Comparison of Uterine Protease and Protease Inhibitors Present in the Pregnant and Non-Pregnant Llama

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Title: Comparison of Uterine Protease and Protease Inhibitors Present in the Pregnant and Non-Pregnant Llama.

Abstract

Approved: Alfred R. Menino

Alfred R. Menino

Llama reproductive physiology is unique when compared with other livestock species. Llamas carry 95-98% of all pregnancies in the left uterine horn. It is believed that there is a physiological difference between the uterine horns that contributes to this phenomenon. This study examined the uterine environment for protease and protease inhibitors, specifically, matrix metalloproteases (MMP) and tissue type (tPA) and urokinase type plasminogen activators (uPA) and their respective inhibitors.

Llama uterine fluid was collected non-surgically from luteal phase pregnant and non pregnant females on days 7, 9, and 11 post-copulation. Uterine fluid was collected a single time from follicular phase (non-ovulatory) females. Fluid was assayed for protein concentration and corrected to 1 mg/mL. Proteases were detected with 10% polyacrylamide gel electrophoresis copolymerized with gelatin for MMP detection or casein and human plasminogen for PA detection. The gels were then analyzed for changes in relative percent of detected proteases.

Both MMPs and PAs were detected. There was no difference due to day, reproductive status or uterine horn for high molecular mass MMPs. Low molecular mass MMPs differed for the luteal phase females due to the combined interaction of day x status and day x status x side. There were multiple interactions for the various molecular mass categories of PA/PAIs. The changes that occurred in protease percentages are consistent with embryonic migration, maternal recognition of pregnancy and preparation for implantation of the embryo.

Furthermore, the evidence revealed in this study suggests that there is a physiological difference due to reproductive status, day and uterine horn, which may support evidence as to why pregnancies are carried in the left uterine horn.

Bachelor of Science thesis of Lisa Bidstrup presented June 13, 2000.

APPROVED:

Major Professor, representing Animal Science

Head of Bioresource Research Program

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Lisa Bidstrup, Author

DEDICATION

This thesis is dedicated to my entire family.
I thank them all for their support and good thoughts.

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Table of Contents

List of Figures

Figure

Comparison of uterine protease and protease inhibitors present in the pregnant and non-pregnant llama.

Chapter 1

Introduction

In the United States (especially Oregon), as well as other areas of the world, llamas have become an increasingly economically important livestock species. Even though llamas have been domesticated longer than nearly any other animal, it hasn't been until the last thirty years that the llama has risen from a relatively unknown animal into a multi-million dollar industry that is growing exponentially (Ebel, 1989). The llama is a versatile animal. It is used for fiber and meat, and as a pack animal and a companion/pet. Reproductive success is crucial for the success of the llama farmer.

Llama reproductive anatomy and physiology is unique. Llamas share some similarities with the horse in that the gestation length is around 335-350 days, placentation is diffuse, females can be bred back shortly after parturition and twinning is uncommon. However, llamas differ from horses and other domestic livestock species in that they are induced ovulators, meaning that stimulation from copulation results in ovulation approximately 26 hours later (Johnson, 1989). Llamas can reproduce at all times of the year. However, in the Andes of South America, wild llamas and alpacas will breed from December to March, which is the warmest season with adequate rainfall (Sumar, 1996).

Females reach sexual maturity at 6 months, depending on when they attain approximately 60% of their adult weight. However, she should not be bred until at least one year. The llama has a bicornuate uterus with a single cervix and two fully functional ovaries. A corpus luteum forms following ovulation. In llamas and alpacas, 95% of the pregnancies are carried in the left uterine horn despite an equivalent ovulation rates

between left and right ovaries (Smith et al. 1994). There are many physiologic contributors to this phenomenon, not all of which are known.

Unfortunately, llamas suffer a much lower reproductive rate than that of other livestock species. Llama breeders would like each of their breeding females to produce a live cria every twelve months. An estimated 50% of females of breeding age fail to produce an offspring each year (Fernandez-Baca, et al. 1970). Conception failure, resorption and abortion greatly reduce pregnancy rates and will extend the birthing interval. From day 0 (time of breeding) to day 14, a theoretical loss will consist of 20% of the females failing to conceive, and 10% of the conceptions will be resorbed (Johnson, 1990). It is the purpose of this study to investigate the uterine environment during early gestation of the llama.

Matrix metalloproteases (MMPs) and plasminogen activators (PAs), as well as their respective inhibitors, tissue inhibitors of metalloproteases (TIMPs) and plasminogen activator inhibitors (PAIs), have been shown to be involved in processes such as maintenance and developmental remodelling of the extracellular matrix (ECM) during uterine implantation and cell migration during embryonic development (Behrendtsen, 1992). The ECM consists of protein fibers and carbohydrate macromolecules. The ECM helps hold cells and tissues together. It provides an organized lattice that cells can use during migration and allows cells to interact with each other (Alberts, et al., 1994).

MMPs have been classified into 4 different groups, dependent upon the preferred substrate. They are collagenases, gelatinases, stromelysins and membrane-type MMPs. Each group has several subclasses. There are two MMPs examined in this study which fall under the gelatinase group. They are gelatinase A (MMP-2), which is approximately 72 kD and gelatinase B (MMP-9), which is approximately 92 kD (Matrisian, 1992). The preferred substrate for these enzymes is type IV collagen and denatured collagens. Type IV collagen is an important component of the ECM. The

ECM is organized into a thin, flexible but very durable sheet called basal lamina. The basal lamina surrounds muscle, fat and Schwann cells. It also underlies epithelial sheets and tubes. During early development of the embryo, there may be little type IV collagen in the basal lamina. There is, however, laminin, which acts similarly to type IV collagen in the ECM (Alberts, et al., 1994).

Plasminogen activators are serine proteases that prefer a plasminogen substrate, but other molecules may be cleaved by the PAs. There are two types of PAs. There is the tissue type plasminogen activator (tPA), ranging from 65-80 kD, and the urokinase type plasminogen activator (uPA), ranging from 50-65 kD. By cleaving plasminogen into plasmin, plasmin is then able to effectively degrade the ECM and activate the MMPs (Vassalli, et al., 1991). Having PAs and MMPs active, the ECM can then be degraded for tissue remodeling, embryonic development and establishment of fetal maternal contact in implantation.

The hypothesis investigated in this study is that there is a physiological difference in distribution of proteases and their inhibitors between the left and right uterine horns of the pregnant, pseudo-pregnant and non-pregnant llama. The objectives are to determine if 1) MMPs and PAs and their respective inhibitors were present in uterine fluid as they are in other livestock species; 2) to quantify changes in the proteases present on different days of gestation; 3) to compare proteases found between left and right uterine horns.

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Chapter 2

A Review of the Literature

History. North America is the origin of the Camelidae family. Migration into Asia and South America was possible via land bridges into those continents. The Camelidae then became extinct in North America. In Asia, the Camelidae evolved into two species. The Bactrian camel or *Camelus bactrianus* is the two-humped camel and the Arabian camel or *Camelus dromedarius* is the one humped camel. Both of these are considerably larger than the four South American species. The South American camelids are classified under the genus *Lama*: the llama (*Lama glama*), the alpaca (*Lama pacos*), the guanaco (*Lama guanico*), and lastly the vicuna (*Vicugna vicugna*) (Novoa, 1970).

Domestication of the South American camelids, the vicuna and guanaco, is believed to have given rise to the llama and alpaca. The llama descended from the guanaco and the alpaca descended from the vicuna. Domestication of the llama and alpaca provided the Andean Indians with food, fiber, fuel as well as a means to transport goods. The economic and cultural influence of the llama and alpaca elevated Incan status. Llama management and husbandry practices were quite advanced, considering the period of time (Ebel, 1989).

It was in the 1500's, with the Spaniard's conquest that llama and alpaca numbers were greatly reduced, nearly to extinction. The Spaniard's eradicated the camelids to reduce competition of the Spaniard-introduced sheep, horses and cattle. Management practices of the llama and alpaca were lost. They survived only in the higher elevations that were not conducive to the other domestics. In the 1800's, the textile market picked up on the fine wool of the alpaca and renewed interest led to improved management and

research. The llama remained obscure until mid-1900. Peru and Bolivia recognized the importance of the llama and alpaca to Andean culture and made efforts to restore the animals population. Today, wild herds are protected and llamas and alpacas are carefully selected and bred. Research on improving reproductive performance has become an important component of the llama/alpaca industry (Ebel, 1989).

Reproductive Biology

The Male. The male llama generally reaches sexual maturity between 2 and 3 years of age. There is considerable variation between mature ages. There have been reports that females have become pregnant from males that are only 6 months of age while others haven't become pregnant until the male has reached 3 years. The male possesses a fibroelastic penis with a sigmoid flexure. There is an embryonic preputial adhesion that inhibits penile protrusion until there has been recurrent sexual stimulation. The length of the extended penis is ~40 cm, of which 18-25 cm extends beyond the prepuce. There is a cartilaginous process at the end of the penis, which dilates the female cervix during copulation. The testicular weight averages ~24 g each, which is relatively small given the size of the animal. The average ejaculate volume is generally no greater than 3 cc (Johnson, 1989).

The Female.

Sexual Maturity. The female llama will reach sexual maturity when she has reached ~60% of her mature body weight. This is dependent upon the nutrition she receives. It can occur anywhere from 5 months to 3 years of age. Determining puberty is not easy because estrus is detected by submissive behavior to the male for copulation. Submissive behavior has been observed in pre-pubertal females that did not reach puberty until the following year (Smith,1994).

Breeding Season. Llamas and alpacas, as well as the guanaco and vicuna, in their natural habitat high in the Andes, will reproduce between December and March, which is the warmest season. On farms where males and females are housed together throughout the year, births will occur during these same months. However if males and females are kept separately, with copulation allowed once per month, both males and females will be sexually active the entire year (Sumar, 1996).

Anatomy. The female llama is equipped with a bicornuate uterus with a single cervix. The cervix has a single spiral fold that makes 2-3 turns giving it the appearance of cervical rings. The uterine body is short. There is a left and right uterine horn leading to two functional ovaries. There is a difference in size between the left and right uterine horns, the left being slightly larger (Smith, et al., 1994). There is also a vascular difference between left and right uterine horns. The right uterine artery is considerably more prominent than the left and has a major crossover branch to the left horn (Del Campo, et al., 1996). The ovaries are oval shaped, similar to the bovine ovary. A pre-ovulatory follicle will range between 7 and 12 mm. The corpus luteum (CL) measures 1-1.5 cm and is detectable upon palpation.

Estrus and Ovulation. Llamas and other South American camelids do not have a regular pattern to their estrous cycles. They will have a continuous estrus in the absence of a male or other ovulatory stimulus. Estrus can last 30-40 days with 1-2 days anestrus in some females (Fernandez-Baca, 1993).

Llamas are classified as induced ovulators, meaning that ovulation is dependent upon the stimulation of copulation. Ovulation occurs 1.8-2 days following copulation. Ovarian activity continues throughout the year. Ovulation will occur when there is a

surge of luteinizing hormone (LH). Ovulation can also be triggered with a single injection of either human chorionic gonadotrophin (hCG) or gonadotrophin releasing hormone (GnRH).

Following ovulation, the CL forms. The CL is usually twice the size of the ovary and the color can vary according to the time of ovulation (Sumar, 1991). On day 3 following ovulation, it is red and hemorrhagic, turning yellow-pink by day 9. In non-pregnant females the maximum size is reached by day 9 and in pregnant females by day 22. The CL's maximum progesterone content peaks by day 4 and maintains a high level until day 12. Regression of the CL is complete by 16. Females will show receptive behavior (if not pregnant) anywhere from 12-18 days following ovulation (Smith et al., 1994).

Pregnancy. Non-pregnancy in the llama can be determined if she shows receptive behavior toward the male. Both pregnant females and females with a non-regressed CL will refuse the male. Other methods to determine reproductive state include palpation, plasma or milk progesterone, ultra-sonagraphy and laparoscopy (Fernandez-Baca, 1993).

Twin embryos are generally not carried to term. As in horses, the second embryo will usually be absorbed during early gestation. Llama fetuses occupy the left uterine horn in greater than 95% of all pregnancies. Both ovaries are known to be functional and ovulation is equal between right and left sides. This indicates that the embryo originating in the right side migrates to the left horn (Sumar, 1996). The exact reason for the preference of the left horn has not been fully elucidated.

Placentation. The llama placenta is epitheliochorial with a diffuse pattern of folded papillation that functions as a placentome. The amnion adheres to either the

allantois or the chorion. The placenta is composed of six layers, which may account for the limited immunoglobulins transferred to the fetus. There is an unusual epidermal membrane that covers the fetus, whose purpose is not fully understood. It is believed that this membrane serves as a water conservation method for the high altitude climate to which they've they are adapted. There is very little uterine fluid during late gestation and this membrane may serve to lubricate the fetus at parturition (Smith, et al., 1994).

Parturition. Parturition for the llama will generally take place during the morning, as mornings are the warmest time of day in their natural habitat. The llama will deliver her young in the standing position, with the entire process lasting approximately two hours. Llamas experience very few occurrences of distocia or retained placenta. Follicular development and CL regression transpire about 6 days following parturition. Regardless of this, she may show receptive behavior toward the male 24 hours post-partum. Uterine involution is complete by day 15 post-partum (Fernandez-Baca, 1993).

Abortion and Resorption. Llama reproductive rates are low compared to other livestock species, excluding horses. Supposing 100 hundred females were bred a single time, one would expect one hundred pregnancies leading to one hundred live births. This is not the case. Of those one hundred, 20 may fail to conceive. By day 14, 10% of the conceptions can resorb. By day 45, 5% may be lost to resorption, abortion, mummification or maceration. As the pregnancy approaches 345 days, 5% may be born prematurely with half not surviving. Of the crias born after 345 days, 5% may be stillborn (Johnson, 1990).

It has been observed that embryos originating from the right uterine horn have a decreased survival rate. Survival rate past day 30 has been associated with the CL

located on the left ovary. No right horn pregnancies have been observed past day 87 of gestation in the alpaca (Fernandez-Baca, et al., 1970).

Mobility of the conceptus has been shown to be essential for the maintenance of pregnancy in the mare (McDowell et al., 1988). Mobility of the embryo allows for maximal interaction and communication with the endometrium prior to implantation. The embryonic intrauterine motility mechanism is not fully understood. It is likely that uterine contractions contribute to embryo propulsion (Ginther, 1984). Failure of recognition of pregnancy leads to the release of prostaglandin $F_{2\alpha}$, regression of the CL and termination of pregnancy (Sharp et al., 1989).

Early Embryonic Development. Fertilization of the ova initiates cell division and development of the embryo. An important component of development involves the extracellular matrix (ECM). The ECM provides a substrate in which cells can adhere and migrate and directs cell form and function via ECM-cell interactions. During embryonic development, ECM is first detectable during early cleavage, at the 4-cell stage. Growth after that requires the remodeling of the ECM. Embryonic cells continue to increase in number as well as degree of differentiation throughout the blastocyst stage, implantation and genesis of embryonic and extra-embryonic tissues (Brenner et al., 1989; Adler et al., 1990).

The ECM is composed of interstitial collagens, basement membrane collagen (type IV), fibrinectin, laminin and proteoglycans (Woessner, 1991). Developmental processes require the maintenance and remodeling of the ECM. In order for this to occur, certain proteases (as well as their inhibitors) are needed. There are two families of enzymes that are functional in degrading the ECM to allow for developmental changes. One is of the plasmin family, plasminogen activator (PA) and related inhibitors

(PAI) and the matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMP).

The MMPs. MMPs belong to a multi-gene group of zinc dependent proteases. There are at least nine different MMPs. Two of the nine are of interest in this study. MMP-2 is a 72 kD gelatinase and MMP-9 is a 92 kD gelatinase. Both degrade type IV collagen (Matrisian, 1992; Woessner, 1991). There are many proteases capable of cleaving ECM molecules, however it is the MMPs that are believed to be the pertinent mediators of matrix degradation. MMPs are secreted proteins, which places them in the proper location for matrix degradation and their enzymatic activity are strongest at near neutral pH. There are many points of regulation of MMP expression and activity, indicating strict control is necessary for normal processes to continue (Matrisian, 1992).

As the embryo readies for implantation, the endometrial tissue will undergo degradation and remodeling at the location where the embryo settles. The degree of invasiveness varies between species. In humans, the blastocyst passes through the epithelium into the sub-epithelial connective tissue to implant. In domestic ungulates, implantation is less invasive. The blastocyst attaches at specific points or caruncles. Fusion occurs between the binucleate cells of the trophoblast and the caruncular epithelial cells, forming a syncytial layer. In the ovine, it has been shown that endometrial cells near the implantation site secrete MMP-2 (Salamonsen et al., 1992).

The invasive phase of implantation of the mouse has been studied to determine which proteases are active during this time. The mouse embryo during pre- and peri-implantation development secretes MMP-9. The maternal decidua immediately adjacent to the implanting embryo produces TIMP-3. Leukemia inhibiting factor and epidermal

growth factor have been implicated in peri-implantation development and both these were found to have regulatory properties on MMP-9 (Harvey et al., 1995).

Synthesis and secretion of TIMPs are regulated by the modification of mRNA transcription rates. There are different mechanisms of regulation for TIMP-1, -2 and –3. Estrogen and progesterone are known to regulate TIMP-1. TIMP-1 and TIMP-2 inactivate MMP-2. TIMP-1 binds to the latent form of MMP-9. TIMP-2 binds to the latent form of MMP-2 (Hampton et al., 1995). Each TIMP family member may have a specific physiologic role in the sequence of tissue degradation and remodeling.

The PAs. Plasminogen activators (PAs) are serine proteases. PAs are released during cell migration and tissue remodeling. There are two types of PAs that have been identified in animals: *urokinase-type PA* (uPA) and the *tissue-type PA* (tPA). uPA has a molecular mass of 50-65 kD and tPA has mass of 65-80 kD. Each PA is the product of a distinct gene and is synthesized as a single-chain protein. tPA is secreted in an active form and uPA is secreted in an inactive form or pro-uPA (Vassalli, et al., 1991). Plasminogen is the preferred substrate for PAs. However, either type of PA can cleave other molecules.

The two PAs are associated with different physiological events. uPA is best known for its participation in cell migration (Bartlett & Menino, 1993). It is able to bind to a plasma membrane receptor as pro-uPA. Once binding occurs, the newly activated uPA can then cleave plasminogen into plasmin. Plasmin is then free to activate pro-MMP, cleave fibrin, laminin and collagen associated with ECM. tPA is associated with clot lysis. It is able to bind directly to fibrin and other ECM molecules. Endothelial cells produce tPA, which will either remain bound to the cell or be released into the blood (Vasselli, et al., 1991).

Embryonic PA production has been identified in many species. Ovine embryos increase production of PA during the morula-blastocyst transformation and PA remains high through blastocoelic expansion and hatching (Bartlett & Menino, 1993). Mouse embryos produce PA in two separate phases. The first phase relates to trophoblastic invasion and the second to parietal endodermal cell migration. (Stricland et al., 1976).

PAs have also been implicated in embryonic hatching from the zona pellucida. Bovine embryos that hatch from the zona pellucida in vitro have been shown to produce more PA than embryos that fail to hatch (Coates & Menino, 1994). This has also been demonstrated in ovine embryo development. It is possible that embryo produced PA may activate uterine plasminogen to plasmin which in turn degrades the zona pellucida (Menino et al., 1989).

In the llama, as in other livestock stock species, there is a cascade of events that the developing embryo must survive in order to implant into the uterus. Since the llama suffers a low survival rate of pre-implantation embryos, it is the purpose of this research to investigate the presence of PAs and MMPs in the uterine fluid of the pregnant and non-pregnant llama.

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Chapter 3

Materials and Methods

A total of 9 reproductively sound females and 2 males (1 fertile and 1 vasectomized) llamas were used in this study. The animals were provided by Oregon State University-College of Veterinary Medicine. All animals employed in this study had no significant health problems. All were maintained on current standard herd health procedures. The llamas were kept on pasture and supplemental hay was provided as needed. Males were kept separated from the females. Free-choice water was available to all animals.

In order to evaluate the effects of reproductive status and day on protease and protease inhibitors produced by the uterus and embryo in the llama, three categories of reproductive status were established. Category A consisted of three confirmed pregnant females. Females were bred to a fertile male and pregnancy was determined using the Estru-check test kit. Category B consisted of three ovulated/non-pregnant females. Females were bred to a sterile male in order to induce ovulation. Category C consisted of 3 females that were non-ovulatory, or in the follicular phase, having no exposure to either male.

Llama uterine fluid was collected non-surgically on days 7, 9 and 11 post copulation (Day 0 = day of mating) of Category A and B females. Uterine fluid from Category C females was collected once. The females were secured in a standard llama chute and injected with 20-40 mg xylazine (Miles Pharmaceuticals, Shawnee, KS) i.m. to obtain standing sedation. The tail head was shaved and surgically scrubbed. For epidural anesthesia, lidocaine (0.22 mg/kg: Anthony Products Co., Arcadia, CA) was injected at the sacrococcygeal junction (Grubb, et al., 1993). Each uterus was flushed with Dulbecco's phosphate buffered saline containing 1% polyvinylpyrrolidine-10 (DPBS

+ PVP) and 10 ml/L antibiotic/antimycin solution (Sigma Chemical Co., St. Louis, MO). The left and right uterine horns were each flushed separately (Powell, 1999).

Collected uterine fluid was concentrated by ultrafiltration and assayed for total protein concentration. Samples were stored in snap-cap vials and frozen at -20°C until analysis. Sample concentrations were corrected to 1 mg/mL with 2X Sample Buffer prior to electrophoresis. Uterine fluid was analyzed for the following proteases: urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activators (t-PA), matrix-metalloprotease -2 and -9 (MMP-2, MMP-9), PA inhibitors -1 and -2, and tissue inhibitors -1, -2 and -3 of matrix-metalloproteases. Matrix-metalloproteases were detected with a 10% SDS polyacrylamide gel, co-polymerized with 1% gelatin. Plasminogen activators were detected with a 10% SDS polyacrylamide gel, copolymerized with casein and human plasminogen.

Electrophoresis was carried out at 4°C for 40 min. using mini-gels. Gels were incubated for one hr. in Triton X-100 (Sigma) at 25°C. MMP gels were transferred to 0.05 M Tris HCl with 10mM CaCl for 48 hrs. at 25°C. Proteins present in each sample were estimated with the use of a molecular weight standard (SDS-PAGE) and collagenase or urokinase, for MMP or PA gels, respectively.

Gels were dried and each lane measured for relative motility. Molecular weights were calculated using the NCSS program. Each gel was scanned using a densitometric scanner to quantify the proteins present. The resulting graphs were measured and relative percents were calculated. Statistical analysis was conducted by ANOVA.

Chapter 4

Results

MMPs. MMPs were categorized by molecular mass (MM) classes into two groups. High band molecular mass mean was 107 kD. Low band molecular mass mean was 91 kD.

High MM MMPs. The relative percent of proteases present in uterine fluid was compared by day, reproductive status and side. In category A and B females, analysis of variance revealed there was no difference due to day, reproductive status or side in the high MW MMPs and category C females had no difference due to side. T-Test results showed no difference between the pooled data of category A & B vs. category C females.

Low MM MMPs. The relative percent of proteases present in uterine fluid was compared by day, reproductive status and side. Category A, B and C females had no significant difference to the main effects of day, reproductive status, or side. However, for category A & B there were a day x status and a day x status x side interaction.

For category A females, there was no significant difference between day 7 and 9 however, analysis of variance showed a significant drop (P=.04) of low band proteases between day 9 & 11 (Fig. 1A). Category B females had showed a steady increase in relative percent of the low band proteases during this time. Between day 7 and 11 there was a significant change (P=.05) of low band proteases on the left side for category A. There was an increase to day 9 and then a drastic decrease to day 11 (Fig. 1B). T-Test results showed no difference between the pooled data of category A & B vs. category C females.

Low MM MMP

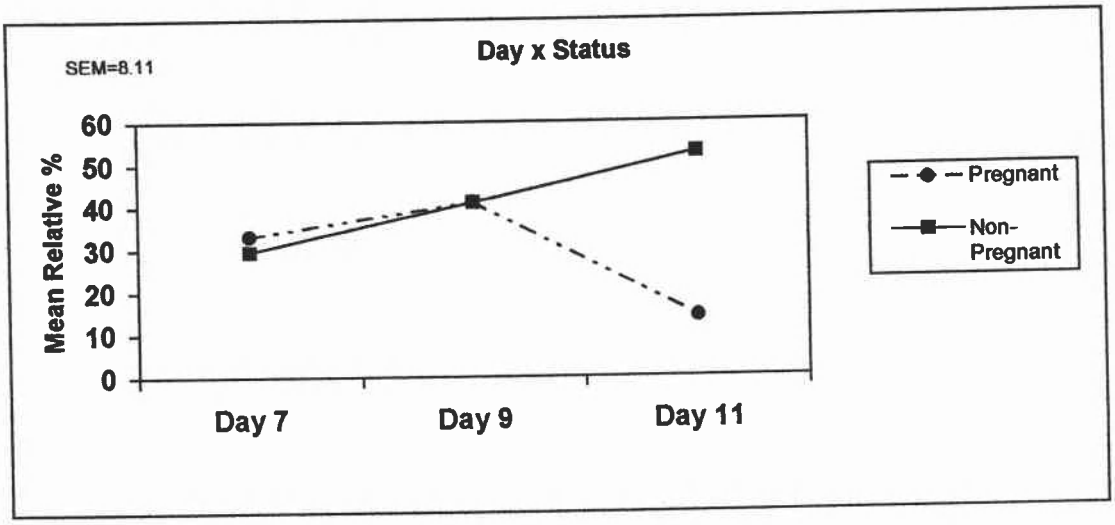

Figure 1A

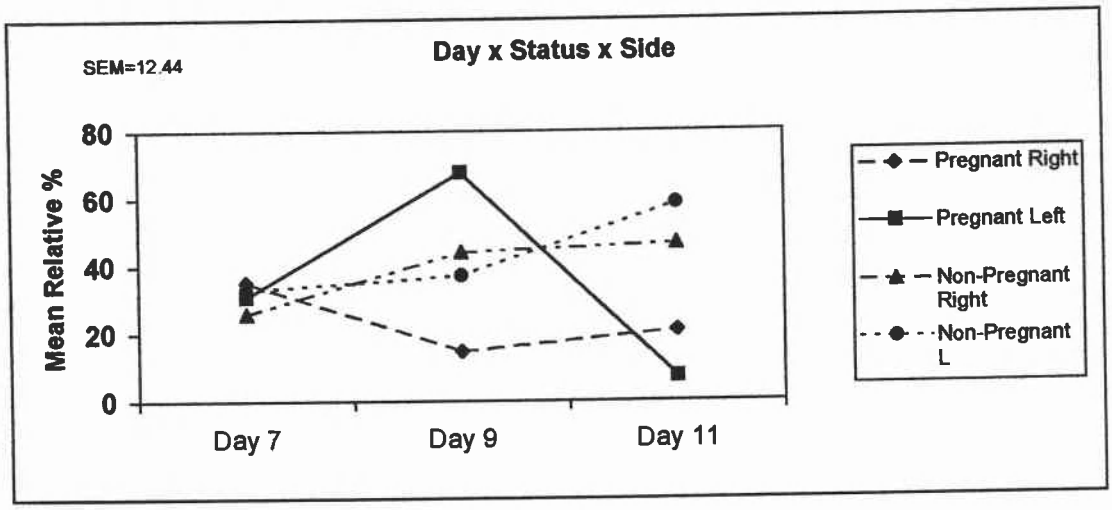

Figure 1B

PAs. The plasmin family proteases were categorized into five groups according to molecular mass: >100 kD encompass tPA-PAI complexes, 80-100 kD encompass uPA-PAI complexes, 65-80 kD encompass tPAs, 50-65 kD encompass uPAs, and <50 kD encompass uPA fragments or unknown proteases Only day 7 and 9 were evaluated. The relative percent of proteases present in uterine fluid was compared by day, reproductive status and side.

>100 kD PAs. Analysis of variance showed no significant difference due to the main effects of day, reproductive status or side for category A & B females. There was a combined effect between day and side (Fig 2). A significant change (P=.04) occurred between the right and left horns between day 7 and 9 of category A & B females. Between day 7 & 9, PAs of the right uterine horn decreased from 11% to 1% whereas the left uterine horn increased from 0% to 7%.

Analysis of variance showed no significant difference between sides for category C females. T-test results showed no difference between category A & B vs. category C females.

80-100 kD PAs. Analysis of variance revealed a significant difference in the main effect of side as well as combined effects of day x status and day x status x side for category A & B females. For the main effect of side, (Fig. 3A), the left uterine horn supports more than twice the amount of 80-100 kD protease (P=.03). The day x status analysis (Fig 3B), indicated an increased relative percent of proteases present between day 7 and 9 of category B and a decrease (P=.05) between these days for category A. The effect of day x status x side (Fig. 3C) indicates a trend for an increase 80-100 kD proteases for category B females between days 7 and 9 as well as a very slight increase in the right side of category A females. There was a significant decrease (P=.03) in category A's left side.

> 100 kD PA: tPA-PAI

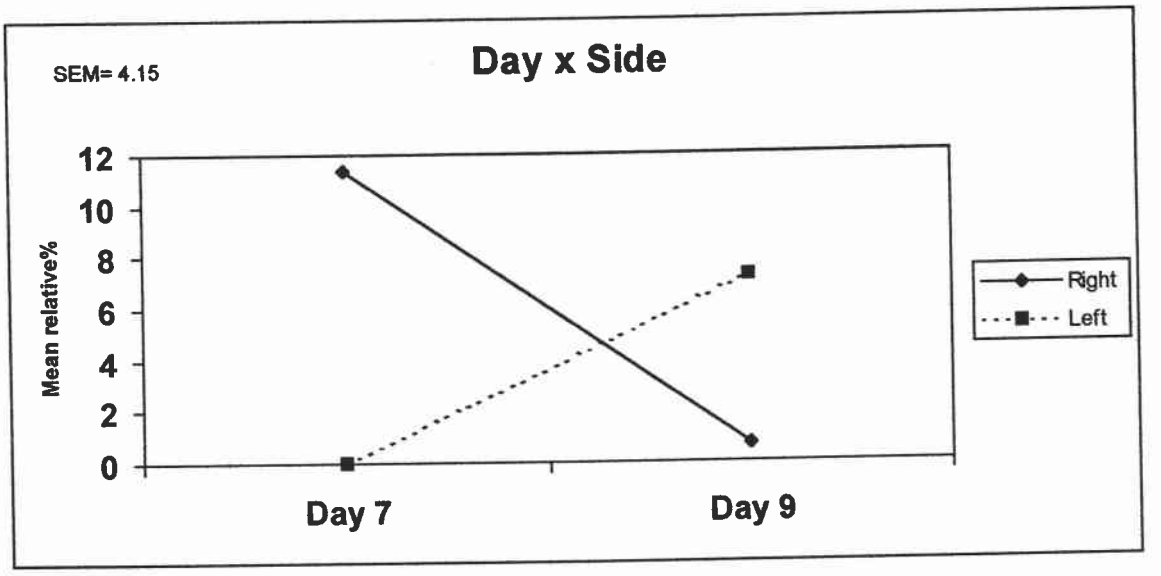

Figure 2

80-100 kD PA: uPA-PAI

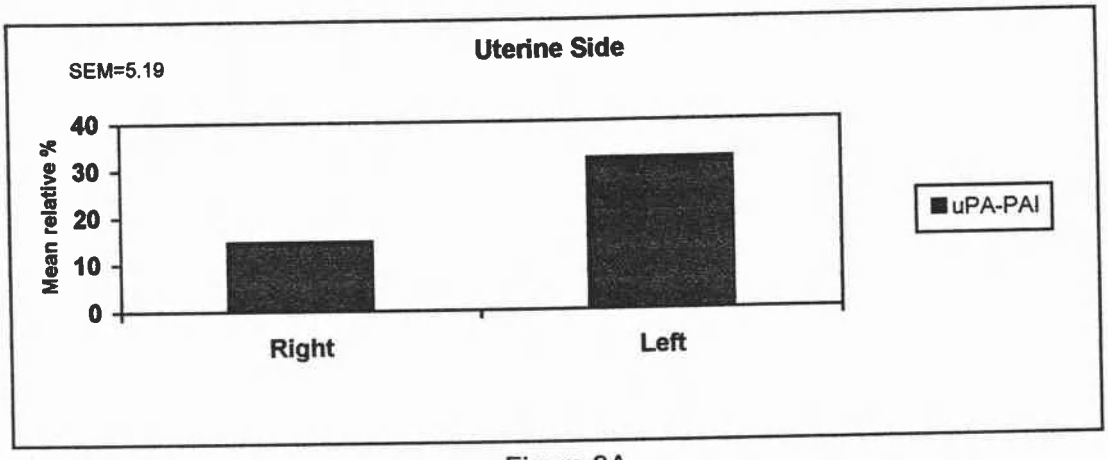

Figure 3A

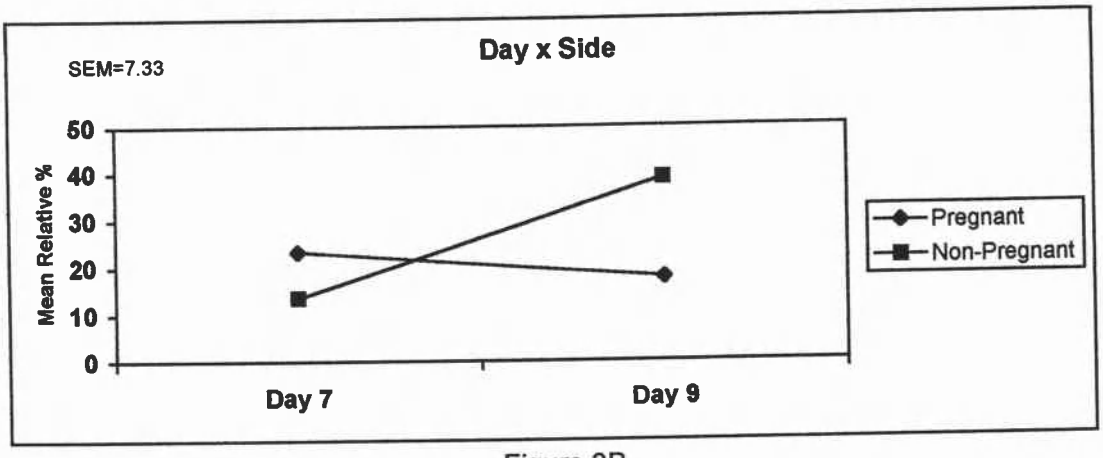

Figure 3B

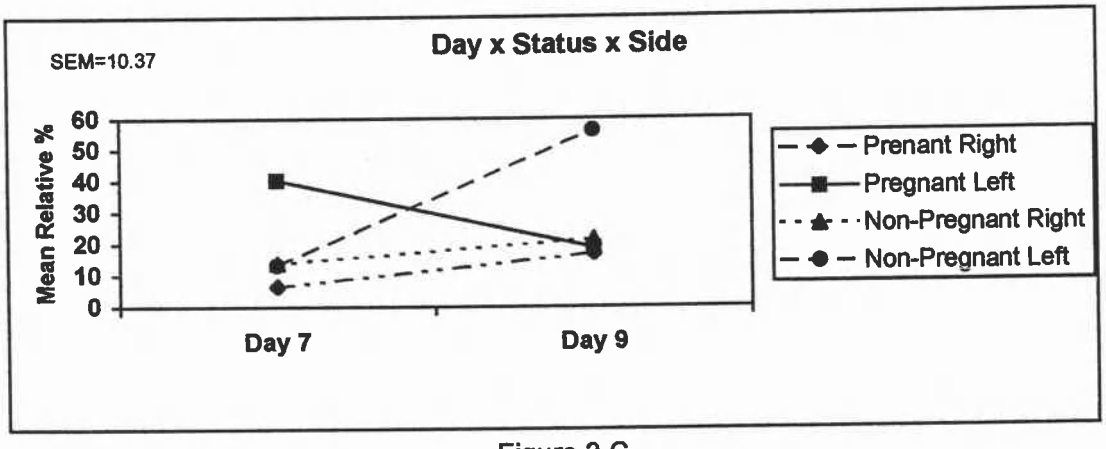

Figure 3 C

Analysis of variance indicated category C had no difference due to the main effect of side. Use of Fisher's Least Significant Difference (LSD) Test (Fig. 7A) revealed no difference between Category A & B vs. category C. It also confirmed analysis of variance test between sides for categories A & B.

65-80 kD PAs. Analysis of variance showed a single main effect of day for category A & B females. No other effects were noted. Day 7 showed a relative percent of 6% while day 9 had sharply increased (P<.001) to 41% (Fig 4).

Analysis of variance indicated no difference between sides for category C females. Fisher's LSD Test (Fig. 7B) confirmed the lack of difference and confirmed the significance between A & B females.

50-65 kD PAs. Analysis of variance showed a main effect of day and a combined effect of day x status for A & B females. On day 7 the relative percent is at 54% and greatly decreases (P=.001) to 13% by day 9 (Fig. 5A). The effect of day x status (fig. 5B) shows the greater decrease of uPA for B females between days 7 and 9 than does the A group (P=.04).

Analysis of variance showed no difference between side for C females. Fisher's LSD Test (Fig. 7B) indicated a difference between C females and day 7 A females. It also confirmed the difference of day x status between A & B females.

<50 kD PAs. Analysis of Variance showed two significant effects for the A & B females: the main effect of day (P=.03) and the combined effect of day x status (P=.002).

The change consisted of an increase from 7% at day 7 to 13% at day 9 (Fig. 6A). The day x status (Fig 6B) analysis shows an increase in relative percent for A females between day 7 & 9 while B females had a decrease in relative percent between these days.

No <50 kD proteases were detected in either uterine horn of category C females.

65-80 kD: tPA

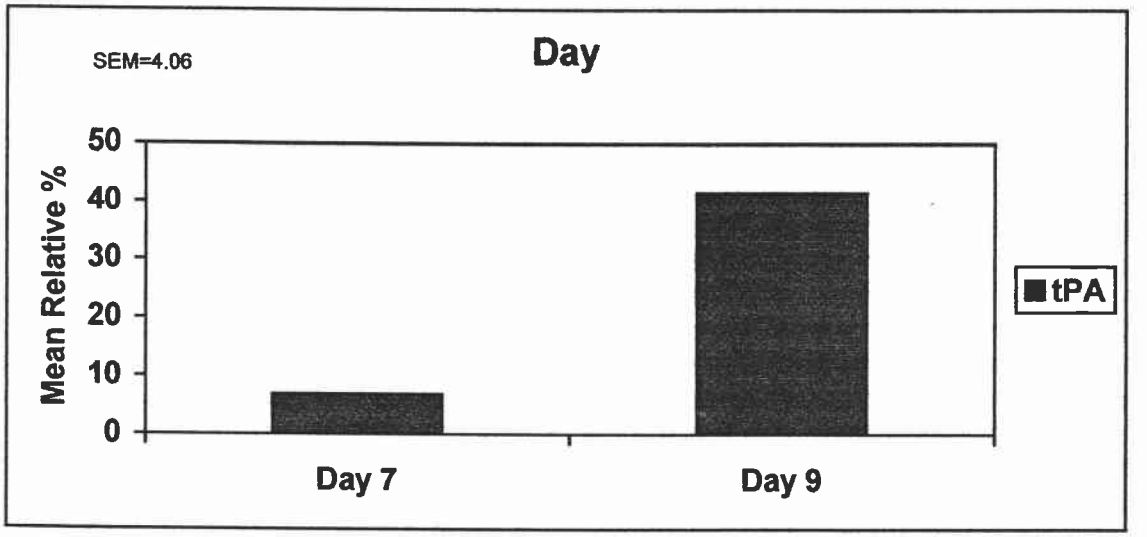

Figure 4

50-65 kD: uPA

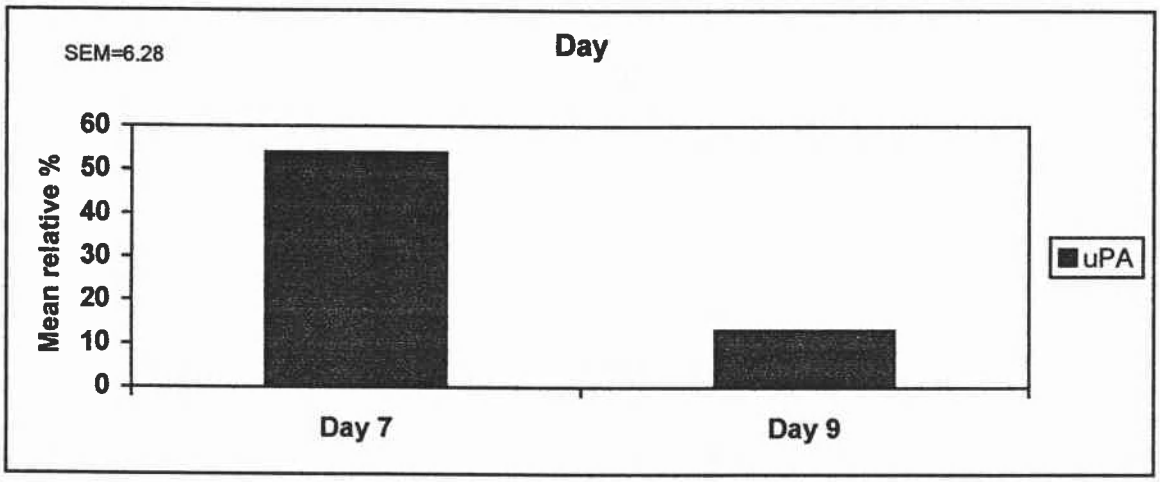

Figure 5A

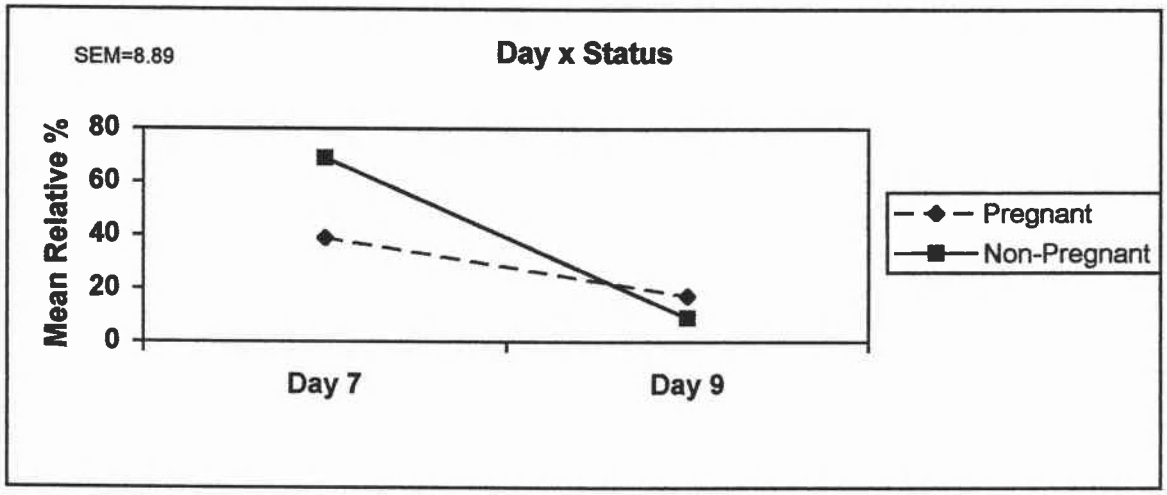

Figure 5B

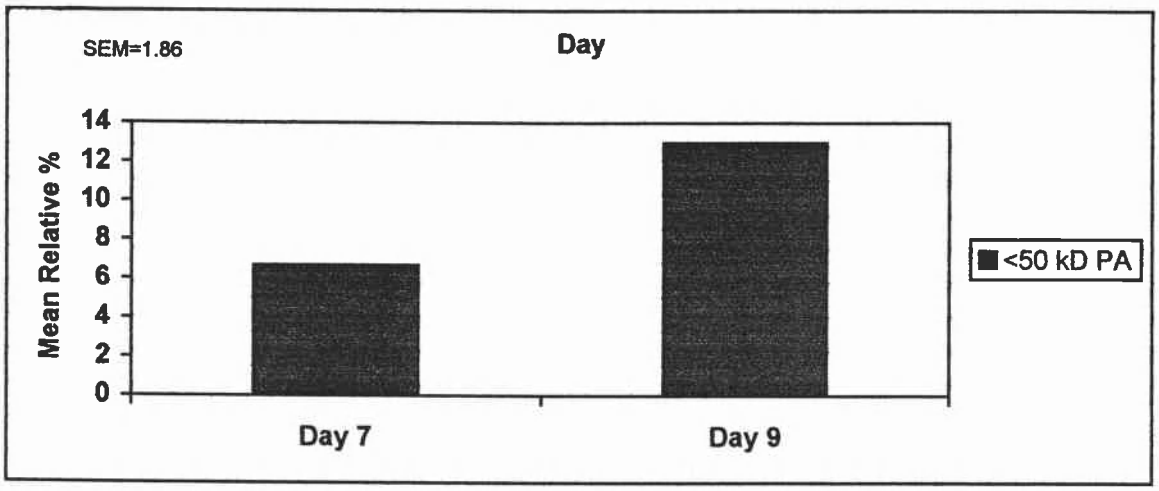

Figure 6A

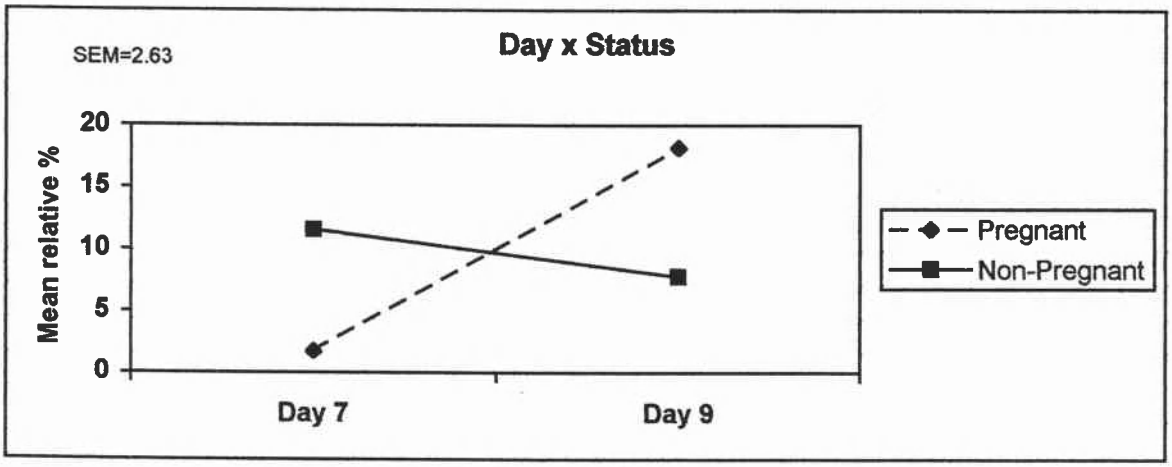

Figure 6B

Fisher's Least Significant Difference Test

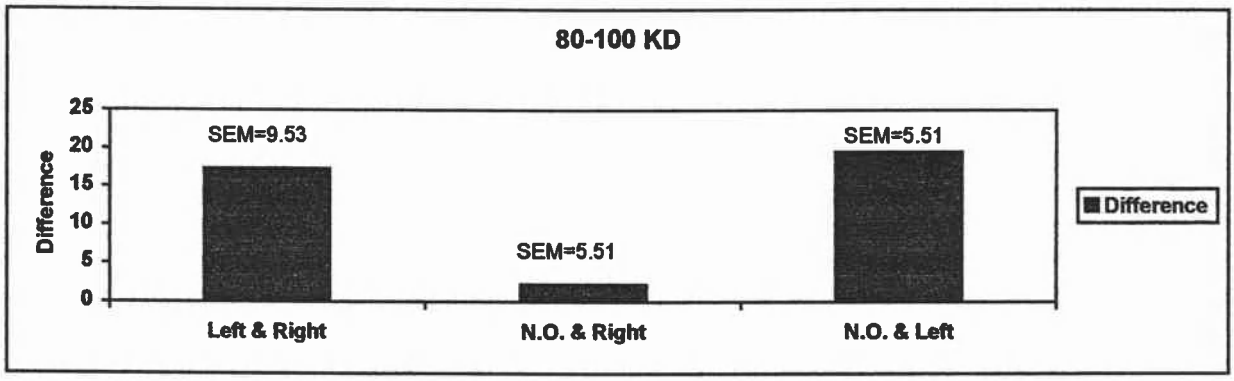

Figure 7A

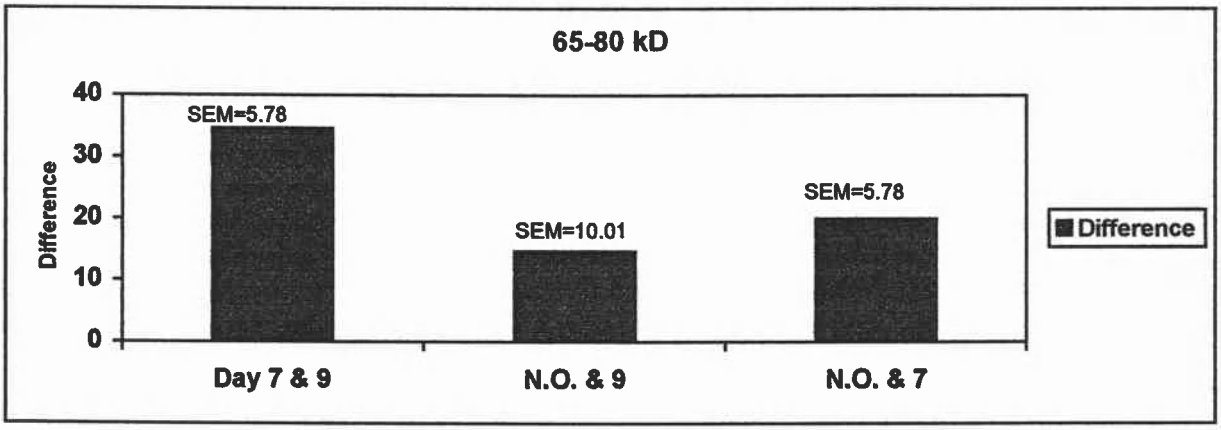

Figure 7B

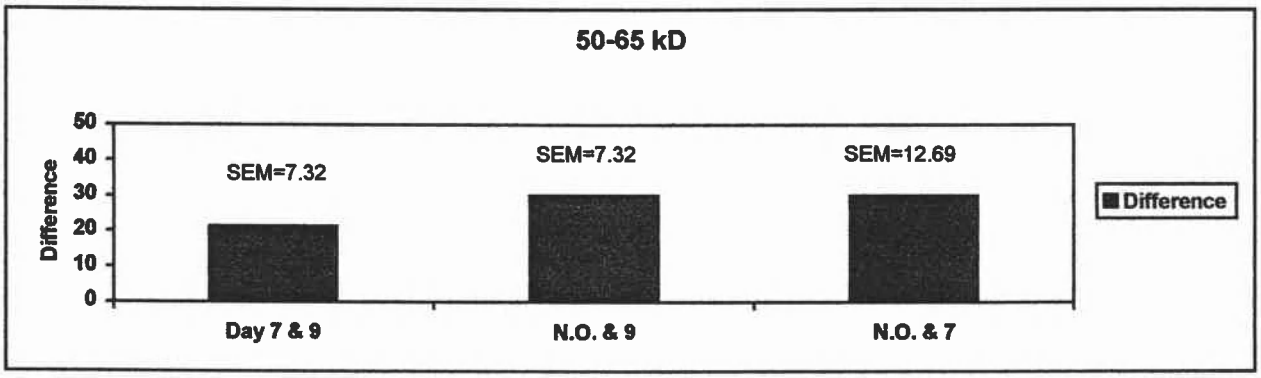

Figure 7C

Chapter 5

Discussion

This study indicates that gelatinases, PA and their respective inhibitors TIMPs and PAIs are present in llama uterine fluid prior to implantation as they are in other livestock species. However this investigation did not determine if the proteases originated maternally or embryonically, but based on other work in our laboratory, it is most likely uterine in origin. The diffuse placentation requires remodeling of the endometrium for embryo-maternal interaction. As the llama embryo does not implant until day 30, there may be substantial remodeling events in the endometrium in preparation for implantation.

The results for the low MM MMP in the pregnant female showed a large drop in percentage in the left uterine horn. This difference between horns may contribute to pregnancies being carried on the left side and/or differences in uterine physiology. It has been demonstrated that sheep endometrial cells produce MMP-2 under the influence of progesterone and estrogen (Salamonsen et al, 1993). It has also been shown that Timp-2 increases in the sheep from day 12 until day 16 and then decreases to undetectable levels by day 20 (Salamonsen et al, 1995). The decrease in the left pregnant horn of the llama may have the same trend as the sheep with the increase and decrease in expression.

The PA/PA-PAI results provide further validation of physiological differences between the right and left uterine horns as well as by reproductive status and days post-mating (category A & B females). Maternal recognition of pregnancy needs to occur by days 8-10 or progesterone levels rapidly decrease and the female llama will return to receptive behavior by day 12 (Powell, 1999). The changes seen on day 9 may indicate,

according to reproductive status, the increase or decrease of PAs/PAIs due to the presence or absence of progesterone.

It has been shown that pig blastocysts produce estrogen as a signal for maternal recognition. This signal causes a maternal release of PAI into the lumen of the uterus thus providing a control for the early events of placentation (Fazleabas et al, 1983). The results of the 80-100 kD proteases illustrate how this may also be true for the llama in that the concentrations increase in both the pregnant and non-pregnant females except for the pregnant female in the left horn. The left horn shows a dramatic decrease. The mechanisms of the PAs and PAIs are not fully understood in the llama at this time.

In conclusion, this study confirms that there is a difference in uterine physiology between the right and left uterine horns of the llama. It was illustrated that changes in protease concentrations occur in the uterus dependant upon day and reproductive status. The changes that occur are consistent with embryonic migration, maternal recognition of pregnancy and preparation for implantation.

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