8-1994

Interaction of oxytetracycline and bacterial collangenase in the treatment of bovine retained placenta

Kellie Ann Fecteau

To the Graduate Council:

I am submitting herewith a thesis written by Kellie Ann Fecteau entitled "Interaction of oxytetracycline and bacterial collangenase in the treatment of bovine retained placenta." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Hugo Eiler, Major Professor

We have read this thesis and recommend its acceptance:

Fred Hopkins, Stephen Oliver

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Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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Date July 22, 1994

INTERACTION OF OXYTETRACYCLINE AND BACTERIAL COLLAGENASE IN THE TREATMENT OF BOVINE RETAINED PLACENTA

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Kellie Ann Fecteau

August 1994

DEDICATION

This thesis is dedicated to my mother for a lifetime of love and giving of herself.

ACKNOWLEDGMENTS

I wish to sincerely thank:

My major professor, Dr. Hugo Eiler, for his guidance in my education, sharing of ideas and knowledge, and the gift of his friendship;

Dr. Fred Hopkins for serving on my committee and for all of his help with the cows at Cherokee Farm;

Dr. Stephen Oliver for serving on my committee and providing supplies and assistance needed for the Bacillus stearothermophilus disc assay;

Dr. Vernon Reich for the statistical analysis of my data and willingness to help;

Mark Lewis for teaching me the Bacillus stearothermophilus disc assay procedure and helping me with the assay;

M. L. Tanner for technical assistance with chemical analyses;

Maria Pyne for her help with formatting this thesis and generating graphs;

My family for their constant love, encouragement, and support;

Melody Crawford and Michael Rowland for their friendship, support, and for making Friday seminar the most enjoyable seminar I've ever attended;

Friends who have lent ears and support;

The Department of Animal Science for the opportunity to continue my education.

ABSTRACT

Retained fetal membranes (RFM) is a postpartum disease that can negatively affect fertility and milk production in the dairy cow population. This disease predisposes the cow to postpartum metritis. Injections of bacterial (Clostridium histolyticum) collagenase have been shown to be highly effective in detaching RFM. Therefore, a combination therapy for RFM and prevention of metritis was hypothesized. This involved mixing collagenase with oxytetracycline, an antibiotic used frequently to treat metritis. However, it has been reported that tetracyclines inhibit collagenase activity. Inactivation of collagenase by oxytetracycline needed to be determined. To test for inactivation of collagenase, isolated bovine placentomes (metabolically active) were infused via umbilical vessels with a mixture of collagenase and oxytetracycline. Eight experimental groups were used: group 1, saline controls; group 2, collagenase 1200 units (U)/ml; groups 3, 4, and 5 contained 1200 U/ml of collagenase plus oxytetracycline (1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, respectively); groups 6, 7, and 8 contained oxytetracycline at 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, respectively. After 5 h of incubation in a warm (39°C) water bath, manometric pressure (pressure needed to separate cotyledon from caruncle), hydroxyproline

(collagenolysis), and total protein (proteolysis) were determined. The antitumor antibiotic doxorubicin was used as a positive control for oxytetracycline. Doxorubicin has been reported to inhibit bacterial (C. histolyticum) collagenase. Experiments with doxorubicin were performed the same as experiments with oxytetracycline except that six groups were used instead of eight. Group 1, saline; group 2, collagenase 1200 U/ml; groups 3, 4, and 5 contained 1200 U/ml of collagenase plus doxorubicin (0.04 mg/ml, 0.02 mg/ml, and 0.01 mg/ml, respectively); group 6 contained doxorubicin at 0.02 mg/ml. It was also the purpose of this study to determine if oxytetracycline residues were present in milk and blood of cows given umbilical injections of collagenase (2×10^5 U/L saline) plus oxytetracycline (0.1 g). This combination therapy was administered to cows that had retained fetal membranes after natural delivery and to cows that had undergone cesarean sections. Samples of blood and milk (when available) were obtained and analyzed for oxytetracycline residues using Bacillus stearothermophilus disc assay.

It was concluded that neither oxytetracycline nor doxorubicin inhibited collagenase in the bovine placentome. All milk and blood (plasma) samples tested negative for oxytetracycline residues.

TABLE OF CONTENTS

LIST OF FIGURES

PART I

INTRODUCTION

STATEMENT OF PURPOSE

Problem

Retained fetal membranes (RFM), more commonly known as retained placenta, is a postpartum disease that affects animals as well as humans. This disease is quite common in dairy cows and can cause economic loss to the producer manifested in milk loss, health problems, and compromised reproductive ability. The etiology of the failure of fetal placental tissue to separate from maternal placental tissue is still not known. There are many methods used for the treatment of RFM, such as manual removal of the membranes and prostaglandin injections, but the most effective, thus far, is use of bacterial collagenase produced by Clostridium histolyticum injected via umbilical vessels of the placenta. This approach is based on the premise that there is a failure in collagen breakdown within the placentome.

Another common disease in the cow is metritis. Metritis occurs frequently in association with retained placenta. A common treatment used by many veterinarians for metritis is oxytetracycline. It is therefore hypothesized that the combination of collagenase and oxytetracycline will have the advantage of loosening the placenta while preventing/fighting infection at the

placentome level. There is concern, however, that oxytetracycline may inhibit collagenase. It has been reported in other studies that tetracyclines inhibit tissue collagenases and bacterial collagenase as observed in human periodontal disease. Inhibition of bacterial collagenase by oxytetracycline needs to be investigated.

It is also thought that by injecting the antibiotic into the placenta there will be little, if any, antibiotic passing into the milk of the cow. It is known that oxytetracycline can cross the placenta. However, since the postpartum placentome is a decaying structure without blood circulation, and perhaps little absorptive capacity, the amount of antibiotic passed into blood and milk should be negligible.

Objectives

The primary objective of this study was to determine if bacterial collagenase was inhibited by oxytetracycline. This was accomplished by injecting isolated placentomes with saline, collagenase, oxytetracycline, and a combination of collagenase and oxytetracycline and recording manometric pressure readings (separation of cotyledon and caruncle), hydroxyproline (collagenolysis) release, and total protein (proteolysis) breakdown. The antitumor antibiotic doxorubicin, which has been reported

to inhibit bacterial collagenase, was used as a positive control.

Another objective of this research was to determine if oxytetracycline residues were present in milk and blood of cows given umbilical injections of collagenase plus oxytetracycline.

LITERATURE REVIEW

Definition of retained fetal membranes

Retained fetal membranes (RFM), more commonly known as retained placenta, is a postpartum disease that affects animals as well as humans. In the bovine, placenta detachment implies detachment or separation of cotyledon from caruncle. This is probably accomplished by hydrolysis of placentome "binding" proteins (Sharpe et al., 1990; Eiler and Hopkins, 1993). The research in this thesis involves the concept of primary retention defined as a failure in cotyledon-caruncle detachment. For research purposes it is important to distinguish a nondetached placenta from one that is detached inside the uterus but has not been expelled. It is not rare to find retained placenta which are detached and loose inside of an atonic uterus. In a study by Eiler and Hopkins (1993), it was observed that of the cows treated with collagenase, 40% of the cows with non-induced RFM and 70% of the cows with experimentally induced RFM had detached fetal membranes that failed to be expelled from the uterus.

Theoretically, all cows that calve have retained placenta. There is always a period of time between parturition and expulsion of the placenta. From data presented by van Werven (1992), most placentas are

expelled within 8 h. Other researchers have reported that approximately 85% of cows release their placentas between 2 and 3 h postpartum (Maas, 1982).

Generally, placentas are considered retained after the passage of a certain amount of time. Definitions of failure of placental expulsion have varied from within 8 to 12 h, within 12 h, within 24 h, and within 48 h postpartum (van Werven et al., 1992). In this thesis, retained placenta is defined as a failure to expel fetal membranes within 12 h postpartum.

Etiology

The basic mechanism for the anatomical detachment of the placenta involves separation of cotyledon from caruncle. This separation is assisted by the weight of the fetal cotyledons along with contractions of the uterus (Roberts, 1986). Unfortunately, detachment of cotyledon from caruncle does not always occur, causing the placenta to be retained. The mechanism for retained placenta is not known. Many factors are known to increase the incidence of RFM, but there is lack of consistent evidence supporting a specific cause-effect relationship.

More recently, etiology of retained fetal membranes has been related to a failure in the breakdown of collagen in the placentome (Sharpe et al., 1989; Eiler and Hopkins,

1992). Collagen is the most abundant fiber in connective tissue. Collagen is inelastic and its triple helical conformation aids in providing tensile strength to this fiber (Junqueira et al., 1992). Collagen can be hydrolyzed by the enzyme collagenase. Collagenase is produced by cells, tissues, and bacteria such as Clostridia. The bacteria that has been most extensively studied is C. histolyticum (Seifter and Gallop, 1962). This enzyme is highly specific for collagens of many animal species. Collagenases may differ little in their composition, however differences may be functionally significant to the extent of determining types of collagen the collagenase will hydrolyze (Seifter and Harper, 1970). Calcium ions promote activity of collagenases and many metal sequestering agents inhibit collagenases (Seifter and Gallop, 1962). Collagenases are proposed to breakdown collagen in the cotyledon matrix in order to open the cotyledon "bag" and to release the "mushroom like" caruncle. Lack of collagenase activation, coupled with an increased amount of type III collagen in the placentome, is hypothesized as a possible cause for RFM (Sharpe et al., 1989; Sharpe et al., 1990; Eiler and Hopkins, 1992; Eiler and Hopkins, 1993).

Another factor associated with the etiology of RFM is a genetic component. Based on analysis of sire influence on the occurrence of retained placenta, Joosten et al.

(1991) proposed a polymorphic genetic system model with a restricted number of genes associated with the etiology of this condition. The idea of genetic control of RFM is consistent with endocrine findings. It is well known that steroid hormones regulate target-cell gene expression (Harrison and Lippman, 1989). The steroid binds to a cell receptor which binds to DNA. Binding of DNA results in a change in transcriptional rate of specific genes (Harrison and Lippman, 1989). Therefore, as an example, progesterone-receptor complex and cortisol-receptor complex could activate different genes. It has been observed that increased levels of progesterone and glucocorticoid can increase incidence of RFM (Chew et al., 1977, 1979; Erb et al., 1981; Agthe and Kolm, 1975)

In studies by Chew et al. (1977, 1979), plasma progesterone was shown to be elevated between days 3-8 prior to parturition in cows that retained fetal membranes. It has been reported extensively in the literature that progesterone inhibits collagenase (Raynes et al., 1988; Halme and Woessner, 1975; Yochim and Blahna, 1976; Jeffrey et al., 1971). Erb et al. (1981) recorded an increase in dystocia in cows whose plasma progesterone levels did not drop before birth. In cows with retained placenta, Agthe and Kolm (1975) observed an increase in estrogen 12 h prior to parturition and a higher progesterone level as compared to controls. Using medium

from cultures of postpartum rat uterus, Jeffrey et al. (1971) found that progesterone at a concentration of 5 x 10^{-5} M produced 100% loss of collagenase activity. It was also found that when progesterone, at minimal effective concentration, was added to medium together with estradiol the activity of collagenase was decreased almost 3-fold. It appears that elevated levels of progesterone, and perhaps estrogen, prior to parturition may be conducive to RFM.

It was reported by Jeffrey et al. (1991) that the neurotransmitter serotonin was required specifically for collagenase production by myometrial cells. It was also observed that myometrial smooth muscle cells in culture produced collagenase in medium containing fetal bovine serum, from which serotonin was isolated, but not in medium containing newborn bovine serum (Jeffrey et al., 1991). It is reasonable to suggest that serotonin serves as a signal to begin the massive collagen degradation and release of fetal membranes in the postpartum uterus. Conversely, lack of serotonin or recognition of serotonin by placentome cells along with a failure of collagenase synthesis/release by placentome tissues could result in retained placenta.

Retention of fetal membranes can be induced by using the synthetic glucocorticoid dexamethasone which causes premature delivery in cows (Mapletoft et al., 1991).

While dexamethasone does not directly inhibit activity of the proteolytic enzyme collagenase, it does inhibit the appearance of the enzyme in uterine cell culture (Koob et al., 1974; Jeffrey et al., 1975). Reduction of collagenase in cell culture parallels the cessation of collagen degradation (Koob et al., 1974).

Shortened gestation length is a factor associated with retained placenta. It is possible that the high incidence of RFM observed in pregnant cows, when induced to deliver by dexamethasone injection, is related to inhibition of placentome collagenase (Sharpe et al., 1990; Eiler and Hopkins, 1993). In non-induced premature delivery, the enzymatic cascade that is possibly needed to release fetal membranes may not be "triggered" in immature placentomes.

Incidence of RFM

Average incidence of RFM in the United States has been estimated to be 11% for all calvings and 8% for uncomplicated calvings (Arthur, 1979). The incidence from various studies has been reported to be from <2% to >50% (Roberts, 1986; Sandals et al., 1979; Bretzlaff et al., 1982).

Several factors have been associated with RFM and appear to increase its incidence. These factors include

shortened gestation length, dystocia, nutritional deficiencies, parity, heat stress, and previous retentions (Sandals et al., 1979; Bretzlaff et al., 1982; Roberts, 1986; Joosten et al., 1991; Dubois and Williams, 1979; Muller and Owens, 1973; Joosten et al., 1987).

Economics

Retained fetal membranes can cause economic loss to the dairy industry. Economic losses associated with RFM and postpartum metritis are mainly seen in four areas: milk production, reproductive performance or fertility, health or treatment of the cow, and culling (Bo et al., 1992; Halpern et al., 1985; Joosten et al., 1988; Deluyker et al., 1991). Economic loss due to reproductive failure in an average Dutch dairy herd has been estimated to be equivalent to 10% of an average farmer's income (Dijkhuizen et al., 1985). It has been reported that fertility of the cow can be compromised by RFM as evidenced by an increase in the number of inseminations per conception; an increase in the number of days open, hence a longer calving interval; and a decrease in pregnancy rate after first service (Halpern et al., 1985; Joosten et al., 1988). Decreased milk production was reported in cases of RFM (Deluyker et al., 1991; van Werven et al., 1992; Joosten et al., 1988) but explanation

of this in the literature is scarce. Joosten et al. (1988) suggested that culling may be involved in decreased milk production. In a study by Schukken et al. (1989), hospitalized cows with retained placenta were three times more likely to develop mastitis than animals without retained placenta. However, in a study by Correa et al. (1993) no significant correlation between RFM and mastitis was reported. It is suspected that mastitis may be largely responsible for the decrease in milk production. If the cow is not productive it will probably be culled from the herd. Economic loss may be attributed to milk that cannot be used and/or sold due to it being contaminated with antibiotic residues from RFM cows treated with antibiotics. As for loss associated with the cow's health, it is not uncommon for a cow with RFM to develop metritis or cystic ovaries (Joosten, 1988) which requires treatment by a veterinarian.

Joosten et al. (1988) estimated financial loss due to retained placenta in Dutch dairy cattle. In a herd with an average incidence of 6.6% the loss was not substantial (£6/cow/year). However, when the rate of retained placenta reached 30% loss was considerable (£22/cow/year). Miller and Dorn (1990) in their survey of sixteen dairy operations in Ohio reported that infertility accounted for 13% of total costs, equalling $23.27/cow/year. In another study, the loss due to a lactation with metritis was

$106.00. This loss represented reproductive efficiency, milk production, cost of medicine, and culling. It was also reported that 44% of lactations which began with retained placenta developed metritis during the lactation (Bartlett et al., 1986). It would seem that retained placenta may have been responsible for part of the $106.00 loss attributed to metritis. Financial loss due to retained placenta for an average United States dairy farm is difficult to find. However, in an unpublished report it has been suggested that economic loss in the United States due to retained placenta at a rate of 7% exceeds $133 million per year (David Thomas, unpublished). This economic impact takes into consideration loss due to metritis.

Retained fetal membranes and metritis complex

Retention of fetal membranes increases the risk of metritis. The occurrence of metritis when fetal membranes are retained has been reported to be as high as 88% (Zamet et al., 1979).

Infection of the uterus or metritis is a commonly diagnosed disorder of the postpartum period in dairy cows. Metritis results from an alteration in the normal clearing of bacteria from the postpartum uterus (Pulfer and Riese, 1991). Normally, these organisms are eliminated by

myometrial contractions, phagocytic leucocyte activity, and by antibacterials produced by glands in the uterus (Paisley et al., 1986). Metritis causes a delay in uterine involution (Pulfer and Riese, 1991) which may be a factor affecting fertility. Metritis may also cause the animal to express signs of toxemia such as dehydration, depression, and anorexia which requires immediate and aggressive medical treatment (Pulfer and Riese, 1991).

Rates of metritis ranging from 32-80% in dairy herds have been reported (Callahan et al., 1987). When metritis was classified as mild or severe, mild cases had no detrimental effect while those classified as severe resulted in reduced fertility. Cows with RFM had a significantly higher incidence of metritis (53%) than cows without RFM (30%). Also, there was a significant difference in conception in cows with RFM and metritis (66%) and those with metritis only (77%) (Callahan et al., 1987).

In a study by Sandals et al. (1979), it was reported that metritis, not RFM, impaired reproductive performance. However, cows experiencing both RFM and metritis are more severely affected than cows with either RFM or metritis by itself.

Metritis is promoted by the decomposing placental tissue which provides a favorable environment for bacterial colonization such as Clostridium spp.,

Escherichia coli, and Pseudomonas aeruginosa (Bretzlaff et al., 1982; Pulfer and Riese, 1991; Roberts, 1986). It is possible that the part RFM plays in metritis may go beyond its role as a favorable substrate for bacterial growth.

Based on a limited number of experiments in our laboratory, excised fetal membranes can survive several days in saline when kept at body temperature. During this time, membranes are metabolically active and are capable of oxygen dependent metabolism and glucose utilization. Thus, RFM may not be dead tissues while attached to the uterus.

This finding of metabolic activity of placentomes suggests that biochemicals similar to those released during inflammatory reactions could be released by RFM further sensitizing the uterus to infection and perhaps generating systemic toxins. If this hypothesis is correct, RFM should be removed as soon as possible.

Treatment

Treatment objectives for bovine RFM are to prevent economic losses due to reduced milk production, impaired reproductive capability, veterinary treatment, and culling (Joosten et al., 1988; van Werven et al., 1992; Deluyker et al., 1991; Bretzlaff et al., 1982). The ideal way of accomplishing these goals would be to remove retained

membranes as soon as possible. It has been reported that for second parity and higher cows, retention exceeding 6 hours had detrimental effects on milk production and reproductive efficiency (van Werven et al., 1992). It would also appear that complications and expenses related to RFM are more likely to increase the longer the membranes are retained. There is no approved rapid way of removing retained membranes. The most effective and efficient method of removal to date is umbilical collagenase injections (Eiler and Hopkins, 1993), but collagenase has not been approved by the Food and Drug Administration (FDA) as a treatment for retained fetal membranes. Traditional methods of treatment include injections of oxytocin and prostaglandin F2-α, treatment with antibiotics, manual removal, and uterine infusion of antiseptics (Paisley et al., 1986; Bolinder et al., 1988; Archbald et al., 1990; Struder et al., 1989; Mapletoft et al., 1991; Putro, 1988). Effectiveness of these treatments is questionable.

Use of prostaglandin F2-α has been reported to be efficacious in promoting uterine involution (Lindell et al., 1982). It has also been reported that PGF2-α reduced the number of services per conception (McClary et al., 1989; Vujosevic et al., 1984). However, Archbald et al. (1990) did not find significant differences in reproductive parameters of PGF2-α treated cows and

untreated cows. Based on their study they would not treat reproductive problems subsequent to retained placenta with PGF2-α. In a study by Kindahl et al. (1984), it was reported that cows with RFM have prolonged release of PGF2-α. Thus, it would appear that prostaglandin administration may not be beneficial to cows with retained membranes. Oxytocin is used to promote uterine contractions and is sometimes effective in expelling RFM (Struder et al., 1989).

Manual removal is no longer a popular method of RFM removal. This method is contraindicated when there is systemic illness and has been found to impair animal fertility (Paisley et al., 1986; Bolinder et al., 1988).

Antibiotics and antiseptics are used mainly to treat metritis that develops subsequent to retained membranes. However, Putro (1988) conducted a study on the effects of intrauterine infusion of dilute iodine solution on the incidence of both retained placenta and endometritis in 40 dairy cows. Within 1-2 h after parturition 30 cows were treated with 500 ml dilute iodine solution, repeated 6 h later. The other 10 cows in the study served as controls. Time of placental expulsion was recorded (>12 h considered retained). Of the treated cows, 13.3% retained placentas as compared to 40% of control cows. Few of the treated cows experienced first degree endometritis while control cows experienced all degrees of endometritis.

A variety of antimicrobial agents such as penicillin, tetracyclines, and sulfonamides are used in the treatment of metritis (Pulfer and Riese, 1991). The antibiotic often used to treat uterine (Dawson et al., 1988) and systemic (Prasad et al., 1987) infections is oxytetracycline. Oxytetracycline is not affected by debris or pus present in the infected uterus (Pulfer and Riese, 1991), thus supporting its effectiveness in fighting the many different bacteria that inhabit the postpartum uterus (Dawson et al., 1988). Tetracyclines can cause irritation to the endometrium of the uterus and have a negative effect on the uterus' defense response (Pulfer and Riese, 1991). This can be alleviated somewhat by using a sufficient amount of saline or water as a carrier for this family of antibiotics (Pulfer and Riese, 1991).

Properties of oxytetracycline

The greatest absorption of oxytetracycline occurs in the blood anywhere from 30 min to several hours after intramuscular administration (Plumb, 1991). However, oxytetracycline is detectable within 15 min after intramuscular injection and maintains significant blood levels for 12 h, declining to trace amounts at 24 h post-injection. The serum half-life of oxytetracycline in

cattle is 9.7 h (Huber, 1988) and the elimination half-life ranges between 4.3 and 9.7 h (Plumb, 1991).

Stability of oxytetracycline when frozen and reconstituted with sterile water was reported to be at least 35 days. However, there was question to the validity of the test due to the use of inaccurate serial dilution method for the assay of potency (Trissel, 1986). This information is necessary for any samples containing oxytetracycline that need to be frozen and analyzed at a later date.

The pH of oxytetracycline ranges from 1.8 to 2.8 when reconstituted with sterile water for intravenous administration. In solutions that range between 3.4 to 7.6, oxytetracycline experiences 10% decomposition within 24 h (Trissel, 1986). The pH is of importance for compatibility with collagenase. Oxytetracycline was added to collagenase solution instead of sterile water and had a pH of 8.75. According to the manufacturer (Sigma Chemical Company), bacterial collagenase has its greatest activity at pH 7.5.

New and promising treatment for retained fetal membranes

Collagenase is the most effective treatment available to date (Eiler and Hopkins, 1993). Injections of 2×10^5 units (U) of bacterial collagenase produced from C.

histolyticum (Sigma Chemical Company, Type XI) are administered into the umbilical arteries to breakdown cotyledon collagen (Eiler and Hopkins, 1993). This technique is simple and described in detail in a paper by Eiler and Hopkins (1993). No adverse side effects were noticed when collagenase was injected either intravenously or via umbilical cord arteries (Eiler and Hopkins, 1993). It is unknown whether an immunogenic reaction is possible if treatment with collagenase is repeated in cows with subsequent retentions.

Umbilical cord injections of 2×10^5 U of collagenase are effective in detaching naturally occurring (85%) and induced (71%) RFM within 36 h (Eiler and Hopkins, 1993). At present, cost of collagenase from Sigma Chemical Company is approximately $23 per 2×10^5 U dose (batch activity of 1750 U/mg).

Injections of collagenase plus antibiotics via umbilical arteries

Based on the effectiveness of collagenase to detach RFM, it was hypothesized that injections of a mixture of collagenase and antibiotic would offer the dual purpose of controlling infection and promoting RFM detachment (Fecteau and Eiler, 1993). Advantages of injecting antibiotics directly into RFM through umbilical arteries

are homogeneous distribution, deep penetration, relatively high tissue concentrations, and long lasting effect of a single dose. The latter two advantages are possible due to membranes being devoid of blood circulation (Roberts, 1986). When membranes are expelled most of the antibiotic should be removed from the body. In the event that oxytetracycline does pass into systemic circulation via umbilical administration, expulsion of the membranes may limit the amount of oxytetracycline that actually passes into blood. Oxytetracycline has not been approved by the FDA for use in lactating dairy cattle, however, it can be used under the guidelines set for veterinarians concerning extra-label use (GAO, 1990). As of 1990 no tolerance level, amount of an animal drug in edible products that is safe in the human diet, had been established for oxytetracycline. Therefore, when oxytetracycline is administered to lactating dairy cows milk must be discarded until free of antibiotic contamination.

Inhibition of collagenase by tetracyclines

In addition to possible antibiotic passage into milk, there was a more immediate concern that needed to be addressed pertaining to the combination of collagenase and oxytetracycline. Numerous reports suggest that tetracyclines inhibit mammalian collagenase (Golub et al.,

1985; Rifkin et al., 1993; Sorsa et al., 1993; Oikarinen et al., 1993; Suomalainen et al., 1992; Lauhio et al., 1992). Tetracyclines are known to inhibit collagenases from several cell types such as neutrophils, macrophages, osteoblasts, and chondrocytes, as well as from tissues like skin, gingiva, cornea, cartilage, and rheumatoid synovium (Rifkin et al., 1993). However, collagenase produced from cultured human fibroblasts appeared to be resistant to tetracycline (Rifkin et al., 1993). The reason being that fibroblasts may be more responsible for tissue remodeling and repair and not predominant in an inflammatory response (Rifkin et al., 1993). Interstitial collagenases promote collagen destruction which is linked to such disorders as rheumatoid and reactive arthritis, periodontal diseases, corneal ulcerations, pathological bone resorption, skin inflammation, and tumor metastasis (Sorsa et al., 1993; Oikarinen et al., 1993; Golub et al., 1987; Lauhio et al., 1992; Suomalainen et al., 1992).

Golub et al. (1985) studied the anticollagenolytic effect of tetracyclines on periodontal disease. Tetracycline loaded monolithic fibers were added to a mixture of collagen and mammalian collagenase in vitro and incubated. There was a 21%-45% inhibition of collagenase activity by tetracycline. This study also resulted in an inhibition of bacterial collagenase. For the in vivo study, gingival crevicular samples were collected, the severity of

inflammation was recorded, and the patient was then put on a two week treatment of low dose minocycline. Result of this study showed a 45%-80% decrease in collagenase activity that lasted for several weeks after treatment stopped.

Lauhio et al. (1992) demonstrated similar results for treatment of reactive arthritis using lymecycline, a form of tetracycline. Through in vitro experiments, it was found that 50% of collagenase activity could be inhibited by using a lymecycline concentration of 310 μM. A study by Suomalainen et al. (1992) found that 15μM-30μM of doxycycline were needed to inhibit 50% of the activity of human neutrophil collagenase. Human fibroblast collagenase was somewhat resistant to tetracycline inhibition.

Golub et al. (1987) showed that the anticollagenolytic effect of tetracyclines was not related to its antimicrobial action. McNamara et al. (1986) reported that by chemically modifying the tetracycline molecule (removing the dimethylamino group from the C4 position on the 'A' ring), the drug's antibiotic efficacy in vitro and in vivo would be eliminated.

Protective mechanisms of the tetracyclines have been postulated. It is thought that these drugs may have the ability to scavenge neutrophil-generated reactive oxygen procollagenase to its actively destructive form (Sorsa et

al., 1993). It has also been suggested that the ability of tetracycline to bind metal ions (Ca^{++}, Zn^{++}) on which collagenase is dependent for activity may influence its inhibitory effect on collagenase (Golub et al., 1987).

In general, this literature review indicated that RFM is a significant postpartum disease in dairy cows. Definitions of failure of placental expulsion have varied from within 8 h to 48 h. In this work, membranes were considered retained if not detached within 12 h postpartum. This time lapse of 12 h has been widely used to define RFM.

Even though there have been many studies correlating nutrition, stress, hormones, infections, physiological, and clinical factors, the etiology of RFM is still unknown. It is of interest that all cows that calve have RFM. There is always a period of time between parturition and expulsion of fetal membranes. Therefore, the incidence of RFM depends very much on the time interval used to define RFM. Based on a 12 h retention, average incidence of RFM has been reported between 6.6% to 11%.

It has been noticed that regardless of the frequent occurrence of this disease in the postpartum cow, very few economic studies have been reported in the United States. However, it has been reported by researchers in The Netherlands that when there is a 30% incidence of RFM the financial loss was substantial. Financial loss is often

attributed not only to RFM, but also to postpartum metritis which is a frequent complication of RFM. Retained fetal membranes are thought to be a favorable tissue for bacterial colonization. Therefore, it seemed reasonable that umbilical injections of oxytetracycline along with collagenase would serve a dual purpose of decreasing bacterial colonization and detaching RFM. Umbilical injections of collagenase have been reported to be highly effective in detaching RFM. However, there have been reports indicating inactivation of collagenase by tetracyclines. For this reason, the primary objective of this research was to investigate the possibility of collagenase inhibition by oxytetracycline.

LITERATURE CITED

Agthe, O. and H. P. Kolm. 1975. Oestrogen and progesterone levels in the blood plasma of cows with normal parturition or with a retained placenta. J. Reprod. Fert. 43:163.

Archbald, L. F., T. Tran, P.G.A. Thomas and S. K. Lyle. 1990. Apparent failure of prostaglandin F2-α to improve the reproductive efficiency of postpartum dairy cows that had experienced dystocia and/or retained fetal membranes. Therio. 34:1025.

Arthur, G. H. 1979. Retention of the afterbirth in cattle: a review and commentary. Vet. Ann. 19:26.

Bartlett, P. C., J. H. Kirk, M. A. Wilke, J. B. Kaneene, and E. C. Mather. 1986. Metritis complex in Michigan holstein-friesian cattle: incidence, descriptive epidemiology and estimated economic impact. Prev. Vet. Med. 4:235.

Bo, G. A., M. Fernandez, A. D. Barth, and R. J. Mapletoft. 1992. Reduced incidence of retained placenta with induction of parturition in the cow. Therio. 38:45.

Bolinder, A. B. Seguin, H. Kindahl, D. Bouley and D. Otterby. 1988. Retained fetal membranes in cows: manual removal versus nonremoval and its effect on reproductive performance. Therio. 30:45.

Bretzlaff, K. N., H. L. Whitmore, S. L. Spahr, and R. S. Ott. 1982. Incidence and treatments of postpartum reproductive problems in a dairy herd. Therio. 17:527.

Callahan, C. J. and L. A. Horstman. 1987. Treatment of early postpartum metritis in a dairy herd: response and subsequent fertility. Bovine Pract. 22:124.

Chew, B. P., H. F. Keller, R. E. Erb and P. E. Malven. 1977. Peripartum concentrations of prolactin, progesterone and the estrogens in blood plasma of cows retaining and not retaining fetal membranes. J. Anim. Sci. 44:1055.

Chew, B. P., R. E. Erb, C. N. Zamet, V. F. Colenbrander, P. V. Malven, and M. F. D'Amico. 1979. Variables associated with peripartum traits in dairy cows. V. hormonal profiles associated with retained fetal membranes. Therio. 12:245.

Correa, M. T., H. Erb, and J. Scarlett. 1993. Path analysis for seven postpartum disorders of Holstein cows. J. Dairy Sci. 76:1305.

Dawson, D. J., E. P. Aalseth, C. H. Hawman, T. Nalsen, and H. J. Howard. 1988. Reproductive performance of dairy cows after early detection and oxytetracycline treatment of postpartum metritis. Bovine Pract. 23:23.

Deluyker, H. A., J. M. Gay, L. D. Weaver, and A. S. Azari. 1991. Change of milk yield with clinical diseases for a high producing dairy herd. J. Dairy Sci. 74:436.

Dijkhuizen, A. A. 1985. Economic aspects of reproductive failure in dairy cattle. I. financial loss at farm level. Prev. Vet. Med. 3:251.

DuBois, P. R., and D. J. Williams. 1980. Increased incidence of retained placenta associated with heat stress in dairy cows. Therio. 13:115.

Eiler, H., and F. M. Hopkins. 1992. Bovine retained placenta: effects of collagenase and hyaluronidase on detachment of placenta. Biol. Reprod. 46:580.

Eiler, H., and F. M. Hopkins. 1993. Successful treatment of retained placenta with umbilical cord injections of collagenase in cows. J.A.V.M.A. 203:436.

Erb, H. N., M. F. D'Amico, B. P. Chew, P. V. Malven, and C. N. Zamet. 1981. Variables associated with peripartum traits in dairy cows. VIII. hormonal profiles associated with dystocia. J. Anim. Sci. 52:346.

Fecteau, K. A., and H. Eiler. 1993. Bovine retained placenta: inhibition of collagenase by oxytetracycline. Biol. Reprod. 48 (Suppl. 1):140.

Golub, L. M., J. M. Goodson, H. M. Lee, A. M. Vidal, T. F. McNamara, and N. S. Ramamurthy. 1985. Tetracyclines inhibit tissue collagenases: effects of ingested low dose and local delivery systems. J. Periodontal. 56:93.

Golub, L. M., T. F. McNamara, G. D. D'Angelo, R. A. Greenwald, and N. S. Ramamurthy. 1987. A non-antibacterial chemically-modified tetracycline inhibits mammalian collagenase activity. J. Dent. Res. 66:1310.

Halme, J., and J. F. Woessner. 1975. Effect of progesterone on collagen breakdown and tissue collagenolytic activity in the involuting rat uterus. J. Endocr. 66:357.

Halpern, N. E., H. N. Erb, and R. D. Smith. 1985. Duration of retained fetal membranes and subsequent fertility in dairy cows. Therio. 23:807.

Harrison, R. W. and S. S. Lippman. 1989. How steroid hormones work. Hosp. Pract. 24:63.

Huber, W. G. 1988. Veterinary Pharmacology and Therapeutics. 6th ed. ed. N. H. Booth and L. E. McDonald. Iowa State University Press. pp. 813-821.

Jeffrey, J. J., R. J. Coffey, and A. Z. Eisen. 1971. Studies on uterine collagenase in tissue culture II. effect of steroid hormones on enzyme production. Biochim. Biophys. Acta. 252:143.

Jeffrey, J. J., T. J. Koob, and A. Z. Eisen. 1975. Hormonal regulation of mammalian collagenases. in Dynamics of Connective Tissue Macromolecules. ed. P. M. C. Burleigh and A. R. Poll, American Elsevier, NY. p.147.

Jeffrey, J. J., L. S. Ehlich, and W. T. Roswit. 1991. Serotonin: an inducer of collagenase in myometrial smooth muscle cells. J. Cell. Physiol. 146:399.

Joosten, I., P. van Eldik, L. Elving, and G. J. W. van der Mey. 1987. Factors related to the etiology of retained placenta in dairy cattle. Anim. Reprod. Sci. 14:251.

Joosten, I., J. Stelwagen, and A. A. Dijkhuizen. 1988. Economic and reproductive consequences of retained placenta in dairy cattle. Vet. Rec. 123:53.

Joosten, I., P. van Eldik, and G. J. W. van der Mey. 1991. Factors affecting occurrence of retained placenta in cattle. Effect of sire on incidence. Anim. Reprod. Sci. 25:11.

Junqueira, L. C., J. Carneiro, and R. O. Kelley. 1992. Basic Histology. 7th ed. Appleton and Lange, Norwalk, CT. pp. 98-104.

Kindahl, H., G. Frederickson, A. Madej, and L. E. Edquist. 1984. Role of prostaglandins in uterine involution. Int. Cong. Anim. Reprod. A. I. 4:9.

Koob, T. J., and J. J. Jeffrey. 1974. Hormonal regulation of collagen degradation in the uterus: inhibition of collagenase expression by progesterone and cyclic amp. Biochim. Biophys. Acta. 354:61.

Lauhio, A., T. Sorsa, O. Lindy, K. Suomalainen, H. Saari, L. M. Golub, Y. T. Konttinen. 1992. The anticollagenolytic potential of lymecycline in the long-term treatment of reactive arthritis. Arthritis Rheum. 35:195.

Lindell, J. O., H. Kindahl, L. Jansson, and L. E. Edqvist. 1982. Postpartum release of prostaglandin F2-α and uterine involution in the cow. Therio. 17:237.

Maas, J. P. 1982. Prevention of retained placenta in dairy cattle. Compend. Cont. Ed. 4:S519.

Mapletoft, R. J., G. A. Bo, A. Garcia, and A. D. Barth. 1991. Controlling the incidence of retained placenta with induction of parturition in the cow. Rev. Bras. Reprod. Anim. 3:140.

McClary, D. G., M. R. Putnam, J. C. Wright and J. L. Sartin. 1989. Effect of early postpartum treatment with prostaglandin F2-α on subsequent fertility in the dairy cow. Therio. 31:565.

McNamara, T. F., L. M. Golub, G. D'Angelo, and N. S. Ramamurthy. 1986. The synthesis and characterization of a non-antibacterial chemically modified tetracycline. J. Dent. Res. 65:226.

Miller, G. Y., and C. R. Dorn. 1990. Costs of dairy cattle diseases to producers in Ohio. Prev. Vet. Med. 8:171

Muller, L. D., and M. J. Owens. 1973. Factors associated with the incidence of retained placentas. J. Dairy Sci. 57:725.

Oikarinen, A., M. Kylmaniemi, H. Autio-Harmainen, P. Autio, and T. Salo. 1993. Demonstration of 72-kDa and 92-kDa forms of type IV collagenase in human skin: variable expression in various blistering diseases, induction during re-epithelialization, and decrease by topical glucocorticoids. J. Invest. Dermatol. 101:205.

Paisley, L. G., W. D. Mickelsen and P. B. Anderson. 1986. Mechanisms and therapy for retained fetal membranes and uterine infections of cows: a review. Therio. 25:353.

Plumb, D. C. 1991. Veterinary Drug Handbook. PharmaVet Publishing. pp.496-507.

Prasad, S., C. Jayachandran, M. K. Singh. 1987. Pharmacokinetics of oxytetracycline in dairy cows. Indian J. Anim. Sci. 57:106.

Pulfer, K. W., and R. L. Riese. 1991. Treatment of postpartum metritis in dairy cows. Iowa State Univ. Vet. 53:27.

Putro, P. P. 1988. Effects of intrauterine dilute iodine solution infusion on the incidence of retained placenta and endometritis in dairy cows. Acta Vet. Scand. 83:58.

Raynes, J. G., F. A. Clarke, J. C. Anderson, R. J. Fitzpatrick, and H. Dobson. 1988. Collagenase inhibitor concentration in cultured cervical tissue of sheep is increased in late pregnancy. J Reprod. Fert. 83:893.

Rifkin, B., A. Vernillo, and L. M. Golub. 1993. Blocking periodontal disease progression by inhibiting tissue-destructive enzymes: a potential therapeutic role for tetracyclines and their chemically-modified analogs. J. Periodontol. 64:819.

Roberts, S. J. 1986. Veterinary Obstetrics and Genital Diseases (Theriogenology). Woodstock, VT. pp.373-383.

Sandals, W. C. D., R. A. Curtis. J. F. Cote, and S. W. Martin. 1979. The effect of retained placenta and metritis complex on reproductive performance in dairy cattle-a case control study. Can. Vet. J. 20:131.

Schukken, Y. H,. H. N. Erb, and J. M. Scarlett. 1989. A hospital-based study of the relationship between retained placenta and mastitis in dairy cows. Cornell Vet. 79:319.

Seifter, S, and P. M. Gallop. 1962. in Methods in Enzymology. Vol. 5: Enzymes of Protein Metabolism. ed. S. P. Colowick and N. O. Kaplan. Academic Press, NY. pp. 659-676.

Seifter, S., and E. Harper. 1970. in Methods in Enzymology. Vol. 19: Collagenases. ed. G. E. Perlman and L. Lorand. Academic Press, NY. pp.613-635.

Sharpe, K. L., H. Eiler, W. C. Cullen, and F. M. Hopkins. 1989. Morphometric analysis of collagen in gestational and retained bovine placentomes. Therio. 32:485.

Sharpe, K. L., H. Eiler, and F. M. Hopkins. 1990. Changes in the proportion of type I and type III collagen in the developing and retained bovine placentome. Biol. Reprod. 43:229.

Sigma Chemical Company. 1994. Biochemicals Organic Compounds for Research and Diagnostic Reagents, pp 282.

Sorsa, T., O. Lindy, Y. T. Konttinen, K. Suomalainen, T. Ingman, H. Saari, S. Halinen, H. M. Lee, L. M. Golub, J. Hall, and S. Simon. 1993. Doxycycline in the protection of serum alpha-1-antitrypsin from human neutrophil collagenase and gelatinase. Antimicrob. Agents Chemother. 37:592.

Struder, E., K. Hirose, A. Holtan, and L. McGinnis. 1989. Efficacy of oxytocin versus fenprostalene for the prevention of retained placenta in the dairy cow. Bovine Pract. 24:81.

Suomalainen, K., S. Halinen, T. Ingman, O. Lindy, H. Saari, Y. T. Konttinen, L. M. Golub, and T. Sorsa. 1992. Tetracycline inhibition identifies the cellular sources of collagenase in gingival crevicular fluid in different forms of periodontal diseases. Drugs Exptl. Clin. Res. 18:99.

Thomas, David. Work unpublished to date. The University of Tennessee.

Trissel, L. 1986. Handbook on Injectable Drugs. 4th ed. pp.426-431.

United States General Accounting Office. 1990. Food Safety and quality: FDA surveys not adequate to demonstrate safety of milk supply: report to the chairman, Human Resources and Intergovernmental Relations Subcommittee, Committee on Government Operations, House of Representatives. Washington, D.C. : The Office. pp. 1-40.

Vujosevic, J., M. Zeremski, I. Korenic, and D. Kuzmanov. 1984. The effect of injection of symthetic prostaglandin F2 alpha (estrumate) in disturbed puerperium on some reproductive measurement in cows. Int. Cong. Anim. Reprod. A. I. Proceed. 3:433.

Werven, T. van, Y. H. Schukken, J. Lloyd, A. Brand, H. Tj. Heeringa and M. Shea. 1992. The effects of duration of retained placenta on reproduction milk production, postpartum disease and culling rate. Therio. 37:1191.

Yochim, J. M. and D. G. Blahna. 1976. Effects of oestrone and progestrone on collagen and ascorbic acid content in the endometrium and myometrium of the rat. J. Reprod. Fert. 47:79.

Zamet, C. N., V. F. Colenbrander, R. E. Erb, C. J. Callahan, B. P. Chew, and N. J. Moeller. 1979. Variables associated with peripartum traits in dairy cows. II. interrelationships among disorders and their effects on intake of feed and on reproductive efficiency. Therio. 11:245.

PART II

DETERMINATION OF BACTERIAL COLLAGENASE INHIBITION BY THE ANTIBIOTICS OXYTETRACYCLINE AND DOXORUBICIN

SUMMARY

The objective was to determine if bacterial collagenase was inhibited by oxytetracycline and doxorubicin. Isolated placentomes were obtained from an abattoir and divided into 8 groups consisting of saline as a control, collagenase (1200 U/cc), 3 different dosages of oxytetracycline (1.0 mg/ml, 0.1 mg/ml (calculated therapeutic dose), and 0.01 mg/ml) plus collagenase (1200 U/cc), and 3 different dosages of oxytetracycline (1 mg/ml, 0.1 mg/ml, and 0.01 mg/ml). For doxorubicin, placentomes were divided into 6 groups consisting of saline as a control, collagenase (1200 U/cc), 3 different dosages of doxorubicin (0.04 mg/ml, 0.02 mg/ml, and 0.01 mg/ml) plus collagenase (1200 U/cc), and doxorubicin at 0.02 mg/ml (calculated therapeutic dose). Placentomes were injected with their respective fluids and incubated for 5 h at 39°C. Neither oxytetracycline nor doxorubicin inhibited bacterial collagenase based on detachment force, hydroxyproline release, and total protein release.

INTRODUCTION

Retention of fetal membranes is a postpartum disease that occurs in an average of 11% of all calvings (Arthur, 1979). This disease is a cause of economic loss manifested in four main areas: milk production, fertility, health, and culling (Bo et al., 1992; Halpern et al., 1985; Joosten et al.,1988; Deluyker et al., 1991). In an attempt to prevent economic loss, various methods of treatment such as injections of PGF2-α, manual removal, antibiotics and antiseptics are used (Paisley et al., 1986; Bolinder et al., 1988; Archbald et al., 1990; Pulfer and Riese, 1991). However, antibiotics and antiseptics are used predominantly as therapy for metritis subsequent to RFM.

Retained fetal membranes and postpartum metritis are highly correlated (Zamet et al., 1979; Callahan and Horstman, 1987; Dawson et al., 1988). Decaying fetal membranes are suspected to be a favorable environment for colonization of microorganisms (Pulfer and Riese, 1991; Paisley et al., 1986). In current antibiotic therapy there may not be distribution of the drug to all areas of infection. This problem may be circumvented by injecting antibiotic into the umbilical arteries of cows with RFM, thus targeting the placentome to receive therapy.

Oxytetracycline is an antibiotic used often to treat metritis in cattle (Dawson et al., 1988; Pulfer and Riese, 1991). It is thought that combining oxytetracycline with collagenase, which is highly effective in detaching RFM, would have the advantage of both detaching membranes and preventing infection at the placentome level. However, oxytetracycline may inhibit the collagenolytic activity of collagenase (Rifkin et al., 1993; Sorsa et al., 1993, Oikarinen et al., 1993; Lauhio et al., 1992; Suomalainen et al., 1992, Golub et al., 1985). The objective of this research was to investigate whether or not bacterial collagenase was inhibited by oxytetracycline. Inhibition was determined by three methods: detachment force (force needed for separation of cotyledon from caruncle), hydroxyproline release (collagenolysis), and total protein breakdown (proteolysis). Recording detachment force allows for the observance of any differences in the pressure needed to separate cotyledon from caruncle in all experimental groups. Hydroxyproline is an amino acid characteristic to collagen (Junqueira et al., 1992). The more collagen that is hydrolyzed (collagenolysis) the greater amount of hydroxyproline that is released. Total protein release is quantitated to insure that collagen breakdown and not overall protein breakdown is responsible for membrane separation. Doxorubicin, a known inhibitor of C. histolyticum collagenase (Bols et al., 1992), was

used as a positive control for oxytetracycline. Doxorubicin is a naturally derived antineoplastic antibiotic. It is hypothesized that disruption of extracellular matrices, such as basement membrane, facilitates tumor invasion and metastasis (Gray et al., 1993; Autio-Harmainen et al., 1993; Stetler-Stevenson, 1990).

In addition to its role as a positive control, the use of doxorubicin allowed for a comparison with oxytetracycline, which does not exhibit antitumor effects, regardless of its anticollagenase activity.

MATERIALS AND METHODS

Tissue collection

Placentomes were obtained from Tennessee Dressed Beef Company, Nashville, TN. Placentomes were from cows ≥7 mo of gestation. Gestation age of fetus was approximated using a fetus development chart (Roberts, 1971). During collection, placentomes (cotyledon and caruncle) were cut from the placental tissue to allow excess cotyledon to show umbilical vessels. Excessively large or small placentomes were discarded. Placentomes were placed in a plastic bag and kept in a cooler filled with ice. In the laboratory, placentomes were kept covered with ice and used within three days. The core temperature of placentomes was approximately 1°C.

Enzyme and antibiotics

Collagenase was purchased from Sigma Chemical Company (St. Louis, MO). Lot numbers and activities used were: 40H6810, 1900 U/mg; 69F68051, 1920 U/mg; 108F6807, 1470 U/mg.

Oxytetracycline hydrochloride (Oxybiotic-100) was obtained from The Butler Company (Columbus, OH). Lot

number and concentration were 9253 and 100 mg oxytetracycline/ml, respectively.

Doxorubicin hydrochloride, USP (Adriamycin PFS) was manufactured by Adria Laboratories (Columbus, OH).

Preparation of collagenase solution

The concentration of collagenase in these experiments was 1200 U/cc saline. This concentration was obtained through calculations based on activity of the enzyme lot being used and the amount of saline needed. Activity was determined by the manufacturer (Sigma Chemical Company) by defining 1 unit as the amount that liberates peptides from collagen equivalent in ninhydrin color to 1.0 μmole of leucine in 5 h at pH 7.4 at 37°C in the presence of calcium ions. Calcium chloride (EM Science, Cherry Hill, NJ), 4 mg/100 cc saline, was added to the collagenase solution for activity purposes and sodium bicarbonate (J.T. Baker Chemical Co., Phillipsburg, NJ), 4 mg/100 cc saline, was added to make the solution more basic. The pH of collagenase solution was corrected for with KOH and HCl and varied from 7.48-7.60 among experiments. Optimum pH of collagenase solution was 7.5.

Validation of collagenase activity

Different lot numbers of collagenase at varying potencies were used. Each batch of collagenase with a different lot number was validated for activity before use. The validation process involved 10 placentomes divided into two groups. There was a saline control group and a collagenase group (1200 U/ml saline). Placentomes were injected with their respective solutions and incubated in a stationary water bath for 5 h at 39°C. After incubation, the pressure needed to separate the cotyledon from the caruncle was recorded and the interface fluid was saved for hydroxyproline and total protein analysis.

Preparation of oxytetracycline solution

The amount of oxytetracycline needed in this experiment was based on dosage recommendation of 3-5 mg/lb body weight (Lewis and Wilken, 1982). The therapeutic dose was calculated by estimating the weight of the bovine placenta.

One ml of oxytetracycline was added to 99 ml of collagenase solution for a final concentration of 1 mg/ml. A ten-fold dilution was used to obtain a concentration of 0.1 mg/ml (therapeutic dose). Another ten-fold dilution

was performed to obtain a concentration of 0.01 mg/ml. Therapeutic dose of oxytetracycline, 0.1 mg/ml, was based on approximated weight of bovine placenta. The pH of these solutions was adjusted with KOH and HCl and varied between 7.4-7.6.

Preparation of doxorubicin solution

One ml of doxorubicin at a concentration of 2 mg/ml was added to 49 ml collagenase solution for a final concentration of 0.04 mg/ml. To obtain a concentration of 0.02 mg/ml (therapeutic dose), 0.50 ml doxorubicin was added to 49.5 ml collagenase solution. To obtain a concentration of 0.01 mg/ml, 0.25 ml doxorubicin was added to 49.75 ml collagenase solution. Therapeutic dose was based on approximated weight of bovine placenta.

Experimental procedure

Eight experimental groups were used with three placentomes per group to test inhibition of collagenase by oxytetracycline: group 1, saline control; group 2, collagenase (1200 U/cc); groups 3, 4, and 5, collagenase (1200 U/cc) plus oxytetracycline at 1.0 mg/cc, 0.1 mg/cc, and 0.01 mg/cc, respectively; groups 6, 7, and 8,

oxytetracycline at 1.0 mg/cc, 0.1 mg/cc, 0.01 mg/cc, respectively.

Six experimental groups were used with three placentomes per group to test inhibition of collagenase by doxorubicin: group 1, saline control; group 2, collagenase (1200 U/cc); groups 3, 4, and 5, collagenase (1200 U/cc) plus doxorubicin at 0.04 mg/cc, 0.02 mg/cc, and 0.01 mg/cc, respectively; group 6, doxorubicin (0.02 mg/cc).

Using a syringe and catheter (Becton-Dickinson 20G 1.5), each placentome was injected via an umbilical vessel with an amount of fluid that was 10% of its weight. Placentomes were housed individually in 10.6 cm x 10.6 cm x 6.0 cm sealed plastic containers with 20 ml of saline to keep them moist. Placentome containers were then placed in an incubating stationary water bath at 39°C for 5 h. After incubation, placentomes were taken from their containers and the force needed to separate cotyledon from caruncle was determined.

Determination of force needed to separate caruncle from cotyledon

Detachment force was accomplished by making an incision in one end of the cotyledon and using Kelly forceps to create a path between the cotyledon and caruncle to the opposite end of the placentome. Through

another incision, and using the Kelly forceps, a 17 cm diameter balloon was pulled gently between membranes of the cotyledon and caruncle. The incision openings were held together by hemostatic forceps and the balloon was inflated by using a hand operated pump on the mercury manometer until the two membranes separated. This was the point at which the pressure was recorded. Saline used to keep the placentomes moist was discarded, the container rinsed, and separated membranes of the placentome were placed in the container with 50 ml of distilled water at 15°C and shaken, 30 revolutions per minute (rpm), for 10 min in a Dubnoff Metabolic Shaking Incubator. After this, the surface fluid of membranes was gently "milked" out and filtered through 10 cm x 10 cm three layer gauze sponges into a beaker. Membranes were discarded and the fluid was swirled and poured into labeled plastic vials and frozen at -20° C until analyzed for hydroxyproline and total protein.

Hydroxyproline standards

Stock solution was prepared by dissolving 10 mg L-hydroxyproline (Sigma Chemical Company) in 100 ml of distilled water.

Standards solutions containing 3, 6, 9, 12, and 15 μg of hydroxyproline were prepared by adding 3, 6, 9, 12,

and 15 ml stock solution to 97, 94, 91, 88, and 85 ml, respectively, of distilled water.

Hydroxyproline analysis procedure

One ml of cotyledon-caruncle interface fluid from each vial was put in a separate Kimax screw capped tube (16 x 125 mm) along with 1 ml of 6 M hydrochloric acid. They were heated overnight (15 h) at 110°C in a Multi-Blok Heater. Samples were analyzed for hydroxyproline by a method described by Kivirikko et al. (1967). After hydrolysis 1 drop of 1% phenolphthalein in ethanol was added to tubes and the sample was adjusted to a light pink color with 10, 1, and 0.1 N KOH. The sample was diluted with distilled water to the 15 ml mark on the hydrolysis tube, mixed, and centrifuged at a speed of 2000 rpm for 10 min.

The hydrolyzed supernatant (0.5 ml) was placed in another screw capped tube and distilled water was added to a final volume of 4 ml. Hydroxyproline standards at 3, 6, 9, 12, and 15 ug were assayed along with samples. One drop of 1% phenolphthalein was added to each of the tubes and the color (indicating pH) was adjusted to pale pink using 0.1 N KOH and HCL. Three gm of KCl, 0.5 ml of 10% alanine solution and 1 ml of potassium borate buffer were added to each tube. Tubes were mixed well and stood at

room temperature for approximately 30 min. One ml of 0.2 M chloramine T solution (made fresh with 1.4 gm chloramine T plus 25 ml ethylene glycol monomethyl ether) was added and tubes stood for another 25 min. After this time, 3 ml of 3.6 M sodium thiosulfate and 5 ml of toluene were added to tubes. Tubes were shaken and centrifuged at a speed of 2000 rpm for 5 min. The toluene layer was suctioned off and discarded and tubes were capped and placed in boiling water for 30 min. Tubes were cooled with running water and 5 ml of toluene were placed in each cooled tube. Tubes were shaken and centrifuged at a speed of 2000 rpm for 5 min. after which time 2.5 ml of toluene were taken from each tube and placed in a test tube along with 1 ml of Ehrlich's reagent and mixed. Samples were then left to stand in the dark for 30 min after which time the absorbance at 560 nm was read on a spectrophotometer (Sequoia-Turner, Model 340).

Total protein analysis procedure

Total protein release was determined by the Kjel dahl method of nitrogen determination as described by Branstreet (1965). In this procedure, 5 ml of cotyledon-caruncle interface fluid and 25 ml concentrated sulfuric acid were added to 200 ml flasks along with a kel-pak that served as a reaction catalyst. Flasks were placed in a

block heater to digest for approximately 45 min. Samples were cooled for several hours and then 50 ml of distilled water were added to each flask. Fifty ml of 4% boric acid and 50 ml distilled water were placed in an Erlenmeyer flask and along with the digested sample flask were placed in Rapid Still II (60 ml NaOH added to sample with this machine) to distill. Sample was allowed to distill for 8 min and then the fluid collected in the Erlenmeyer flask was titrated with 0.1 N HCl until the color changed back to its original purple. Amount of HCl used for each sample was placed in a calculation to determine total protein breakdown.

Statistical analysis

Statistical analysis was performed by use of the computer program SAS. General Linear Models Procedure and Student-Newman-Keuls test were used. Probability of significance was reported at $P \leq 0.01$.

RESULTS

Detachment force

There were no significant differences found in the pressure needed to separate cotyledon from caruncle in the collagenase group and collagenase plus oxytetracycline groups, at any of the dosages. These groups required significantly less pressure to separate the membranes than did the saline control group or any of the oxytetracycline groups (Figure 2.1).

No differences were found between the collagenase and any of the collagenase plus doxorubicin groups in the force needed to separate cotyledon from caruncle. Saline and doxorubicin groups needed more pressure to separate membranes than that required by collagenase or collagenase plus doxorubicin groups (Figure 2.2).

Collagenolysis

A significant difference in the amount of hydroxyproline that was released from the placentomes was noticed between the collagenase group and collagenase plus oxytetracycline group at 1.0 mg/ml. Collagenase plus oxytetracycline group reported higher hydroxyproline values per cc. Collagenase and all collagenase plus

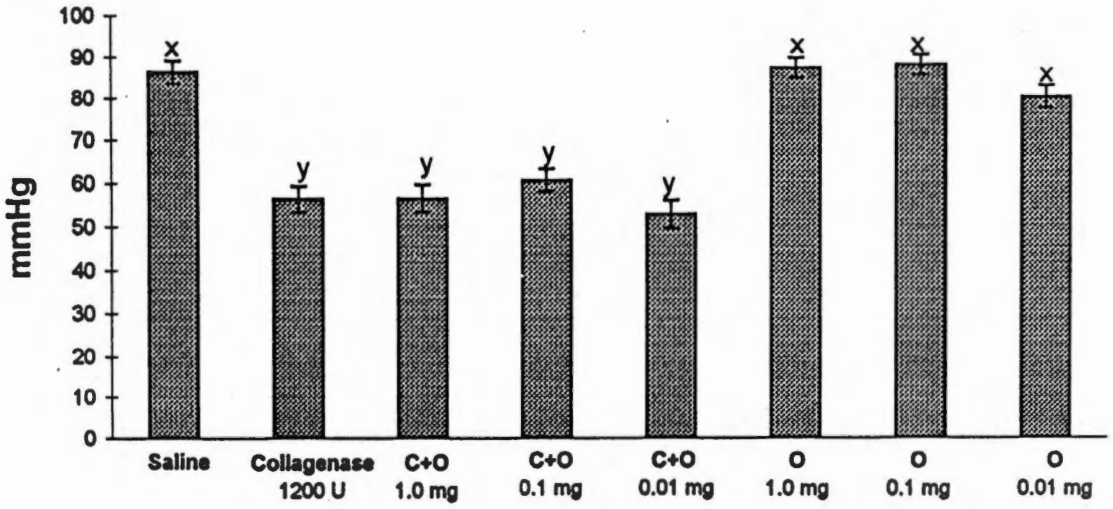


Figure 2.1 - Effect of collagenase and oxytetracycline on detachment force needed to separate cotyledon from caruncle. Columns are mean ± SE for a minimum of 26 placentomes. Different letters (x, y) represent significant difference (P<0.01) among groups. Letters C and O indicate treatments collagenase and oxytetracycline, respectively. Dosage is expressed in units/ml for collagenase and in mg/ml for oxytetracycline.

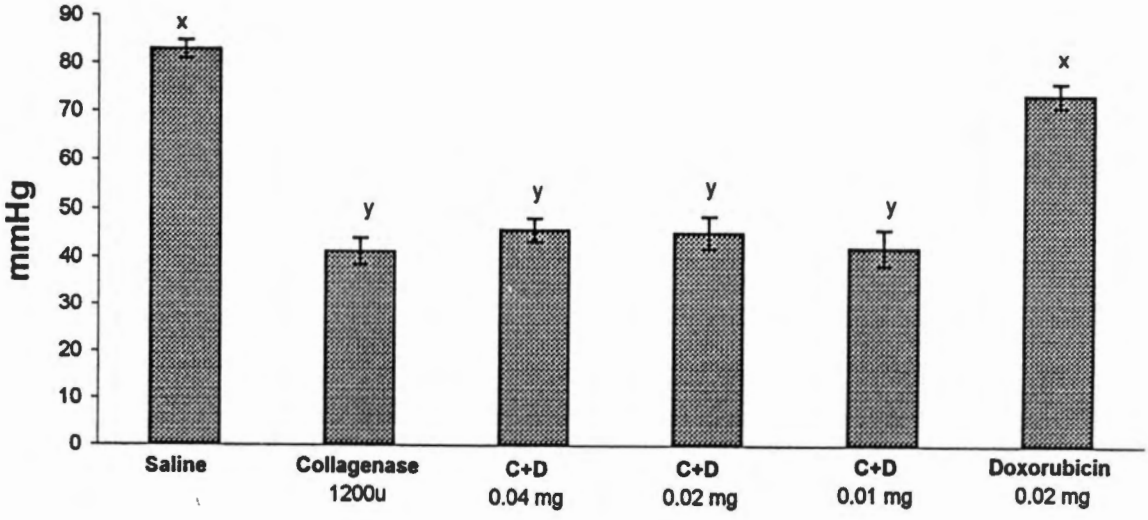


Figure 2.2 - Effect of collagenase and doxorubicin on detachment force needed to separate cotyledon from caruncle. Columns are mean ± SE for a minimum of 20 placentomes per group. Different letters (x, y) represent significant difference ($P<0.01$) among groups. Letters C and D indicate treatments collagenase and doxorubicin, respectively. Dosage is expressed in units/ml for collagenase and in mg/ml for doxorubicin.

oxytetracycline groups released significantly more hydroxyproline than the saline control group and any of the oxytetracycline groups. In general, oxytetracycline did not affect collagenase activity (Figure 2.3).

There were no significant differences in the amount of hydroxyproline released from the collagenase group and any of the collagenase plus doxorubicin groups. There were differences between these groups and the saline and doxorubicin groups, where significantly less hydroxyproline was released (Figure 2.4).

Total protein

There were no significant differences in the amount of total protein released among any of the groups in either experiment (Figure 2.5 and Figure 2.6).

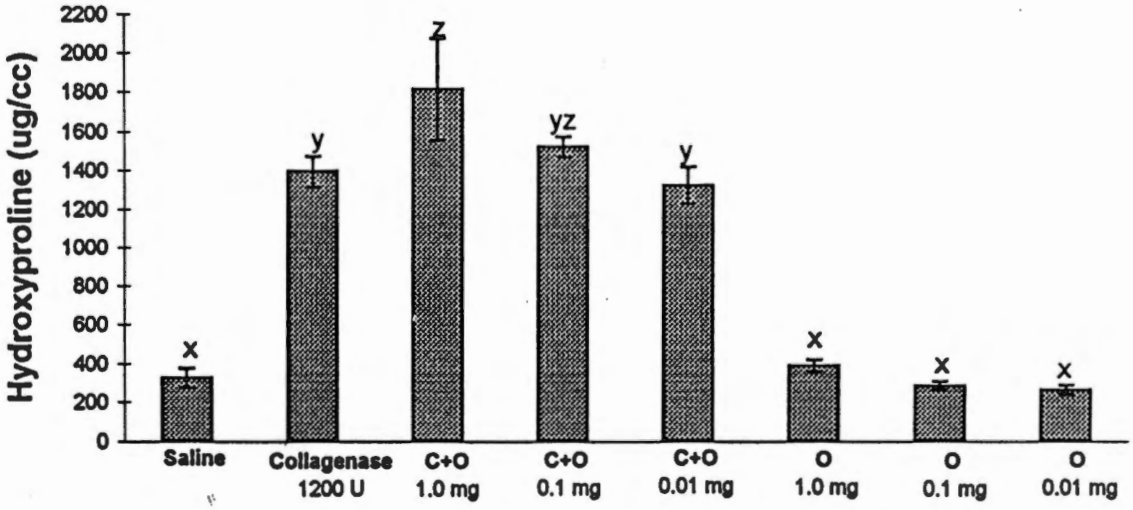


Figure 2.3 - Effect of collagenase and oxytetracycline on release of hydroxyproline. Columns are mean ± SE for a minimum of 24 placentomes per group. Different letters (x, y, z) represent significant difference ($P<0.01$) among groups. Letters C and O indicate treatments collagenase and oxytetracycline, respectively. Dosage is expressed in units/ml for collagenase and in mg/ml for oxytetracycline.

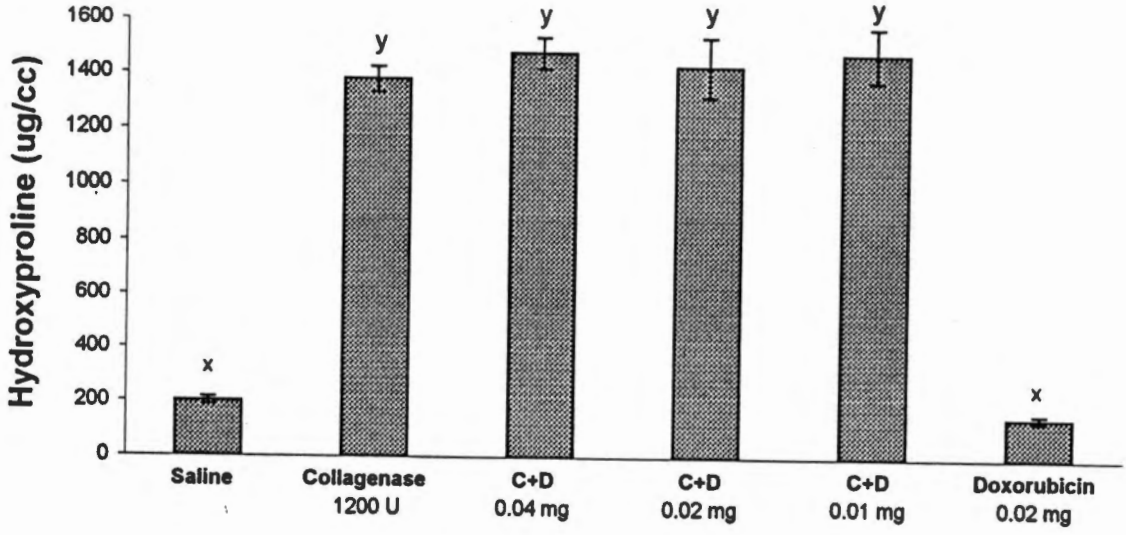


Figure 2.4 - Effect of collagenase and doxorubicin on release of hydroxyproline. Columns are mean ± SE for a minimum of 20 placentomes per group. Different letters (x, y) represent significant difference ($P<0.01$) among groups. Letters C and D indicate treatments collagenase and doxorubicin, respectively. Dosage is expressed in units/ml for collagenase and in mg/ml for doxorubicin.

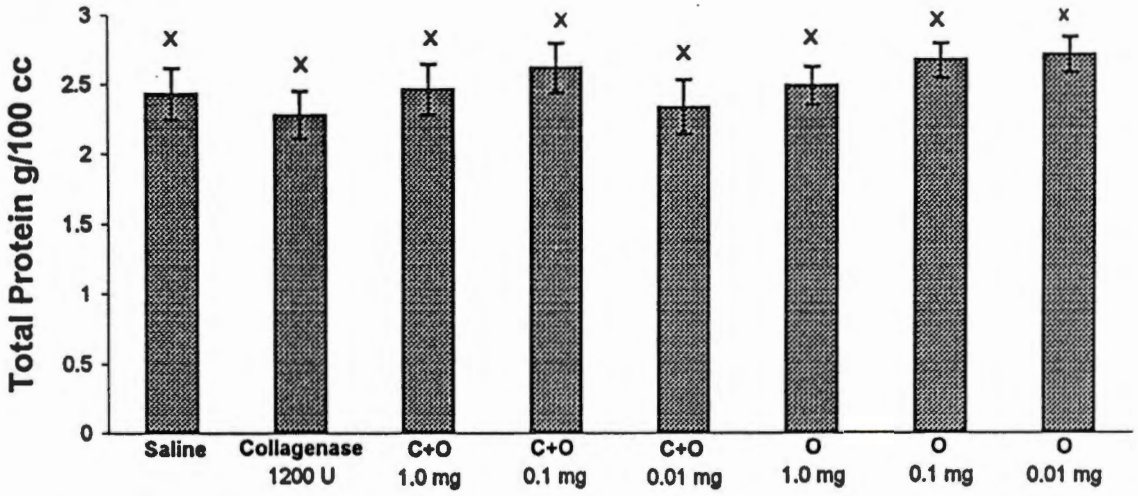


Figure 2.5 - Effect of collagenase and oxytetracycline on total protein breakdown. Columns are mean ± SE for a minimum of 23 placentomes per group. The same letter (x) represents no significant difference (P>0.01) among groups. Letters C and O indicate treatments collagenase and oxytetracycline, respectively. Dosage is expressed in units/ml for collagenase and in mg/ml for oxytetracycline.

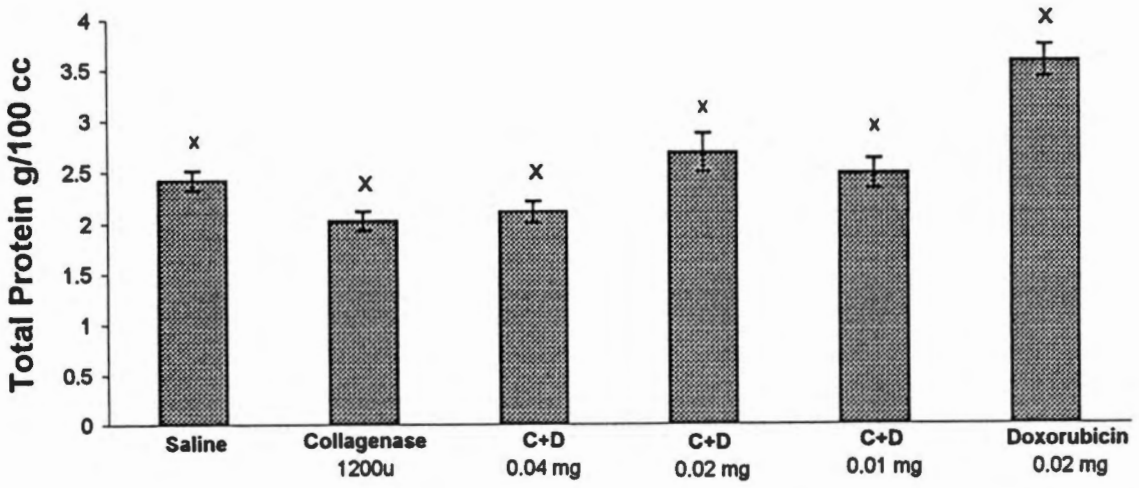


Figure 2.6 - Effect of collagenase and doxorubicin on total protein breakdown. Columns are mean ± SE for a minimum of 21 placentomes per group. The same letter (x) represents no significant difference (P>0.01) among groups. Letters C and D indicate treatments collagenase and doxorubicin, respectively. Dosage is expressed in units/ml for collagenase and in mg/ml for doxorubicin.

DISCUSSION

In these experiments neither oxytetracycline nor doxorubicin inhibited collagenase activity. This indicates that oxytetracycline and collagenase are compatible for the treatment of RFM. The activity of collagenase is highly dependent upon pH. This is important when mixing with oxytetracycline because this antibiotic is very acidic when reconstituted with sterile water (Trissel, 1986) and very basic when not reconstituted.

The fact that oxytetracycline did not inhibit collagenase in the placentome is in contrast with research conducted in humans. Both in vivo and in vitro studies have been performed using mammalian collagenase and tetracyclines that showed that tetracyclines inhibited tissue collagenase (Golub et al., 1985; Suomalainen et al., 1992; Lauhio et al., 1992; Sorsa et al., 1993; Oikarinen et al., 1993). However, the anticollagenase effect of tetracyclines seemed to be relatively specific to different types of collagenase. For example, collagenase from gingival crevicular fluids, but not collagenase from human fibroblasts, was inhibited by tetracyclines (Suomalainen et al., 1992). Lack of collagenase inhibition in the placentome may be due to structural differences in the bacterial collagenase that

perhaps will not allow tetracyclines to bind. Another possibility is that the amount of tetracycline added to the collagenase was too low to cause inhibition. As reported by Golub et al. (1985), it took two weeks to inhibit collagenase 45%-80% by administering 40-80 mg/day. The experiment described in this thesis used, at most, 100 mg of oxytetracycline that was administered once. However, it is known that members of the tetracycline family differ in their inhibition of collagenase (Rifkin et al, 1993). Oxytetracycline may not be as potent an inhibitor of collagenase as other tetracyclines. Another explanation may be that perhaps the extra calcium (calcium chloride) added to the collagenase solution protected the active site of collagenase, which is very likely to be occupied by a zinc ion (Seifter and Harper, 1970). One of the suggested mechanisms of inhibition by tetracyclines was its ability to bind metal ions such as zinc (Golub et al., 1987). It appears that calcium ions may aid collagenase in conforming around zinc. If calcium is not present, not all chelating positions of zinc are occupied leaving positions open for other substances to bind (Seifter and Harper, 1970). Thus, it seems reasonable to postulate that excess calcium promoted collagenase binding to all chelating positions of zinc leaving no position open for oxytetracycline to bind and inhibit collagenase.

Doxorubicin did not inhibit collagenase which is inconsistent with results of a study by Bols et al. (1992). Lack of inhibition did not appear to be related to a dose-effect relationship between doxorubicin and collagenase. Based on molarity, the amounts of doxorubicin used in this work were greater, ranging from 20 μM to 68 μM as compared to 1.1 μM used in the study by Bols et al. (1992). Therefore, it does not appear that the molar amount of doxorubicin used in this current work was responsible for lack of collagenase inhibition. It is difficult to explain lack of collagenase inhibition by doxorubicin. It is known that collagenases are specific for different types of collagen. A possible explanation to the difference in results of the two experiments could be the different systems used. Collagen in the experiment conducted by Bols et al. (1992) was obtained from Sigma Chemical Company (St. Louis, MO). In contrast, collagen in the current study was native to the placentome. Collagenase may have been more specific for the native collagen producing greater hydrolization of collagen and was not significantly affected by doxorubicin. In addition, there may have been tissue inhibitors present in the placentomes that blocked doxorubicin's effect on collagenase. However, this was not investigated. Another possibility to explain the lack of inhibition is that in this work calcium chloride was added to the collagenase

solution. Perhaps all chelating positions were occupied leaving no position for doxorubicin to bind and promote inhibition of collagenase.

The fact that doxorubicin did not act as a positive control does not invalidate the main conclusion of this experiment that oxytetracycline and collagenase are compatible substances for the treatment of RFM. The rationale is that the inhibitory action of doxorubicin may have itself been inhibited by the environment or system used in this experiment. Further investigation would be needed to better understand the discrepancy of results.

LITERATURE CITED

Archbald, L. F., T. Tran, P. G. A. Thomas, and S. K. Lyle. 1990. Apparent failure of prostaglandin F2a to improve the reproductive efficiency of postpartum dairy cows that had experienced dystocia and/or retained fetal membranes. Therio. 34:1025.

Autio-Harmainen, H., T. Karttunen, T. Hurskainen, M. Hoyhtya, A. Kauppila, and K. Tryggvason. 1993. Expression of 72 kilodalton type IV collagenase (gelatinase A) in benign and malignant ovarian tumors. Lab. Invest. 69:312.

Bo, G. A., M. Fernandez, A. D. Barth, and R. J. Mapletoft. 1992. Reduced incidence of retained placenta with induction of parturition in the cow. Therio. 38:45.

Bolinder, A., B. Seguin, H. Kindahl, D. Bouley, and D. Otterby. 1988. Retained fetal membranes in cows: manual removal versus nonremoval and its effect on reproductive performance.Therio. 30:45.

Bols, M., L. Binderup, J. Hansen, and P. Rasmussen. 1992. Inhibition of collagenase by aranciamycin and aranciamycin derivatives. J. Med. Chem. 35:2768.

Branstreet, R. B. 1965. The Kjel dahl Method for Organic Nitrogen. Academic Press, NY.

Callahan, C. J., and L. A. Horstman. 1987. Treatment of early postpartum metritis in a dairy herd: response and subsequent fertility. Bovine Pract. 22:124.

Dawson, D. J., E. P. Aalseth, C. H. Hawman, T. Nalsen, and H. J. Howard. 1988. Reproductive performance of dairy cows after early detection and oxytetracycline treatment of postpartum metritis. Bovine Pract. 23:23.

Deluyker, H. A., J. M. Gay, L. D. Weaver, and A. S. Azari. 1991. Change of milk yield with clinical diseases for a high producing dairy herd. J. Dairy Sci. 74:436.

Golub, L. M., J. M. Goodson, H. M. Lee, A. M. Vidal, T. F. McNamara, and N. S. Ramamurthy. 1985. Tetracyclines inhibit tissue collagenases: effects of ingested low dose and local delivery systems. J. Periodontol. 56:93.

Golub, L. M., T. F. McNamara, G. D. D'Angelo, R. A. Greenwald, and N. S. Ramamurthy. 1987. A non-

antibacterial chemically-modified tetracycline inhibits mammalian collagenase activity. J. Dent. Res. 66:1310.

Gray, S. T., K. Yun, T. Motoori, and Y. M. Kuys. 1993. Interstitial collagenase gene expression in colonic neoplasia. Amer. J. Pathol. 143:663.

Halpern, N. E., H. N. Erb, and R. D. Smith. 1985. Duration of retained fetal membranes and subsequent fertility in dairy cows. Therio. 23:807.

Joosten, I, J. Stelwagen, and A. A. Dijkhuizen. 1988. Economic and reproductive consequences of retained placenta in dairy cattle. Vet. Rec. 123:53.

Junqueira, L. C., J. Carneiro, and R. O. Kelley. 1992. Basic Histology. 7th ed. Appleton and Lange. Norwalk, CT. pp. 98-104.

Kivirikko, K. I., O. Laitinen, and D. J. Prockop. 1967. Modifications of a specific assay for hydroxyproline in urine. Anal. Biochem. 19:249.

Lauhio, A., T. Sorsa, O. Lindy, K. Suomalainen, H. Saari, L. M. Golub, Y. T. Konttinen. 1992. The anticollagenolytic potential of lymecycline in the long-term treatment of reactive arthritis. Arthritis Rheum. 35:195.

Lewis, B. P., and L. O. Wilken. 1982. Veterinary Drug Index. W. B. Saunders Company. p. 54.

Oikarinen, A., M. Kylmaniemi, H. Autio-Harmainen, P. Autio, and T. Salo. 1993. Demonstration of 72-kDa and 92-kDa forms of type IV collagenase in human skin: variable expression in various blistering diseases, induction during re-epithelialization, and decrease by topical glucocorticoids. J. Invest. Dermatol. 101:205

Paisley, L. G., W. D. Mickelsen, and P. B. Anderson. 1986. Mechanisms and therapy for retained fetal membranes and uterine infections of cows: a review. Therio. 25:353.

Pulfer, K. W., and R. L. Riese. 1991. Treatment of postpartum metritis in dairy cows. Iowa State Univ. Vet. 53:27.

Rifkin, B., A. Vernillo, and L. M. Golub. 1993. Blocking periodontal disease progression by inhibiting tissue-destructive enzymes: a potential therapeutic role for tetracyclines and their chemically modified analogs. J. Periodontol. 64:819.

Roberts, S. J. 1971. Veterinary Obstetrics and Genital Diseases. 2nd ed. Ithaca, NY. p. 19.

Seifter, S, and E. Harper. in Methods in Enzymology. Vol. 19: Collagenases. ed. G. E. Perlman and L. Lorand. Academic Press, NY. pp. 613-635.

Sorsa, T. O. Lindy, Y. T. Konttinen, K. Suomalainen, T. Ingman, H. Saari, S. Halinen, H. M. Lee, L. M. Golub, J. Hall, and S. Simon. 1993. Doxycycline in the protection of serum alpha-1-antitrypsin from human neutrophil collagenase and gelatinase. Antimicrob. Agents Chemother. 37:592.

Stetler-Stevenson, W. G. 1990. Type IV collagenases in tumor invasion and metastasis. Cancer Metastasis Rev. 9:289.

Suomalainen, K, S. Halinen, T. Ingman, O. Lindy, H. Saari, Y. T. Konttinen, L. M. Golub, and T. Sorsa. 1992. Tetracycline inhibition identifies the cellular sources of collagenase in gingival crevicular fluid in different forms of periodontal diseases. Drugs Exptl. Clin. Res. 18:99.

Trissel, L. 1986. Handbook on Injectable Drugs. 4th ed. pp.426-431.

Zamet, C. N., V. F. Colenbrander, R. E. Erb, C. J. Callahan, B. P. Chew, and N. J. Moeller. 1979. Variables associated with peripartum traits in dairy cows. II interrelationships among disorders and their effects on intake of feed and on reproductive efficiency. Therio. 11:245.

PART III

DETERMINATION OF OXYTETRACYCLINE RESIDUES IN BLOOD AND MILK OF COWS GIVEN UMBILICAL INJECTIONS OF COLLAGENASE PLUS OXYTETRACYCLINE

SUMMARY

The objective of this experiment was to determine if oxytetracycline residues were present in blood and milk of cows given umbilical injections of collagenase plus oxytetracycline. Cows (n=4) near term pregnancy were obtained from a local stockyard and divided into two groups: natural delivery and cesarean section. Parturition was induced by injection of prostaglandin and dexamethasone. Cows that delivered naturally retained fetal membranes. Arteries of the umbilical cord were injected with a mixture of collagenase (2×10^5 U) and oxytetracycline (1 cc or 0.1 g). Blood samples (collected through a jugular catheter) and milk samples, if available, were obtained prior to injection and at preset times post-injection. Plasma and serum were obtained from blood samples. Plasma and milk were analyzed for antibiotic residues by the Bacillus stearothermophilus disc assay.

Results from the bioassay showed that both milk and plasma samples tested negative for antibiotic residues.

INTRODUCTION

Postpartum metritis is a frequent complication of retained fetal membranes (RFM) (Sandals et al., 1979; Roberts., 1986; Bretzlaff et al., 1982; Callahan and Horstman, 1987). The reason for this is not known. It is thought that because fetal membranes are a decaying structure they provide a favorable environment for bacterial colonization (Pulfer and Riese, 1991; Bretzlaff et al., 1982; Roberts, 1986). Uterine infusions of oxytetracycline are used frequently to prevent uncontrolled bacterial growth and subsequent complications (Dawson et al., 1988). However, uterine infusions may not reach all areas of a uterus with RFM (Bretzlaff et al., 1982). Therefore, it is doubtful that oxytetracycline would penetrate deep into placentomes. It is known that antibiotics infused into the uterus gain blood circulation and pass into milk (Oliver et al., 1990; Kaneene et al., 1986). This could pose a hazard to human health and be economically detrimental to the dry milk and cheese industry among others (GAO, 1990; Marth and Ellickson, 1959). A goal in administering antibiotics would be to treat RFM metritis while preventing antibiotic passage into milk.

Presently, the most effective treatment for detachment of RFM is umbilical injections of collagenase

(Eiler and Hopkins, 1993). This technique lead to the idea of combining collagenase solution with a relatively small, but effective amount of oxytetracycline. This method would have three hypothetical advantages: to loosen RFM, to prevent infection, and minimize passage of oxytetracycline into blood and milk. With this approach, most oxytetracycline would be deposited in RFM without exposing the antibiotic to peripheral elimination by the kidney and liver (Plumb, 1991; Huber, 1988).

The objective of this experiment was to determine if oxytetracycline would pass into blood and milk when injected into umbilical arteries retrieved from hanging RFM and also into umbilical arteries isolated during cesarean section.

MATERIALS AND METHODS

Animals

Four beef cows near term pregnancy (≥8 mo) were purchased from a local stockyard. The four cows were divided in two groups of two cows each. Group 1 was natural delivery and group 2 was cesarean section delivery.

Group 1, natural delivery

The two cows (#39 and #34) that composed this group were induced to deliver by an injection of 20 mg of dexamethasone (Anthony Products, Arcadia, CA) and 25 mg of prostaglandin F2-α (Lutalyse, The Upjohn Company, Kalamazoo, MI). The two cows delivered normally approximately 36 h and 48 h after dexamethasone/prostaglandin F2-α injection. Both cows retained fetal membranes, and between 24 and 36 h postpartum the umbilical arteries of the hanging membranes were retrieved in each cow.

In cow #39, umbilical arteries were catheterized and injected with a mixture of 2×10^5 U of collagenase and 10 cc (1.0 g) of oxytetracycline (Oxybiotic-100) in one liter of saline. In cow #34, the umbilical arteries were

catheterized and injected with a mixture of 2 x 10^5 U of collagenase and 1.0 cc (0.1 g) of oxytetracycline in one liter of saline. An indwelling jugular catheter was placed in each cow immediately prior to the experiment. Blood samples were taken prior to injection of collagenase/oxytetracycline and every 30 min for the first 2 h post-injection and then every h for 5 h. Blood samples were also taken at 24 and 48 h after injection. Blood was collected in vacutainers with and without EDTA to allow for both serum and plasma samples to be used in antibiotic residue determination. Milk samples were obtained from cow #39 at 5.5, 6.0, 29.5, and 54 h after injection. Milk from cow #34 was obtained at 6.0 h only. Serum, plasma, and milk samples were stored at -20° C until assayed for antibiotic residues.

Group 2, cesarean section delivery

The two cows (#24 and #29) were injected with dexamethasone (20 g) and prostaglandin F2-α (25 g). Approximately 36 h post dexamethasone/prostaglandin F2-α injection, a cesarean section was conducted in each cow. Umbilical arteries were catheterized and injected with a mixture of collagenase (2 x 10^5 U) and oxytetracycline (0.1 g) in one liter of saline. A jugular catheter was placed in each cow after the cesarean operations were

completed. Blood samples were taken at preset times consistent with group 1. Serum and plasma samples were kept at -20° C until performance of antibiotic residue analysis. Milk samples were not obtained from either cow due to lack of colostrum or milk secretions. The pH of collagenase/oxytetracycline injections was adjusted to 7.5 units for optimum collagenase activity.

Procedure used for cesarean section

The cow was placed in right recumbency. Lidocaine 2% solution (Bristol Laboratories, Syracuse, NY) was used for a line block located approximately 4 cm to the left of midline extending caudally to within 8 cm of the udder and 5 cm cranial to the umbilicus. Betadine solution (Perdue Federick Co., Norwalk, CT) was used as a sterile preparation. A paramedian incision was made in the position of the line block and continued through the abdominal musculature and peritoneum. The live (premature) fetus was removed from the uterine horn and euthanized immediately. The two umbilical arteries of the placenta were located, catheterized, and each injected with 500 ml of collagenase solution (2×10^5 U collagenase/L saline plus 1.0 cc oxytetracycline). The uterus was closed using a simple continuous pattern with #4 catgut. A three layer closure followed starting with

the peritoneum and fascia, then the muscular and subcutaneous tissues, and ending with closure of the skin.

Bacillus stearothermophilus disc assay procedure

The Bacillus stearothermophilus disc assay was used to detect oxytetracycline residues in milk and plasma. This test was designed and used for determination of antibiotic residues in milk (GAO, 1990; Anonymous, 1982) and has been reported to detect >0.20 μg/ml of oxytetracycline (Haaland et al., 1984). The principle of this assay is inhibition of bacterial growth by the antibiotic. In this study, the procedure described by Ouderkirk (1979) was used for milk samples and plasma samples. The B. stearothermophilus disc assay was not designed to detect antibiotic residues in plasma, therefore parameters of this assay are unknown for plasma. Plasma that was free of antibiotic was spiked with oxytetracycline to serve as a control for comparison with plasma samples obtained from cows.

Media were made by adding 200 ml deionized water to a flask with 6.4 g PMI agar (Difco Laboratories, Detroit, MI) and 0.012 g bromocresol purple (J.T. Baker Chemical Co., Phillipsburg, NJ). The flask was placed on a stir/heat plate to mix and dissolve the agar and then autoclaved, along with a Cornwall pipette, to 250°F for 15

min. Media were cooled to <80°C then two 1.0 ml vials of B. stearothermophilus spores (Difco Laboratories, Detroit, MI) were added via a sterile transfer pipette and mixed to distribute spores. A Cornwall pipette was used to deliver 6.0 ml of media to each sterile petri dish. Media were distributed evenly and allowed to solidify. Petri dishes were incubated at 64°C for 30 min, then cooled to room temperature, and refrigerated for 24 h before use. To plate samples, petri dishes, milk, and plasma samples were brought to room temperature and the dishes were numbered starting with 1 and each was also numbered 1-4 to correspond where discs were placed. All samples were run in duplicate. The sample was mixed gently and, using clean forceps, a non-sterile disc (13 mm diameter) was touched to the sample and filled by capillary action. Excess sample was removed by blotting the disc on a paper towel. The disc was placed on the agar surface. Neomycin discs were used on each plate and served as a positive control. Plates were incubated for 3 h at 64°C. After incubation, agar turned from purple to yellow except where inhibition occurred. Diameter of zones of growth inhibition were measured and duplicates were averaged. A sample was considered positive for oxytetracycline residues if the zone of inhibition was >16.

RESULTS

Release of retained fetal membranes

Group 1, the cows that delivered naturally, failed to release RFM after injection of collagenase plus oxytetracycline. Membranes remained firmly attached for 8 days in cow #39, which received 1.0 g oxytetracycline, and for 6 days in cow #34, which received 0.1 g oxytetracycline. Within 36 h after injection, cow #39 developed a severe metritis that produced an abundance of a whitish secretion that was expelled spontaneously during defecation or when the membranes were pulled manually.

Group 2, the cows that delivered by cesarean section, had retained membranes hanging from their vulvas by 24 h and had expelled placentas within 36 h after the collagenase/oxytetracycline injection.

Bioassay

Based on the knowledge that a zone of inhibition is considered positive for antibiotic residue if >16 mm in diameter (Anonymous, 1982), milk samples screened were negative. Zones of inhibition for these samples ranged from 13.0 mm (size of disc, no observable zone) to 14.6 mm (Table 3.1). The B. stearothermophilus disc assay was not

Table 3.1 Bacillus stearothermophilus disc assay determination of oxytetracycline in milk of cows given umbilical injections of collagenase plus oxytetracycline

Cow No. and quarter (a)	Time post-injection (hours)	Zone of inhibition (mm diameter) (b,c)
39 LF	6	13.6
39 RF	30	13.0
39 LF	30	13.0
39 RF	54	14.6
39 LF	54	14.1
34 RF	6	13.0

(a) Milk available only from designated left front (LF) and right front (RF) quarters from cow 39 and cow 34
(b) Numbers are mean for duplicates with a variation of ≤1 mm in diameter. Overall mean and SD were 13.6 ± 0.7 mm
(c) Neomycin control was 13 mm.

designed to detect antibiotics in plasma. This disc assay may not be limited to detection in milk, but this has not been officially determined. The criteria for detection of residues in plasma using this assay are unknown. Therefore, the size standard of >16 mm used for zones of inhibition in milk was also applied to plasma. Zone size of plasma samples did not exceed 14.75 mm and were interpreted as negative for oxytetracycline detection. Samples that had been spiked with oxytetracycline produced zone sizes of 29 to 30 mm in diameter. These were interpreted as positive for oxytetracycline residues (Table 3.2, Figures 3.3 and 3.4).

Table 3.2 *Bacillus stearothermophilus* disc assay determination of oxytetracycline in plasma of cows given umbilical injections of collagenase plus oxytetracycline

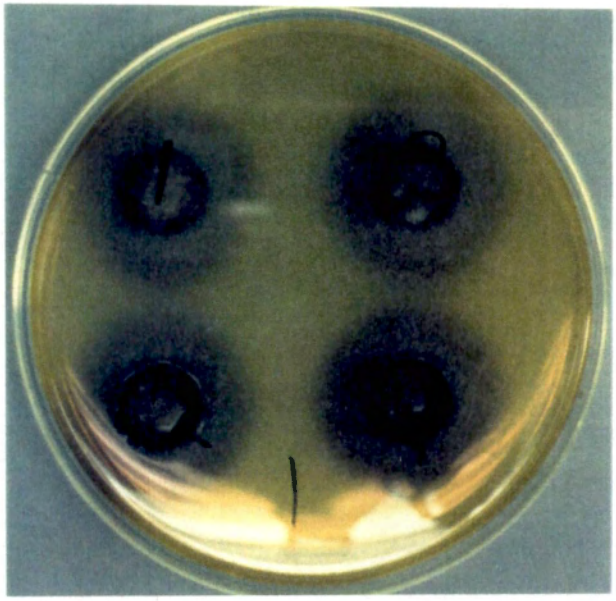
Cow No.	Time post-injection (hours)	Zone of inhibition (mm diameter) (a, b)
39	0	13.0
	3	13.0
	24	14.5
	54	14.8
34	0	13.0
	3	13.0
	24	ND
	54	13.0
24	0	13.0
	3	13.0
	24	13.0
	54	ND
29	0	13.0
	3	13.0
	24	ND
	54	ND

(a) Numbers are means for duplicates with a variation of ≤1 mm in diameter. Overall mean and SD were 13.3 ± 0.6 mm

(b) Control plasma plus 0.0, 5.0, 10.0, and 20.0 µg/ml oxytetracycline resulted in zone of inhibition of 13.0, 29.5, 30.0, and 29.0 mm, respectively.
Neomycin control was 13.0 mm.

ND Not determined

Figure 3.3
Detection of oxytetracycline in plasma samples using Bacillus stearothermophilus disc assay. Plate 1 is a known amount of oxytetracycline added to plasma. Discs 1 and 2 contain 0.01 mg/ml, and discs 3 and 4 contain 0.1 mg/ml. Plate 2 is a known amount of oxytetracycline added to plasma. Discs 1 and 2 contain 1.0 mg/ml, and discs 3 and 4 are plasma with no oxytetracycline added.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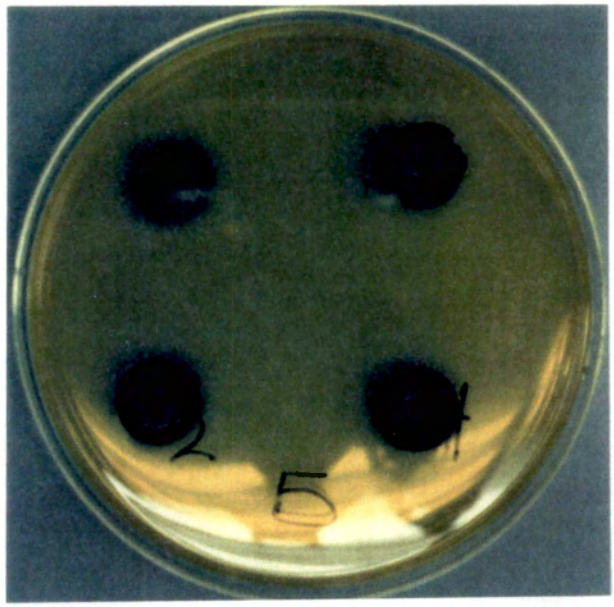
2

Figure 3.4
Lack of detection of oxytetracycline in plasma samples using Bacillus stearothermophilus disc assay. Plate 5 contains plasma samples from cow #34. Discs 1 and 2 contain plasma at 0 h, and discs 3 and 4 contain plasma at 3 h. Plate 6 contains plasma samples from cows #34 and #24. Discs 1 and 2 (cow #34) contain plasma at 48 h, and discs 3 and 4 (cow #24) contain plasma at 0 h.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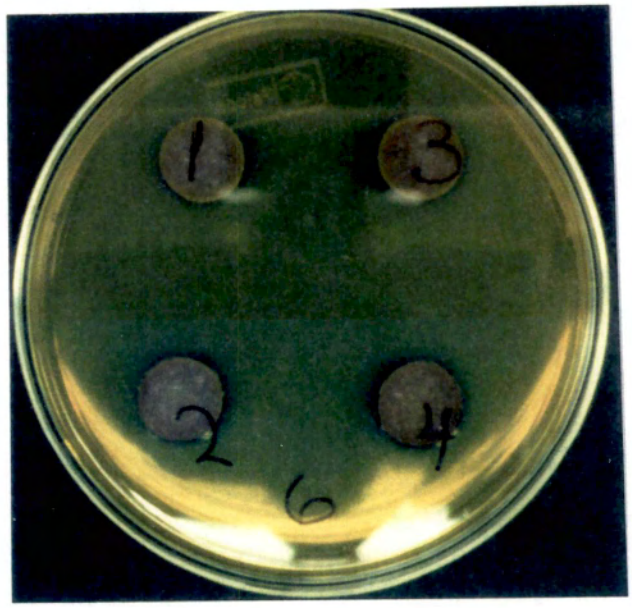
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2
4
6

DISCUSSION

Results of this experiment should be interpreted cautiously due to limited numbers of cows and samples. It would seem reasonable to think that significant passage of oxytetracycline from fetal membranes into the blood stream would be more likely to occur when the antibiotic is injected during cesarean section than when it is injected into hanging membranes. However, this study showed that whether injected into umbilical arteries during cesarean section or from arteries retrieved from the hanging membranes, oxytetracycline did not pass from placentomes into systemic blood circulation. This conclusion is based solely on the B. stearothermophilus disc assay and requires confirmation. Results of this experiment are not surprising though, since placentomes, caruncles, and cotyledons are decaying tissues without blood circulation (Roberts, 1986).

Since there is seemingly no passage of oxytetracycline into the blood and milk, due in part to the expulsion of the fetal membranes which contain most of the injected oxytetracycline, the question of whether or not milk should be withheld is raised. Obviously, this question cannot be answered at present. There are many issues that need to be addressed and much more data

collected. However, the question is valid and requires consideration beyond this study.

This study also raised a question to whether or not oxytetracycline administered via the umbilical cord is effective in preventing infection or in fighting an existing infection. Unfortunately, the answer is not contained within this research. Another study with more cows is needed to make an attempt in answering this question.

It is recognized that fetal membranes and placentomes are decaying tissues, providing a favorable environment for bacterial growth (Pulfer and Riese, 1991). The intraumbilical injection route confines the antibiotic deep within the placentome and to the bulk of the RFM. However, the interplacentome uterine tissues are deprived of oxytetracycline unless minor diffusion takes place. Therefore, this route of administration may not be beneficial to all infected or potentially infected tissues. Supplemental therapy may be necessary.

A consideration to this route of administration is the irritating effect oxytetracycline has on tissues (Pulfer and Riese, 1991). This may be the effect that was seen in cow #39 when she developed a severe metritis within 36 h of a 10 cc injection of oxytetracycline. It is possible that the metritis was caused, in part, by the

release of biochemicals secondary to the irritation of the fetal membranes by static oxytetracycline.

Fetal membranes are metabolically active when excised and stored at body temperature for three days. This suggests that fetal membranes may be capable of inflammatory response which may be caused by oxytetracycline irritation. An inflammatory response may facilitate metritis complications. This needs to be investigated.

Due to the metritis experienced by cow #39 and the consideration of irritation caused by oxytetracycline, the other three cows were injected with 1.0 cc oxytetracycline/L saline rather than with 10 cc oxytetracycline.

The failure in loosening retained membranes in cows injected via umbilical arteries from hanging membranes is difficult to explain. This result is inconsistent with the in vitro study in which collagenase was not inhibited by oxytetracycline. However, statistically, 15% of cows with RFM do not respond to collagenase injections (Eiler and Hopkins, 1993). The reason for this is unknown.

Results of this study indicate that oxytetracycline did not pass into milk and blood (plasma). This was determined by Bacillus stearothermophilus disc assay. This assay was designed to detect antibiotics in milk not

plasma. Therefore, results of the plasma samples should be confirmed by another method of antibiotic detection.

The findings of this experiment should be interpreted conservatively due to the small number of cows used. Questions were raised that could not be answered based on the number of cows in the study. Further investigation is needed.

LITERATURE CITED

Anonymous. 1982. Uniform antibiotics test OKd for pasteurized milk ordinance. Dairy Rec. 83:25.

Bretzlaff, K. N., H. L. Whitmore, S. L. Spahr, and R. S. Ott. 1982. Incidence and treatments of postpartum reproductive problems in a dairy herd. Therio. 17:527.

Callahan, C. J. and L. A. Horstman. 1987. Treatment of early postpartum metritis in a dairy herd: response and subsequent fertility. Bovine Pract. 22:124.

Dawson, L. J., E. P. Aalseth, C. H. Hawman, T. Nalsen, and H. J. Howard. 1988. Reproductive performance of dairy cows after early detection and oxytetracycline treatment of post-partum metritis. Bovine Pract. 23:24.

Eiler, H. and F. M. Hopkins. 1993. Successful treatment of retained placenta with umbilical cord injections of collagenase in cows. J.A.V.M.A. 203:436.

Haaland, M. A. J. E. Manspeaker, and T. W. Moreland. 1984. Antibiotic residues in milk after intrauterine infusion. Vet. Med. 79:382.

Huber, W. G. 1988. Veterinary Pharmacology and Therapeutics. 6th ed. Iowa State University Press. pp. 813-821.

Kaneene, J. B., P. H. Coe, J. H. Smith, P. Rapnicki, C. L. Smith, B. Gerloff, and D. A. Morrow. 1986. Drug residues in milk after intrauterine injection of oxytetracycline, lincomycin-spectinomycin, and povidone-iodine in cows with metritis. Am. J. Vet. Res. 47:1363.

Marth, E. H. and B. E. Ellickson. 1959. Problems created by the presence of antibiotics in milk and milk products - a review. J. Milk Food Technol. 22:266.

Oliver, S. P., J. L. Maki, and H. H. Dowlen. 1990. Antibiotic residues in milk following antimicrobial therapy during lactation. J. Food Prot. 53:693.

Ouderkirk, L. A. 1979. Bacillus stearothermophilus disc assay for detection of residual penicillins in milk: collaborative study. J. Assoc. Offic. Anal. Chem. 62:985.

Plumb, D. C. 1991. Veterinary Drug Handbook. Pharmavet Publishing. pp. 496-507.

Pulfer, K. W. and R. L. Riese. 1991. Treatment of postpartum metritis in dairy cows. Iowa State Univ. Vet. 53:27.

Roberts, S. J. 1986. Veterinary Obstetrics and Genital Diseases (Theriogenology). Woodstock, VT. pp.373-383.

United States General Accounting Office. 1990. Food safety and quality: FDA surveys not adequate to demonstrate safety of milk supply: report to the chairman, Human Resources and Intergovernmental Relations Subcommittee, Committee on Government Operations, House of Representatives. Washington, DC: The Office.

PART IV

SUMMARY

This thesis has provided new information in the study of tetracycline inhibition of collagenase. In contrast to the many reports of tetracyclines inhibiting mammalian collagenases, it appears that oxytetracycline does not inhibit bacterial collagenase (*C. histolyticum*). It is also evident from this work that the antitumor antibiotic doxorubicin, which has been reported to inhibit *C. histolyticum* collagenase, does not inhibit all *C. histolyticum* collagenases.

Based on the lack of inhibitory effect oxytetracycline has on collagenase, this antibiotic and enzyme could be used as a combined treatment for RFM. However, further investigation is needed on the behavior of oxytetracycline and collagenase in vivo.

VITA

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