone acetate) using a variety of regimens, then induced mastitis using *Aerobacter aerogenes* bacteria introduced into the mammary gland. In that study, doses of 50 mg and 100 mg of 9-alpha fluoroprednisolone were given once intramuscularly, 250 mg was given twice intramuscularly at a ten-hour interval, 200 mg was given thrice intramuscularly at 12-hour intervals, or 500 mg was given by the intramammary route into each front quarter once. Single dose regimens were administered concurrently with the intramammary administration of bacteria, while multiple dose regimens had the final dose administered concurrently with the induction of mastitis. Nine-alpha fluoroprednisolone decreased mammary swelling following the induction of mastitis and caused decreased milk production prior to the induction of mastitis in multiple-dose regimens. Milk production beyond 48 hours following the mastitic episode was not recorded, but might not be relevant to today's high producing dairy cows in any case; the cows enrolled in this study 37 years ago were producing between 25 and 45 pounds of milk per day, much less than typical modern dairy cows.

Administration of 30 mg dexamethasone intramuscularly to cows concurrently with the induction of mastitis using *Escherichia coli* bacteria affected physiological parameters and milk production in treated cows (Lohuis et al, 1988).

When compared to saline-treated controls, cows receiving dexamethasone had reduced neutropenia, reduced local signs of inflammation, diminished decreases in rumen amplitude, and increased rectal temperature during the acute phase of mastitis. Milk production over the 14 days following induction of mastitis was markedly increased in cows receiving dexamethasone treatment, when compared to saline-treated controls. Dexamethasone also decreased the shift in milk production ratio from rear to front quarters in this study in which all cows were infected in a rear quarter. This suggests that the drug directly affected the mastitic gland, rather than facilitating a shift in production to the unaffected glands.

Further work by Lohuis et al (1989) examined three different steroidal drugs. Flumethasone at a dose of 5mg and dexamethasone at a dose of 30 mg were given intramuscularly at the same time as induction of mastitis using purified bacterial endotoxin. Prednisolone was given at a dose of 40 mg by the intramammary route at zero, two or four hours following the administration of endotoxin. Intramammary administration of prednisolone two hours after administration of endotoxin abolished the febrile response and diminished the endotoxin-induced rise in heart rate. Cows given intramuscular steroid drugs in this experiment had less mammary swelling, smaller increase in rectal temperature, smaller increase in heart rate, and less severe neutrophilia and lymphopenia when compared to untreated mastitic controls. Milk production effects were not reported.

An endotoxin-induced mastitis model was used to investigate the effect of a very high intravenous dose of 0.44 mg/kg dexamethasone; for a typical 500 kg dairy cow this would be 220 mg, more than seven times the dose used by Lohuis et

al (Anderson, KL and Hunt, E, 1989). Dexamethasone was administered two hours after the introduction of endotoxin into the mammary gland. It was stated that there was clinically detectable mammary swelling at the time of drug administration, but not that there were detectable systemic abnormalities at that time. Cows given dexamethasone in this study did not achieve mean rectal temperatures above 39.2 °C, in marked contrast to the increased rectal temperatures observed in cows receiving a much lower dose of dexamethasone in the previously mentioned study.

The purpose of the study reported here was to investigate whether a moderate dose of the steroidal drug isoflupredone acetate could ameliorate milk production losses and clinical signs when used as it might be used in a clinical setting- at the approved label dose, in a cow with systemic signs of illness. Isoflupredone acetate does not have a labeled indication for intravenous administration, but such use is not prohibited. The results obtained when the drug was given after the development of clinical signs were not entirely consistent with the results of the earlier studies, which generally administered steroid drugs prior to the development of clinical signs. Unlike previous studies, inhibition of rumen motility (measured in this study using the crude method of auscultation) and increased mammary gland surface area were not alleviated. Isoflupredone acetate also did not affect febrile changes in rectal temperature in treated cows with mastitis; it neither increased the magnitude of change as a moderate dose of dexamethasone had done previously, nor decreased the magnitude of change as a large dose of dexamethasone had done. Isoflupredone did not decrease heart rate in treated cows with mastitis as compared to untreated cows with mastitis. An

unanticipated finding in this study was that cows receiving drug treatment only, without mastitis, had a higher heart rate over the observation period than did untreated controls. This may be partly or entirely due to stress, as the cows receiving drug treatment had jugular venipuncture performed eight times during the 14-hour observation period for a concurrent pharmacokinetic study.

Isoflupredone acetate did not affect milk production in mastitic or healthy cows in this study. Mastitic cows with or without drug treatment had significantly decreased milk production for one day following the induction of mastitis. This drop in mean production was approximately 30 percent of baseline for both groups on the first day after the trial, and it had rebounded to approximately a ten percent loss for both groups by the next day. This is a considerably milder effect than was achieved with live bacterial model used by Lohuis et al in 1988. In that study, the induction of mastitis using live *Escherichia coli* bacteria resulted in milk production being 48 to 100% reduced in untreated cattle fourteen days after the initiation of mastitis. Because the model used in the current study caused only mild and transient milk production loss, drug treatment effects would have to be very strong or study power (cow numbers) would have to be quite high to detect an effect of drug treatment on milk production. Isoflupredone acetate has less than half the anti-inflammatory potency of dexamethasone (Langston, 1993). The unfortunate loss of all data for two cows due to lameness and milk production data for two cows due to computer malfunction reduced the power of this study to detect any significant treatment effects.

In conclusion, the data from this study suggest that the steroidal drug isoflupredone acetate, when administered at the label dose after the onset of

clinical signs in an endotoxin-induced mastitis model, does not affect the course of clinical signs in the acute phase of mastitis or subsequent milk production.

Acknowledgements

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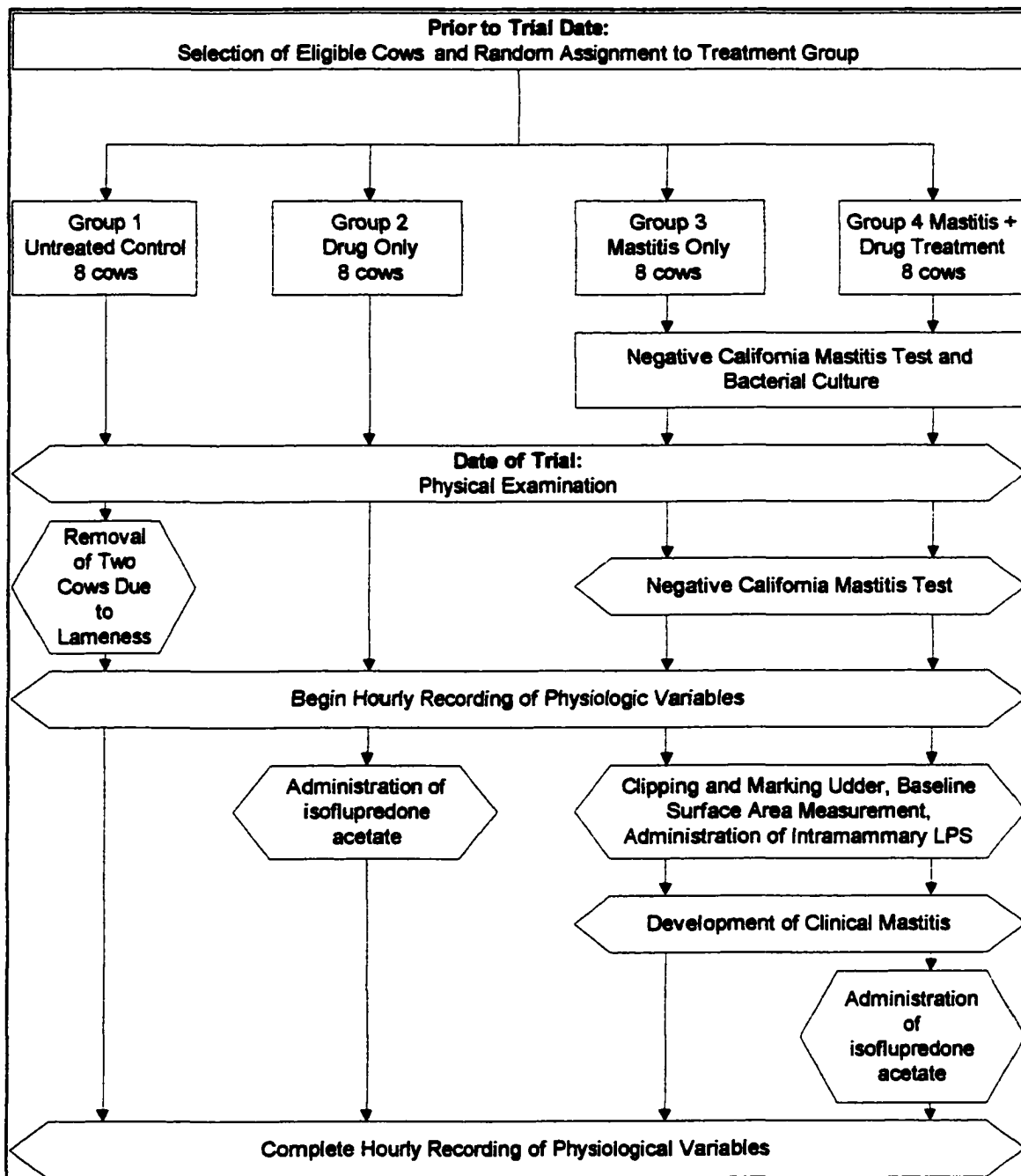


Figure 1.1 Flowchart of Study Procedures

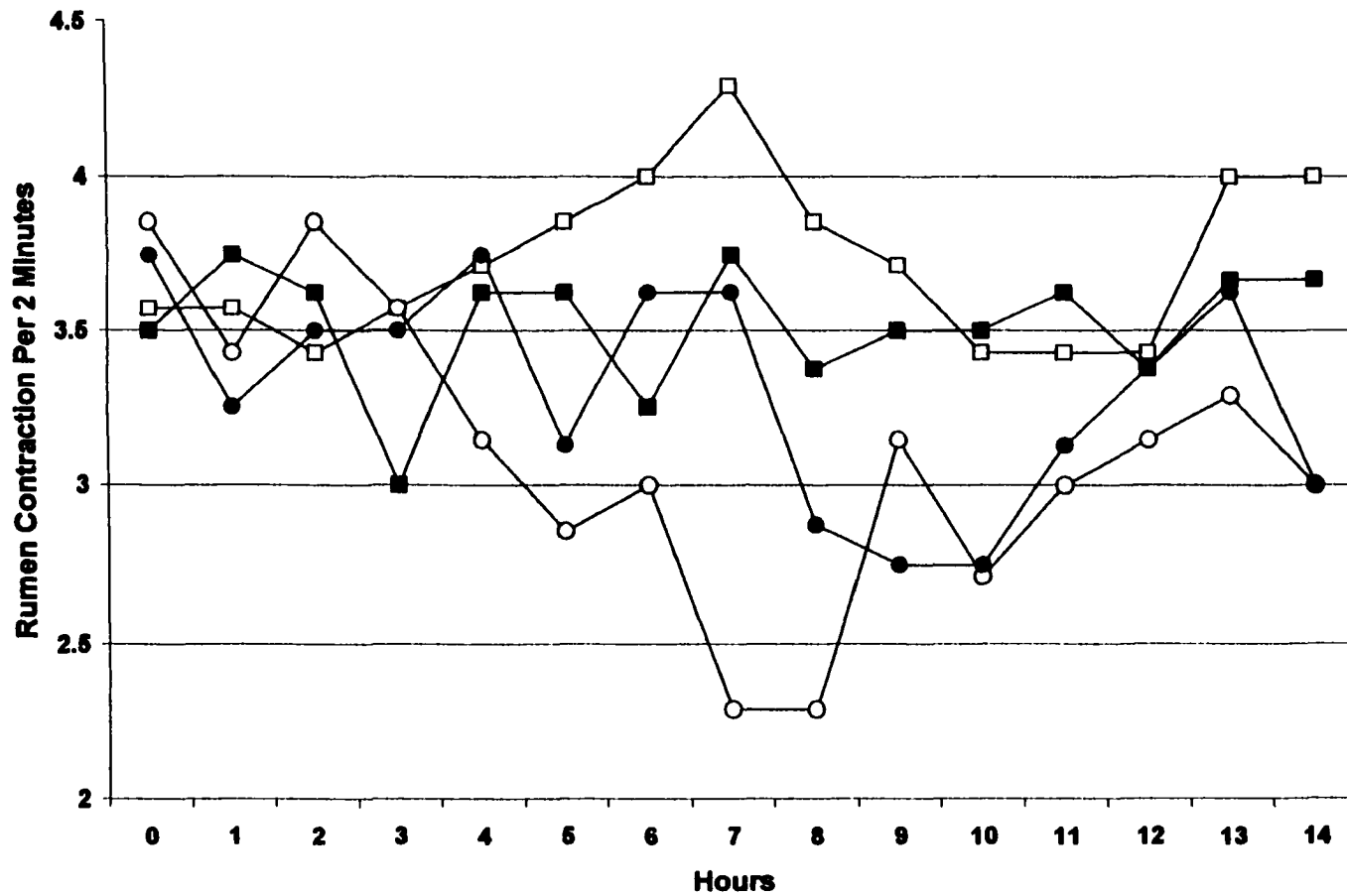


Figure 1.2 Effects of Various Treatments on Rumen Motility. Hour 1 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: □ Untreated Control; ■ Drug Treatment Only; ○ Untreated Endotoxin-Induced Mastitis; ● Endotoxin-Induced Mastitis and Drug Treatment

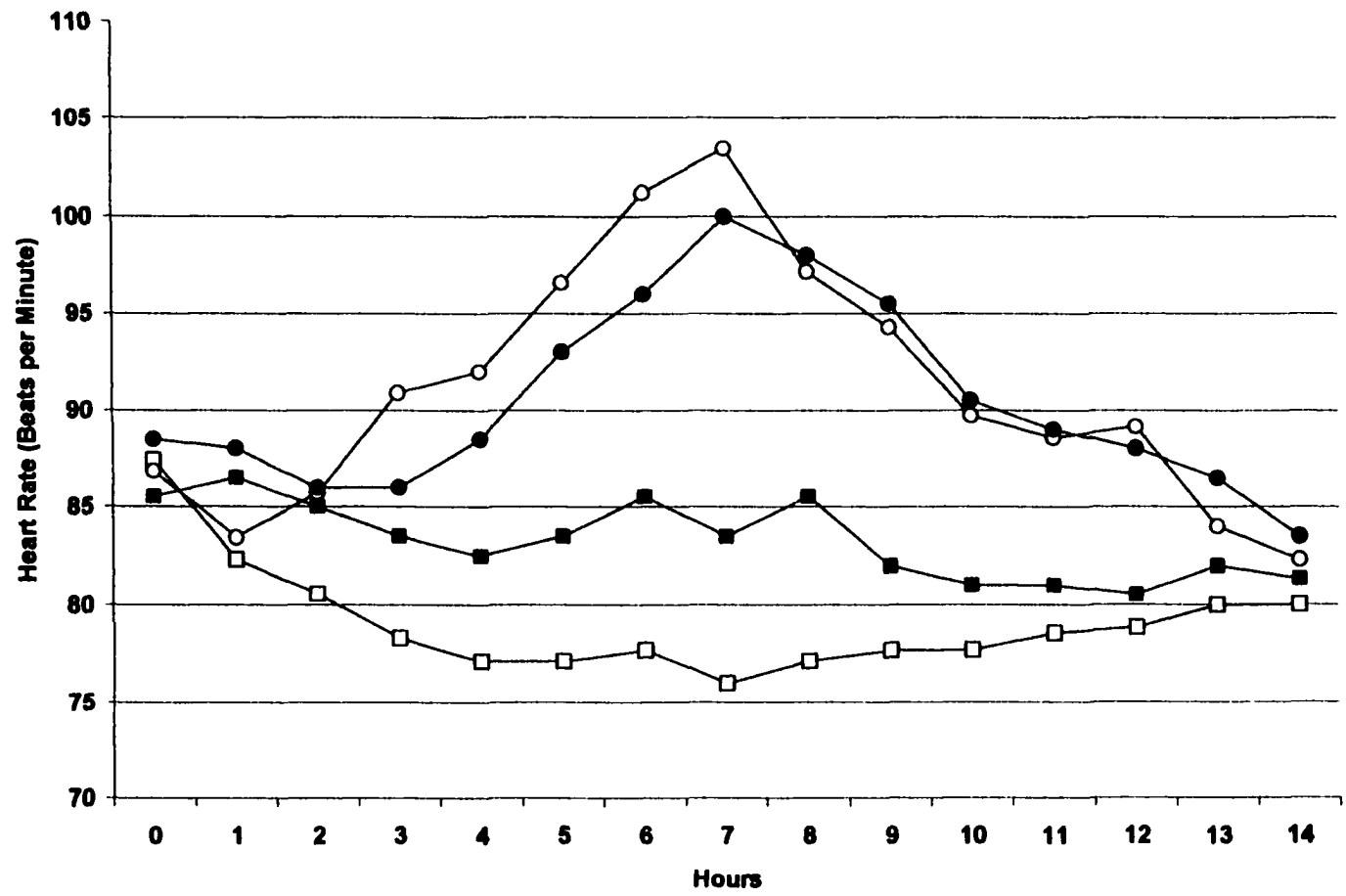


Figure 1.3 Effects of Various Treatments on Heart Rate. Hour 1 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: □ Untreated Control; ■ Drug Treatment Only; ○ Untreated Endotoxin-Induced Mastitis; ● Endotoxin-Induced Mastitis and Drug Treatment

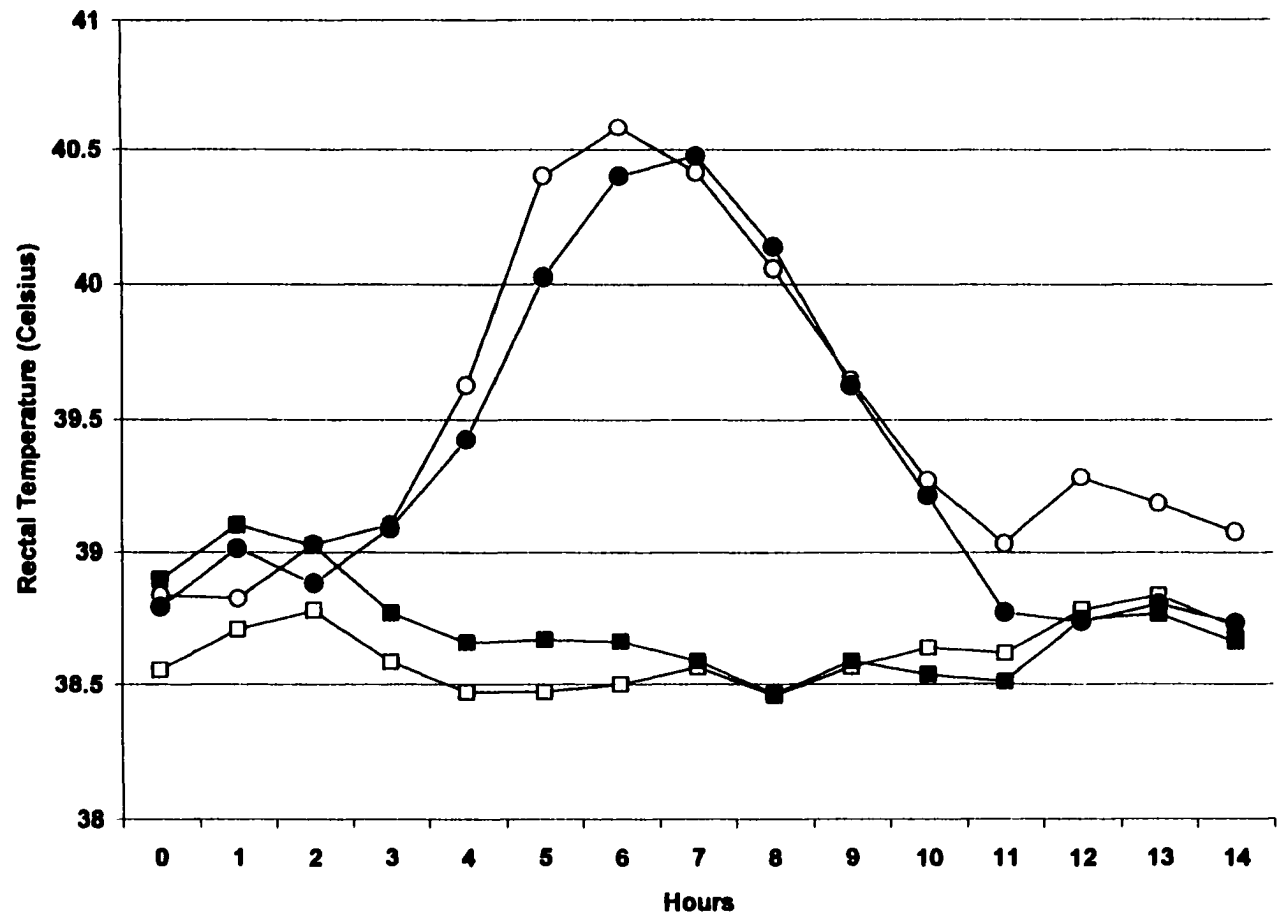


Figure 1.4 Effects of Various Treatments on Rectal Temperature. Hour 1 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: □ Untreated Control; ■ Drug Treatment Only; ○ Untreated Endotoxin-Induced Mastitis; ● Endotoxin-Induced Mastitis and Drug Treatment

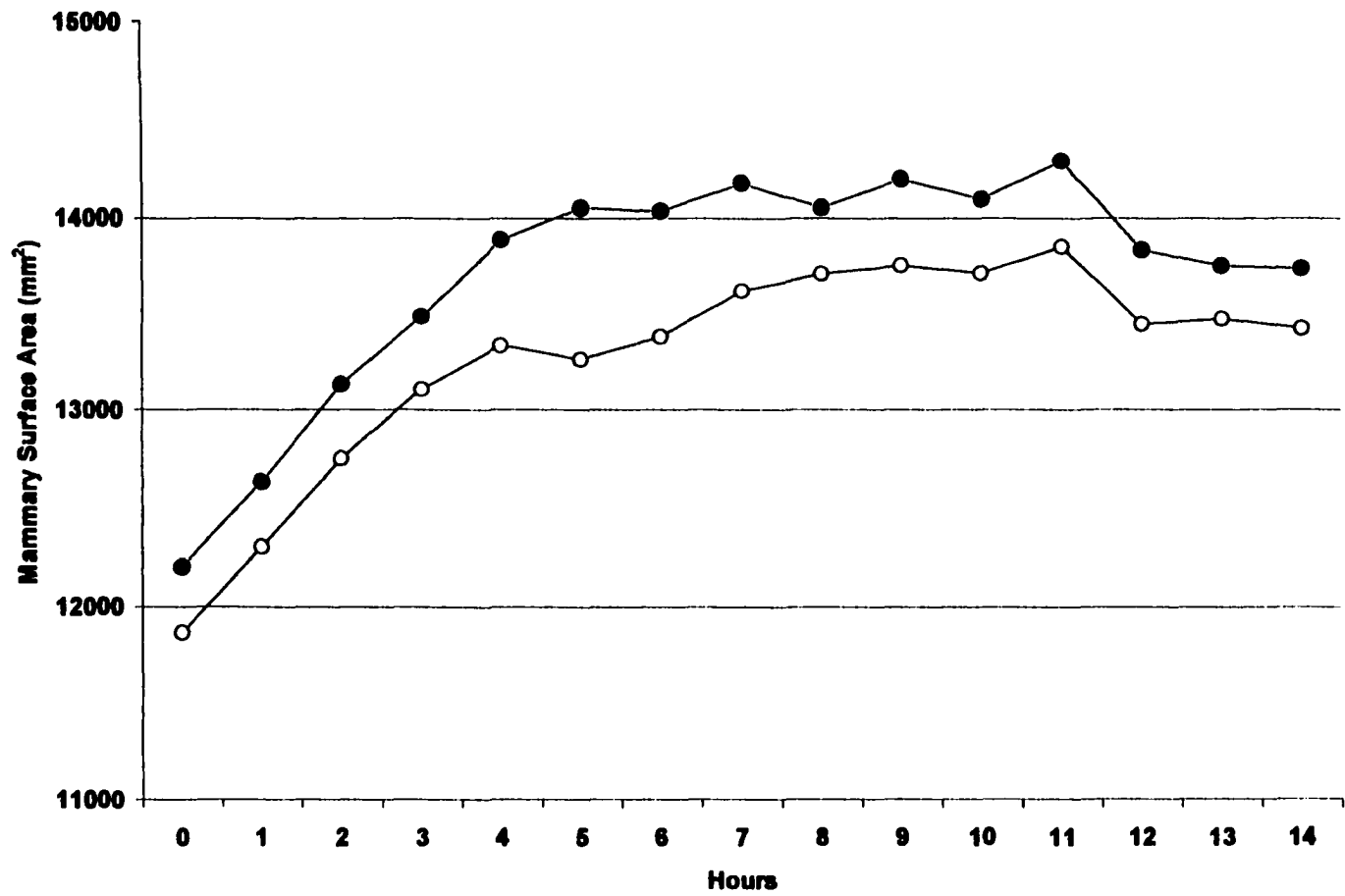


Figure 1.5 Effects of Various Treatments on Mammary Swelling. Hour 1 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: □ Untreated Control; ■ Drug Treatment Only; ○ Untreated Endotoxin-Induced Mastitis; ● Endotoxin-Induced Mastitis and Drug Treatment

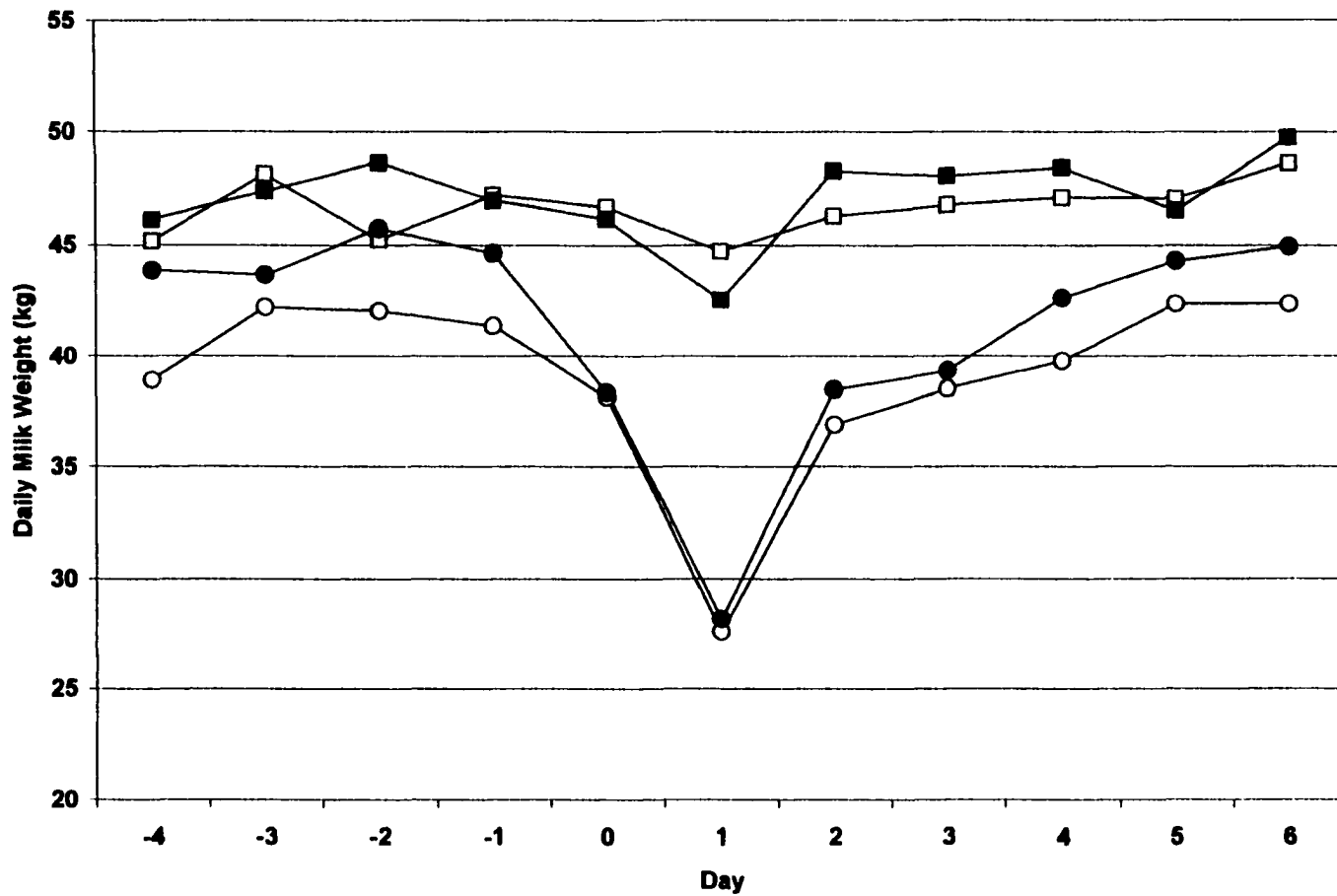


Figure 1.6 Effects of Various Treatments on Milk Production. Treatments Were Administered on Day 0. Each Value represents the Group Mean. Legend: □ Untreated Control; ■ Drug Treatment Only; ○ Untreated Endotoxin-Induced Mastitis; ● Endotoxin-Induced Mastitis and Drug Treatment

**CHAPTER 3. FLOW CYTOMETRIC ANALYSIS OF CYTOKERATIN-POSITIVE
CELLS IN MILK FOLLOWING ENDOTOXIN-INDUCED MASTITIS**

A Paper Submitted to the Journal of Dairy Science

S. A. Wagner, D.E. Jones, and M. D. Apley

Abstract

Somatic Cell Counts and flow cytometric analysis of cytokeratin-positive cells in the milk of lactating Holstein cows were performed before and 12 hours after the induction of mastitis using purified bacterial endotoxin. Epithelial cells are the only cells in bovine milk that are expected to be cytokeratin-positive. Prior to the induction of mastitis, the mean percentage of cells that were cytokeratin-positive was 4.18%, and the mean total number of cytokeratin-positive cells per milliliter of milk was 7,711. Twelve hours after the induction of mastitis, cytokeratin-positive cell percentages and numbers in bovine milk were significantly increased; the mean percentage of cytokeratin-positive cells was 40.63 %, and the mean number of cytokeratin-positive cells per milliliter of milk was 2,954,554. Treatment with steroidal or non-steroidal anti-inflammatory drugs after the development of clinical signs of mastitis did not mitigate the increase in somatic cell count or cytokeratin-positive cells in milk in the first 12 hours following endotoxin administration.

Text

Epithelial cells are considered to be a normal component of milk from cows without mastitis; estimates of the prevalence of epithelial cells as a percentage of

total somatic cells in normal milk have varied from 0 to 65%. When a cow develops endotoxic mastitis, epithelial cells in the mammary gland are damaged and may be sloughed into the milk (Frost and Hill, 1982). It was hypothesized that treatment of cows with anti-inflammatory drugs following the development of clinical endotoxic mastitis might result in decreased damage to epithelial cells in the mammary gland, resulting in a decreased number of epithelial cells sloughed into the milk during the acute phase of mastitis.

Twelve non-pregnant cows between 30 and 60 days in milk in their second or greater lactation were selected for use in this study based on a history of no mastitis in the current lactation, no drug treatment in the previous 30 days, negative California Mastitis Test on all four quarters, and negative bacterial culture on the front 2 quarters. Milk samples for analysis of Somatic Cell Count and cytokeratin-positive cells were collected at the morning milking, and again just prior to the next milking 12 hours later.

Endotoxic mastitis was induced in one front quarter of each cow as soon as possible after the morning milking by intramammary administration of 100 µg purified lipopolysachharide (LPS) from *Escherichia coli* strain 0111:B4 (Sigma-Aldrich, St. Louis, MO) in 20 ml sterile isotonic saline solution.

Each cow was randomly assigned to a treatment group. No drug treatment was administered to the control group, flunixin meglumine (Banamine®, Schering-Plough, Union, NJ) at a dose of 2.2 mg/kg was administered to the second group, and isoflupredone acetate (Predef® 2x, Pharmacia Animal Health, Kalamazoo, MI) at a dose of 20 mg was administered to the third group. Drug treatments were administered intravenously by jugular venipuncture as soon as possible after the

development of clinical mastitis, which was defined as measurable mammary swelling combined with a rectal temperature of at least 40° Celsius. Clinical mastitis developed between 4 and 7 hours following LPS administration in all cows.

Milk samples for Somatic Cell Count were preserved using bronopol and sent by mail to the Iowa Dairy Herd Improvement Association laboratory in Dubuque, Iowa, for analysis using a Fossomatic 400 cell counter. Analysis of cytokeratin-positive cells was performed at the College of Veterinary Medicine at Iowa State University. On the day of collection, samples were washed with phosphate buffered saline solution with 0.1% bovine serum albumin and 0.1% sodium azide, to remove milk fat and whey. These cell samples were then preserved using 1 % paraformaldehyde and refrigerated until staining and flow cytometric analysis, which was performed within 48 hours. Just before flow cytometric analysis, intracellular staining for cytokeratin was performed by incubating the samples with a 0.1% saponin solution and FITC-conjugated monoclonal mouse anti-human cytokeratin IgG. Cytokeratin is considered to be a marker of epithelial cells in bovine milk (Paape and Miller, 1988). Each sample incubated with anti-cytokeratin IgG had a paired isotype control sample incubated with FITC-conjugated mouse IgG. Flow cytometric analysis of forward scatter, side scatter and fluorescent staining properties of milk samples was performed using a FACScan Flow Cytometer (Becton-Dickinson, Franklin Lakes, NJ). Percentage of cytokeratin-positive cells in each sample was multiplied by the total Somatic Cell Count in order to calculate the total number of cytokeratin-positive cells per milliliter in each milk sample. Data from two cows were discarded from cell analysis because their initial Somatic Cell Count was greater than 500,000, so

their mammary glands could not be assumed to be free of inflammation prior to the administration of LPS. Cell analysis results are presented in Table 1.

The mean percentage of cells that stained positive for cytokeratin prior to the induction of mastitis was 4.177%. Mean Somatic Cell Count prior to mastitis induction was 185,700 cells/ml. The calculated mean number of cytokeratin-positive cells per milliliter of milk prior to induction of mastitis was 7711. The percentage of cytokeratin-positive cells found in normal milk in this study was within the range of values previously reported for epithelial cells in normal milk. Paape and Miller (1988) reported a mean value of 16.5% epithelial cells in normal milk using Wright's stain and light microscopy, and 17.5% using cytokeratin monoclonal antibody staining and light microscopy, while Lee, Wooding and Kemp (1979) reported values ranging from 0% to 7% epithelial cells in normal milk using electron microscopy.

Changes in cell populations in the milk in the first 12 hours following the administration of LPS were significant. Except for one cow in the isoflupredone acetate-treated group, Somatic Cell Count and total number of cytokeratin-positive cells per ml were increased in all cows. Mean Somatic Cell Count increased to 6,332,600 cells/mL, and the percentage of cytokeratin-positive cells increased to 40.6%. The mean total number of cytokeratin-positive cells per milliliter of milk after LPS administration was 2,954,554. These findings are at odds with previous work that describes a drop in percentage of epithelial cells in mastitic milk as a result of a large influx of neutrophils (Miller et al, 1991). The amount of time that had elapsed between the onset of infection and the sampling of milk was unknown in the study by Miller et al, and may have been as long as six days. The work of

Frost and Hill (1982), which focused on acute changes to the mammary epithelium 10 or 14 hours after the intramammary administration of *Escherichia coli*, is congruent with the findings of this study. Frost and Hill found that ten hours following the introduction of 50 or 200 *Escherichia coli* organisms into the mammary gland, microscopic examination revealed only mild changes in the epithelial layer, but 14 hours post-infection there were extensive areas where mammary epithelium had been completely denuded and the basement membrane was exposed. These desquamated areas were more prevalent lower in the gland, with damage concentrated in the teat sinus, less epithelium lost from the lactiferous sinuses and little apparent damage to the glandular tissue.

The behavior of epithelial cells in the current study is consistent with the observation of extensive damage to mammary epithelium following administration of *Escherichia coli* into the mammary gland. Damage to the epithelium could be expected to occur more quickly with the direct administration of LPS than with the administration of 50 or 200 endotoxin-producing bacteria, as it would take time for the bacteria to multiply to high enough numbers to produce damaging amounts of endotoxin. The high numbers of cytokeratin-positive cells in milk obtained 12 hours following the administration of endotoxin are likely a result of extensive epithelial cell damage and sloughing. Observation of this effect may have been enhanced in the current study, as post-mastitis milk samples were obtained by stripping a small amount of milk onto the floor, then milking the sample for analysis by hand. This may have resulted in the post-endotoxin milk samples containing primarily milk from the more greatly affected sinus areas of the gland, and very little or no milk from higher up in the less-damaged alveoli of the gland.

No effects of drug treatment on the magnitude of change in somatic cell counts or percentage of cytokeratin-positive cells in the milk were identified using the student's t-test for matched pairs in the JMP software program (SAS Institute, Cary, NC).

In summary, in the first 12 hours following the administration of LPS into the mammary gland, the percentage and total number of cytokeratin-positive cells in milk increased dramatically. This finding is consistent with severe damage and sloughing of epithelium in the mammary gland early in the course of endotoxic mastitis. Treatment with flunixin meglumine or isoflupredone acetate following the development of clinical signs of mastitis did not limit the magnitude of the increase in cytokeratin-positive cells in the milk. This lack of treatment effect may be associated with the drugs not being given until 4 to 7 hours following the intramammary administration of endotoxin, when clinical signs were detected. Future research may be directed at determining when in the course of endotoxic mastitis relative numbers of neutrophils begin to exceed relative numbers of cytokeratin-positive cells milk, and how sloughing of epithelial cells from the mammary gland may affect the course of the disease and subsequent milk production.

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<u>Cow ID</u>	<u>Treatment Group</u>	<u>Before LPS Administration</u>			<u>After LPS Administration</u>			<u>Net Change</u>
		<u>Cytokeratin Positive (% of cells)</u>	<u>Somatic Cell Count (x 10³)</u>	<u>Cytokeratin Positive (cells/mL)</u>	<u>Cytokeratin Positive (% of cells)</u>	<u>Somatic Cell Count (x 10³)</u>	<u>Cytokeratin Positive (cells/mL)</u>	<u>Cytokeratin Positive (cells/mL)</u>
4830	Control	2.25	38	855	4.27	582	24851	+23,996
4920	Control	7.22	293	21,155	37.28	9421	3512149	+3,490,994
5319	Control	3.61	284	10,252	88.35	5004	4421034	+4,410,782
5301	Control	1.62	159	2576	28.29	4632	1310393	+1,307,817
4599	Flunixin	12.47	20	2494	1.25	12,794	159925	+157,431
5051	Flunixin	7.39	348	25,717	68.19	4809	3279257	+3,253,540
4924	Isoflupredone	1.47	17	250	68.45	11,036	7554142	+7,553,892
5294	Isoflupredone	2.12	231	4897	2.69	69	1856	-3041
4985	Isoflupredone	1.7	25	425	91.68	9108	8350214	+8,349,789
5307	Isoflupredone	1.92	442	8486	15.87	5871	931728	+923,241
	Mean	4.177	185.7	7711	40.632	6332.6	2954555	+2,946,844
	Standard Error of the Mean	1.05	44.67	2575	10.23	1208.33	877,719	877,792

Table 2.1 Results of Somatic Cell Count and Flow Cytometric Analysis of Milk Samples Before and After the Administration of Intramammary Lipopolysaccharide (LPS)

**CHAPTER 4. PHYSIOLOGIC AND MILK PRODUCTION EFFECTS OF TWO ANTI-
INFLAMMATORY DRUGS IN COWS WITH ENDOTOXIN-INDUCED MASTITIS**

A Paper Submitted to the Journal of Dairy Science

S. A. Wagner, M. D. Apley

Abstract

The effects of two commonly used anti-inflammatory drugs were investigated in lactating Holstein dairy cows with mastitis induced by the administration of purified bacterial endotoxin. Flunixin meglumine, a non-steroidal anti-inflammatory drug, or isoflupredone acetate, a steroidal anti-inflammatory drug, were administered to cows following the development of mammary swelling in the affected gland and a rectal temperature of 40 °C or greater. Heart rate, rectal temperature, and rumen motility were recorded hourly for 14 hours following the intramammary administration of endotoxin. Milk production was evaluated for five days before and ten days following the day of mastitis induction and drug treatment. Neither drug decreased milk production loss nor mammary surface swelling when compared to untreated mastitic controls. Both drugs reduced the mean heart rate in treated groups over the 14 hours following endotoxin administration, when compared to untreated mastitic controls. Animals treated with flunixin meglumine also had increased rumen motility and decreased rectal temperature over the same time period, when compared to untreated mastitic controls or mastitic cows treated with isoflupredone acetate. This study suggests that both drugs may provide some relief from the pain of mastitis. Until it is known whether relief of fever is beneficial or detrimental in naturally occurring cases of

infectious mastitis, it is not possible to infer that one agent would be superior to the other in the treatment of such cases.

Introduction

Mastitis is the most costly disease on modern dairy farms. In addition to the expenses of treatment and discarded milk, income is lost because inflammatory damage to the mammary gland may temporarily or permanently reduce milk production. Limiting inflammation in the mammary gland during an episode of clinical mastitis might also limit damage to the gland and preserve milk production.

Previous work has demonstrated that anti-inflammatory drugs may ameliorate milk production loss in cows with mastitis induced by the intramammary administration of *Escherichia coli* bacteria or bacterial endotoxin, when the drugs are administered prior to the development of clinical signs (Harmon, Shuster et al. 1985; Lohuis, Leeuwen et al. 1988). Unfortunately, in practice it is not possible to anticipate mastitis and administer anti-inflammatory therapy prior to the development of clinical signs. The study reported here compared the effects of two commonly used anti-inflammatory drugs on milk production and clinical signs when they were administered to lactating dairy cows after the development of clinical signs of mastitis resulting from the intramammary administration of purified bacterial endotoxin.

Materials and Methods

This protocol was approved by the Committee on Animal Care at Iowa State University.

Study Population

Thirty Holstein cows from the Iowa State University Dairy in Ankeny, Iowa, were selected for use in this study. They were selected based on the following criteria: 30 to 60 days into second or greater lactation, no clinical mastitis in the current lactation, no drug treatment in the previous 30 days, and no abnormalities on physical examination.

Group Assignment and Randomization Procedures

Cows were randomly assigned to one of three treatment groups: mastitis with no drug treatment, mastitis treated with isoflupredone acetate (Predef 2x™, Pharmacia Animal Health, Kalamazoo, Michigan) or mastitis treated with flunixin meglumine (Banamine®, Schering-Plough, Union, NJ). In order to minimize day effects on clinical outcomes, one cow from each of the three treatment groups was enrolled in the trial on each trial day. Randomization was achieved by assigning a two digit random number from a random number list, read in sequence from a random starting point, to each treatment group on each trial day. Selected cows were ranked from lowest to highest farm identification number and assigned to the group with the random number of the same rank. All clinical work for this project was conducted between September 2001 and April 2002.

Housing and Husbandry

On trial days, cows were kept in a small barn where they were accustomed to spending time while undergoing treatments and routine examinations such as

pregnancy diagnosis. They had unlimited access to water and a total mixed ration composed primarily of corn silage and dry hay, formulated for lactating dairy cows. Cows were caught in headlocks for the hourly recording of clinical observations, but were allowed to roam freely in the barn at other times. The cows were milked at their normal 12-hour interval.

Induction of Mastitis

Cows selected for use in this study had a negative bacterial culture 72 hours before the induction of mastitis and a negative California Mastitis Test in the treated quarter on the day of their participation the study. The California Mastitis Test on the other three quarters had either negative or trace reaction immediately before the induction of mastitis.

Mastitis was induced in the left front quarter by intramammary administration of 0.1 mg purified Lipopolysaccharide (**LPS**) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St.Louis, MO) in 20 mL sterile isotonic saline solution.

Clinical mastitis was defined as a measurable increase in mammary surface area above the baseline measurement, combined with a rectal temperature of 40° C or higher. One cow from the untreated control group and one cow from the isoflupredone acetate-treated group were removed from the study because they failed to develop clinical mastitis as defined above.

Clinical Variables

Clinical variables recorded hourly for all treatment groups included rectal temperature by electronic thermometer, heart rate by auscultation for 15 seconds with a stethoscope, number of rumen contractions per two minutes by auscultation of the left paralumbar fossa for two minutes with a stethoscope, and

mammary surface area. Mammary surface area was measured as follows: after removing the hair from the lateral aspect of the left front quarter with electric clippers, four marks were made on the udder skin with a permanent marker using a rectangular plastic template measuring 100 mm by 120 mm. The distance between the marks for each side of the rectangle and both diagonals were measured using a flexible plastic ruler. In order to calculate mammary surface area, the rectangular area being measured was divided by the two diagonals into four triangles, and the area of each triangle was calculated based on the length of its three sides using Heron's Formula, which states that the area of a triangle with sides of lengths a , b , and c is equal to $\sqrt{s(s-a)(s-b)(s-c)}$, where s equals $\frac{a+b+c}{2}$. The areas of the four triangles were summed to total the overall rectangular surface area. This method allowed surface area measurements to reflect expansion that occurred outward from the initial plane of measurement, in addition to expansion within the two dimensions of the created rectangle.

Study Design

Following the morning milking, four black marks were made on the udder skin of the left front quarter as described above. Each cow was examined and had baseline clinical observation values recorded, then LPS was infused into the left front quarter. Clinical observations and mammary surface area measurements were recorded at fourteen additional hourly time points thereafter.

Anti-inflammatory treatment was administered to cows in the drug treatment groups as soon as possible after the hourly observation at which they were noted to have developed clinical mastitis. Both drugs were given intravenously by jugular venipuncture; isoflupredone acetate was dosed at 20 mg per cow and

flunixin meglumine was dosed at 2.2 mg/kg body weight per cow. Body weight for all cows was estimated using a girth weight tape.

Milk Production

Milk weight data used in this study were collected electronically. Each cow in the study wore a computer chip on a neck strap bearing her farm identification number. The farm identification number was read from the computer chip by a scanner as each cow entered the milking parlor and used to record the cow's production at each milking. Cows enrolled in this study had twice-daily milk weights recorded for the week prior and three weeks following the day of participation in the study.

Statistics

All statistical analyses for this study were performed using the JMP statistical software program (SAS Institute, Cary, North Carolina). The study design provided a power of greater than 0.80 to detect a one-day difference of 10 pounds in daily milk weight between groups. Analysis of baseline clinical variables was performed using analysis of variance. Subsequent clinical variables and five-day baseline milk production data were analyzed using multivariate analysis of variance, which accounts for repeated measurements over time. Statistical significance was designated a priori as a *P* value less than or equal to 0.05. Baseline clinical observations were considered covariates for statistical analysis of clinical variables following treatment, in order to compensate for variation in mean baseline measurements between groups.

Initial analysis of milk production during the recovery period using multivariate analysis of variance for repeated measures revealed an interaction

between time and treatment. Further statistical analysis was therefore done using analysis of variance separately for each day of the recovery period. Dunnett's method was used to correct the tendency toward type 1 error when comparing treatment groups individually to the control group. Baseline milk production for the five days prior to the study was considered a covariate for statistical analysis of milk production recovery following mastitis, in order to compensate for variation in mean baseline milk production between groups.

Results

Physiological Variables

Rumen contractions. (Figure 1) There was no significant difference between groups in baseline rumen contraction frequency ($P = 0.5996$). Cows treated with flunixin had a higher frequency of rumen contractions during the fourteen hours following endotoxin administration than the control group ($P = 0.0357$) or the isoflupredone-treated group ($P = 0.0121$). There were no significant differences in rumen contraction frequency between the control group and the isoflupredone-treated group ($P = 0.4310$)

Heart rate. (Figure 2) Baseline heart rate for the three groups was not different ($P = 0.2931$). Cows receiving drug treatment had a lower heart rate over time than untreated controls ($P = 0.0019$ for flunixin, $P = 0.0274$ for isflupredone). There were no significant differences in heart rate between the two drug treatments ($P = 0.1626$)

Rectal Temperature. (Figure 3) Mean baseline temperatures for all groups were not significantly different ($P = 0.5262$). Cows treated with flunixin had lower

rectal temperatures over time than did isoflupredone-treated ($P = 0.0023$) or untreated ($P = 0.0017$) cows. Differences in rectal temperature between the isoflupredone-treated and control groups did not achieve statistical significance ($P = 0.0960$)

Mammary Surface Area. (Figure 4) There were no significant differences between any of the three groups in baseline measurements of mammary surface area ($P = 0.6971$) or changes in mammary surface area over the 14 hours following the introduction of LPS into the mammary gland ($P = 0.1757$).

Milk Production. (Figure 5) Baseline milk production for the five days preceding the induction of mastitis was not significantly different between groups ($P = 0.2436$). By the 10th day following the induction of mastitis, each treatment group had recovered to pre-challenge mean milk production. There were no significant differences between groups in the rate of recovery of milk production ($P > 0.05$ for all days during the recovery period).

Discussion

Flunixin meglumine and isoflupredone acetate are commonly used drugs in the treatment of inflammatory conditions of dairy cows in the United States, and both drugs are available in preparations that are approved for such use. Flunixin meglumine is a non-steroidal anti-inflammatory drug, while isoflupredone acetate is a corticosteroid drug with primarily glucocorticoid anti-inflammatory activity.

It has previously been established that flunixin meglumine provides relief of clinical signs such as fever, local swelling, decreased rumen motility and depression in mastitis induced using *Escherichia coli* bacteria or bacterial

endotoxin (Anderson, Smith et al. 1986; Lohuis, Leeuwen et al. 1989). In toxic mastitis due to natural infection with Escherichia coli, Klebsiella species, and other organisms, however, flunixin meglumine was not shown to affect the rate of bacterial cure or subsequent milk production (Dascanio, Mecher et al. 1995).

Isoflupredone acetate, in contrast, has received little published attention as a therapy for endotoxic mastitis, despite its labeled approval for use to treat inflammation and infection in cattle, and evidence that other steroidal anti-inflammatory drugs may relieve clinical signs in endotoxin-induced mastitis (Lohuis, Leeuwen et al. 1989) and reduce milk production loss in cows with mastitis induced by the intramammary administration of Escherichia coli bacteria (Lohuis, Leeuwen et al. 1988). One previous study did not show a reduction in milk production loss due to endotoxin-induced mastitis when isoflupredone acetate was administered after the development of clinical signs (Wagner and Apley In Press).

The objective of the current study was to directly compare these two commonly used drugs using an endotoxin-induced mastitis model. Unlike much previous research involving anti-inflammatory drugs and mastitis, treatment was not administered until after local and systemic clinical signs of mastitis had developed. Cows which failed to develop mastitis of a severity that would warrant treatment were it seen in practice were discarded from the study.

Even when administered following the development of clinical signs, flunixin meglumine did alleviate most signs over the remainder of the acute phase in which they were measured. Despite providing no measurable reduction in local mammary swelling, flunixin did preserve rumen motility and reduce fever and heart rate in cows with endotoxic mastitis, when compared to untreated controls. Isoflupredone

did not affect local swelling, rumen motility, or rectal temperature when compared to untreated controls, but it did reduce heart rate equally to flunixin. Neither drug had a significant effect on milk production following the mastitic episode.

Making inferences about anti-inflammatory treatment of naturally occurring endotoxic mastitis based on the results of this study and other studies involving these two drugs is difficult. There are differences between endotoxin-induced and infectious disease; fatal disease may occur due to infection with *Escherichia coli* bacteria but is extremely unlikely using the endotoxin-induced model, and endotoxin-induced mastitis has different effects on cells and cytokine production than natural infection (Oostveldt, Tomita et al. 2002). In naturally occurring disease, neither therapy has been shown to affect the clinical outcome, but there are other worthwhile therapeutic objectives that may be investigated using the endotoxin model, such as the relief of pain, reduction of local swelling, and preservation of milk production.

Both of the agents investigated here reduced mean heart rate in treated mastitic cows, when compared to untreated control groups. Heart rate may rise due to fever or pain; however, only flunixin meglumine significantly reduced fever in this study, so one might conclude that heart rate reduction in this study is an indicator of pain relief. Previous research using isoflupredone acetate to treat endotoxic mastitis demonstrated a rise in heart rate in treated cows compared to untreated controls; however, in the previous study only treated cows were having repeated jugular venipuncture, the stress of which is likely the reason for increased heart rate (Wagner and Apley In Press).

Fever reduction has traditionally been a goal of anti-inflammatory therapy, but recent human literature has suggested that this may not be a valid goal, and in humans antipyretic therapy may actually extend the duration of infectious disease (Mackowiak 2000). Fever reduction in the therapy of mastitis in dairy cows may be beneficial or detrimental; there is no published research to support either proposition. It is therefore difficult to judge the value of the fever-reduction properties of flunixin meglumine or the absence of such activity by isoflupredone acetate in endotoxic mastitis in dairy cows.

In conclusion, the results of this study, when considered in concert with previous research into the effects of these two drugs in cows with endotoxic mastitis, may raise more questions than they answer. Flunixin meglumine preserves rumen motility while isoflupredone acetate does not, but this difference did not translate into improved milk production following either treatment. These two drugs differ significantly in their effects on fever, with flunixin meglumine quickly reducing fever in treated cows and isoflupredone acetate-treated cows showing no reduction of fever when compared to untreated controls. Whether fever reduction is beneficial in the therapy of endotoxic mastitis is unknown; it can be said that reduction of fever is not correlated with accelerated recovery of milk production in cows with endotoxin-induced mastitis. Despite the absence of measurable reduction in mammary gland swelling for either agent, both isoflupredone acetate and flunixin meglumine did reduce heart rate in treated animals, which may indicate that both agents provide some relief of pain in cows with endotoxin-induced mastitis.

Acknowledgements

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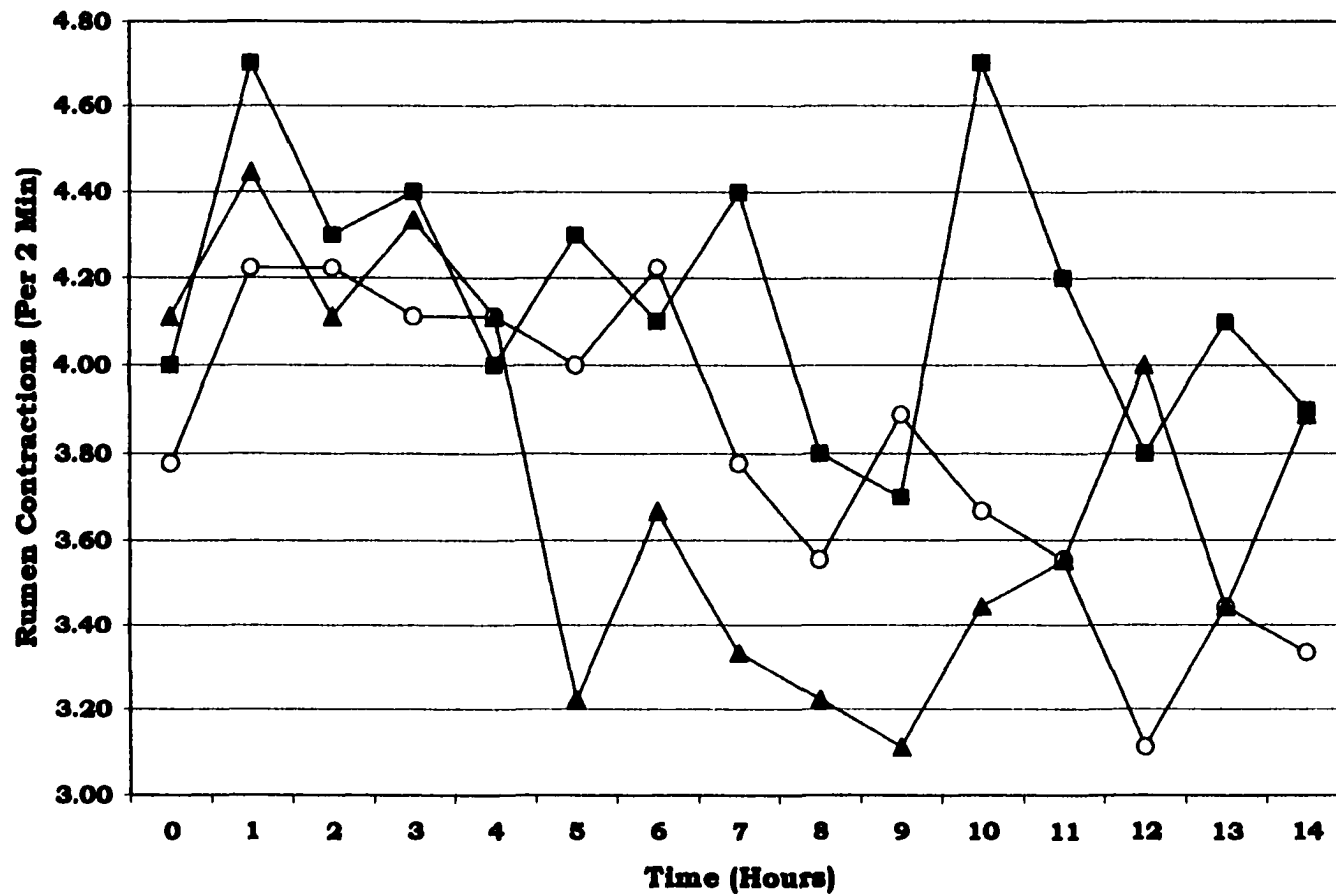


Figure 4.1 Effects of Drug Treatment on Rumen Motility. Hour 0 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: ○ Untreated Endotoxin-Induced Mastitis, ▲ Isoflupreonde-Treated Endotoxin-Induced Mastitis, ■ Flunixin-Treated Endotoxin-Induced Mastitis

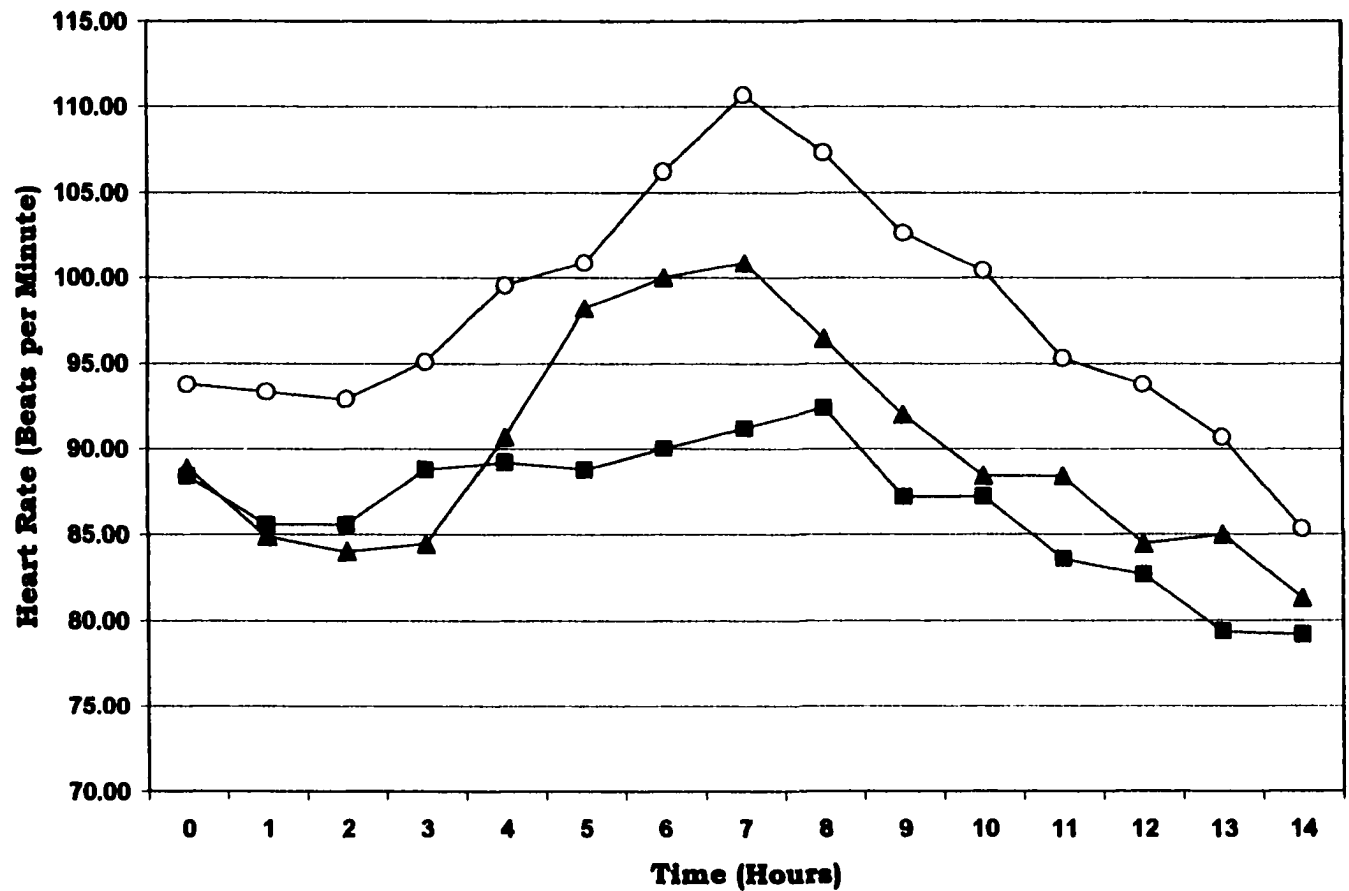


Figure 4.2 Effects of Drug Treatment on Heart Rate. Hour 0 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: ○ Untreated Endotoxin-Induced Mastitis, ▲ Isoflupreonde-Treated Endotoxin-Induced Mastitis, ■ Flunixin-Treated Endotoxin-Induced Mastitis

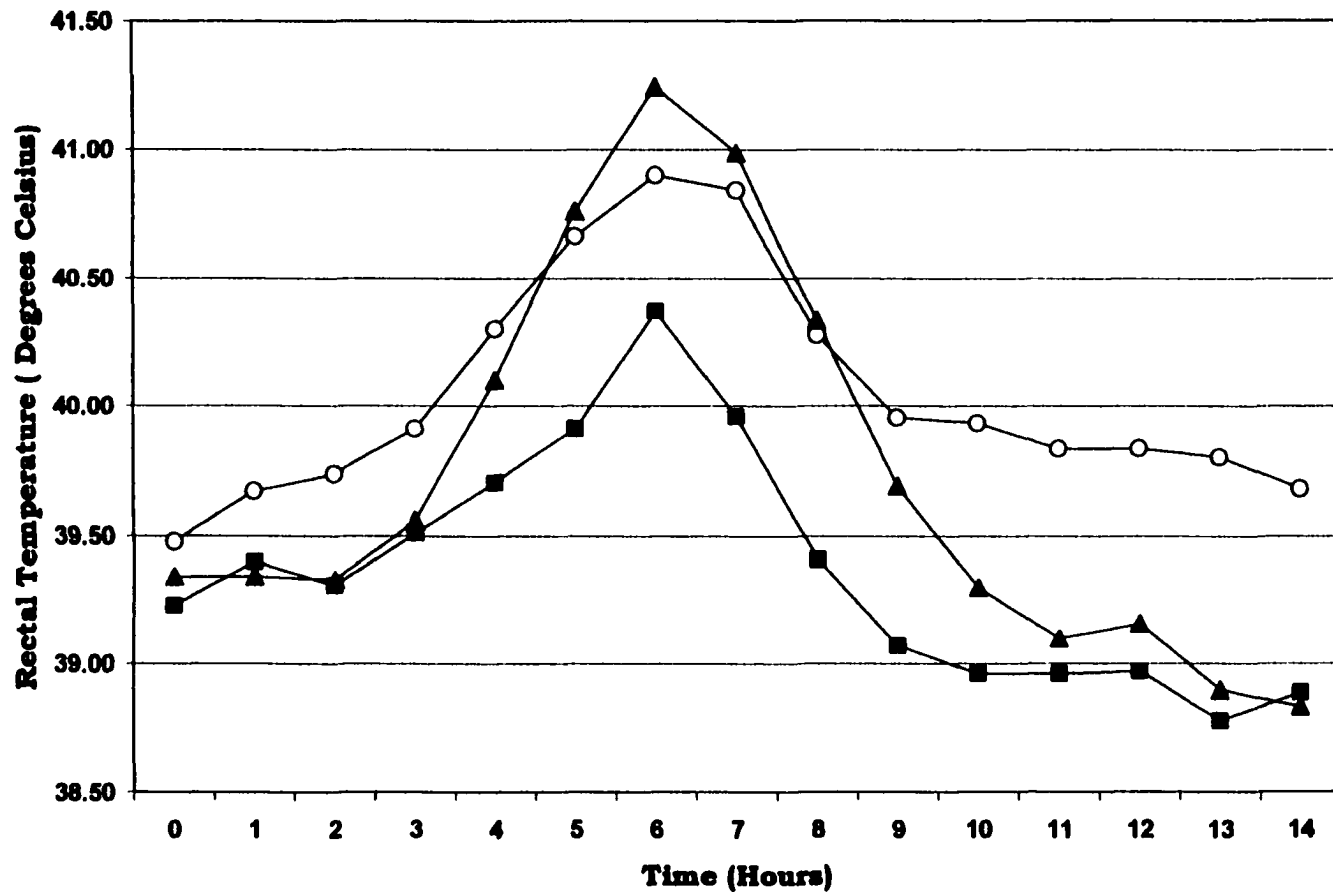


Figure 4.3 Effects of Drug Treatment on Rectal Temperature. Hour 0 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: ○ Untreated Endotoxin-Induced Mastitis, ▲ Isoflupreonde-Treated Endotoxin-Induced Mastitis, ■ Flunixin-Treated Endotoxin-Induced Mastitis

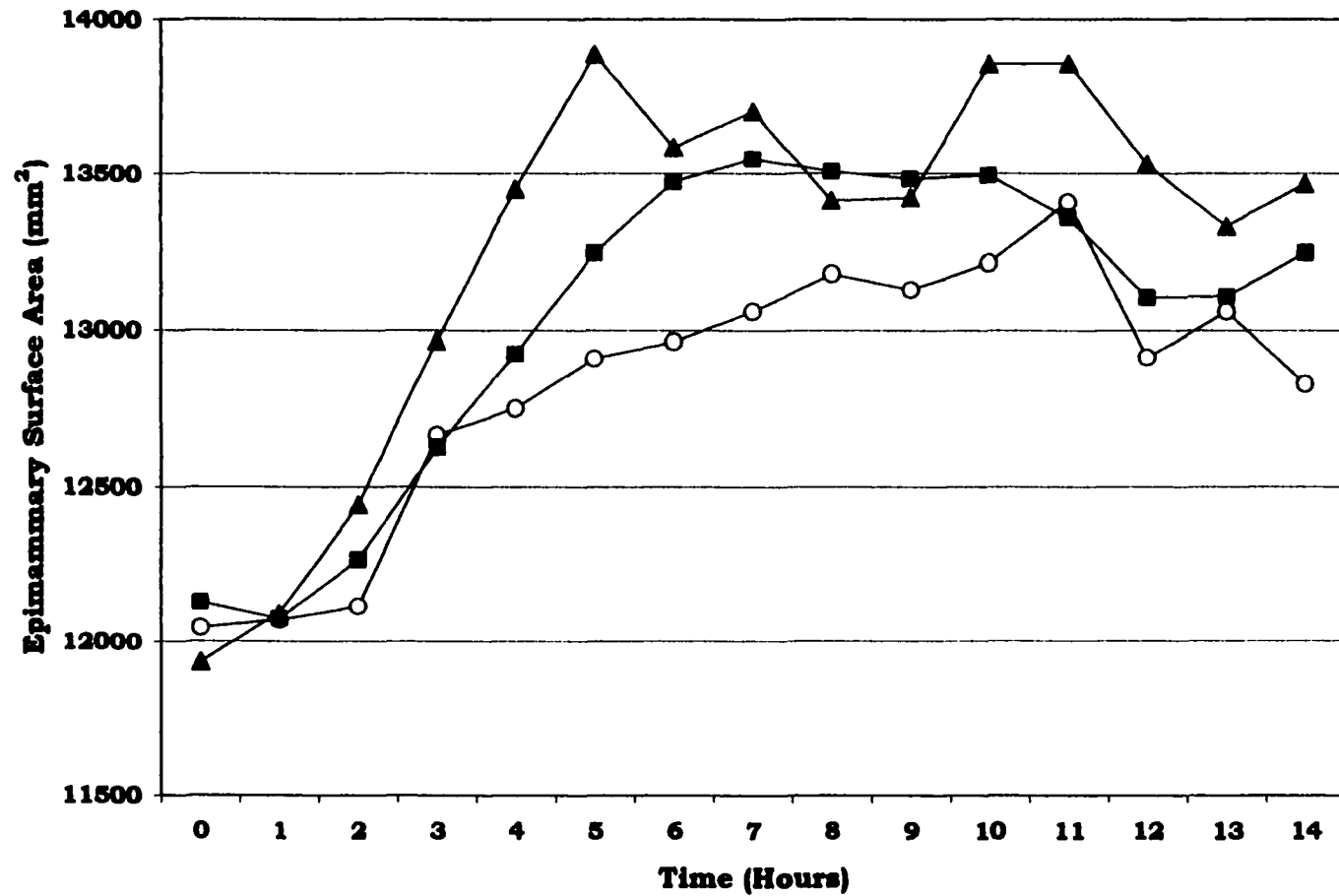


Figure 4.4 Effects of Drug Treatment on Epimammary Surface Area. Hour 0 Represents Baseline Measurements. Each Value Represents the Group Mean. Legend: ○ Untreated Endotoxin-Induced Mastitis, ▲ Isoflupreonde-Treated Endotoxin-Induced Mastitis, ■ Flunixin-Treated Endotoxin-Induced Mastitis

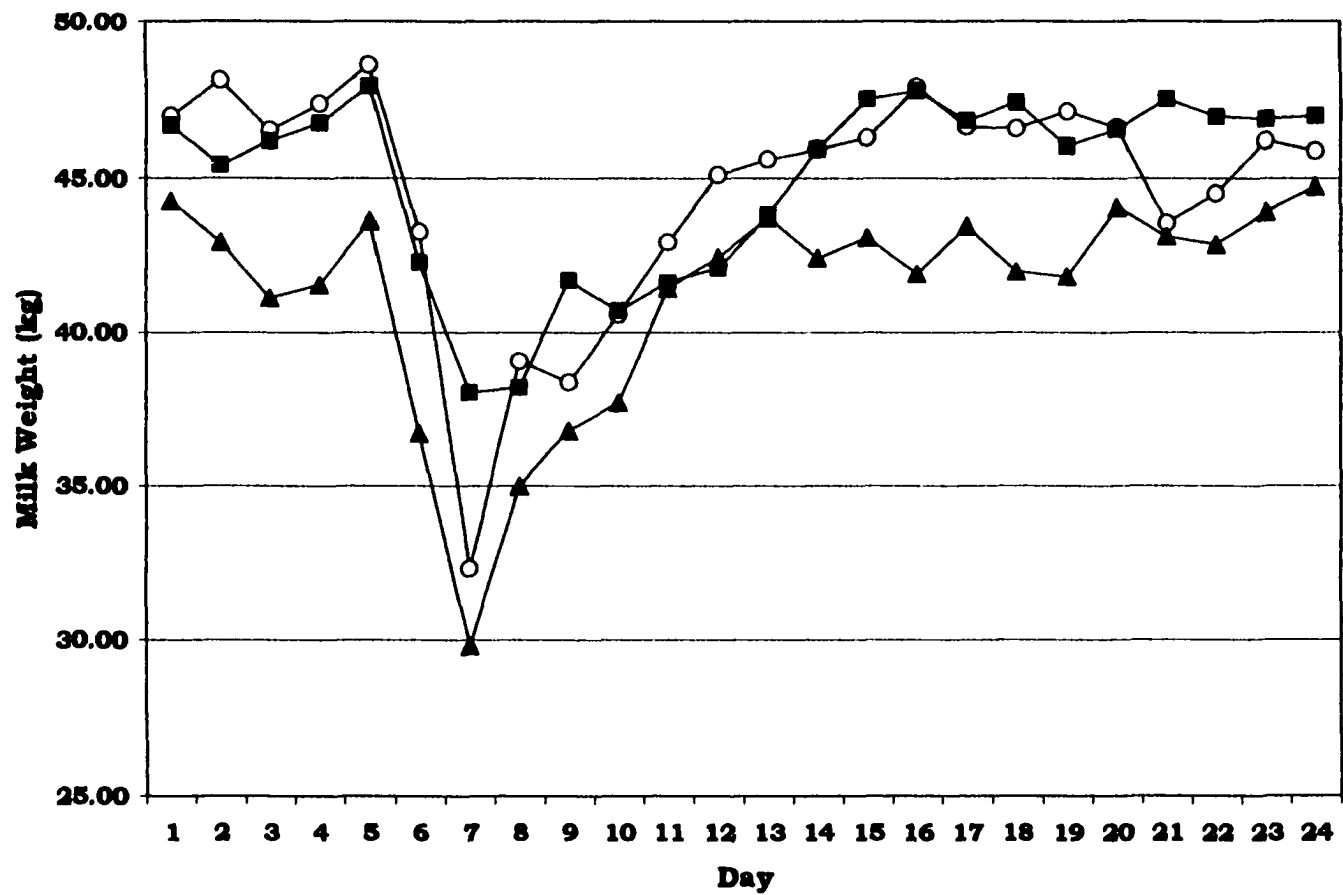


Figure 4.5 Effects of Drug Treatment on Milk Production. Endotoxic Mastitis was Induced on Day 0. Each Value represents the Group Mean. Legend: ○ Untreated Endotoxin-Induced Mastitis, ▲ Isoflupreonde-Treated Endotoxin-Induced Mastitis, ■ Flunixin-Treated Endotoxin-Induced Mastitis

CHAPTER 5. GENERAL CONCLUSIONS

The objectives of the research presented herein were to describe the effects of flunixin meglumine, a non-steroidal anti-inflammatory drug (NSAID) and isoflupredone acetate, a corticosteroid drug, on the clinical and cellular changes caused by endotoxic mastitis in dairy cows, using an endotoxin-induced mastitis model. Unlike much previous work done to investigate anti-inflammatory therapy of endotoxic mastitis, in the studies reported here the administration of drug therapy followed the development of local and systemic clinical signs. In many of the previous studies done using an infectious or endotoxic model of naturally occurring endotoxic mastitis, anti-inflammatory drugs were administered either concurrently with the agent used to induce the mastitis or at a set time after the administration of the agent, instead of in response to clinical signs, as would be done in a clinical setting.

In the first study, the steroidal drug isoflupredone acetate was given to healthy cows and cows with endotoxin-induced mastitis, at the label dose of 20 mg per cow. Unlike the steroidal drug dexamethasone, which has been shown to ameliorate milk production losses when given to cows with induced mastitis prior to the development of systemic clinical signs, isoflupredone administered after the development of clinical signs did not have a protective effect on milk production. The drug also did not relieve local or systemic clinical signs of endotoxic mastitis.

In the second study, isoflupredone acetate and the NSAID drug flunixin meglumine were investigated for their ability to limit the degree to which mammary epithelial cells were sloughed into the milk during the acute phase of endotoxin-induced mastitis. This study demonstrated a marked increase in the numbers and percentage of epithelial cells in the milk of cows within the first 12 hours following the administration of endotoxin, but the magnitude of the increase was not affected by administration of either drug.

The final study compared the effects of flunixin meglumine and isoflupredone acetate when administered following the development of clinical signs to cows with endotoxin-induced mastitis. Neither drug relieved mammary gland swelling or milk production loss. Both drugs reduced heart rate in treated cows, suggesting that both drugs provided pain relief. Isoflupredone acetate did not relieve elevated heart rate in the first study, but this was likely due to the concurrent serial venipuncture performed on treated cows, to which untreated cows were not subjected. Flunixin meglumine also reduced rectal temperature and increased rumen motility during the acute phase when compared to untreated controls.

No well-designed or well-reported study has documented increased survival of naturally occurring endotoxic mastitis following treatment with any anti-inflammatory agent. Until it is known whether reduction of fever is beneficial, detrimental, or of no influence in the outcome of infectious mastitis, it cannot be concluded that the fever reduction property of flunixin meglumine makes it a better

choice for such therapy. Neither can isoflupredone be recommended as a superior treatment, as its administration at the label dose in response to clinical signs of mastitis has not demonstrated benefits to milk production, as were observed when the steroidal drug dexamethasone was given prior to the onset of clinical mastitis. It can, however, be concluded from the research described herein that either flunixin meglumine or isoflupredone acetate will provide humane relief of the pain associated with mastitis. The use of one of these agents in the treatment of mastitis may be recommended based on this finding alone.

Although neither drug was found to protect milk production in cows with endotoxin-induced mastitis when given at the label dose after the development of clinical signs, further investigation, especially of isoflupredone acetate, might be warranted. Future work might be directed at investigating a higher dose of isoflupredone acetate, as it is not as potent as dexamethasone, which was found to protect milk production when given prior to the development of clinical signs of induced mastitis at a dose of 12.5 to 30 mg for a 500 kg cow. Isoflupredone is described as being about one third the potency of dexamethasone, so an equivalent amount of glucocorticoid potency would be achieved by a dose of 37.5 to 90 mg for a 500 kg cow. Another approach would be to investigate dexamethasone itself, at a similar dose to those used in previous studies on induced-mastitis models, but administered after the development of clinical signs instead of before clinical signs, as done by previous researchers.

Further research into the question of whether fever reduction in cows with endotoxic mastitis is beneficial to survival and subsequent milk production would also be warranted, ideally in cows with naturally-occurring infectious mastitis. The observation that the NSAID drug flunixin meglumine reduces fever in cows with endotoxic mastitis while the steroidal drug isoflupredone acetate does not, suggests that these two agents might be appropriate for use in such a study.

APPENDIX A. REPRESENTATIVE FLOW CYTOMETRY DATA

Forward Scatter vs. Side Scatter Plots

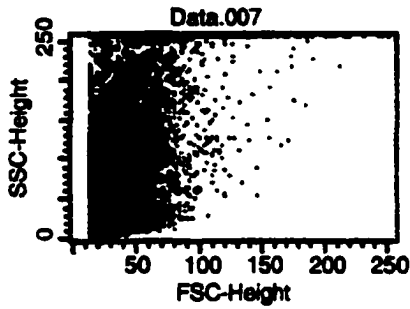


Figure A1.
Control Milk Cells (Unstained)
Before Endotoxin Administration

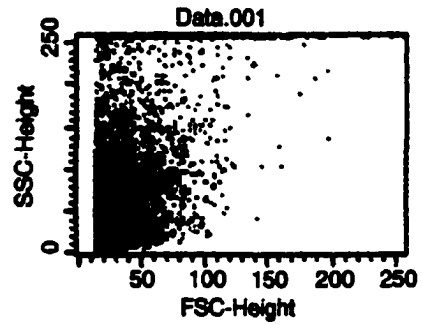


Figure A2.
Cytokeratin-linked FITC Stain
Before Endotoxin Administration

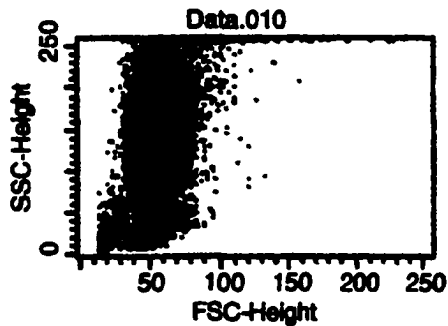


Figure A3.
Control Milk Cells (Unstained)
After Endotoxin Administration

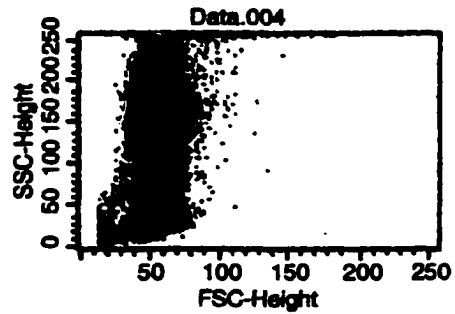


Figure A4.
Cytokeratin-linked FITC Stain
After Endotoxin Administration

Forward Scatter (FSC), plotted here on the X axis, is considered a measurement of cell size. Side Scatter (SSC), plotted here on the Y axis, is considered a measurement of the granularity of cell contents. Plots FSC vs. SSC, as seen above, did not depict clearly differentiated cell populations in any of the milk samples. All data presented in this appendix are from the same cow.

Natural Fluorescence vs. Fluoroisothiocyanate (FITC) Fluorescence Plots
 Before Endotoxin Administration

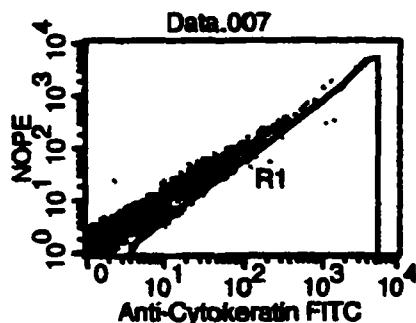


Figure A5.
 Control Milk Cells (Unstained)
 Before Endotoxin Administration

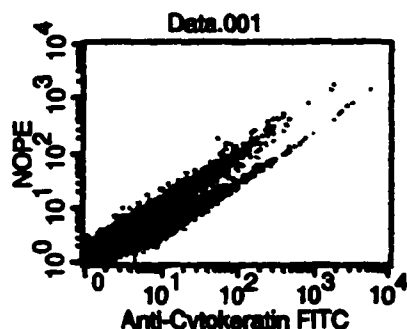


Figure A6.
 Cytokeratin-linked FITC Stain
 Before Endotoxin Administration

Unlike FSC vs. SSC plots, these plots of natural fluorescence (on the Y axis) versus intensity of FITC fluorescence do clearly identify different cell populations. The FITC stain was linked to anti-cytokeratin antibody in samples identified as "Cytokeratin-linked FITC Stain", and to mouse IgG in samples identified as "Control Milk Cells". R1, shown in Figure A5, is a gate drawn by the investigator to measure the number of cells in one population. One can see from these plots that the population of cells contained within the gate is present in the stained sample but not in the control sample. The population of cells within gate R1 is therefore considered to be mammary epithelial cells, as they are the only cells in milk which can be expected to bind anti-cytokeratin antibody. This is borne out by the count of cells within the gate measured by the flow cytometer. The percentage of gated cells in the sample containing anti-cytokeratin linked FITC stain is 7.39%, while only 0.53% of cells in the control sample with FITC linked to unspecific mouse IgG are contained within the gated area. Gate 1, as drawn in Figure A5, remains the same throughout all of the data presented here.

Natural Fluorescence vs. Fluoroisothiocyanate (FITC) Fluorescence Plots
After Endotoxin Administration

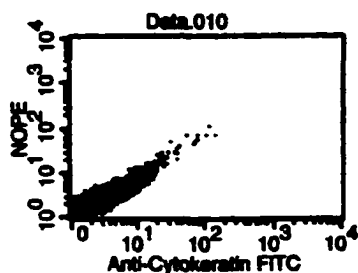


Figure A7.
Control Milk Cells (Unstained)
After Endotoxin Administration

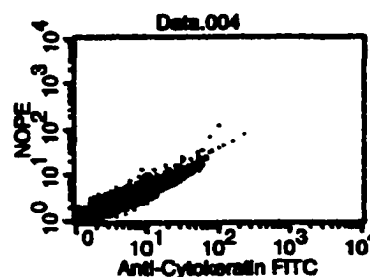


Figure A8.
Cytokeratin-linked FITC Stain
After Endotoxin Administration

Following endotoxin administration, the natural fluorescence vs. FITC fluorescence plots do not depict separate cell populations clearly, as they did in the same cow prior to the onset of mastitis (Figures A5 and A6). Still, analysis of cell counts inside gate R1, which was drawn using the pre-endotoxin samples, reveals that there is a population of cells present in the stained sample that is not present in the control sample; 68.19% of cells in the stained sample are within the gate, while only 0.32% of cells in the control sample from the same cow are within the gate.

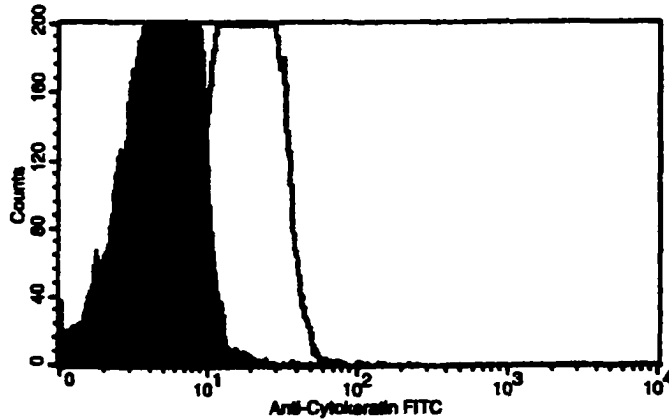
Histograms

Figure A9.

Histograms are another useful way to examine flow cytometry data. Figure A9 is a histogram plot of cells in milk samples taken after endotoxin administration. The solid area is the count of cells vs. FITC fluorescence for the control sample. The lighter grey line is an outline of the same plot for the anti-cytokeratin stained sample. The mean amount of fluorescence by the cells in the stained sample is clearly greater than that of the control sample, as the area contained in the plot is further along the X axis, FITC fluorescence.

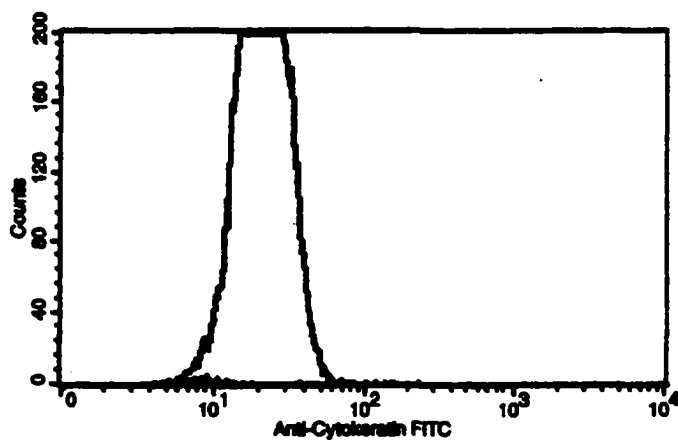
Histograms

Figure A10.

Figure A10 is a histogram plot in the same format as Figure 9, but including only the cells contained in gate R1. The solid area containing the count of gated cells in the control sample is very small. The area contained in the lighter grey line, representing gated cells in the stained sample, is much larger and centered farther along the X axis than the gated cells in the control sample. This reflects the higher number of gated cells in the stained sample, and their greater intensity of FITC fluorescence.

**APPENDIX B. SUMMARY OF ATTEMPTS TO QUANTIFY ISOFLUPREDONE IN
THE SERUM OF DAIRY COWS FOLLOWING INTRAVENOUS ADMINISTRATION
OF ISOFLUPREDONE ACETATE**

As mentioned briefly in Chapter 1, one of the original objectives of the first study described in this dissertation was to describe the pharmacokinetic behavior of isoflupredone acetate in healthy cows and cows with endotoxin-induced mastitis following the administration of 20 mg of the drug intravenously. The pharmacokinetic portion of the study was never executed due to difficulties encountered with the quantification of drug in bovine serum. This appendix describes the procedures that were performed in the laboratory in an attempt to quantify the drug in bovine serum.

In the autumn of 2000, a pilot study was done in which one cow had blood samples drawn before and after the administration of isoflupredone acetate. These samples were centrifuged and serum was removed and saved for analysis. Quantification of isoflupredone was performed using High Performance Liquid Chromatography (HPLC) and Immunohistochemistry (IHC). Results of these analyses are given in Tables B1 and Table B2.

Unknown (ng/ml)

Hb

9/28/00
mr

Sample	Wells	Values	R	Result	MeanResult	Std.Dev.	CV%
UNK01	A1	0.363		1.069	1.108	0.054	4.9
	A2	0.362		1.148			
UNK02	B1	0.270		31.708	26.525	4.502	15.8
	B2	0.282		25.341			
UNK03	C1	0.259		38.857	41.194	3.588	8.7
	C2	0.252		43.731			
UNK04	D1	0.245		49.380	41.439	11.243	27.1
	D2	0.267		33.489			
UNK05	E1	0.280		26.324	23.796	3.575	15.0
	E2	0.291		21.288			
UNK06	F1	0.320		11.363	9.559	2.551	26.7
	F2	0.335		7.755			
UNK07	G1	0.357		3.882	3.882	0.000	0.0
	G2	0.415	R	Range?			
UNK08	H1	0.453	R	Range?	Range?	Range?	Range?
	H2	0.441	R	Range?	Range?	Range?	Range?
UNK09	H7	0.071	R	2275.941	2275.941	0.000	0.0
UNK10	H8	0.370		2.259	2.259	0.000	0.0
UNK11	H9	0.416	R	Range?	Range?	Range?	Range?
	H10	0.432	R	Range?	Range?	Range?	Range?

R - Outside standard range
Mean Adjusted Result: Error

POS control (flunithason)
Neg control
Blk Plasma

Table B1. Results of Immunohistochemical Analysis of Serum Samples From The Pilot Cow (in Parts Per Billion).

In this table, "Unk 01" is the serum sample taken prior to the administration of isoflupredone acetate, and "Unk 02" through "Unk 08" are serial serum samples taken following drug administration. Measured drug concentration is negligible for the sample taken prior to drug administration, then there is an increase followed by a decrease in a drug levels over time, with drug levels at the time of sample "Unk 08" undetectable. Positive and negative control and blank plasma measurements appear to have appropriate analytical results.

serum sample	1st analysis	2nd analysis	3rd analysis	Average
1	23.2	21		22.1 ✓
2	29.3	37.5		33.4
3	49.9	53.2		51.2
4	48.2	45.8		47
5	29.5	24.1		26.8
6	13	18.9		16
7	10.5	8.4		9.5
8	0	5.8	4.3	3.4

Table B2. Results of High Performance Liquid Chromatography Analysis of Serum Samples From The Pilot Cow (in Parts Per Billion).

As with the samples analyzed using Immunohistochemistry, sample 1 was taken prior to the administration of isoflupredone acetate, and samples 2 through 8 were taken in sequence following drug administration. The results using HPLC were similar to those obtained using IHC, with the notable exception that the samples taken prior to drug administration appeared to have significant levels of the drug. This was considered an artifact of the analysis, as the cow in question had been carefully screened to be sure she was not exposed to the drug prior to its administration as a part of this project. Based on results of HPLC and IHC analysis in the pilot study, it was decided that IHC would provide the most accurate analysis of serum drug levels.

In the late spring of 2001, the first serum samples for use in the study were obtained from cow number 4598 and analyzed using Immunohistochemistry. Results of that analysis are given in Table B3.

Unknown (ng/ml)

Sample	Wells	Values	R	Result	MeanResult	Std.Dev.	CV%	Susp
UNK01	A1	0.778	R	Range?	Range?	Range?	Range?	
	A2	0.814	R	Range?				
UNK02	B1	0.674	R	Range?	Range?	Range?	Range?	
	B2	0.744	R	Range?				
UNK03	C1	0.714	R	Range?	Range?	Range?	Range?	
	C2	0.777	R	Range?				
UNK04	D1	0.811	R	Range?	Range?	Range?	Range?	
	D2	0.892	R	Range?				
UNK05	E1	0.978	R	Range?	Range?	Range?	Range?	
	E2	0.818	R	Range?				
UNK06	F1	0.914	R	Range?	Range?	Range?	Range?	
	F2	0.802	R	Range?				
UNK07	G1	0.859	R	Range?	Range?	Range?	Range?	
	G2	0.802	R	Range?				
UNK08	H1	0.808	R	Range?	Range?	Range?	Range?	
	H2	0.840	R	Range?				
UNK09	A3	0.689	R	Range?	Range?	Range?	Range?	
	A4	1.002	R	Range?				
UNK41	A6	0.267		8.721	8.347	0.529	6.3	
	A7	0.274		7.973				
UNK42	B6	0.361		3.275	3.495	0.312	8.9	
	B7	0.347		3.716				
UNK43	C6	0.336		4.116	3.505	0.863	24.6	
	C7	0.375		2.895				

R - Outside standard range

Table B3. Results of IHC Analysis of Serum From Cow 4598.

As in previous figures, sample 1 was taken prior to drug administration and samples "Unk 02" through "Unk 09" samples were taken in sequence following drug administration. "Unk 41" through "Unk 43" are samples with known amounts of isoflupredone added to them. The samples with isoflupredone added to them did have measurable drug levels in them, however IHC analysis did not detect the drug in any of the serum samples.

Because IHC analysis failed to detect isoflupredone in any of the samples taken from the drug treated cow 4598, the same samples were re-analyzed using HPLC. Results of that analysis are given in Table B4 and Figure B5.

serum sample	1st analysis	2nd analysis	3rd analysis	Average
1	0	0		0
2	8.6	10	13.1	10.6
3	16.2	11.8	13.6	13.9
4	6.8	9.8	4.5	7
5	3	4.3	3.9	3.7
6	4.4	2.2	2.1	2.9
7	3	0	4.3	2.4
8	4.4	0	1.9	2.1
9	1.5	8.4	10.1	6.7

Table B4. Results of HPLC Analysis on Cow 4598 (in Parts Per Billion)

Unlike IHC, HPLC did detect isoflupredone in the serum of cow 4598. Drug levels were lower overall than those found using HPLC in the pilot cow, but the problem of detecting the drug in samples taken prior to drug administration appeared to have been resolved.

Table B5 and Figure B6 compare HPLC analysis of sera from the pilot cow and from cow 4598.

serum sample	Pilot Average	4598 Average
1	22.1	0
2	33.4	10.6
3	51.2	13.9
4	47	7
5	26.8	3.7
6	16	2.9
7	9.5	2.4
8	3.4	2.1
9		6.7

Table B5. Comparison of HPLC Results from Pilot Cow and Cow 4598 (in Parts Per Billion)

Isoflupredone Analysis

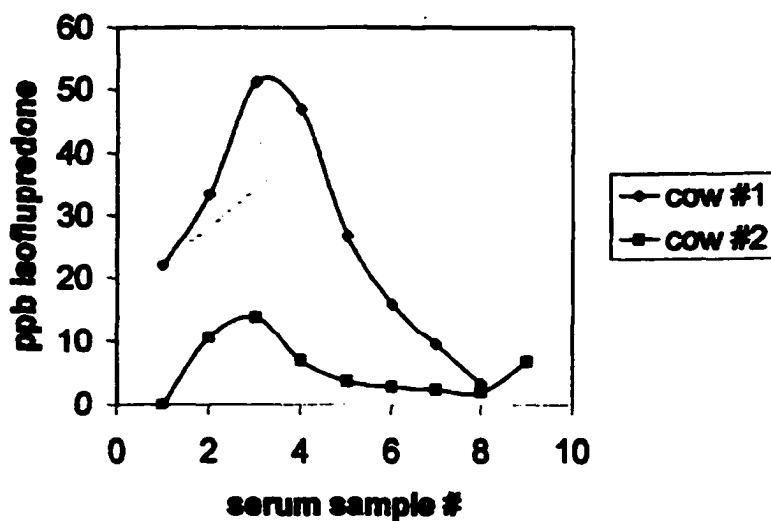


Figure B6. Graphic Comparison of HPLC Results From Pilot Cow (#1) and Cow 4598 (#2)

As described above, HPLC on Cow 4598 gave results of overall lower drug concentrations, but the problem of “false positive” results appeared to have been resolved. It was therefore decided that analysis of sera from cows in the pharmacokinetic study would begin in earnest using HPLC. Results from the first batch of sera analyzed using HPLC are given in Table B7.

Cow Number	Isoflupredone Levels in Parts Per Billion								
	1	2	3	4	5	6	7	8	9
4104	nda	nda	nda	nda	8.2	nda	nda	nda	nda
4623	9.9	12.7	15.9	5.6	2.4	nda	nda	nda	nda
4787	10.8	19.9	23.4	11.6	nda	nda	nda	nda	nda
4836	13.7	13.4	18	2.7	nda	nda	nda	nda	nda
4723	nda	5	4.3	0.7	nda	nda	nda	nda	nda
4963	13.3	11.7	14.5	10.9	4	nda	nda	nda	nda
4577	10.6	16.4	15.2	9.1	0.9	nda	nda	nda	nda
5012	nda	nda	nda	nda	nda	nda	nda	nda	nda
4620	20.9	13.5	14.3	11	1.6	nda	nda	nda/2.5	nda

Table B7. Results of HPLC on First Batch of Pharmacokinetic Study Cow Sera (in Parts Per Billion)

The results shown in Table B7 were problematic. The primary concern was that HPLC analysis detected quantities of isoflupredone in the first serum sample in 6 of 9 cows. As with previous examples, this serum sample was obtained prior to administration of the drug. It was considered extremely unlikely that the samples were contaminated or that the cows already had the drug in their system. Great care was taken with sample handling on the farm and in the laboratory, and good records of drug treatment were kept for the cows and carefully reviewed prior to their admission to the study. It was therefore concluded that these false positive results were most likely due to some other analyte registering as isoflupredone in the serum upon analysis with HPLC. It was also unusual that one cow never had detectable levels of the drug according to HPLC and one cow only had detectable drug levels at one time point, but this concern has not yet been addressed, as the issue of false positives was made first priority. It was decided that some of the cows

with false positive results would have serum drawn and retested using HPLC, without any concurrent administration of isoflupredone acetate. Results are given in Table B8.

sample ID	area	ng from curve	ppb	% rec.
ACN	nda			
water blank	nda			
water spk	8	3.15	31.50	63.00
plasma 1	1.2	0.47	4.70	
plasma 1 spk	7.8	3.07	30.70	52.00
plasma 2	1.3	0.51	5.10	
plasma 2 spk	9	3.54	35.40	60.60
plasma 3	2	0.79	7.90	
plasma 3 spk	9.9	3.69	36.90	62.00
serum 1A	1.3	0.51	5.10	
serum 1B	nda		0.00	
serum 1B spk	6.2	3.23	32.30	64.60
serum 2A	1.9	0.75	7.50	
serum 2B	1.4	0.65	5.50	
serum 2B spk	6.9	3.50	35.00	59.00
serum 3A	3.4	1.34	13.40	
serum 3B	1.1	0.43	4.30	
serum 3B spk	9.5	3.74	37.40	68.20
serum 4A	nda		0.00	
serum 4B	1.8	0.71	7.10	
serum 4B spk	10.5	4.13	41.30	68.40

Table B8. Results of HPLC Analysis on Serum From Cows That Previously Tested Positive For Isoflupredone Prior to Drug Administration (in Parts Per Billion)

Upon re-analysis using HPLC, most of the serum samples from these cows again showed the presence of isoflupredone, despite the cows having no known timely exposure to the drug. It was therefore decided that serum from two of the cows (which provided samples 2A and 4A in Table B8) would be further analyzed using Mass Spectrometry, in an attempt to isolate the analyte causing the false positive results in HPLC analysis.

When analyzed using Mass Spectrometry, serum from both of the cows was found to have a spike at the molecular weight of isoflupredone, despite sound evidence that neither cow had been exposed to isoflupredone recently enough to have detectable levels in her serum. The first test, in which the drug was administered as part of the initial pharmacokinetic study, had been several months prior to the Mass Spectrometry analysis, and the cows had not, according to farm records, been treated with the drug since. There is no reason to believe the farm records were incomplete, and furthermore isoflupredone is uncommonly used on the farm in any case.

Results of Mass Spectrometry are shown in Figures B11a through B11d.

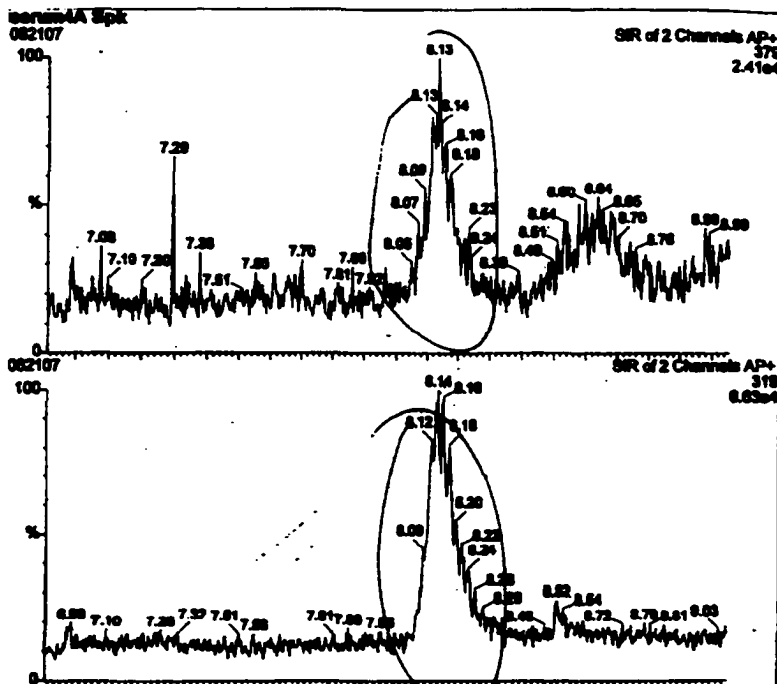


Figure B9. Mass Spectrometry of a Serum Sample With Isoflupredone Added

When Isoflupredone is added to serum, a “spike” in the Mass Spectrometry output is seen at 8.13 to 8.14 (circled areas).

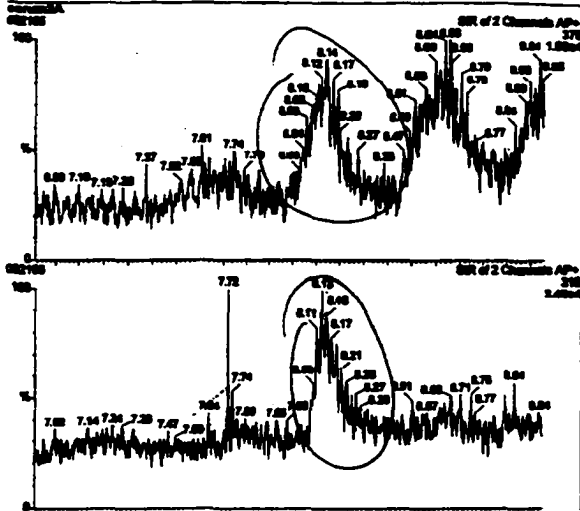


Figure B11b. Mass Spectrometry on Serum From a Cow (2A) With False Positive Results Using HPLC

The results of Mass Spectrometry on this cow show spikes at exactly the same molecular weight as is seen in serum samples with added isoflupredone (circled areas).

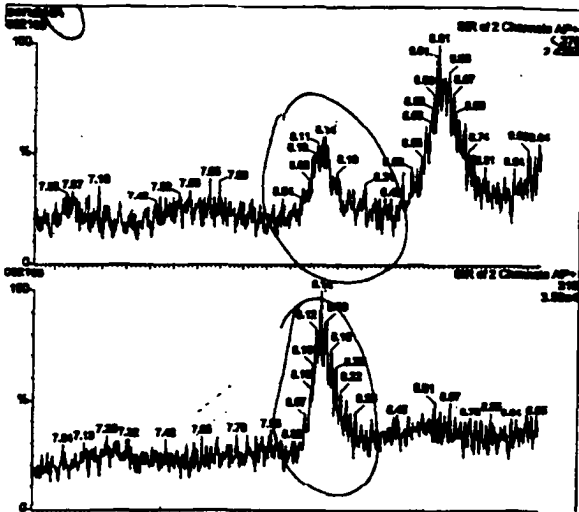


Figure B11c. Mass Spectrometry on Serum From a Cow (4A) With False Positive Results Using HPLC

Again, this cow has spikes at the same points that they would be expected in a serum sample to which isoflupredone had been added

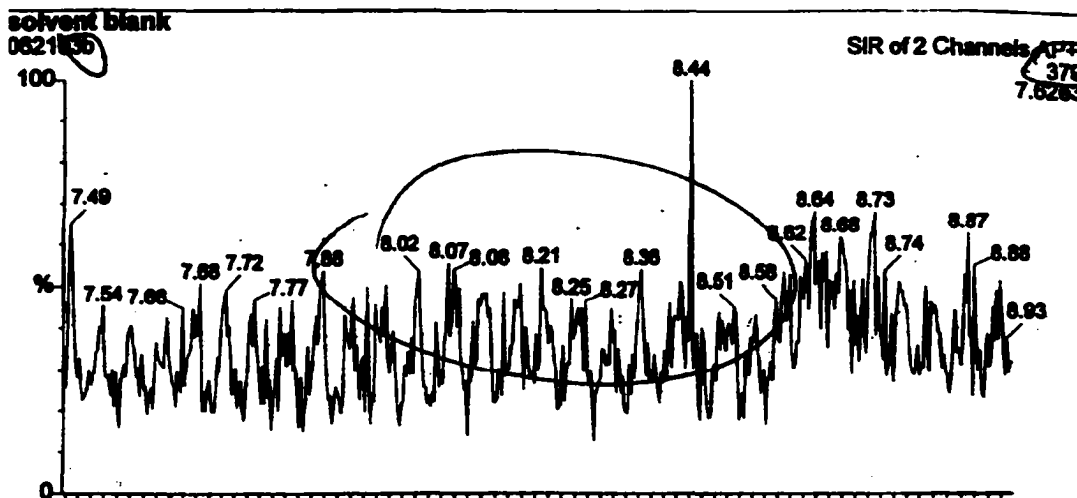


Figure B11d. Mass Spectrometry on Solvent Without Isoflupredone Added

The spike around molecular weight 8.13-8.14 is not seen in this sample of solvent with no isoflupredone added (circled areas).

As with HPLC, Mass Spectrometry could not distinguish between an analyte in the serum of certain untreated cows and isoflupredone acetate. Current work is focused on finding a way to separate the false analyte from the serum prior to HPLC analysis for isoflupredone, using a specialized solid-phase cleanup column.