

DETECTION OF GLANDULAR KALLIKREIN AND  
LOW MOLECULAR WEIGHT KININOGEN IN THE  
PORCINE UTERUS THROUGHOUT THE ESTROUS  
CYCLE AND EARLY PREGNANCY

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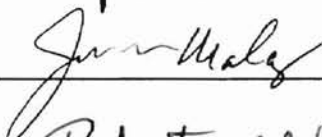
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## CHAPTER I

### INTRODUCTION

Mammalian embryos undergo a transitional sequence of biologic developmental changes during the first week of pregnancy, which include cleavage, compaction, blastocoele formation, and hatching from the zona pellucida. Unlike species such as rodents and primates, which immediately implant after hatching, a prolonged pre-implantation period occurs in domestic farm animals (Stroband and Van der Lende, 1990). Following hatching from the zona pellucida, the porcine conceptus continues a period of rapid growth and development in preparation for expansion of its extraembryonic membranes. The porcine conceptus undertakes an incredible alteration in morphology during the second week of gestation. This dynamic period is the time accounting for seventy-five percent of embryonic loss that can occur during the first thirty days after fertilization. Therefore, the most critical period for conceptus survival is between days 12 and 18 of pregnancy (Day et al., 1959; Perry and Rowlands, 1962; Pope, 1994). Since conceptus trophoblastic elongation, attachment, and placentation occur during the time of greatest embryonic mortality, it is essential that we thoroughly understand the biology of conceptus-maternal interactions necessary for establishment of pregnancy in swine. Efforts made to understand

the integrated system of porcine embryogenesis and implantation are currently underway in attempt to hopefully alleviate early conceptus death in the future. The following review of literature will cover developmental events that occur during the second week of pregnancy in the pig that include: late peri-implantation conceptus development and elongation, factors contributing to estrogen production from the conceptus, effects of estrogen on uterine secretion and maintenance of the corpora lutea, early conceptus mortality, and endometrial changes essential for conceptus survival. Finally, the kallikrein-kininogen-kinin system and its relationship to reproductive processes will be reviewed.

## CHAPTER II

### LITERATURE REVIEW

#### **Late Peri-Implantation Conceptus Development, Estradiol-17 $\beta$ Synthesis and Elongation**

As the porcine conceptus achieves a spherical diameter of 4-6 mm, enzymes essential for conversion of uterine luminal steroid precursors to estradiol-17 $\beta$  (E<sub>2</sub>) are present (Gadsby et al., 1980; Fischer et al., 1985; Mondschein et al., 1985; Pusateri et al., 1990; Conley et al., 1992, Ko et al., 1994). Two enzymes of particular importance are 17 $\alpha$ -hydroxylase (17 $\alpha$ -OH), for conversion of progesterone to 17 $\alpha$ -hydroxyprogesterone, and aromatase, for conversion of testosterone to E<sub>2</sub>. Seventeen- $\alpha$ -hydroxylase, expressed only in the trophoctoderm, is the rate-limiting steroidogenic enzyme needed for E<sub>2</sub> production (Conley et al., 1992, Wilson and Ford, 1997), whereas aromatase is expressed in the inner cell layer (hypoblast) of the trophoblast (Ko et al., 1994).

The conceptus continues to expand into a large spherical (10 mm) shape through a mitotic increase in trophoctodermal cells. Estradiol production by the porcine conceptus increases with the increase in trophoctodermal cell number.



Twenty-four hours after the initiation of  $E_2$  synthesis (5 mm spherical conceptus), the conceptuses will expand to a 10 mm in diameter with the trophoctoderm cells displaying a typical cuboidal morphology (Anderson, 1978; Geisert et al., 1982a; Mattson et al., 1990). A sharp increase in conceptus estrogen secretion occurs on day 11-12 (10 mm spherical conceptuses) with increased  $E_2$  concentrations also detectable in the uterine vein and lymph (Magness and Ford, 1982). This increase in  $E_2$  is correlated with an increase in blood flow (Ford and Christenson, 1979; Ford et al., 1982b; Ford, 1983), alterations in endometrial secretion (Corner, 1921), and is responsible for the signal for the maternal recognition of pregnancy in the pig (Bazer and Thatcher, 1977). Pope et al. (1982) demonstrated that the early secretion of  $E_2$  is involved with the late equidistant spacing of the conceptus within the uterine horns.

Following the attainment of a 10 mm diameter, the conceptus begins its rapid morphologic change in trophoctodermal morphology, with the embryonic disk remaining at  $\sim 300\mu\text{m}$  during the rapid transformation of the trophoblast (Mattson et al., 1990). The conceptus now takes on an ovoid shape with the trophoctoderm cells remaining cuboidal in shape (Heuser and Streeter, 1929). Conceptuses less than 9-10 mm do not appear to elongate at the same time as their 10 mm littermates suggesting elongation is regulated somewhat by individual conceptus development (Morgan et al., 1987).

Shortly after attaining an ovoid morphology, conceptuses transform further into a tubular (13-40 mm in length) form (Heuser and Streeter, 1929;

Anderson, 1978; Geisert et al., 1982b). Tubular conceptuses have polygonal shaped trophoderm cells (Geisert et al., 1982b; Mattson et al., 1990). As tubular conceptuses continue to undergo this morphological change into a thin, filamentous form (100-150 mm in length), there is a rapid increase in conceptus length with no increase in mitotic rate (Geisert et al., 1982b; Pusateri et al., 1990). The conceptus rapidly elongates within 2-4 hours from initiation of the tubular form. The elongated conceptus will become convoluted within the uterine folds of the endometrium where the initial points of attachment begin to occur across the endometrial surface (Danstzer, 1985; Keys and King, 1990).

Initiation of estrogen secretion and elongation is closely timed with the emergence of mesodermal outgrowth (as reviewed in Geisert, et al. 1990). Yelich et al. (1997b) indicated that the time of mesodermal outgrowth (4-5 mm spherical conceptus) coincides with the first conceptus gene expression for brachyury, a transcription factor needed for mesodermal differentiation. Aromatase gene expression also increases in early 4-5 mm spherical embryos. Presence of brachyury, thus mesodermal differentiation, may be involved in regulating steroidogenic events in the conceptus.

Other factors may initiate estrogen production besides brachyury. Presence of high concentrations of insulin-like growth factor-I (IGF-I) in the uterine lumen during early conceptus development has been proposed to enhance steroidogenesis as well as aid in the continued growth and expansion of the conceptus (Simmen et al., 1989, Hofig et al., 1991a; Ko et al., 1994;

Simmen et al., 1995). Presence of IGF-I receptors on the trophoctoderm surface during peri-implantation (Corps et al., 1990) would suggest that IGF-I has a direct action as a mitogenic factor (Lewis et al. 1992) and possible inducer of protein synthesis *in vitro* (Estrada et al., 1991) by the conceptus. However, Chastant et al. (1994) was unable to detect IGF-I receptors through immunocytochemical localization, although IGF-II receptors were very evident. Lack of the IGF-I receptor would negate the IGF-I binding on the conceptus, as IGF-II receptors lack high affinity for IGF-I. Nonetheless, Hofig et al. (1991a) demonstrated increased conversion of testosterone to estradiol when IGF-I is supplemented in culture media. The question still remains whether IGF-I can act directly on the conceptus (through a receptor) to increase conceptus estrogen release, or indirectly through endometrial release of other growth factors, as IGF-I receptors are present in the endometrium and myometrium (Hofig et al., 1991b).

Factors that regulate the trophoblast remodeling are conceivably caused by the cellular morphogen, retinoic acid (Harney et al., 1990). One of the major proteins in the uterus during the time of conceptus estrogen synthesis is retinol binding protein (RBP) (Trout et al., 1992; Harney et al., 1994). Retinol binding protein serves to deliver retinol from plasma across the uterine endometrium to the lumen for the developing conceptus. The developing porcine conceptus also produces RBP (Harney et al., 1990). Harney and co-workers (1994) indicated that RBP mRNA was detectable in the endometrium, the myometrium and

conceptus. Low amounts of endometrial RBP mRNA on day 12-15 suggest that uterine luminal RBP detected after day 12 is of conceptus origin. Conceptus RBP may serve to protect the conceptus from toxic levels of retinol, as well as serving to distribute retinol to all available cells. In order for retinol to have its biologic effects on the conceptus, such as production of extracellular matrix proteins (De Luca, 1991) and cell adhesion molecules (Agura et al., 1992), it must act through its nuclear receptor. Yelich and co-workers (1997a) demonstrated that retinoic acid receptor (RAR) mRNA is expressed in porcine conceptuses. Gene expression for RAR- $\alpha$ , - $\beta$ , and - $\gamma$  is detectable in day 10-12 conceptuses with RAR- $\alpha$  increasing linearly in 4-8 mm spherical conceptuses (consistent with RBP expression) and remaining elevated until another sharp increase occurs at the filamentous stage of development. Furthermore, transforming growth factor- $\beta$  (TGF $\beta$ ), a regulator of cellular growth and differentiation and extracellular matrix protein modifiers, also increased in a similar fashion to that of RAR $\alpha$  gene expression (Yelich et al., 1997a). The presence of RBP, RAR, and TGF $\beta$  provide an attractive model for initiation and/or remodeling that occurs during conceptus elongation in the pig. Geisert and Yelich (1998) proposed a model for trophoblastic elongation in the pig. Conceptus estrogen, acting on its receptors in the endometrium provides the initial release of uterine RBP. Retinol binding protein carries retinol to the conceptus directly or may relay the retinol to conceptus RBP. Retinol that is not bound activates its receptors located in the trophectoderm. Through activation of RAR, TGF $\beta$  gene expression (and other possible morphogens) is turned on,

resulting in remodeling of the extracellular matrix allowing migration of endodermal cells and restructuring of trophoctoderm.

### **Embryonic Mortality in the Pig**

In 1923, George Corner determined a good measure of ova shed at ovulation was to count the number of CL present on the ovary. He went on to note that as gestation proceeded, the number of conceptuses were almost always less than the number of CL (Corner, 1923). Since the time of Corner, significant effort has been concentrated on the time period and cause of embryonic loss in the pig (Pope, 1994).

Fertilization rate in the pig is approximately 95%, and with the exception of chromosomal aberrations, few embryos are lost prior to initiation of steroidogenesis (Polge, 1982). Between days 12-18 of gestation, nearly 30% of conceptuses are estimated to be lost (Pope, 1994). Initial conceptus loss has been proposed to be due to littermate diversity (i.e. differences in developmental stages) (Pope et al., 1982a). Embryonic loss is thought to stem from lack of adequate uterine space for each conceptus, and therefore, uterine capacity dictates number of conceptuses that will survive. Treatments to exceed uterine capacity include superovulation, superinduction and unilateral-hysterectomy-ovariectomy (UHO) (Christenson et al., 1987). Superovulation increases the number of ova shed while superinduction is the transfer of additional embryos into the uterine lumen. In the UHO female, the remaining ovary compensates for the lost ovary and ovulates the same number of oocytes to be fertilized that will

be contained in half the uterine space. Approximately 30% of embryos are lost in limited uterine capacity studies (Dzuik, 1968; Christenson et al., 1987; Dzuik, 1987). Sometime after day 30, uterine capacity will begin to take effect, so superovulated or superinduced litters will have the same number of fetuses as in untreated controls (except UHO females, where they will have  $\frac{1}{2}$  fetuses normally present) (Dzuik, 1968; Pope et al., 1972; Fenton et al., 1972; Huang et al., 1987).

Evidence exists suggesting that embryonic diversity is at the root of the majority of embryonic loss, where the smaller, less developed conceptuses perish in response to their larger, more developed littermates. Asynchronous embryo transfer experiments were the first to implicate littermate diversity as a cause of embryonic mortality in the pig (Pope et al., 1982a).

Pope and co-workers (1982a) transferred day 5 or 7 embryos into day 6 recipients and allowed them to develop until day 11.5, 60 or 70. More older embryos survived compared to the younger embryos on day 11.5 and particularly at day 60 and 70. Further, in superinduction experiments, Pope et al. (1986a) demonstrated that embryos transferred into a less advanced uterine environment are more competitive for survival, versus the less competitive position of embryos when placed into a more advanced uterine environment with endogenous embryos. Wilde et al. (1988) reported that smaller embryos are not less viable than their larger littermates and can survive if placed together in the same uterus void of larger conceptuses.

Further evidence that littermate diversity is the cause of early embryonic mortality stems from experiments artificially accelerating uterine secretions. Geisert et al. (1982c) demonstrated that uterine secretions could be advanced approximately 24 hours in cyclic gilts to mimic pregnant uterine secretion, if they were administered exogenous estrogen. Altering the uterine milieu with estrogen sets up a hostile environment for developing conceptuses. Other experiments have demonstrated that asynchronous administration of estradiol results in embryonic loss by alterations in uterine secretions (Pope et al., 1982b) as well as loss of the uterine glycocalyx (Blair et al., 1991).

### **Maternal Recognition of Pregnancy in the Gilt**

Maternal recognition of pregnancy, first coined by Short in 1969, is defined as the method derived from the conceptus to prolong the life span of the corpora lutea (CL) and therefore maintain peripheral plasma concentrations of progesterone. Normal luteal regression during the estrous cycle of the gilt occurs on day 15 through release of the luteolytic agent, prostaglandin  $F_{2\alpha}$  (PGF) (Moeljono et al., 1976). The signal for maternal recognition of pregnancy in the pig is triggered by conceptus estrogen secretion on day 12 (Perry et al., 1973). Initial estrogen production on day 12 is reinforced between day 12-15 with a second sustained episode of conceptus estrogen release (Zavy et al., 1980; Geisert et al., 1982a; Stone and Seamark, 1985). This two-phase secretion of conceptus estrogen sustains pregnancy until term. Bazer and Thatcher



(1977) proposed the theory of maternal recognition of pregnancy in the gilt. Under progesterone stimulation, in cyclic gilts on day 12-18, PGF is released from the glandular and surface epithelium into the peripheral circulation via uterine capillaries (endocrine direction) (Bazer and Thatcher, 1977). In the presence of the porcine conceptus (or exogenous estrogens) the direction of endometrial PGF release is shifted from the endocrine direction, to an exocrine direction, into the uterine lumen. Luminal PGF increases tremendously during early pregnancy. Furthermore, the conceptus is also producing PGF and prostaglandin E (PGE), of which PGE is a vasodilator. Although most PGF is sequestered in the uterine lumen, there may be enough that escapes into the bloodstream to cause luteolysis. In the presence of the conceptus secretion of estrogen and PGE, there may be another system that protects the porcine CL from luteolysis by PGF. Sulfated estrogens (estrone and estradiol) are also released from the uterine lumen, that may reach the CL which has sulfatase activity (see review Ford, 1985). Estrone and estradiol can be hydroxylated into catechol estrogens that serve to further increase blood flow to the CL (Stice et al., 1987). In an in vivo study, Ford and Christenson (1991) demonstrated that estrogen alone can not prevent luteolysis. Unlike intraluteal E<sub>2</sub> administration, intraluteal administration of PGE protected individual CL against concomitant PGF<sub>2α</sub> administration (Ford and Christenson, 1991). At PGE: PGF<sub>2α</sub> ratios of 4:1, CL weight and progesterone content was significantly greater than administration of PGF<sub>2α</sub> alone (Ford and Christenson, 1991). Sequestering of PGF in the uterine lumen, the ability of catechol estrogens to sustain blood flow



to the CL, and luteal protection of PGE maintains progesterone levels necessary for pregnancy.

### **Endometrial Changes During the Estrous Cycle and Early Pregnancy**

As early as 1921, Corner documented that the porcine uterine luminal and glandular epithelial morphology changed throughout the estrous cycle and early pregnancy. As the estrogen to progesterone ratio changed due to ovarian hormones, uterine growth occurs (Davis and Blair, 1993). With the initiation of conceptus estrogen on approximately day 11 of pregnancy, the uterine epithelium becomes markedly different from its non-pregnant counterpart (Corner, 1921; Geisert et al., 1982b; Keys and King, 1990). Prior to the time of conceptus elongation, the uterus prepares itself by producing components (i.e. growth factors, ions, and hormones) whose release is advanced under conceptus control in early pregnant versus cyclic gilts. In order to better understand uterine changes associated with the time of conceptus elongation and estradiol production, many investigators have compared cyclic versus early pregnant uterine flushings, endometrial explant cultured media, and endometrial monolayer cell cultures from gilts to study some of the components that may be playing a role in the orchestration of the conceptus with the uterine environment (Squire et al., 1972; Knight et al., 1973; Knight et al., 1974a, 1974b; Bazer, 1975; Moffatt et al., 1980; Murray et al., 1980; Basha et al., 1980a, 1980b; Geisert et al., 1982a; Fazleabas et al., 1984; Young et al., 1987; Roberts and

Bazer, 1988; Stroband and van der Lende, 1990; Roberts et al., 1993; Murphy and Ballejo, 1994; Geisert and Yelich, 1998)

To better understand and characterize uterine differences between cyclic and early pregnant gilts caused by the conceptus, Geisert et al. (1982a) conducted a series of experiments. First, uterine flushing concentrations of estrone ( $E_1$ ),  $E_2$ ,  $E_1$  sulfate ( $E_1S$ ),  $E_2S$ , PGE, PGF, total protein and calcium were measured in cyclic and pregnant gilts. Unlike the unchanging estrogens found in cyclic females, the uterine lumen of pregnant gilts contained high concentrations of  $E_2$ ,  $E_1S$  and  $E_2S$  that was correlated with increasing conceptus size on days 11-12. Uterine estrogen content decreased after day 13 following the completion of trophoblastic elongation. Calcium remained unchanged in nonpregnant flushings, but there was a marked increase on day 11 consistent with the initial peak increase in conceptus estrogen secretion. Geisert and coworkers (1982b) also reported a 2-4 fold increase in total protein in day 12-14 pregnant gilts and Murray et al. (1972) reported a peak of uterine protein content on day 15, which declined to low concentrations by day 18 of the estrous cycle. The release and increase in total protein from the uteri of cyclic gilts was delayed approximately one day, in comparison with pregnant uterine flushings (Geisert et al., 1982b). Prostaglandin E and PGF concentrations remain between 1-4 ng between days 10.5 to 14 in cyclic gilts. However, although identical to non-pregnant flushings on day 10.5, PGE and PGF levels increase 6-8 fold on day 11, further increasing on day 12 until becoming 100 fold greater by day 14 (Geisert et al., 1982b).

Further studies to clarify conceptus influence on the uterine milieu were conducted by Geisert et al. (1982c). By administering estradiol valerate (EV) to cyclic gilts, direct effects of estrogens on the uterine secretory activity could be studied. Administration of EV to gilts on day 11 produced similar concentrations of free estrogen within the uterine lumen that were found in pregnant gilts (Geisert et al., 1982a). A transient 24 hour increase in luminal uterine content of calcium was detected 12 hours after the injection of EV, as was seen in pregnant gilts with elongating conceptuses on day 12.

These experiments clearly illustrate that the estrogen that is conceptus derived is responsible for the change in the porcine uterine environment (Geisert et al., 1982a,b,c). Other investigators (Ford et al., 1982a, Pusateri et al., 1990) have also demonstrated similar findings, which corresponded with conceptus growth and changes in uterine environment. Increases in estradiol in the pig is necessary for maintenance of pregnancy due to its luteostatic effect through sequestering PGF within the uterine lumen on day 15 (Bazer and Thatcher, 1977). Estradiol is also responsible for increasing blood flow to the pregnant uterus needed for delivery of nutrients and oxygen to the developing conceptuses (Ford and Christenson, 1979; Ford et al., 1982b).

### **Conceptus-Endometrial Interactions**

Initially, the early, unattached conceptus can obtain all of its nutrients from the uterine milk, or histotrophe. Histotrophe is composed of a large quantity of the purple acid phosphatase, uteroferrin, as well as serpins and lysozyme

(see Roberts et al, 1993). Uteroferrin serves to transport iron while uterine serpins are known to be serine protease inhibitors. Uterine lysozyme, identical to porcine spleen and stomach lysozyme, is bacteriostatic (see Roberts et al., 1993). However, once the conceptus attains the length of a meter in length by day 15 of gestation, the necessity for increased nutrients can not be provided from histotrophe alone. Unlike other species that actively invade into the endometrium (e.g. the human and rat), porcine conceptuses attach to the endometrium to set up an epithelial-chorial placenta (Keys and King, 1990). Interestingly, if the porcine conceptus is placed outside the uterine lumen, it will invade into the surrounding tissue to form a syncytium (Samuel, 1971; Samuel and Perry, 1972) suggesting the porcine conceptus is highly proteolytic. Therefore, porcine endometrium must contain inhibitors to the proteolytic factors from the conceptus. Trophoblastic attachment to the uterine surface epithelial glycocalyx present on the microvilli provides close contact between the maternal endometrial surface and the conceptus to establish the placenta attachment and development for nutrient transport (Keys and King, 1990). However, before the conceptus can attach, the uterine surface epithelium must be altered for contact with the conceptus. Therefore, the opening of the "implantation window" is under ovarian hormone regulation.

### **Peri-Implantation Window**

Prior to the period of implantation in the pig, the apical border of surface epithelium contains microvilli that have a thick glycocalyx that becomes

diminished during the time of trophoblastic attachment (Dantzer, 1985; Keys and King, 1990) or exogenous estrogen treatment in the pig (Blair et al., 1991). The glycocalyx present on the epithelial microvilli is essential for the conceptus to adhere, and if absent, does not appear to allow attachment to occur and conceptuses degenerate (Blair et al., 1991). Even though the glycocalyx is essential for conceptus survival, some glycoproteins that are part of this matrix may serve as steric hindrance to adherence molecules during the period prior to opening the "implantation window". Mucin-1 (Muc-1) is a large (200-1000 kD) integral transmembrane glycoprotein that is associated with the porcine glycocalyx (Bowen et al., 1996, 1997). Bowen et al. (1996) detected expression of Muc-1 during the estrous cycle and early pregnancy, which is consistent with the theory that Muc-1 is an anti-adhesive molecule first indicated in the rat (Surveyor et al., 1995). Muc-1 is present on the apical surface of uterine epithelial cells in ovariectomized gilts receiving estradiol, but absent in gilts treated with progesterone or progesterone and estradiol for ten days, indicative of the time of first progesterone receptor down-regulation in the uterine epithelium (Geisert et al., 1994). Muc-1 is down regulated during the time that the conceptus initiates attachment to the uterine epithelium. This is consistent with mouse data demonstrating that Muc-1 disappears during the time of implantation in the rodent (Braga and Gendler, 1993; Surveyor et al., 1995). Disappearance of Muc-1 may reduce steric hindrance to other molecules of the glycocalyx and allow for the conceptus interaction with the epithelium for trophoblastic attachment.

The extracellular matrix of the conceptus, as in all cells, has more of a role than just support. Some of the extracellular matrix proteins that are known to be expressed in the conceptus are fibronectin, laminin, vitronectin, and collagen type IV (see review Murphy and Ballejo, 1994). For cell to cell contact between the conceptus and uterine epithelium, there must be some type of receptor on the epithelial cell surface of the uterus. Integrins are cell surface glycoproteins that serve to act as receptors to extracellular cellular matrix proteins (see Hynes, 1992). All integrins are composed of an  $\alpha$  and  $\beta$  subunit, whose various combinations dictate cell function (Hynes, 1992). There are currently eight known  $\beta$  subunits and fourteen known  $\alpha$  subunits (see Hynes, 1992). Bowen and coworkers (1996, 1997) investigated the endometrial and conceptus expression of  $\alpha_1$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_v$ ,  $\beta_1$ , and  $\beta_3$  throughout the estrous cycle and early pregnancy of the pig. Evidence that both  $\beta$  and  $\alpha$  subunits occurred in the same cell type suggests possibilities of heterodimers. Subunits that appeared in both the trophectoderm and the uterine epithelium were  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_v$ ,  $\beta_1$ , and  $\beta_3$ , with peak expression of  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_v$ , and  $\beta_1$  appearing on days 11-15 of pregnancy. Furthermore,  $\alpha_3$ ,  $\alpha_v$ , and  $\beta_3$  subunits were not altered by steroid treatment on ovariectomized gilts, with levels of  $\alpha_v$  and  $\beta_3$  remaining high at all days. However, where estrogen treatment down-regulated  $\alpha_4$  and  $\alpha_5$ , progesterone treatment up regulated  $\alpha_1$  and  $\beta_1$ . Heterodimers  $\alpha_4\beta_1$ ,  $\alpha_5\beta_1$  and  $\alpha_v\beta_1$  are known to bind to fibronectin (Hynes, 1992). Fibronectin was only detected on the porcine conceptus trophectoderm (Bowen et al., 1996). Richoux et al. (1989) reported fibronectin to be present on day 5 porcine blastocysts with laminin first

appearing in day 10 conceptuses. Tuo and Bazer (1996) reported oncofetal fibronectin on conceptuses on days 12 and 15. Vitronectin is also present in the trophoctoderm, uterine epithelium, and interestingly at the site of implantation (Bowen et al., 1996). Vitronectin has been shown to bind to the heterodimers of  $\alpha_v\beta_1$  and  $\alpha_v\beta_3$  (Hynes, 1992). The expression of the integrins present on the uterine epithelium, as well as their ligands on the trophoctoderm, which are highly expressed at the time of implantation, suggests a potential role for integrin involvement in the attachment process in pigs.

As mentioned earlier, the glycocalyx is made up of a plethora of glycoproteins. In 1995, Geisert and coworkers reported the presence of a basic 30 kD glycoprotein (pGP30) fragment in the porcine lumen. Through immunocytochemical analysis, pGP30 was detected within and on the surface epithelium of the uterus with maximal expression on day 12 of the estrous cycle and days 12-18 of pregnancy. Conceptus uptake of pGP30 was also apparent; suggesting that pGP30 may be utilized by the conceptus. Geisert et al. (1995) demonstrated that pGP30 was steroid regulated as progesterone increased pGP30 in ovariectomized gilts.

Endometrial explant cultures secreted pGP30 but there was a detectable alteration in size from day 10 to day 12 of the estrous cycle, with further alteration in band number in day 12 pregnant tissue. Geisert et al. (1995) suggested that conceptus estrogen and/or other conceptus derived components are responsible for this reduction in  $M_r$ . Porcine GP30 was later found to be



homologous to a member of the inter- $\alpha$ -trypsin-inhibitor ( $\alpha_1$ I) family (Geisert et al., 1996).

As stated previously, when placed in an ectopic site outside the uterine lumen, the porcine conceptus invades into the surrounding tissues forming a syncytium (Samuel, 1971; Samuel and Perry, 1972). The endometrium has incorporated into its secretions a series of protease inhibitors (see review Roberts et al., 1993). Fazleabas et al. (1983) first reported the trophoblast secreted plasminogen activator at the time of trophoblast elongation which releases plasmin from plasminogen. Plasmin has been shown to be responsible for cell migration, cellular remodeling and extracellular matrix regulation (Bode and Dziadek, 1979; Fazleabas et al., 1983; Werb et al., 1990). Fazleabas et al. (1983) and Stallings-Mann et al. (1994) reported the presence of a protease inhibitor specific for plasmin, chymotrypsin and trypsin in the uterus, which keeps this proteolytic system in-check. Furthermore, an antileukoproteinase, possibly regulated by  $TGF\alpha$ , is present in the porcine uterine secretions (Farmer et al., 1990; Reed et al., 1998). Antileukoproteinases are potential inhibitors of neutrophil elastase and cathepsin G (Thompson and Ohlsson, 1986) and are unique to epithelial-chorial placental species (Badinga et al., 1994).

The serine protease inhibitor, bikunin, was also detected in porcine endometrium (Diederich et al., 1997). Via RT-PCR, message for bikunin was detected in cyclic and pregnant endometrium on days 10 and 12. However, gene expression was significantly greater on days 15 and 18 of pregnancy. Individual



conceptuses from day 10 and 12 also had detectable amounts of bikunin (Diederich et al., 1997).

Bikunin is also a member of the  $\alpha_1$  family. The  $\alpha_1$  family consists of 5 members; 4 heavy chains and one light chain (see review Salier et al., 1996). The light chain, bikunin, can be found alone or bound to heavy chain 1, heavy chain 2, heavy chain 3, or heavy chains 1 and 2, or heavy chains 2 and 3 by linkage of the glycosaminoglycan, chondroitin sulfate (see review Salier et al., 1996). Inter- $\alpha$ -trypsin inhibitor heavy chain 1 has also been reported to be expressed and localized in the porcine endometrium (Geisert, unpublished data).

Inter- $\alpha$ -trypsin-inhibitors are major members of Kunitz-type protease inhibitors (review in Salier et al., 1996). A Kunitz-type inhibitor is one that has a low molecular mass, a basic isoelectric point, and has at least one domain that is inhibitory to serine proteases, such as trypsin, cathepsin G, and plasmin. Although bikunin has 2 Kunitz-type domains, plasmin inhibitors more effectively inhibit the above-mentioned proteases, thus the role of  $\alpha_1$  as a protease inhibitor is still being investigated (Salier et al., 1996).

Inter- $\alpha$ -trypsin-inhibitor family members play an important role in extracellular matrix (ECM) stabilization (see Bost et al., 1998). The ECM is a complex network of several macromolecules that allow for tissue cohesiveness. The ECM also allows for cell migration and interaction between different cell

types. A major component of ECM is hyaluronic acid (HA). The presence of HA is often associated with HA-binding proteins (HABP) (see Bost et al., 1998). The linkage of  $\alpha_1$ I proteins with HA was first discovered in the ovary and inflamed synovial fluids (see Bost et al., 1998). Inter- $\alpha$ -trypsin-inhibitors have now been viewed as a major player in stabilization of ECM, as well as having a role in protease inhibition.

Not only does  $\alpha_1$ I play a role in ECM stabilization,  $\alpha_1$ I also is involved with inflammatory processes.  $\alpha_1$ I gene expression is regulated by interleukin-6, an important inflammatory mediator (Daveau et al., 1993; Sarafan et al., 1995.) Inter- $\alpha$ -trypsin-inhibitor heavy chains (H) -1, -2, -3, and -4 all are derived from separate genes with heavy chain mRNA 1-3 being known for some time, but it wasn't until recently that  $\alpha_1$ IH4 was known (Nishimura et al., 1995). Bikunin shares its gene with  $\alpha_1$ -microglobulin, which separates as it becomes a matured polypeptide (Diarra-Mehrpour et al., 1989).

Not all heavy chains can bind bikunin. Inter- $\alpha$ -trypsin inhibitor H4 does not contain the consensus sequence needed to covalently bind bikunin. It was therefore first termed as  $\alpha_1$ I related protein, in the human (Saguchi et al., 1995; Nishimura et al., 1995) and major acute phase serum protein in the pig (Gonzalez-Ramon, 1995). It wasn't until recently that these two proteins were determined to be homologous, (Hashimoto et al., 1996) and since gene structure was similar, was classified as  $\alpha_1$ I H4.

Although the Kunitz-type domains are the only functional regions of the protein that possess protease inhibitory activity, recent progress has determined putative functional domains in the heavy chains. First, the von Willibrand-type A domain has been located on  $\alpha$ IH1, -H2, -H3, and -H4, which binds to integrins, collagen, proteoglycans and heparin (Colombatti and Bonaldo, 1991). Secondly, heavy chains 1 and 3 also contain a multicopper oxidase domain (Chan et al., 1995) whose ability to bind copper seems to have disappeared through evolution, and therefore does not appear to be biologically active. Thirdly, human  $\alpha$ IH4's C-terminal sequence is rich in a proline, bradykinin-like domain. The proline-rich region is surrounded by arginine residues and therefore make this region sensitive to kallikrein cleavage (Nishimura et al., 1995; Gonzalez-Ramon, et al. 1995).

Recently, the N-terminal sequence of pGP30 detected in the porcine endometrium (Geisert et al., 1995), was determined to be homologous to  $\alpha$ IH4 (Geisert et al., 1998). Gene expression of porcine uterine  $\alpha$ IH4-like glycoprotein was specific to the endometrium and absent in conceptuses (Geisert et al., 1998). Subjecting endometrial  $\alpha$ IH4 to kallikrein has not been done to determine if a 30 kD fragment (pGP30) is released. However, Hashimoto and co-workers (1995) detected 55 and 25 kD polypeptides after incubating porcine plasma  $\alpha$ IH4 with kallikrein. Evidence that both pGP30 and  $\alpha$ IH4 are present in the porcine uterine lumen suggests kallikrein cleavage does occur in porcine

endometrium. Besides the role that kallikrein may play in cleaving  $\text{I}\alpha\text{H4}$ , kallikrein is more noted for its regulation of kinin release.

### **Kininogen-Kallikrein-Kinin System**

The mammalian blood contains three major protease cascades that are activated by the Hageman Factor. These cascades include the blood coagulation, fibrolysin and the kallikrein-kininogen-kinin (K-K-K) system. The focus of this section will concentrate on the K-K-K system. The K-K-K system is composed of several members that include the kininogens, kallikreins, kinins, kallikrein binding proteins, and kininases (see review Bhoola et al., 1992). Briefly, high and low molecular weight kininogen are cleaved by plasma or tissue (glandular) kallikrein, respectively, to liberate kinins, namely bradykinin. Bradykinin is the active molecule in the system that functions to release histamines, acts as a vasodilator, potentiates prostaglandin release as well as other vasoactive events. Kinins are quickly inactivated by kininases that are present within the tissues (See figure 2.1).

There are two pathways that make up the K-K-K system (see reviews Bhoola et al., 1992; Carhini et al., 1993; Rusiniak and Back, 1995; Margolius, 1996). The first is found in the peripheral vascular system. In the blood, when trauma is caused or activated by coming in contact with collagen (intrinsic

pathway), Hageman Factor is activated. Hageman Factor converts pre-kallikrein into kallikrein that has a positive feedback on activating more Hageman factor. Activated kallikrein acts on its normal substrate, high molecular weight kininogen, to liberate bradykinin (see reviews Bhoola et al., 1992; Carhini et al., 1993; Rusiniak and Back, 1995; Margolius, 1996).

An alternative pathway for the K-K-K system occurs in the tissue. Here, plasma kallikrein, or other proteases in the tissue activate pro-kallikrein into kallikrein. Glandular kallikrein acts on its normal substrate, low molecular weight kininogen to release lysyl-bradykinin, or kallidin, that is further converted to bradykinin by amino-peptidase. Bradykinin is rapidly broken down to an inactive form by kininases (see reviews Bhoola et al., 1992; Carhini et al., 1993; Rusiniak and Back, 1995; Margolius, 1996).

## Plasma

## Tissue

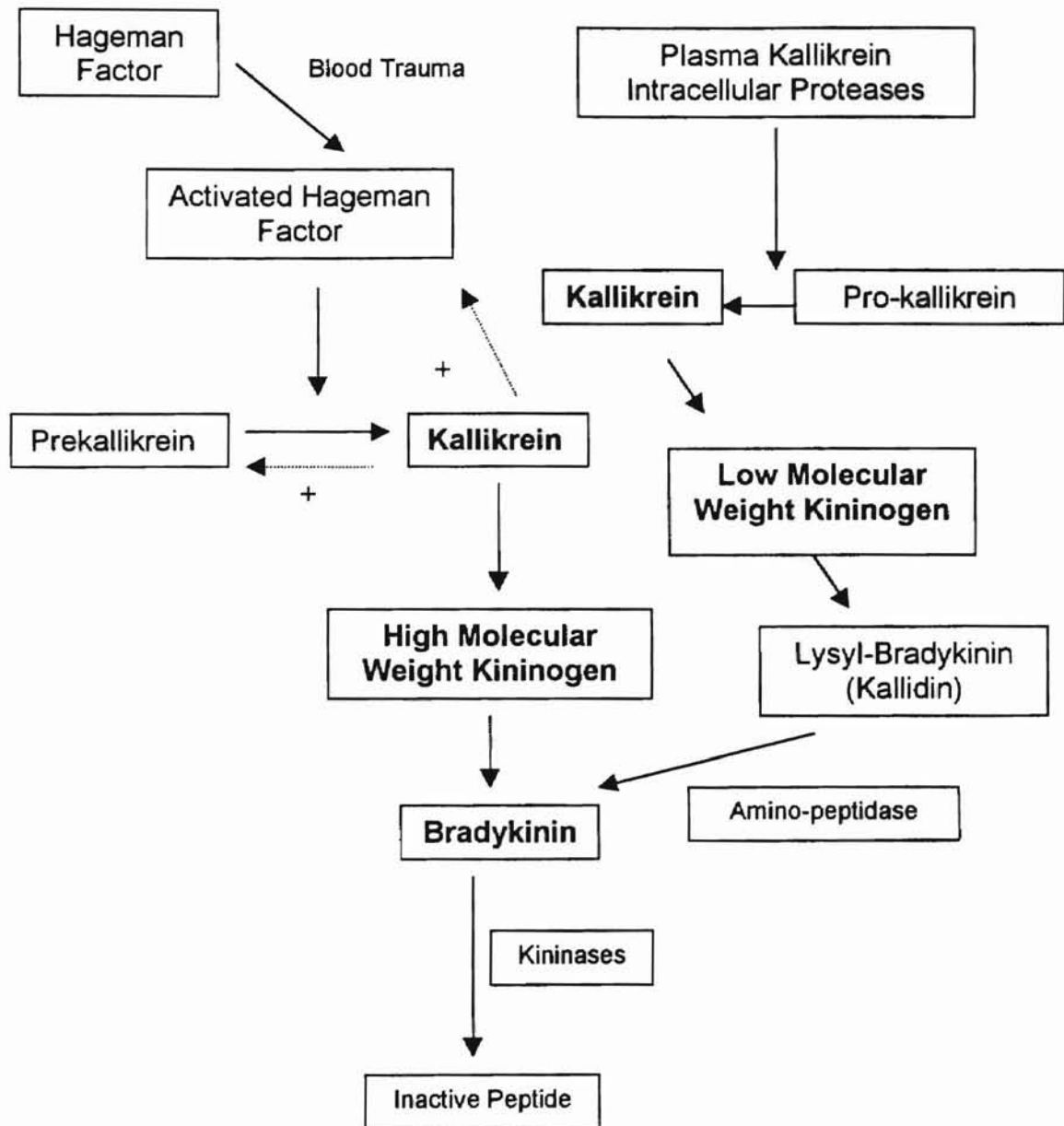


Figure 2.1. Plasma and tissue (glandular) kallikrein proteolytic pathways involved in bradykinin production and degradation. Hatched arrows and + indicate positive feedback loops.

## Kininogens

As mentioned previously, kininogens are glycoproteins that serve as substrates for the serine proteases, kallikreins. Plasma kallikrein cleaves HMW kininogen to release the nonapeptide, bradykinin. Tissue, or glandular, kallikrein cleaves LMW kininogen to release lysyl-bradykinin, or kallidin (see reviews Bhoola et al., 1992; Carbini et al., 1993; Rusiniak and Back, 1995; Margolius, 1996).

Splicing of a single human kininogen gene gives rise to high molecular weight (HMW; 88-120 kD) and low molecular weight (LMW; 50-68kD) kininogen (Margolius, 1996). The first nine exons are the same for both HMW and LMW kininogens, giving rise to the same amino acid sequence. The difference between HMW and LMW kininogen lies beyond the bradykinin sequence (exon 9), where HMW contains part of exon 10, and LMW, although lacking exon 10, is unique for containing exon 11. There is another type of low molecular weight kininogen found only in the rat, named T-kininogen (see reviews Bhoola et al., 1992; Carbini et al., 1993; Rusiniak and Back, 1995; Margolius, 1996; Schmaier, 1997), which will not be discussed.

Kininogens are composed of a heavy chain and a light chain, of which the heavy chain (composed of domains 1-3 and the bradykinin moiety) is identical in both HMW and LMW kininogen (Schmaier, 1997). The light chain is unique to each kininogen, and gives each its own unique physiologic function. Comprising the shared heavy chain, domains 1, 2 and 3 are known to bind calcium (Ishiguro et al., 1987), inhibit calpain, papain and cathepsin L, respectively with domains 2

and 3 sharing inhibition of papain and cathepsin L (see Colman, 1996; Schmaier, 1997). Domains 2 and 3 also appear to play a role in cysteine protease inhibition as they contain three disulfide loops and a highly conserved QVVAG sequence (Coleman, 1996). Domain 4 is the bradykinin region, and serves as the cleavage site for kallikrein.

The light chain of HMW kininogen includes a histidine rich domain 5 and C-terminal domain 6 (see Schmaier, 1997). The histidine rich domain is essential in HMW kininogen's role in the coagulation cascade involved with the intrinsic pathway. Domain 6 has sites that bind pre-kallikrein as well as the Hageman Factor, which are essential for initiation of the cascade (see Coleman, 1996; Schmaier, 1997). LMW kininogen has a very small light chain, comprised of only a portion of domain 5, which lacks the histidine-rich segment (see Schmaier, 1997; Coleman, 1996).

It was once thought that the liver was the only source of kininogen. However, LMW kininogen has also been demonstrated to be present in the adrenal (Wang et al., 1996), human placenta (Hermann et al., 1996) and rat uterus (Brann et al., 1995) while HMW kininogen is expressed in endothelial cells (see review Reddigari et al., 1995). Since kininogens can be synthesized by and have actions on various cell types, Takano and coworkers (1995) used mouse fibroblasts to study LMW kininogen in response to dibutyryl cAMP (dbcAMP), forskolin, PGE and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). Kininogen release was stimulated by dbcAMP; and forskolin, an activator of adenylate cyclase, indicating that kininogen secretion occurs through a cAMP second messenger



system. In order to further test this hypothesis, kininogen secretion was tested in the presence of PGE, which stimulates cAMP production in fibroblasts. After 24 hours, levels of kininogen were significantly increased in the presence of PGE. Since  $\text{TNF}\alpha$  and IL-1 induced PGE production in fibroblasts, they were also examined. Fibroblasts receiving  $\text{TNF}\alpha$  stimulus, but not IL-1 or IL-6, increased kininogen secretion (Takano et al., 1995). Kininogen not only acts to serve as kallikrein's substrate for kinin production, but also serves as a cysteine protease inhibitor. Evidence that fibroblasts can mediate kininogen secretion in response to inflammatory agents such as  $\text{TNF}\alpha$  and PGE, suggest that the connective tissue may be playing a role at the site of inflammation.

### **Kallikreins**

Kallikreins are serine proteases that liberate kinins from kininogen (see reviews Bhoola et al., 1992; Carhini et al., 1993; Rusiniak and Back, 1995; Margolius, 1996). Investigators have found two types of kallikrein, plasma kallikrein and tissue, or glandular kallikrein. Each can be characterized by differences in substrate types, tissue localization, function and molecular structure. Plasma kallikrein cleaves HMW kininogen and is located in the peripheral blood where it is involved in the intrinsic pathway, whereas tissue kallikrein acts on LMW kininogen, and functions to release kinins from many tissues (see review Bhoola et al., 1992).

As reported by Chao et al. (1996), Abelous and Bardier in 1909, first identified tissue kallikrein activity in a human patient's urine who experienced a dramatic lowering of blood pressure. Similar hypotensive activity was

demonstrated in dogs and rabbits by independent researchers (see Bhoola et al., 1992). As Bhoola et al. (1992) indicate, Wehle and co-workers studied an active factor present in blood, pancreas and salivary glands, which they named kallikrein, from the Greek word, kallikreas meaning pancreas.

The gene families of plasma and tissue kallikrein differ, as plasma kallikrein is derived from a single gene in the liver and glandular kallikrein is a multiple gene family of which 3 genes have been described in the human, 20 in the rat and 24 in the mouse (see reviews Bhoola et al., 1992; Carhini et al., 1993; Rusiniak and Back, 1995; Margolius, 1996). Regardless of species and specific tissue, all tissue kallikreins consist of five exons and 4 introns (see Margolius, 1996). Members of the kallikrein gene family are a subfamily of serine proteases that are specific for cleaving arginine residues (see Bhoola et al., 1992). True tissue kallikrein (rGK-1) and tonin (rGK-2) are present in the rat, human and mouse (see Margolius, 1996). Tonin is a member of the kallikrein family, with specific function to cleave angiotensinogen to release angiotensin II (Boucher et al., 1994). True kallikrein genes are designated as rGK-1 in the rat, hRKALL in the human, and mGK-6 in the mouse (see Bhoola et al., 1992). Tissue kallikreins are acidic glycoproteins of 24-45 kD which is consistent across many species and tissue types (figure 2).

Species	Tissue/ Organ	Reference
Pig	Pancreas	Fiedler et al., 1981
	Salivary Gland	Lemon et al., 1979
	Anterior Pituitary	Powers and Nasjletti, 1984
Rat	Pancreas	Hojima et al., 1975
	Salivary Gland	See Richards et al., 1996
	Intestinal Tissues	See Bhoola et al., 1992
	Anterior Pituitary	Jones et al., 1990
	Kidney	Bascands et al., 1995
	Uterus	Brann et al., 1995
Human		Valdes et al., 1996
	Kidney	Chen et al., 1995
	Pituitary	Clements et al., 1996
	Prostate	Herrala et al., 1997
	Endometrium	Clements and Mukhtar, 1994
	Adrenal Gland	Wang et al., 1996

Figure 2. Tissue kallikrein in various tissue types and species.

Besides serving to cleave its natural substrate, kininogen, kallikreins have also been noted to act on other molecules. Plasma kallikrein converts pro-renin to renin, which is the initiator of the renin-angiotensin system (Sealey et al., 1978). Plasma kallikrein can also convert plasminogen to plasmin (Mandle and Kaplan, 1977). Studies conducted with the rat pituitary indicate that tissue kallikrein cleaves prolactin to its active form, *in vitro*, suggesting a possible role in the biosynthesis of prolactin (Powers, 1993). Powers (1987) demonstrated that expression of rGK-1 in the pituitary gland is regulated by estrogen. Furthermore, tissue kallikrein is induced by estrogen and inhibited by dopamine in females attaining puberty, with no change noted in male rats (Hatala and Powers, 1988). Rat pituitary kallikrein did not change during the estrous cycle or early pregnancy, but on day 15 of gestation there was a sharp increase, declining to

normal levels 5 days post-partum. Jones et al. (1991) indicated that only lactotrophs are associated with tissue kallikrein in the anterior pituitary.

Other proteins may be cleaved by kallikrein are the binding proteins of insulin-like growth factors. Insulin-like growth factors (IGF) are important for cellular growth and various other processes. Regulators of IGF are its binding proteins, IGF-BPs. IGF-BP are known to have proteases that serve to release IGF, thereby allowing IGF to have its cellular action. Currently there are three classes of IGF-BP proteases: kallikreins, cathepsins, and matrix metalloproteases. Cohen and co-workers (1992) demonstrated that seminal plasma kallikrein, prostate-specific antigen (PSA), is an IGF-BP protease that cleaves IGF-BP3. PSA is a member of the kallikrein gene family in the human (see review Bhoola et al., 1992). Furthermore, another member of the kallikrein gene family has been shown to modulate the IGF system. In 1996, Rajah and co-workers noted that 7S nerve growth factor (NGF) displayed protease activity against IGF-BP4 and -BP6. These authors suggest that the synergistic relationship between IGF and NGF may help mediate growth promoting activities including cell development and repair (Rajah et al., 1996).

The K-K-K system has also been detected in reproductive processes in both male and females. Schill and Haberland (1974) reported that when porcine pancreatic kallikrein was added to human sperm, motility was increased. Kallikrein is present in prostate (in humans only) and sertoli cells of the testis (Schill and Haberland, 1974). Presence of kallikrein in Sertoli cells suggests a role in spermatogenesis (see Bhoola et al., 1992). Kinins acting to increase

glucose uptake may be one of the reasons for an increase in sperm motility (see Bhoola et al., 1992).

A number of reproductive processes have similarity to inflammatory reactions. The K-K-K system is present and active during ovulation (Gao et al., 1992), implantation (Valdes et al., 1996; Corthorn et al., 1997), menses (Clements and Mukhtar, 1994) and parturition (Brann et al., 1995). Gao and co-workers (1992) studied parallelism between the inflammatory response and ovulation in the rat. Kallikrein levels and estrogen concentration were highest at time 0 (injection of hCG) and then dramatically decreased with a slight elevation at 20 hours (ovulation). Total kininogen levels increased at 10 hours, dropped at 20 hours and peaked at 30 hours post-injection. Ovarian cathepsin B activity was lowest at 10 hours post-injection, corresponding to the high levels of kininogen. Changes in kininogen and kallikrein during the ovulatory process suggest the K-K-K system is present and active at ovulation.

Kallikrein activity has also been detected in the rat uterus as well. Valdes et al. (1996) previously reported finding kallikrein activity in rat uteri and kallikrein gene expression present in endometrium from cyclic and early pregnant rats. During the estrous cycle, immunoreactivity to a polyclonal rat kallikrein antibody, was localized in the apical surface of glandular and luminal epithelium, with greater intensity occurring during proestrus. During pregnancy, kallikrein staining intensified by day 5 with staining increasing slightly and in some cases, protruding into the lumen on day 6. These results may possibly indicate a release of kallikrein into the uterine lumen. Epithelial cells in the implantation chamber

stained on day 7. Following completion of placentation, on day 9-10 kallikrein staining was absent. On day 12, staining for kallikrein was evident in granules contained within decidual cells, intensifying by day 14. Days 16-21, staining was detected on the maternal blood vessels, just under the placenta throughout the remainder of gestation. No staining was detected in the stromal fibroblasts, myometrium or basal placenta. The authors suggest that presence of kallikrein within cells of the implantation chamber maybe mediating the decidualization process. On days 6-7, there is an increase of permeability, possibly due to kinin release.

Detection of kallikrein throughout the estrous cycle and pregnancy in the rat suggests that it may be mediated by steroid hormones. To get a better understanding of how hormones could be influencing kallikrein release, Corthorn et al. (1997) evaluated hormonal and intraluminal stimulation on kallikrein release. Uterine weight increased as well as kallikrein activity with increased amounts of estradiol administered to ovariectomized rats. Progesterone supplemented ovariectomized rats had no significant increase in uterine weight, and there was a decrease in kallikrein activity. In pseudopregnant rats, as uterine weight and protein content decreased, kallikrein content rose from day 1 to day 5, reaching a plateau on day 7. Induction of decidualization with intraluminal oil injection resulted in a 3-4 fold greater increase of kallikrein within the decidualized horn compared to the untreated contralateral horn. Furthermore, in unilateral pregnant rats, the decidualized horn had 2 to 3 fold more kallikrein than its non-pregnant counterpart. These studies provide clear indication that estrogen

stimulates kallikrein release whereas kallikrein secretion is impeded in presence of progesterone.

### **Kallikrein Inhibitors**

Although the roles of kininogens, kallikreins, kinins and kininases have been studied extensively, there is limited literature on kallikrein inhibitors. In the early 1980s,  $\alpha$ 1-antitrypsin, a serine protease inhibitor, was reported (Geiger et al. 1981). Geiger et al. (1981) indicated that  $\alpha$ 1-antitrypsin did inhibit kallikrein activity, however, the coupling of the two proteins leading to inhibition, is a very slow process. Chao and coworkers (1996) reported a new kallikrein binding protein, which they characterized and cloned. These authors named the kallikrein binding protein, kallistatin. Kallistatin binds more strongly to kallikrein than other proteases. Chen et al. (1995) indicated that kallikrein and kallistatin mRNA are localized in the same cells in the human kidney, suggesting a potential role in kallistatin regulating kallikrein activity.

### **Kinins**

Kinins are vasoactive peptides produced from kallikrein activity. Kallidin and bradykinin are the mammalian sources that will be discussed. Plasma kallikrein releases the nonapeptide, bradykinin, from HMW kininogen. Kallidin comes from tissue kallikrein action and is 10 amino acids long (lys<sup>1</sup>-bradykinin) that is converted to bradykinin by aminopeptidases (see reviews Bhoola et al., 1992; Rusiniak and Back, 1995; Carhini et al., 1993; Margolius, 1996). There are two other kinin sources in mammals that are only detected in the rat at this time.



These are T-kinin (Ile-Ser-bradykinin), and Met-T-kinin (Met-Ile-Ser-bradykinin) that have been limited to plasma (see Bhoola et al., 1992). Kinins are rapidly degraded in the body by kininases (see reviews Bhoola et al, 1992; Rusiniak and Back, 1995; Carhini et al, 1993; Margolius, 1996). Kininases remove the terminal arginine by carboxypeptidase. This carboxypeptidase, kininase II, also is responsible for converting the inactive angiotensin I to the potent angiotensin II (Rusiniak and Back, 1995). Estimated half life of bradykinin and kallidin in blood is < 30 seconds (McCarthy et al., 1965).

Kinins have been known to be involved in a variety of biological functions that include increasing vascular permeability, contract smooth muscle of the lungs, intestine and uterus, increasing in glucose uptake and increase sperm motility (see Bhoola et al., 1992). At the cellular level, kinins have been known to stimulate by release of neurotransmitters (norepinephrine), histamine, activating platelet-activator factor, leukotrienes, and prostaglandins (see review Bhoola et al., 1992). Bradykinin appears to stimulate prostaglandin synthesis by a calcium/calmodulin mechanism (Cooper et al., 1985). Kinins can also stimulate release of renin from the kidney and catecholamines from the adrenal medulla. Kinins carry out their actions by attaching to their receptors that are bound to a G-protein-coupled second messenger system (see Bhoola et al., 1992).

### **Statement of the Problem**

The process of porcine conceptus elongation and attachment to the uterine epithelium remains an enigma. Many components contribute to the milieu



of the porcine uterus and must be synchronized with the conceptus for successful early embryonic development and placental establishment. Uterine protein profiles are dictated by estrogen; the conceptus derived signal for the maternal recognition of pregnancy in the pig (Bazer and Thatcher, 1977).

Identification of  $\alpha$ LH4 in the porcine uterus, with its unique characteristic of kallikrein cleavage, and detection of pGP30 in the porcine uterine lumen, suggest that kallikrein may be synthesized by the porcine endometrium (Geisert et al., 1995). Regulation of  $\alpha$ LH4 may be necessary for conceptus attachment and establishment of placentation. Furthermore, presence of uterine kallikrein suggests a functional kininogen-kallikrein-kinin system in the porcine uterus. Events that occur in other tissues via kallikrein (i.e. prostaglandin production, histamine release, increase in vascular permeability, increase in blood flow) all occur during the critical time of conceptus elongation and attachment in the pig.

Presence of kallikrein in the porcine uterine lumen needs to be evaluated. If kallikrein is detected, changes in enzymatic activity, tissue source, and gene expression throughout the estrous cycle and early pregnancy should be investigated.

## CHAPTER III

### Detection of Kallikrein gene expression and enzymatic activity in porcine uterus during the estrous cycle and early pregnancy

#### Introduction

During early pregnancy, porcine conceptuses undergo a rapid morphological transformation from a 10 mm sphere to a long filamentous thread within the course of a few hours on Day 12 of gestation (Geisert et al., 1982b). This process allows the release of conceptus estrogen to act as the signal for maternal recognition of pregnancy across the endometrium in the pig (see Geisert et al., 1990). Conceptus synthesis and release of estrogen alters uterine secretion of proteins (Roberts et al., 1993), prostaglandins (Bazer and Thatcher, 1977), uterine blood-flow (Ford et al., 1982), and uterine cellular morphology (Geisert et al., 1982b; Keys and King, 1990). Although a great amount of information is available for conceptus estrogen influence on uterine activity, factor(s) induced by conceptus estrogen to orchestrate uterine changes conducive to conceptus development and survival are not fully understood.

Implantation by pig conceptuses is typically non-invasive forming the diffuse, epitheliochorial placenta of this species (see Keys and King, 1990). Although non-invasive within the uterine lumen, pig conceptuses produce

several proteolytic enzymes that can erode cells when they are translocated to tissues outside the uterine lumen (Samuel and Perry, 1972). Invasive implantation of the proteolytic porcine conceptus is inhibited through endometrial production of several protease inhibitors such as uterine plasmin/trypsin inhibitor (Stallings-Mann et al., 1994), antileukoproteinease (Simmen et al., 1996) and bikunin (Diederich et al., 1997). Previously, our laboratory isolated and characterized a glycoprotein that is homologous to inter- $\alpha$ -trypsin inhibitor heavy chain 4 ( $\alpha$ IH4) (Geisert et al., 1998).  $\alpha$ I family members consist of four heavy chain glycoproteins (H1, H2, H3, H4) and a light chain called bikunin (see review Salier et al., 1990). Bikunin binds with  $\alpha$ IH1, -H2, and -H3 and acts as a serine protease inhibitor (see review Salier et al., 1990). Interestingly,  $\alpha$ IH4 does not appear to function as a carrier for the protease inhibitor activity as it does not bind bikunin. However,  $\alpha$ IH4 does contain a cleavage site for the serine protease, kallikrein (Nishimura et al., 1995). Kallikrein has been shown to cleave porcine plasma  $\alpha$ IH4 into 100 and 35 kD fragments, further cleaving the 100 kD into a 70 kD fragment (Hashimoto et al., 1996). A 30 kD fragment corresponding to the C-terminal of  $\alpha$ IH4, has been detected within the uterine lumen during the time of porcine conceptus elongation and attachment (Geisert et al., 1995). These results suggest that kallikrein may be present within the uterine lumen of the pig, possibly functioning to regulate alterations in  $\alpha$ IH4 necessary for conceptus attachment and placentation. Therefore the objective of the current study was to determine if endometrial kallikrein enzymatic activity, protein and

endometrial gene expression are detectable during the estrous cycle and early pregnancy in the pig.

## Materials and Methods

### Experiment I: Evaluation of Kallikrein Activity in Cyclic and Pregnant Gilts

#### Animals

Cyclic, crossbred gilts of similar age (8-10 mo) and weight (100-130 kg) were checked twice daily for the onset of estrous behavior by intact boars. Onset of estrus was considered Day 0 of the estrous cycle. Gilts assigned to be mated were bred naturally with fertile boars at the onset of estrus and 12 hours later. Cyclic gilts (n=12) and pregnant gilts (n=11) were hysterectomized on either Day 10, 12, and 15 as previously described by Gries et al. (1989). Following initial induction of anesthesia with a 1.5 cc i.m. administration of a cocktail consisting of 2.5 cc Rompum (xylazine; 100 mg/ml) (Miles, Inc., Shawnee Mission, KS), and 2.5 cc Vetamine (ketamine HCl; 100 mg/ml) (Mallickrodt Veterinary, Mundelein, IL) in 500 mg of Telazol (tiletamine HCl and zolazepam HCl) (Fort Dodge, Syracuse, NE), anesthesia was maintained with a closed-circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ) and oxygen (1.0 liter/min). After exposure by midventral laparotomy, the uterine horns and ovaries were surgically removed. The incision site was closed using routine surgical procedures, and gilts were treated i.m. with procaine penicillin G (20,000 IU/kg BW).

## Collection of Uterine Flushings and Endometrium

Uterine flushings (UTF) and endometrium were obtained by isolating one horn and flushing with 20 ml of phosphate buffered saline (PBS; (pH 7.4). Flushings were examined to confirm pregnancy in mated gilts. Conceptus diameters were measured, recorded and conceptuses snap frozen in liquid nitrogen. Uterine flushings were placed on ice, until centrifugation (2500 x g, 10 min; 4°C), and stored at -80°C at the laboratory. After flushing, the horn was cut along its anti-mesometrial border and endometrium was exposed for removal with sterile scissors. Endometrium was collected, snap frozen in liquid nitrogen and stored at -80°C until RNA was extracted. The remaining uterine horn was immediately placed in a sterile container and transported on ice to a sterile horizontal flow hood. The uterine horn was opened along its anti-mesometrial border and endometrium was removed from the myometrium using sterile scissors. Endometrial strips were diced into 4x4 mm sections and a total of 0.5 g explant tissue was placed in a 100x15 mm standard sterile petri dish (Fisher Scientific, Pittsburgh, PA) containing 15 ml Dulbecco's modified Eagle medium (MEM) (Gibco/ Life Sciences, Gaithersburg, MD) and 2% (v/v) antibiotic-antimycotic (Gibco/ Life Sciences). To remove serum leaching from tissue, endometrial explant cultures were initially incubated for 3 hours when media was removed and replaced with fresh media. Endometrial explant cultures were incubated in air on a rocking platform (4 cycles/min) for an additional 24 h in MEM at 37°C. Endometrial explant culture media (ECM) was centrifuged (2500 x g; 10 min) and supernatant stored at -80°C until analyzed.

An additional group of animals was utilized to collect endometrium for RNA extraction. Endometrium was obtained from cyclic gilts (n=11) on Day 0, 5, 10, 12, 15 and 18 and Day 10, 12, 15, and 18 of pregnancy (n=8) as previously described.

## Experiment II: Evaluation of Conceptus Effects on Uterine Kallikrein.

### Animals

Cyclic, Yorkshire gilts (n=43) of similar age and weight (100-130 kg) were checked twice daily for the onset of estrous behavior by intact boars. Onset of estrus was considered Day 0 of the cycle. Gilts were bred at onset of estrus and 12 hours later. In order to get a range of conceptus sizes, uterine horns were flushed on days 10.5, 11, 11.5 and 12 of pregnancy. Gilts were anesthetized with 12 cc Pentathol (~6 mg/ kg BW; Abbott Laboratories, North Chicago, IL) via the jugular vein. Anesthesia was maintained on a closed-circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ) and oxygen (1.0 liter/min). After exposure by midventral laparotomy, the uterine horns were flushed *in situ* with 20 ml PBS. Conceptus diameters were recorded, UTF were centrifuged, supernatant immediately placed on dry ice, transported back to the laboratory and stored at -80°C until analyzed for kallikrein activity and estradiol-17 $\beta$  content. The incision site was closed using routine surgical procedures, and gilts were treated i.m. with procaine penicillin G (20, 000 IU/kg BW).

## Estradiol-17 $\beta$ Radioimmunoassay

Estradiol-17 $\beta$  within UTF was quantified by radioimmunoassay as previously described by Anderson et al. (1993) and validated for conceptus estrogen in UTF by Wilson and Ford (1997). Samples (100  $\mu$ l) were assayed in duplicate. The sensitivity of the assay was 2 pg/ tube. Multiple aliquots of a conceptus pool were included in each assay resulting in intra- and inter-assay coefficients of variation of 4.5% and 12.2%, respectively.

## Microconcentration and Protein Determination

Uterine flushings and ECM samples were prepared for enzyme assay by concentrating 4 ml of sample using Centricon 10 microconcentrators (Amicon, Beverly, MA) with a molecular weight cut-off of 10,000 daltons. Protein concentrations were determined by the method of Lowry et al. (1951). Concentrated samples were stored at -80°C until analyzed.

## Enzyme Assay

To determine the  $K_m$  and  $V_{max}$  of kallikrein, 5  $\mu$ l (3.75 mg/ml) porcine pancreatic kallikrein (Calbiochem, La Jolla, CA) was added at varying concentrations (21, 50, 100, 250, 500  $\mu$ M) of the fluorescent substrate, Pro-Phe-Arg-methylcoumarylamide (MCA; 0.87 mM) (Sigma, St. Louis, MO). The reaction was brought to a final volume of 500  $\mu$ l with the enzyme buffer, 0.1 M Tris-HCL buffer containing 0.15 M NaCl, pH 8.0. The assay was performed at room temperature in a fluorescence spectrophotometer (Perkin Elmer 650-40

Fluorescence Spectrophotometer) with excitation at 370 nm and emission at 460 nm to measure the hydrolysis of the fluorescent product, amino methylcoumarin. A chart recorder (Cole Palmer, Chicago, IL) recorded fluorescent activity with a setting of 6 cm/min. Each reaction was recorded for at least one minute. The  $K_m$  and  $V_{max}$  of kallikrein was determined to be 300–450  $\mu\text{M}$  and  $2.3 \pm 0.3 \mu\text{M}/\text{min}$ , respectively.

Prior to analysis, samples, standards and substrate were kept on ice. A standard curve was performed prior to each assay with the addition of aminomethyl coumarin (Sigma, St. Louis, MO) at concentrations of 0.20, 0.50, 0.75, 1.00, and 1.50  $\mu\text{M}$  in enzyme buffer. The number of fluorescent units per standard concentration was recorded. Fluorescence of the 1.50  $\mu\text{M}$  standard was recorded on the chart recorder for determination of fluorescent units to mM/mV. To assay samples, 40  $\mu\text{l}$  concentrated UTF or ECM was added with 10  $\mu\text{l}$  (0.87 mM) MCA and 200  $\mu\text{l}$  enzyme buffer was added for a total volume of 250  $\mu\text{l}$ . The assay was performed in duplicate at room temperature with excitation at 370 nm and emission at 460 nm to measure the hydrolysis and release of the fluorescent product, amino methylcoumarin. Chart recorder speed was 6 cm/min. Each reaction was recorded for at least one minute. Values of kallikrein activity (mV/min) were then divided by the sample's protein content in 20 ml of uterine flushing to give specific kallikrein activity expressed as  $\mu\text{M min}^{-1}/\text{mg total protein/ horn}$ .

#### Specificity of Enzyme Assay



Kallikrein activity in UTF and ECM was validated through addition the synthetic kallikrein inhibitor, cyclohexylacetyl-phe-arg-ser-val-gln amide (Sigma, St. Louis, MO). The inhibitor was added to a UTF sample of known activity. Briefly, increasing concentrations of inhibitor (0, 1, 25, 50, 75, 100 to 200  $\mu$ M) were added to enzyme buffer containing 10  $\mu$ l of 0.87 mM MCA. After 40  $\mu$ l of UTF sample was added, the cuvette was immediately placed in the fluorescent spectrophotometer, and enzyme activity recorded as previously described.

#### Western Blot Analysis

Uterine flushings and ECM (Experiment I) were analyzed by Western blotting for the presence of immunoreactivity to antiserum against human plasma kallikrein (Calbiochem, La Jolla, CA). Polypeptides in UTF and ECM (50  $\mu$ g total protein) were separated by 12.5% 1D SDS-PAGE (Laemmli, 1970) and immediately transferred to PVDF membrane (Millipore Corporation, Bedford, MA) at 150 mA constant current for 35 min. After electroblotting, the membranes were washed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) and incubated for 1 h with the first blocking solution of 3% gelatin in TBS. After washing in Tween-TBS (TTBS; 20mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) for 10 minutes, membranes were incubated overnight with the 1st antibody (1:200 dilution) in 1% gelatin TTBS. The next day, membranes were washed twice in TTBS, twice in TBS, and then immunoreactive polypeptides were detected using the Bio-Rad Immuno-Blot kit (Bio-Rad, Hercules, CA) according to manufacturer's specifications.

## RNA Extraction

TRIzol reagent (Gibco/ Life Sciences, Gaithersburg, MD) was utilized to extract total RNA from endometrium. Briefly, 0.5 g of endometrium was homogenized in 5 ml TRIzol reagent using a Virtishear homogenizer (Virtis Co. Inc., Gardiner, NY). After the addition of 1 ml of chloroform, samples were centrifuged for 30 min at 3500 x g (4°C). The aqueous layer was harvested and placed into a new tube. RNA was precipitated by addition of 2.5 ml isopropanol at room temperature. for 10 min. After centrifugation (3500 x g; 4°C) for 10 min, supernatant was poured off. RNA pellets were washed with 5 ml 75% ethanol and centrifuged (3500 x g) for 5 min at 4°C. RNA was rehydrated with 10mM Tris 1mM EDTA (pH 7.4) and stored at -80°C until further analyzed. Total RNA was quantified spectrophotometrically by absorbance at 260 nm. RNA purity was determined from calculations of 260/280 ratios. The extraction procedure consistently yielded 260/280 ratios of 1.7-2.0 indicating very low DNA contamination of total RNA preparation.

Total RNA was also extracted from conceptuses from three different litters using TRIzol reagent. Two pools of Day 12 filamentous conceptuses and one pool of Day 12 spherical conceptuses (5 and 7 mm diameters) were analyzed. RNA extraction was performed as previously described with the following modifications. Filamentous conceptuses were homogenized in 1 ml TRIzol reagent using a sterile, bulbed-end glass Pasteur pipet while spherical conceptuses were homogenized by simply passing through a pipette tip. After

homogenizing, 200  $\mu$ l of chloroform was added and centrifuged for 20 minutes at 12,000 rpm. The aqueous layer was harvested and placed into a new tube. RNA was precipitated by addition of 500  $\mu$ l isopropanol. After centrifugation at 12,000 rpm for 5 min, isopropanol was removed and RNA pellets were washed with 1 ml of 75% ethanol. Following centrifugation at 12,000 rpm for 5 min, ethanol was removed and RNA was rehydrated in 50  $\mu$ l of 10 mM Tris 1 mM EDTA (pH7.4) and stored at -80°C until further analyzed. RNA purity was analyzed as previously described.

#### Complementary DNA Preparation

Total RNA was reverse transcribed to cDNA in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler Model 480. The reactions were carried out in a total volume of 20  $\mu$ l containing 200 U of Moloney murine leukemia virus reverse transcriptase-RNase H- (M-MLV-RT), 1.0  $\mu$ g of oligo(dT)<sub>15</sub> primer, 0.5 mM each of dATP, dCTP, dGTP, dTTP, 50mM Tris-HCl (pH8.3), 75 mM KCl, 3 mM MgCl, 10 mM dithiothreitol, 20 U of RNasin, and 1.0  $\mu$ g total RNA brought to volume with DEPC water. The sample preparation was incubated at 22°C for 15 min and then at 42°C for 30 min. The reaction was terminated by heating at 95°C for 5 min and cooling to 4°C at which temperature it was stored. The M-MLV-RT, reaction buffer, RNasin, and oligo(dT)<sub>15</sub> primer were obtained from Promega Corporation (Madison, WI).

## Kallikrein Primer, Optimization, and Sequencing

Kallikrein primers were first designed to regions of homology between the amino acid sequence of porcine pancreatic kallikrein (Bode et al., 1983) and human kidney kallikrein (Baker and Shine, 1985). The nucleotide sequence of human kidney kallikrein (bp169-603) (Baker and Shine, 1985) coinciding to homologous amino acid regions between porcine pancreas and human kidney was utilized to design the 5' TGACTACAGTCCACGACCTCATG and 3' GCAGGTTGGCAGGTGCTGCC primers. All PCR reactions were carried out in 25  $\mu$ l volumes covered with 25  $\mu$ l mineral oil. To optimize PCR conditions, pooled cDNA (from endometrium of all days cyclic and pregnant) was amplified with 0.6 U of Taq DNA polymerase and its supplied  $MgCl_2$ -free buffer (Promega, Madison, WI) and a 3x2x3 factorial (see Table 3.1) combination of primer (50, 150, or 250 nM), deoxynucleotide triphosphates (dNTPs 50 or 100  $\mu$ M) and  $MgCl_2$  (1.25, 2.50, or 3.75 mM). All samples were loaded directly from ice into the 95°C heat block to minimize time required for samples to reach denaturation temperature. The first cycle used denaturation at 95°C for 2 min, annealing at 55°C for 1 min, and a 2 min extension at 72°C. This was followed by 34 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and a 1 min extension at 72°C. Finally, a 9 min extension at 72°C and cooling to 4°C completed the PCR reaction. The resulting PCR product was resolved in a 3% gel at 85 volts for 1.5 h, followed by 30 min stain in ethidium bromide (1.0 mg/ml). The optimal conditions used for kallikrein gene amplification were 25 mM  $MgCl$ , 100  $\mu$ M dNTPs, 250 nM primer (Figure 3.1). To

verify PCR product as glandular kallikrein, pooled cDNA was amplified with the previously described optimal conditions, run on a 4% agarose gel, stained with ethidium bromide and bands were cut from the gel with a razor blade. The PCR product was then extracted using Qiaquick (Qiagen, Santa Clarita, CA), and sequenced by the Recombinant DNA/Protein Research Facility at Oklahoma State University. The 109 base pair PCR product was determined to be 89% homologous to baboon glandular kallikrein (Perelygina et al., 1995) (Figure 3.2). Endometrial and conceptus cDNA samples (3  $\mu$ g) obtained across the estrous cycle and early pregnancy were PCR as previously described. Resultant bands were scanned in duplicate with NIH Image, Version 1.6 (NIH) densitometer.

To quantitate PCR synthesis procedure for comparable amounts of starting cDNA in samples, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (Yelich et al., 1997) were utilized. G3PDH PCR products were run in 3% agarose gels, stained and bands scanned with densitometer. A ratio of kallikrein to G3PDH was utilized to standardize cDNA loading in each PCR reaction.

### Statistical Analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models of SAS (SAS, 1990). In Experiment I, the model used to analyze UTF enzymatic activity and protein concentration included the effects of day, reproductive status, the day x reproductive status interaction, gilt within day x reproductive status and the residual (horn within gilt x day x reproductive

status). The effects of day, reproductive status and the day x reproductive status interaction were tested using gilt within day x reproductive status as the error term. ECM enzymatic activity and PCR densitometry scans were each analyzed with a model that included the effects of day, reproductive status, and the day x reproductive status interaction.

In Experiment II, conceptuses were classified by size as follows: <5 mm (n=7), 5-10 mm (n=14), ovoid and tubular (n=7) and filamentous (n=15). Kallikrein activity, estradiol-17b content and protein concentration were each analyzed with a model that included the effects of conceptus classification. In addition to the least-squares analyses, a Pearson correlation between estradiol and total enzymatic activity was calculated using the Correlation procedure of SAS (SAS, 1990).

## Results

### Experiment I:

#### Kallikrein Enzymatic Activity

Specificity of the assay for kallikrein was determined through addition of the kallikrein inhibitor, cyclohexylacetyl-phe-arg-ser-val-gln amide. Addition of inhibitor caused a linear decrease in kallikrein activity (Figure 3.3). Fifty percent inhibition of enzyme activity was observed at 35  $\mu$ M, with kallikrein activity completely abolished with addition of 200  $\mu$ M inhibitor.

There was no day x reproductive status interaction ( $p=0.68$ ) in UTF protein concentration (Figure 3.4). There was a day effect as will increases in

total protein concentration on Day 15 ( $p<0.01$ ). Total protein in UTF was similar between Days 10 and 12 with a two fold increase in total protein on Day 15 (Figure 3.4).

There was a significant day x reproductive status interaction ( $p<0.001$ ) in enzymatic activity in UTF (Experiment I) (Figure 3.5). Kallikrein activity in UTF increased on Day 15 of the estrous cycle and pregnancy. However, a two fold greater activity was detected in UTF from cyclic compared to pregnant gilts. No day x reproductive status interaction occurred in the ECM enzymatic assay ( $p=0.2$ ). However, enzymatic activity in ECM (Figure 3.6) was affected by day ( $p<0.09$ ) and reproductive state ( $p<0.05$ ). Endometrial secretion of kallikrein from endometrial explants was approximately 2.5 fold greater on Day 12 of pregnancy compared to cyclic gilts. Enzyme activity in ECM declined on Day 15 of the estrous cycle and pregnancy. Although numerically greater, ECM enzymatic activity was not statistically different between cyclic and pregnant gilts on Day 15.

#### Western Blot Analysis:

Antiserum to human plasma kallikrein detected a 50 kD immunoreactive product in UTF and ECM across Day 10 to 15 of the estrous cycle and early pregnancy. Intensity of immunostaining was similar in Day 10, 12 and 15 UTF of either cyclic or pregnant gilts (Figure 3.7). In UTF, a 23 kD product was usually absent on Day 10 of the estrous cycle or pregnancy, but appeared regardless of reproductive status on Day 12 and 15 (Figure 3.7). The 23 kD reactive product



appears to correspond to kallikrein activity as enzymatic activity in UTF was associated with appearance of the immunostained band. Only the 50 kD immunoreactive product was detected with kallikrein antiserum in ECM (Figure 3.8). Intensity of the 50 kD band was similar between cyclic and pregnant gilts.

### Kallikrein Gene Expression

Endometrial gene expression of glandular kallikrein was detected on Day 0, 5, 10, 12, 15 and 18 of the estrous cycle and early pregnancy (Figure 3.9a). Since there was only one Day 0 sample, it was deleted from the statistical analysis. There was no reproductive status x day interaction ( $p=0.83$ ) or reproductive status effect in endometrial kallikrein gene expression. Gene expression of endometrial kallikrein was different ( $p<0.05$ ) across the days of the estrous cycle and pregnancy (Figure 3.9b). Densitometry analysis demonstrates kallikrein gene expression an increase of kallikrein gene expression on Days 10 and 15. Gene expression for kallikrein was also detected in Day 12 porcine conceptuses, muscle and kidney (Figure 3.10).

### Experiment II

Content of estradiol-17 $\beta$  in UTF (Figure 3.11) increased ( $p<0.01$ ) with conceptus development from <5 mm spherical morphology to tubular and filamentous conceptuses. Uterine content of estrogen increased slightly when the uterus contained 6-10 mm spherical conceptuses with a three fold increase when conceptuses reached a tubular morphology. Estradiol-17 $\beta$  content in UTF



was similar between UTF which contain either tubular or filamentous conceptuses were present in the uterine lumen. Kallikrein enzymatic activity (Figure 3.11) was correlated ( $r=0.3$ ) with estradiol-17 $\beta$  content ( $p<0.05$ ). As content of estradiol-17 $\beta$  in UTF increased, kallikrein enzymatic activity also increased. Although not statistically different ( $p=0.7$ ), kallikrein activity in UTF increased two fold between <5 mm spherical to ovoid and tubular conceptuses. However, there was almost a four fold increase ( $p<0.05$ ) of kallikrein activity when the conceptuses rapidly expanded to a filamentous morphology. Increase in kallikrein enzymatic activity was temporally associated with an increase in UTF total protein (Figure 3.12). Total protein in UTF increase two fold ( $p<0.01$ ) when conceptuses rapidly expanded from tubular to the filamentous stage of development.

## Discussion

Porcine conceptuses undergo a rapid morphological change from a 10mm sphere to a thin, filamentous thread within 2-4 hours (Anderson, 1978; Geisert et al., 1982b) on Day 12 of gestation. During this expeditious transformation, conceptuses produce and release estrogens to alter the uterine environment (Geisert et al., 1990). Alteration of endometrial secretion of proteins (Roberts et al., 1993), prostaglandins (Bazer and Thatcher, 1977), uterine blood-flow (Ford et al., 1982), uterine cellular morphology and vascular permeability (Dantzer, 1985; Keys and King, 1990) occur during conceptus elongation and estrogen release. Despite information available on conceptus influence on the uterine

environment, uterine factors induced by conceptus estrogen to stimulate changes necessary for maternal recognition of pregnancy and placental attachment have not been completely elucidated.

We have previously determined that  $\alpha$ LH4 is present in the endometrium of the pig (Geisert et al., 1998). Because of the function of  $\alpha$ LH4 in acute phase reactions, Geisert et al. (1998) proposed that  $\alpha$ LH4 may be involved with attachment of the conceptus to the uterine epithelium through  $\alpha$ LH4 association with hyaluronate. Cleavage of  $\alpha$ LH4 by kallikrein (Hashimoto et al., 1996) could be involved with the timing of conceptus attachment to the uterine epithelium. It is possible that release of kallikrein from the endometrium and/or conceptus could regulate a number of biological events essential to conceptus development.

The present study detected the presence of endometrial kallikrein enzymatic activity, protein and gene expression during the estrous cycle and early pregnancy of the pig. Kallikrein activity in UTF increased from low activity on Day 10 to a three fold increase in activity on Day 15. Enzymatic activity was greater in cyclic compared to pregnant gilts on Day 15. Increased levels of kallikrein activity on Day 15 of the estrous cycle maybe caused by declining progesterone concentrations with regression of the corpora lutea and increase in follicular estrogen. Progesterone has a negative effect on uterine kallikrein activity in the rat (Valdes et al., 1996). Therefore, as plasma progesterone concentrations decrease, kallikrein activity may increase in the pig. It is possible that a kallikrein inhibitor may also be released from the endometrium to regulate

kallikrein activity during pregnancy. Kallistatin, the kallikrein binding protein, has been shown to significantly hinder kallikrein's action (Chao et al., 1996). In ECM, there was a significant increase of kallikrein enzymatic activity on Day 12 of pregnancy compared to the estrous cycle. Differences in endometrial activity suggest conceptus regulation of local kallikrein release. Kallikrein activity was temporally related to the increase estradiol secretion from the filamentous conceptuses. The four fold increase in kallikrein activity was associated with conceptus elongation through the uterine horns as there was little increase in UTF with tubular conceptuses that contained high concentrations of estrogen.

Uterine kallikrein gene expression suggests that the endometrium is the source of kallikrein in the uterine lumen on Day 12 of the estrous cycle and early pregnancy. Increase of kallikrein message could cause an increase of kallikrein synthesis and storage within the uterine epithelium. Valdes et al. (1996) detected glandular kallikrein in secretory vesicles in the rat endometrium. Kallikrein was released from storage vesicles after the stimulation of ovarian estrogen on Day 5 (Valdes et al., 1996). Production of estrogen by porcine conceptuses would suggest a similar mechanism to release kallikrein into the uterine lumen. The second increase in kallikrein gene expression occurs on Day 15. This time period corresponds to the second sustained increase in conceptus estrogen secretion necessary for maintenance of corpora lutea function beyond Day 30 in the pig (see Geisert et al., 1990). The release of kallikrein suggests that estrogen stimulates endometrial kallikrein gene expression as the conceptus expands through the uterine horns. Estrogen stimulated release of

kallikrein in the rat has also been proposed to be involved with blastocyst implantation (Valdes et al., 1996; Corthorn et al., 1997).

Presence of kallikrein in the uterus between Day 12 to 18 may function to cleave  $\alpha$ 1H4.  $\alpha$ 1H4 does not bind to the serine protease inhibitor, bikunin, as occurs with the other  $\alpha$ 1 heavy chains (see Salier et al., 1990). Rather,  $\alpha$ 1H4 alone contains a kallikrein cleavage site (Nishimura et al., 1995).  $\alpha$ 1H4 contains a von Willebrand Type-A domain which is known to bind to integrins, proteoglycans and heparin (see Salier et al., 1990). It is possible that cleavage of  $\alpha$ 1H4 by kallikrein may allow trophoblastic attachment to the uterine epithelial surface. Detection of kallikrein gene expression in the conceptus suggests conceptus secretion of kallikrein could further regulate local effects on the endometrium.

Presence of kallikrein activity in the uterus indicates a functional kallikrein-kininogen-kinin system exists in the porcine uterus as been documented in the uterus of rodents (Brann et al., 1995; Valdes et al., 1996). Tissue kallikrein cleaves low molecular weight kininogen and releases kinins, namely bradykinin (see Bhoola et al., 1992). Kinins have many bioactive properties, including increased blood flow, decreased membrane permeability, contraction of smooth muscle, release of calcium and release of prostaglandins (Cooper et al., 1985; Bhoola et al., 1992) all of which occur during the time of porcine conceptus elongation and implantation (see Geisert et al., 1990). Bradykinin has also been known to stimulate interleukin (IL) -6 and IL-8 secretion from human decidua derived cells (Rehbock et al., 1997). Mathialagan

et al. (1992) indicated that IL-6 is secreted from porcine conceptuses between Day 13-17. Furthermore, IL-6 upregulates  $\alpha$ I heavy chains -1, -2, and -3 which also contain a von Willibrand type A domain (see Salier et al., 1996). Therefore, it is possible that kallikrein functions to cleave low molecular weight kininogen releasing bradykinin on Day 12, which stimulates IL-6 release from porcine conceptuses to upregulate  $\alpha$ I members for conceptus attachment to the uterine epithelial surface.

Increase in kallikrein activity could also play a role in regulating insulin-like growth factor (IGF) activity within the uterine lumen. Simmen et al. (1989) indicated a substantial increase in IGF-I occurs within the uterine lumen on Day 12 of pregnancy in the pig. Activity of IGFs is regulated by IGF binding proteins (IGFBP) (see Simmen et al., 1995). Kallikrein has been demonstrated to cleave IGFBPs (Cohen et al., 1992; Rajah et al., 1996). Prostate specific antigen (PSA) is form of glandular kallikrein in the human (see Bhoola et al., 1992) that cleaves IGFBP-3 (Cohen et al., 1992). Insulin-like growth factor BP3, BP4, BP5 and BP6 is cleaved by 7S nerve growth factor, another form of glandular kallikrein (Rajah et al., 1996).

Lee and coworkers (1998) demonstrated the disappearance of high affinity IGFBPs in UTF of pigs on Days 11 and 12 of pregnancy. As porcine conceptuses reach the filamentous stage, IGFBP-3 is completely abolished. When aprotinin, a serine protease inhibitor, is added to reactions, IGFBP protease activity is inhibited. Furthermore, when a specific inhibitor for plasminogen activator (a serine protease) is added, IGFBP protease activity is

not hindered. IGFBP have also been shown to be cleaved by metalloproteinases (MMPs; Fowlkes et al., 1994), but UTF IGFBP proteolysis was not stopped when MMP inhibitors were added (Lee et al., 1998). By eliminating plasminogen activator as a candidate for the serine protease involved, as well as MMPs, kallikrein activity may very well be the protease controlling IGFBP proteolysis.

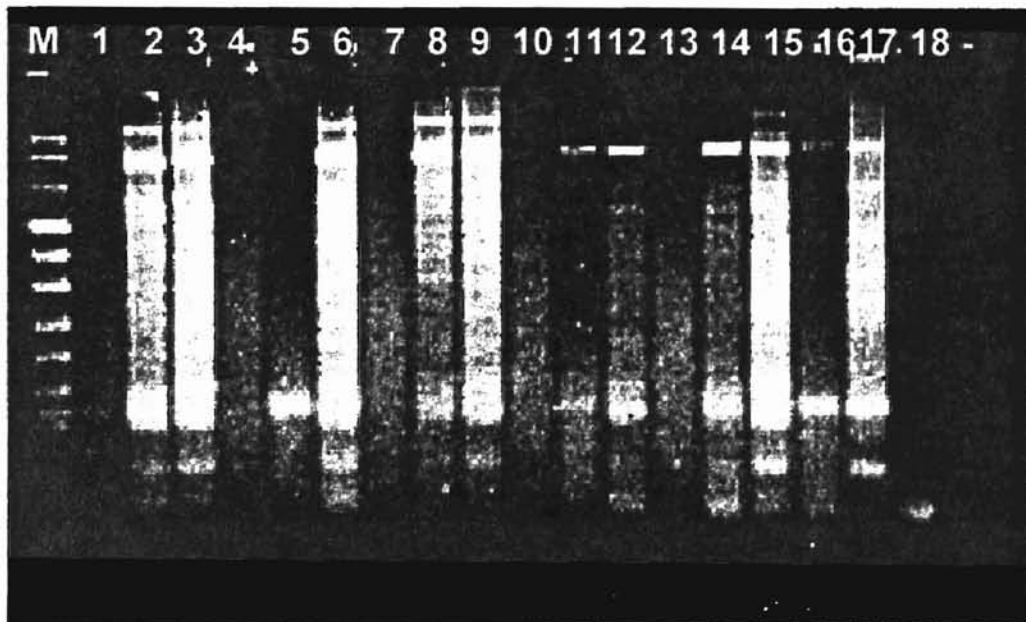
Evidence that kallikrein in the porcine lumen increases in activity at crucial stages in conceptus survival, suggests kallikrein may be a key regulator in biological processes coincident with conceptus elongation and trophoblast attachment. Estrogen appears to dictate kallikrein gene expression and kallikrein release in the porcine uterus as has been reported in the rat (Valdes et al., 1996; Corthorn et al., 1997). Further investigations are needed to determine the processes that kallikrein is involved with during the time of maternal recognition of pregnancy in the porcine uterus.

Table 3.1. Conditions for PCR optimization with changes in primer, dNTPs, and MgCl.

Lane	Primer (5 $\mu$ M stock)	dNTPs (10 mM stock)	10 x MgCl
1	0.5 $\mu$ l	0.5 $\mu$ l	1.25 $\mu$ l
2	0.5	0.5	2.50
3	0.5	0.5	3.75
4	0.5	1.0	1.25
5	0.5	1.0	2.50
6	0.5	1.0	3.75
7	1.5	0.5	1.25
8	1.5	0.5	2.50
9	1.5	0.5	3.75
10	1.5	1.0	1.25
11	1.5	1.0	2.50
12	1.5	1.0	3.75
13	2.5	0.5	1.25
14	2.5	0.5	2.50
15	2.5	0.5	3.75
16	2.5	1.0	1.25
17	2.5	1.0	2.50
18	2.5	1.0	3.75

Figure 3.1. Photograph of an ethidium bromide stained 3% gel with PCR products from kallikrein primer optimization. Conditions utilized for amplification of kallikrein in Lane 5 (see Table 3.1) were selected for analysis of gene expression in porcine endometrium. Arrow indicates proper band size (~150 bp). (-) indicates negative control (no cDNA added). M represents the marker lane.





←  
147

Figure 3.2. Nucleotide sequencing of the 150 bp PCR product is 89% homologous to baboon glandular kallikrein. p indicates the porcine sequence; b indicates the baboon sequence with black color as homologous base pairs.

p3            actacag**tcc** acgacctcat gctgctccgc  
b353        actacag-cc acgacctcat gctgctccgc

p33           ctg**acg**cagc ccgccgcc**aa** gatcacagac  
b383        ctg**cag**--agc ccgccgcc**ga** gatcacagac

p63           gctgtg**aagg** **tcct**ggag**ct** gcccacccag  
b403        gctgtg**cagg** **ttgt**ggag**tt** gcccacccag

p93           gaacccgaac**c** tggg**c**agcacc tg  
b433        gaacccgaag**g** tggg**g**agcacc tg

Figure 3.3. Kallikrein activity in a Day 15 cyclic gilt UTF following addition of various concentrations of the kallikrein inhibitor, cyclohexylacetyl-phe-arg-ser-val-gln amide.

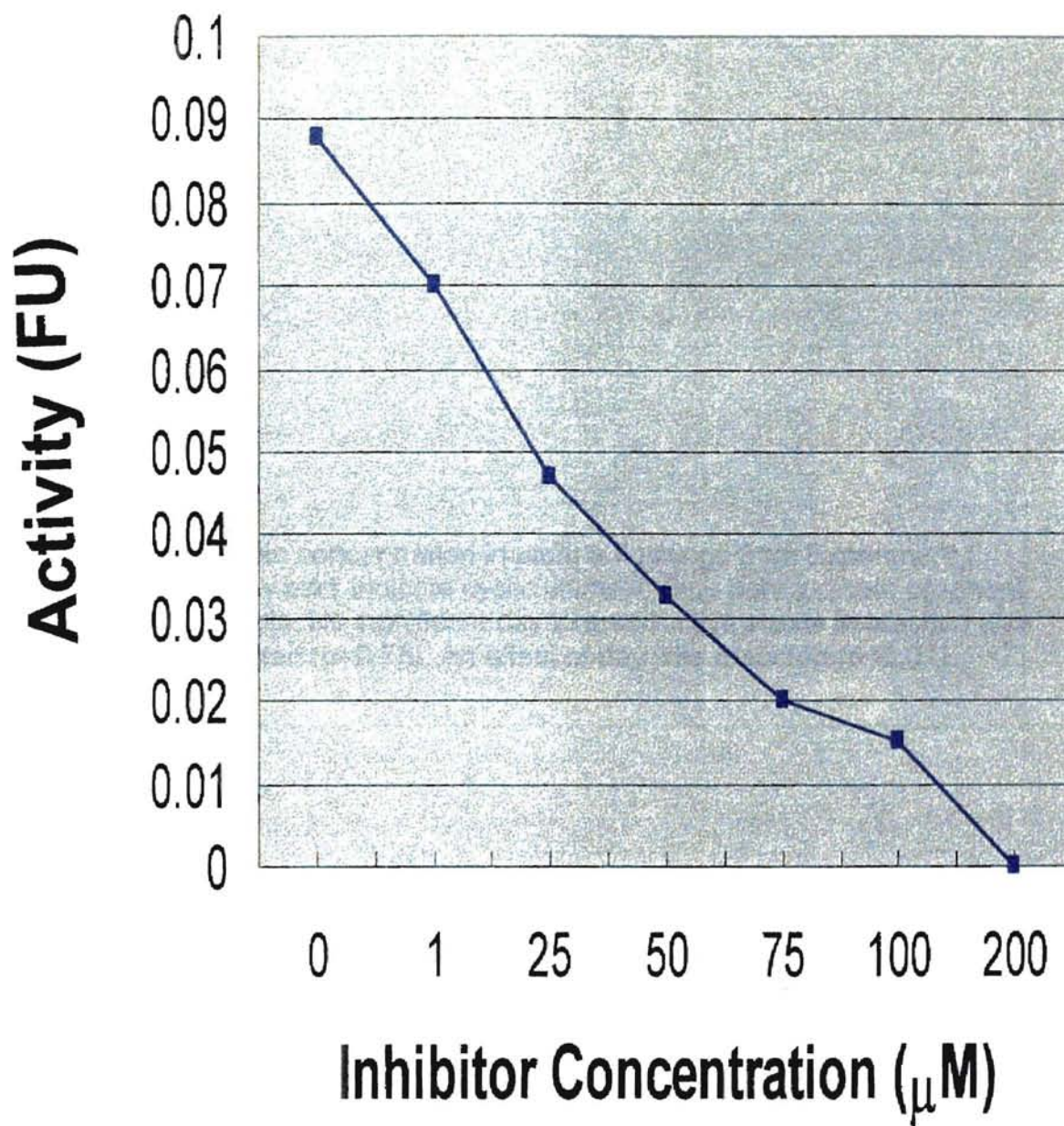


Figure 3.4. Protein concentration in uterine flushings from Experiment I. Yellow bars indicate cyclic animals; Blue bars indicate pregnant animals. No significant day x reproductive status interaction was detected ( $p=0.78$ ). An effect of day was detected ( $p<0.01$ ).

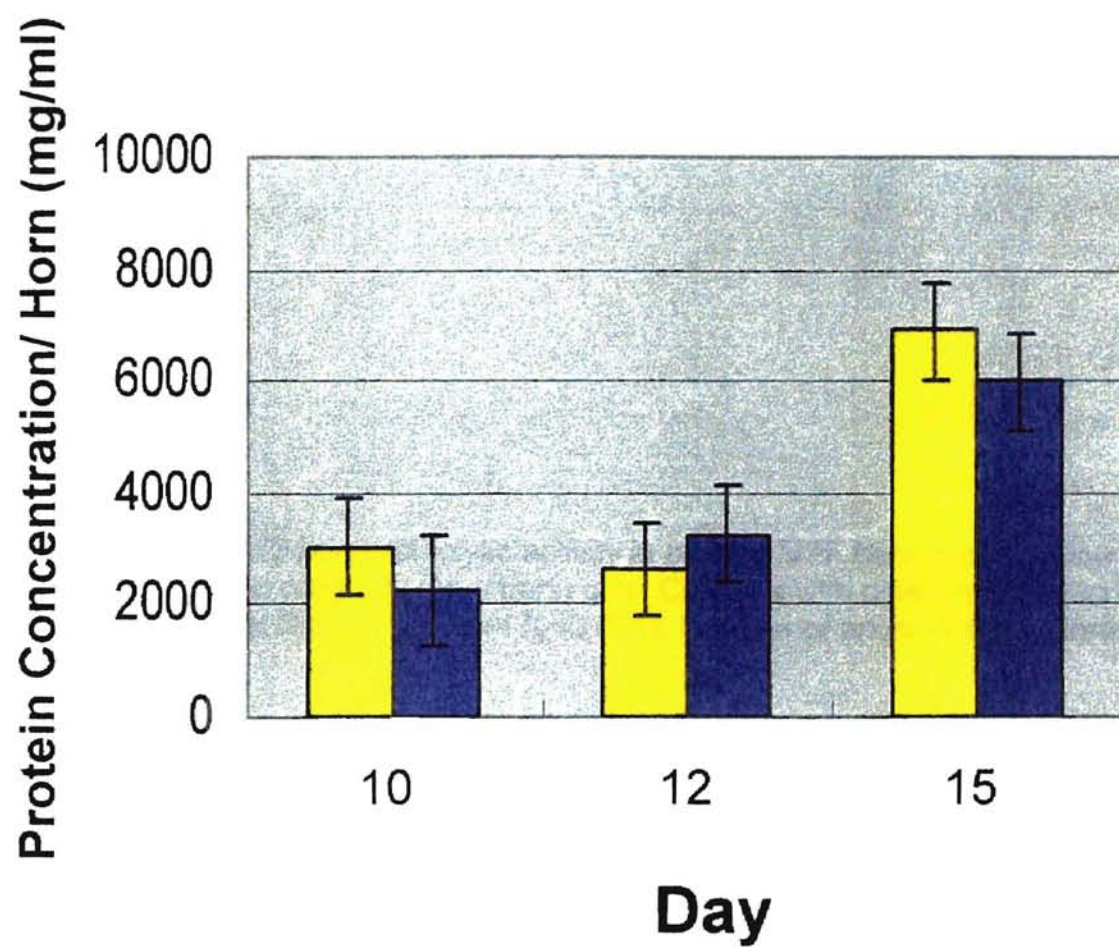


Figure 3.5. Kallikrein proteolytic activity in porcine UTF from cyclic (yellow bars) and pregnant (blue bars) gilts. Columns with different superscripts are significantly different ( $p < 0.001$ ). Number of animals are in parenthesis.



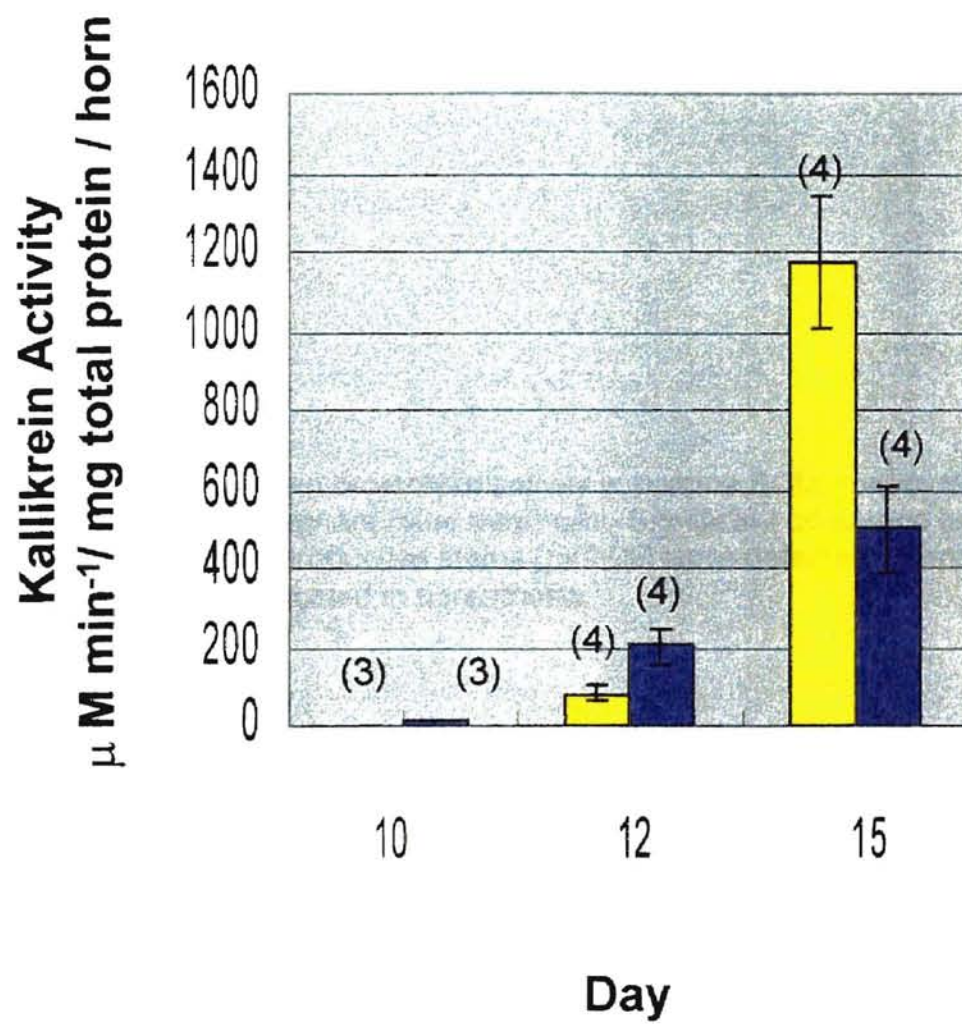


Figure 3.6. Kallikrein proteolytic activity in porcine ECM from cyclic (yellow bars) and pregnant (blue bars) gilts. Significant difference by Day ( $p < 0.09$ ) and reproductive status ( $p < 0.05$ ) were detected. Number of animals are indicated in parenthesis.

**Kallikrein Activity**  
 $\mu\text{M min}^{-1} / \text{mg total protein / horn}$

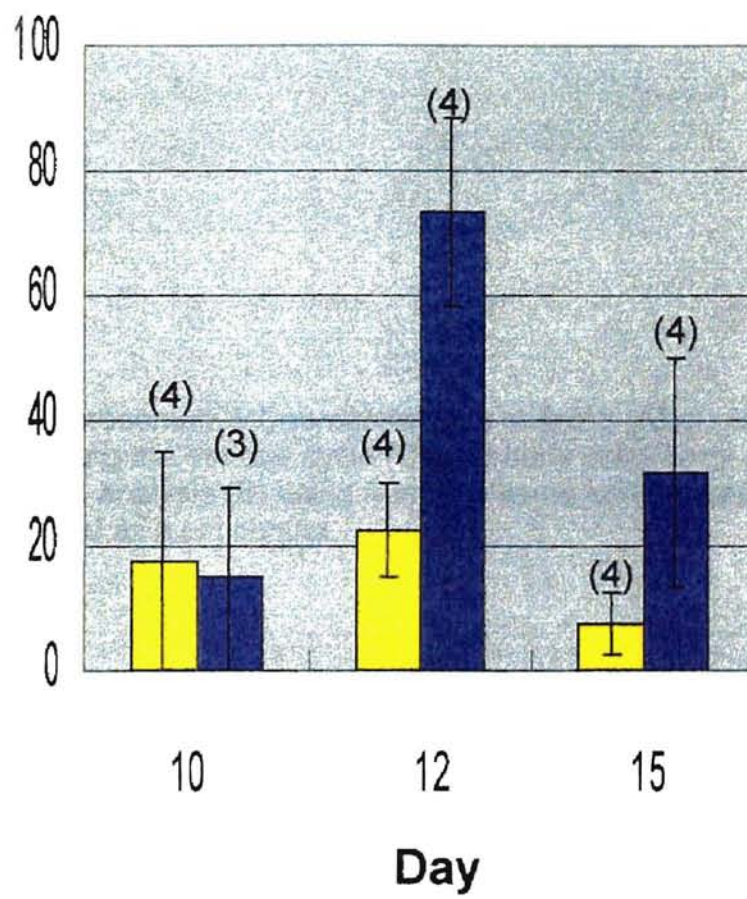


Figure 3.7. Representative Coomassie Blue staining (A) of uterine flushings during the estrous cycle (C) and early pregnancy (P) and Western Blot analysis (B) using human plasma anti-kallikrein. Human plasma used as a (+) control.

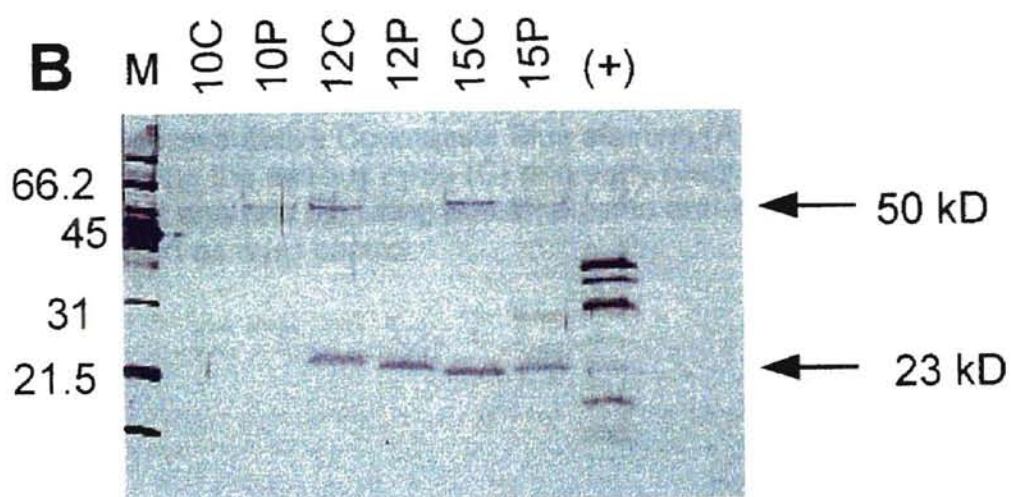
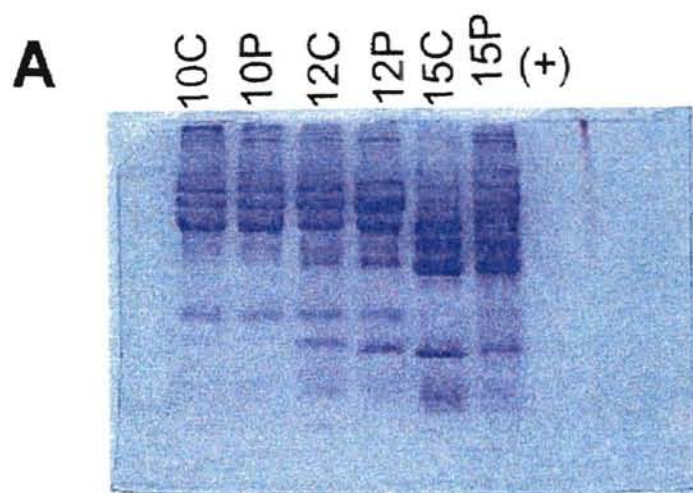
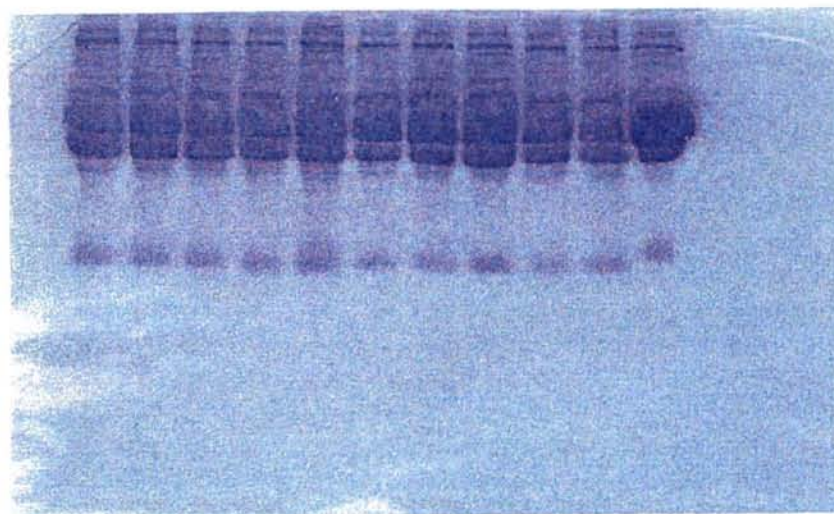


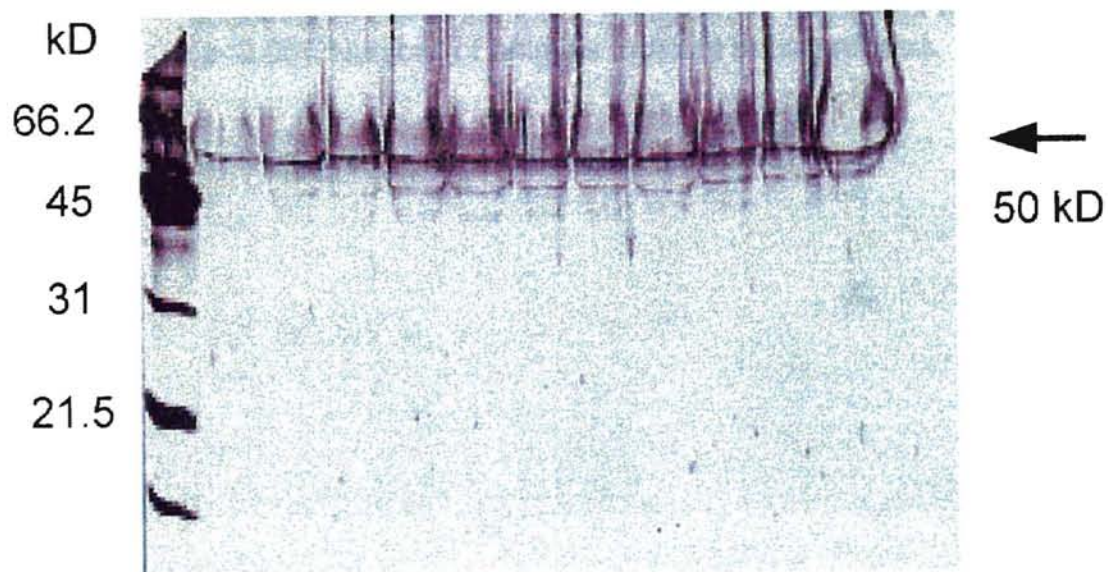
Figure 3.8. Representative Coomassie Blue staining (A) of explant culture media during the estrous cycle (C) and early pregnancy (P) and Western Blot analysis (B) using human plasma anti-kallikrein. Human plasma used as a (+) control.



**A**      10C   10C   10P   12C   12C   12P   12P   15C   15P   15P   (+)



**B**      M   10C   10C   10P   12C   12C   12P   12P   15C   15P   15P   (+)



- Figure 3.9a. Representative PCR using kallikrein primers to express endometrial cDNA across the estrous cycle (C) and early pregnancy (P). (-) indicates negative control (no cDNA added).
- b. Densitometry analysis of PCR using kallikrein primers on porcine endometrial cDNA samples. A significant day effect was present ( $p < 0.05$ ). Number of animals indicate in parenthesis.



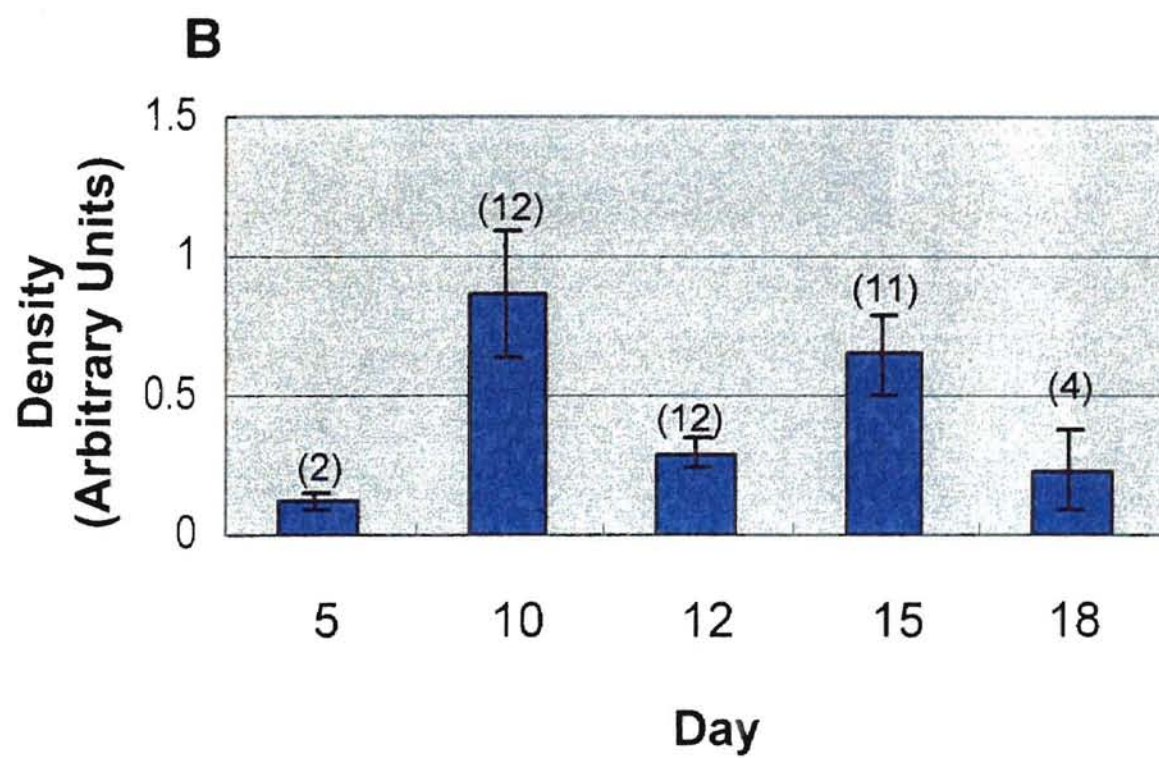
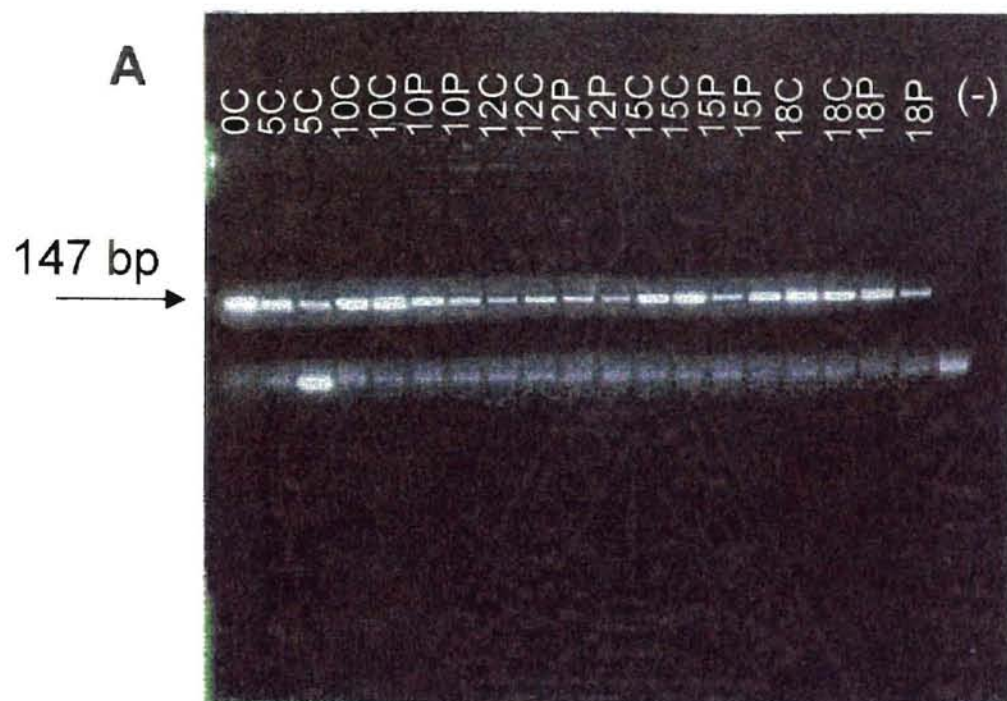


Figure 3.10. PCR using kallikrein primers on porcine endometrium (E), conceptus (C), muscle (M) and kidney (K) cDNA. (-) indicates negative control (no cDNA added).

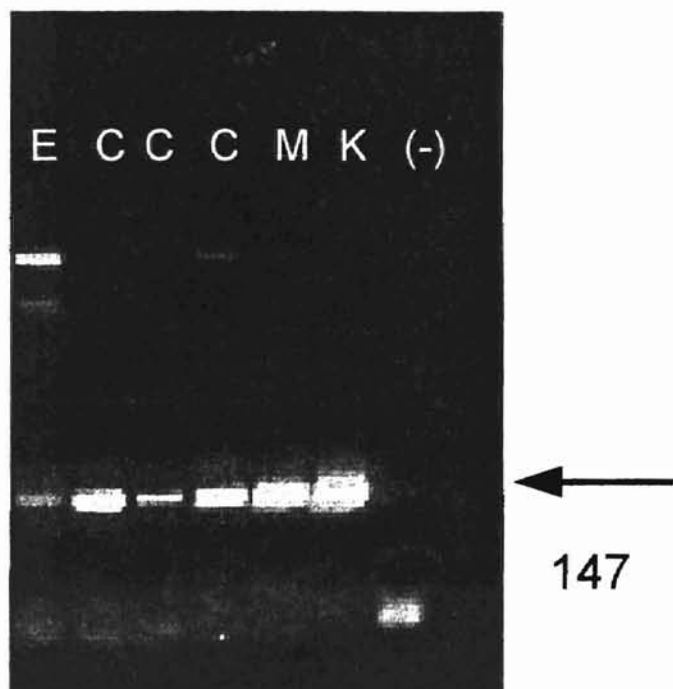
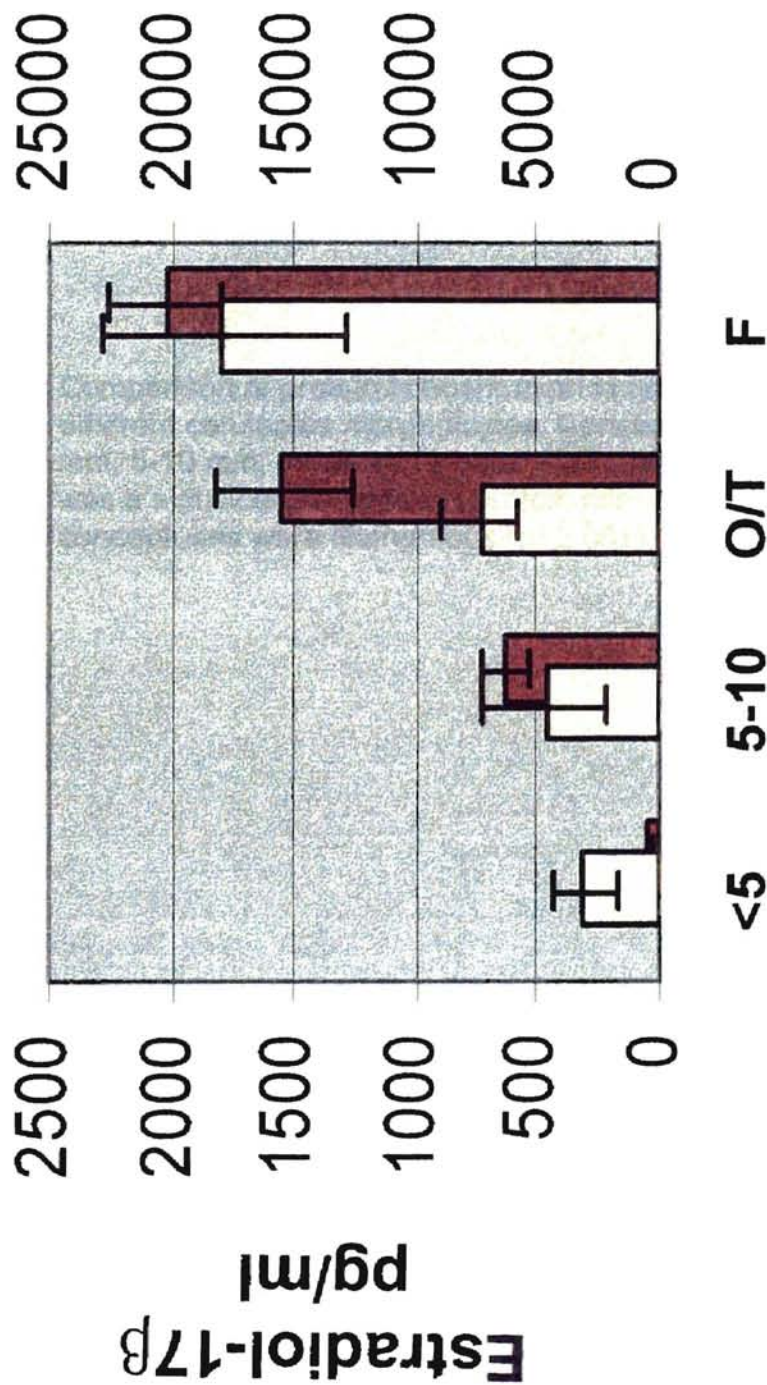


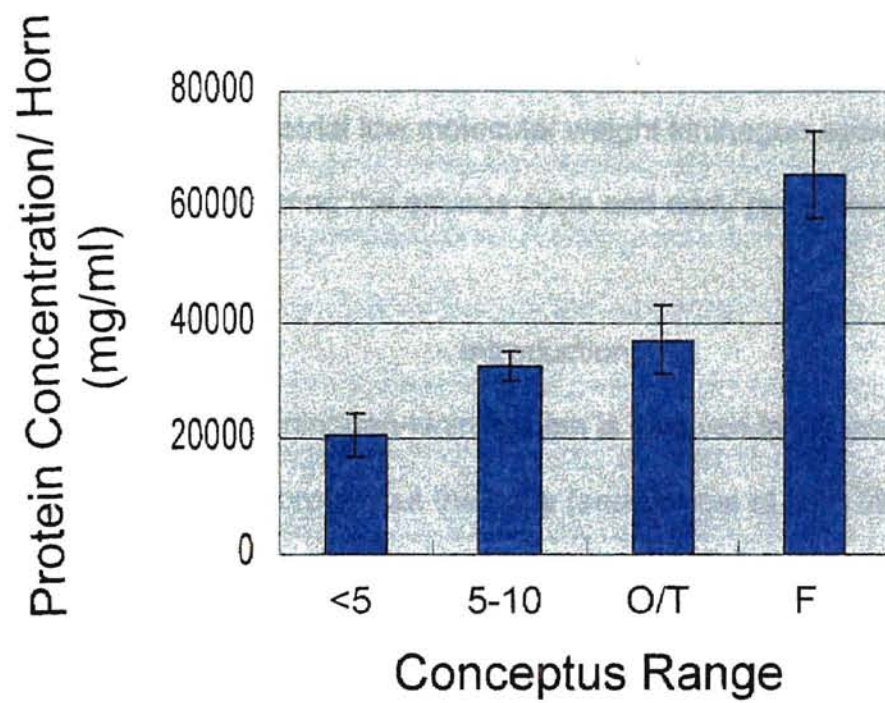
Figure 3.11. Comparison of kallikrein activity and estradiol-17 $\beta$  content in UTF containing different conceptus morphologies. Conceptuses ranges include: <5 mm, 5-10 mm, ovoid and tubular (O/T), and filamentous (F). There was a significant increase in estradiol-17 $\beta$  (red bars) ( $p<0.01$ ) and kallikrein activity (yellow bars) ( $p<0.05$ ).

# **Kallikrein Activity** $\mu\text{M min}^{-1}/\text{mg total protein/ horn}$



**Conceptus Range**

Figure 3.12. Comparison of protein concentration in uterine flushings containing different conceptus morphologies. Conceptuses ranges include: <5 mm, 5-10 mm, ovoid and tubular (O/T), and filamentous (F). There was a significant increase in protein concentration when conceptuses were filamentous ( $p<0.001$ ).





## CHAPTER IV

### Detection of endometrial low molecular weight kininogen secretion and gene expression during the estrous cycle and early pregnancy in gilts.

#### Introduction

The kallikrein-kininogen-kinin system is involved in inflammatory processes that occur throughout the body (see Bhoola et al., 1992; Clements et al., 1997). An inflammatory response in tissue is characterized by increased vasodilation, vascular permeability and edema (Clements et al., 1997). Along with mast cell activation and neutrophil invasion, prostaglandins and various growth factors play a role in kallikrein-kininogen-kinin mediated events (Bhoola et al., 1992).

Many reproductive processes including endometrial proliferation, ovulation and decidualization have been suggested to mirror inflammatory reactions (Clements et al., 1997). Various growth factors, prostaglandins and cytokines are known to play a role in these reproductive actions (Bhoola et al., 1992; Clements et al., 1997). It is logical that biologic processes involved in endometrial proliferation, ovulation (Gao et al, 1992) and decidualization (Valdes et al., 1996) may be similar to those involved in inflammatory responses.



During the period of maternal recognition of pregnancy until the time of conceptus attachment, many changes occur within the porcine uterine lumen to permit conceptus attachment and growth. Porcine conceptuses morphologically change from 10 mm spheres to long, filamentous threads in 2-4 hours on Day 12 of pregnancy (Geisert et al., 1982b). During this period of conceptus growth and elongation, conceptuses synthesize and release estrogens (Gadsby et al., 1980; Fischer et al., 1985; Mondschein et al., 1985) to initiate maintenance of CL function and uterine secretion necessary for conceptus attachment and growth. Endometrial prostaglandin release is changed from an endocrine to an exocrine direction (Bazer and Thatcher, 1977), as well as there is an increase in uterine blood-flow (Ford et al., 1982), uterine smooth muscle contraction, and vascular permeability (Keys and King, 1990). Kallikrein enzymatic activity, protein and gene expression have been detected within the porcine uterus (Chapter 3). Presence of kallikrein may regulate cleavage of inter- $\alpha$ -trypsin inhibitor heavy chain 4 ( $\alpha_1\text{IH4}$ ), which has been proposed to assist with conceptus attachment to the uterine epithelium (Geisert et al., 1998). Tissue kallikrein may also function to utilize its natural substrate, low molecular weight kininogen, to release the potent vasoactive peptide, bradykinin (Bhoola et al., 1992). Kinin production has been associated with increased prostaglandin production, increased blood flow and increased smooth muscle contraction (see review Bhoola et al., 1992), all of which occur during establishment of pregnancy in the pig. The objective of the current study is to investigate gene expression of low molecular weight kininogen in porcine endometrium as well as presence of low molecular weight kininogen in

the uterine flushings and explant culture media throughout the estrous cycle and early pregnancy in the pig.

## Materials and Methods

### Animals

Cyclic, crossbred gilts of similar age (8-10 mo) and weight (100-130 kg) were checked twice daily for the onset of estrus behavior by intact boars. Onset of estrus was considered Day 0 of the estrous cycle. Gilts assigned to be mated were bred naturally with fertile boars at the onset of estrus detection and 12 hours later. Cyclic (n=12) and pregnant gilts (n=11) were hysterectomized on either Day 10, 12, and 15 as previously described by Gries et al. (1989). Following initial induction of anesthesia with a 1.5 cc i.m. administration of a cocktail consisting of 2.5 cc Rompum (xylazine; 100 mg/ml) (Miles, Inc., Shawnee Mission, KS), 2.5 cc Vetamine (ketamine HCl; 100 mg/ml) (Mallickrodt Veterinary, Mundelein, IL) in 500 mg of Telazol (tiletamine HCl and zolazepam HCl) (Fort Dodge, Syracuse, NE), anesthesia was maintained with a closed-circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ) and oxygen (1.0 liter/min). After exposure by midventral laparotomy, the uterine horns and ovaries were surgically removed. The incision site was closed using routine surgical procedures, and gilts were treated i.m. with procaine penicillin G (20,000 IU/kg BW).

## Collection of Uterine Flushings and Endometrium

Uterine flushings (UTF) and endometrium were obtained by isolating one horn and flushing with 20 ml of phosphate buffered saline (PBS; pH 7.4). Flushings were examined to confirm pregnancy in mated gilts. Conceptus diameters were measured, recorded and conceptuses snap frozen in liquid nitrogen. Uterine flushings were placed on ice, until centrifugation (2500 x g, 10 min; 4°C), and stored at -80°C at the laboratory. After flushing, the horn was cut along its anti-mesometrial border and endometrium exposed for removal with sterile scissors. Endometrium was collected, snap frozen in liquid nitrogen and stored at -80°C until RNA extraction was performed. The remaining uterine horn was immediately placed in a sterile container and transported on ice to a sterile horizontal flow hood. The uterine horn was opened along its anti-mesometrial border and endometrium was removed from the myometrium using sterile scissors. Endometrial strips were diced into 4x4 mm sections and a total of 0.5 g explant tissue was placed in a 100x15 mm standard sterile petri dish (Fisher Scientific, Pittsburgh, PA) containing 15 ml Dulbecco's modified Eagle medium (MEM) (Gibco/ Life Sciences, Gaithersburg, MD) and 2% (v/v) antibiotic-antimycotic (Gibco/ Life Sciences). To remove serum leaching from tissue, endometrial explant cultures were initially incubated for 3 hours when media was removed and replaced with fresh media. Endometrial explant cultures were incubated in air on a rocking platform (4 cycles/min) for an additional 24 h in MEM at 37°C. Endometrial explant culture media (ECM) was centrifuged (2500 x g; 10 min) and supernatant stored at -80°C until analyzed.

Additional animals were hysterectomized as previously described from cyclic gilts (n=11) on Day 0, 5, 10, 12, 15 and 18 and Day 10, 12, 15, and 18 of pregnancy (n=8). Endometrium was extracted from all animals for RNA analysis, whereas UTF from Day 0 and 5 cyclic were used in Western Blot Analysis.

#### Microconcentration and Protein Determination

Uterine flushings and ECM samples were prepared for enzyme assay by concentrating 4 ml of sample using Centricon 10 microconcentrators (Amicon, Beverly, MA) with a molecular weight cut-off of 10,000 daltons. Protein concentrations were determined by the method of Lowry et al. (1951). Concentrated samples were stored at -80°C until analyzed.

#### Western Blot Analysis

Uterine flushings and ECM were analyzed by Western blotting for the presence of immunoreactivity to antiserum against human low molecular weight (LMW) kininogen (Biogenesis, England, UK). Polypeptides in UTF and ECM (50 µg total protein) were separated by 12.5% 1D SDS-PAGE (Laemmli, 1970) and immediately transferred to PVDF membrane (Millipore Corporation, Bedford, MA) at 150 mA constant current for 35 min. After electroblotting, the membranes were washed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) and incubated for 1 h with the first blocking solution of 3% gelatin in TBS. After washing in Tween-TBS (TTBS; 20mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) for 10 minutes, membranes were incubated overnight with the 1st antibody (1:200 dilution) in 1%

gelatin TTBS. The next day, membranes were washed twice in, twice in TBS, and then immunoreactive polypeptides were detected using the Bio-Rad Immuno-Blot kit (Bio-Rad, Hercules, CA) according to manufacturer's specifications.

### RNA Extraction

TRIzol reagent (Gibco/ Life Sciences, Gaithersburg, MD) was utilized to extract total RNA from endometrium. Briefly, 0.5 g of endometrium was homogenized in 5 ml TRIzol reagent using a Virtishear homogenizer (Virtis Co. Inc., Gardiner, NY). After the addition of 1 ml of chloroform, samples were centrifuged for 30 min at 3500 x g (4°C). The aqueous layer was harvested and placed into a new tube. RNA was precipitated by addition of 2.5 ml isopropanol at rt. for 10 min. After centrifugation (3500 x g; 4°C) for 10 min, supernatant was poured off. RNA pellets were washed with 5 ml 75% ethanol and centrifuged (3500 x g) for 5 min at 4°C. RNA was rehydrated with 10mM Tris 1mM EDTA (pH 7.4) and stored at -80°C until further analyzed. Total RNA was quantified spectrophotometrically by absorbance at 260 nm. RNA purity was determined from calculations of 260/280 ratios. The extraction procedure consistently yielded 260/280 ratios of 1.7-2.0 indicating very low DNA contamination of total RNA preparation.

Total RNA was also extracted from conceptuses from three different litters using TRIzol reagent. Two pools of Day 12 filamentous conceptuses and one pool of Day 12 spherical conceptuses (5 and 7 mm diameters) were analyzed.

RNA extraction was performed as previously described with the following modifications. Filamentous conceptuses were homogenized in 1 ml TRIzol reagent using a sterile, bulbed-end glass Pasteur pipet while spherical conceptuses were homogenized by simply passing through a pipette tip. After homogenizing, 200  $\mu$ l of chloroform was added and centrifuged for 20 minutes at 12,000 rpm. The aqueous layer was harvested and placed into a new tube. RNA was precipitated by addition of 500  $\mu$ l isopropanol. After centrifugation at 12,000 rpm for 5 min, isopropanol was removed and RNA pellets were washed with 1 ml of 75% ethanol. Following centrifugation at 12,000 rpm for 5 min, ethanol was removed and RNA was rehydrated in 50  $\mu$ l of 10 mM Tris 1mM EDTA (pH7.4) and stored at -80°C until further analyzed. RNA purity was analyzed as previously described.

#### Complementary DNA Preparation

Total RNA was reverse transcribed to cDNA in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler Model 480. The reactions were carried out in a total volume of 20  $\mu$ l containing 200 U of Moloney murine leukemia virus reverse transcriptase-RNase H- (M-MLV-RT), 1.0  $\mu$ g of oligo(dT)<sub>15</sub> primer, 0.5 mM each of dATP, dCTP, dGTP, dTTP, 50mM Tris-HCl (pH8.3), 75 mM KCl, 3 mM MgCl, 10 mM dithiothreitol, 20 U of RNasin, and 1.0  $\mu$ g total RNA brought to volume with DEPC water. The sample preparation was incubated at 22°C for 15 min and then at 42°C for 30 min. The reaction was terminated by heating at 95°C for 5 min and cooling to 4°C at which temperature it was stored. The M-MLV-RT,

reaction buffer, RNasin, and oligo(dT)<sub>15</sub> primer were obtained from Promega Corporation (Madison, WI).

#### LMW Kininogen Primer, Optimization, and Sequencing

LMW kininogen primers were designed to bovine (Nawa et al., 1983; bp 401-954) and mouse (Takano et al., 1996; bp 295-851) low molecular weight kininogen (5': TGGCAGGACTGTGACT; 3'GCTGGCTCTGCCCCTGCCT). All PCR reactions were carried out in 25  $\mu$ l volumes covered with 25  $\mu$ l mineral oil. To optimize PCR conditions, pooled cDNA (from endometrium of all days cyclic and pregnant) was amplified with 0.6 U of Taq DNA polymerase and its supplied MgCl<sub>2</sub>-free buffer (Promega, Madison, WI) and a 3x2x3 factorial combination (see Table 3.1) of primer (50, 150, or 250 nM), deoxynucleotide triphosphates (dNTPs 50 or 100  $\mu$ M) and MgCl<sub>2</sub> (1.25, 2.50, or 3.75 mM). All samples were loaded directly from ice into the 95°C heat block to minimize time required for samples to reach denaturation temperature. Touchdown PCR was utilized to amplify the LMW kininogen gene. The first cycle used denaturation at 95°C for 2 min, annealing at 65°C for 1 min, and a 2 min extension at 72°C. This was followed by 28 cycles of denaturation at 94°C for 1 min, annealing starting at 64°C and dropping 0.5°C each cycle for 1 min and a 2 min extension at 72°C. Nine more cycles were performed with denaturing for 1 min at 94°C, annealing for 1 min at 50°C and extension for 2 min at 72°C. PCR was completed by cooling to 4°C. The resulting PCR product was resolved in a 3% gel at 85 volts for 1.5 h, followed by 30 min stain in ethidium bromide (1.0 mg/ml). The optimal



conditions used for kallikrein gene amplification were 25 mM MgCl<sub>2</sub>, 50  $\mu$ M dNTPs, 250 nM primer (Figure 4.1). To verify PCR product as low molecular weight kininogen, pooled cDNA was amplified with the previously described optimal conditions, run on a 3% agarose gel, stained with ethidium bromide and bands were cut from the gel with a razor blade. The PCR product was then extracted using Qiaquick (Qiagen, Santa Clarita, CA), and sequenced by the Recombinant DNA/Protein Research Facility at Oklahoma State University. The 409 base pair PCR product sequence was 83% homologous (Figure 4.2) to bovine LMW kininogen (Nawa et al., 1983). Endometrial cDNA samples (1  $\mu$ g) obtained across the estrous cycle and early pregnancy were PCR as previously described. Resultant bands were scanned in duplicate with NIH Image, Version 1.6 (NIH) densitometer.

To quantitate PCR synthesis procedure for comparable amounts of starting cDNA in samples, glyceraldehyde-3-phosphodehydrogenase (G3PDH) primers (Yelich et al., 1997) were utilized. G3PDH PCR products were run in 3% agarose gels, stained and bands scanned with densitometer. A ratio of LMW kininogen to G3PDH was utilized to standardize cDNA loading in each PCR reaction.

### Statistical Analysis

PCR and Western blot densities were analyzed with least squares methods using the General Linear Models procedure of SAS (SAS, 1990). The



model included the effects day, reproductive status, and the day x reproductive status interaction.

## Results

### Western Blot Analysis

Western blot analysis detected a ~66 kD immunoreactive product in both the ECM (Figure 4.3) and UTF (Figure 4.4) across all days of the estrous cycle and early pregnancy. No visual differences of LMW kininogen were observed in ECM did between days of the estrous cycle or pregnancy (Figure 4.3). Although densitometry analyses of UTF LMW kininogen approached a tendency for a reproductive status x day interaction ( $p=0.2$ ), there was an effect of day ( $p<0.05$ ). Although not statistically different, LMW kininogen density was numerically lower in pregnant versus cyclic UTF on Day 15.

### Touchdown PCR

The 400 bp product for LMW kininogen gene expression was detected across all days of the estrous cycle and early pregnancy (Figure 4.6). No difference in gene expression was detected when densitometry scans of the LMW kininogen gene product were adjusted for the housekeeping gene, G3PDH. Gene expression for LMW kininogen was also detected in porcine conceptus pools, muscle and kidney (Figure 4.7).

## Discussion

Many reproductive events mimic immunological responses (Clements et al., 1997). Biologic processes involved in endometrial proliferation, ovulation

(Gao et al, 1992) and decidualization (Valdes et al., 1996) reflect the inflammatory response. During the time of maternal recognition of pregnancy until the time of conceptus attachment, many events occur in the porcine uterine lumen, which are similar to inflammatory responses. The porcine conceptus morphologically changes from a 10 mm sphere to a long filamentous thread in 2-4 hours on Day 12 of pregnancy (Geisert et al., 1982b). With rapid elongation and growth of the conceptus, estrogen synthesis by conceptuses can alter uterine secretion of proteins (Roberts et al., 1993), prostaglandins (Bazer and Thatcher, 1977), uterine blood-flow (Ford et al., 1982), uterine cellular morphology (Geisert et al., 1982b) and vascular permeability (Keys and King, 1990) as it covers the uterine surface. Porcine endometrial release of kallikrein (Chapter 3) may be involved with cleavage of inter- $\alpha$ -trypsin inhibitor heavy chain 4 ( $\alpha$ IH4), which is found in the porcine uterus (Geisert et al., 1998). Cleavage of  $\alpha$ IH4 may be important for opening the implantation window for conceptus attachment. Tissue kallikrein may also cleave its natural substrate, low molecular weight kininogen, to release the potent vasoactive peptide, bradykinin. Kinin production has been associated with increased prostaglandin production, increased blood flow and increase smooth muscle contraction (see review Bhoola et al., 1992), all of which occur during maternal recognition of pregnancy and conceptus attachment in the gilt.

The present study clearly demonstrates that LMW kininogen is present in porcine uterine flushings, and endometrial tissue throughout the estrous cycle and early pregnancy. Endometrial gene expression of LMW kininogen does not

differ throughout the estrous cycle or pregnancy when adjusted for G3PDH gene expression. Although not statistically different, Day 15 pregnant LMW kininogen levels appeared to decline. Levels of detectable LMW kininogen may be decreased due to an increase in kallikrein activity on day 15 (Chapter 3). Increase in kallikrein would cause more kinin to be liberated, and therefore providing the important biological effects necessary for establishing an environment permissive to conceptus development.

Endometrial gene expression and protein secretion of LMW kininogen appears to be constitutive as no difference was detected across the days of the estrous cycle or pregnancy. Kininogen must always be available in a moment of trauma and/ or inflammatory response in order for kinins to be liberated (see Bhoola et al, 1992). Therefore, the key regulator of LMW kininogen function to release kinin in the porcine uterus is the release of kallikrein. Through kallikrein regulation, kinin acts to perform the biological processes that are present in the uterus (i.e. increased blood-flow, vascular permeability, smooth muscle contraction). Blood flow to the pregnant uterine horn is increased on Day 12 and 13 compared to the nongravid horn in unilateral pregnancies (Ford and Christenson, 1979). Ford et al. (1982) documented that Day 13 gravid horns had higher uterine blood flow, as well as greater uterine luminal concentrations of estrogen compared to nonpregnant females. In the rat there is an increase in estrogen (Brann et al., 1995; Valdes et al., 1996) that is associated with an increase in uterine kallikrein activity, thus increased kinin release. The increase

in blood flow that is detected on Days 12 of pregnancy, may be due to estrogen's action to stimulate kinin release.

Porcine conceptuses were also demonstrated to have LMW kininogen gene expression as detected through PCR analysis. If LMW kininogen is translated, along with glandular kallikrein (see Chapter 3), the conceptus itself may liberate kinins, thus having a local effect on the uterine epithelium in which it is contacting. Thus, endometrial as well as conceptus sources may be contributing to kinins in the uterine lumen.

Kininogens contain a domain known to inhibit cathepsin L (Coleman, 1996; Schmaier, 1997). Cathepsin L is proteolytic and has a high affinity for collagen (Kirschke et al., 1982). Geisert et al. (1997) demonstrated an increase of cathepsin-L activity in porcine UTF on Day 15 of the estrous cycle and pregnancy. This corresponds to the time when LMW kininogen was decreased in porcine UTF in this study. The increase that Geisert et al. (1997) documented may have been due to a decline in cathepsin L's inhibitor, LMW kininogen.

Presence of LMW kininogen and kallikrein (see Chapter 3) in the porcine uterus indicate that the kininogen-kallikrein-kinin system is biologically active during establishment of pregnancy in the pig. The role that the kallikrein-kininogen-kinin system is playing in the porcine uterus may direct numerous events that are necessary for conceptus survival. Future studies will establish if conceptus estrogen acts through the kallikrein-kininogen-kinin system to establish pregnancy and provide an environment for continued conceptus growth and survival.

Figure 4.1. Photograph of an ethidium bromide stained 3% gel with PCR products from LMW kininogen primer optimization. Conditions used in further analysis are those of Lane 4 (see Table 3.1). Arrow indicates proper band size (~400 bp). (-) indicates negative control (no cDNA added). M represents the marker lane.

400  
→

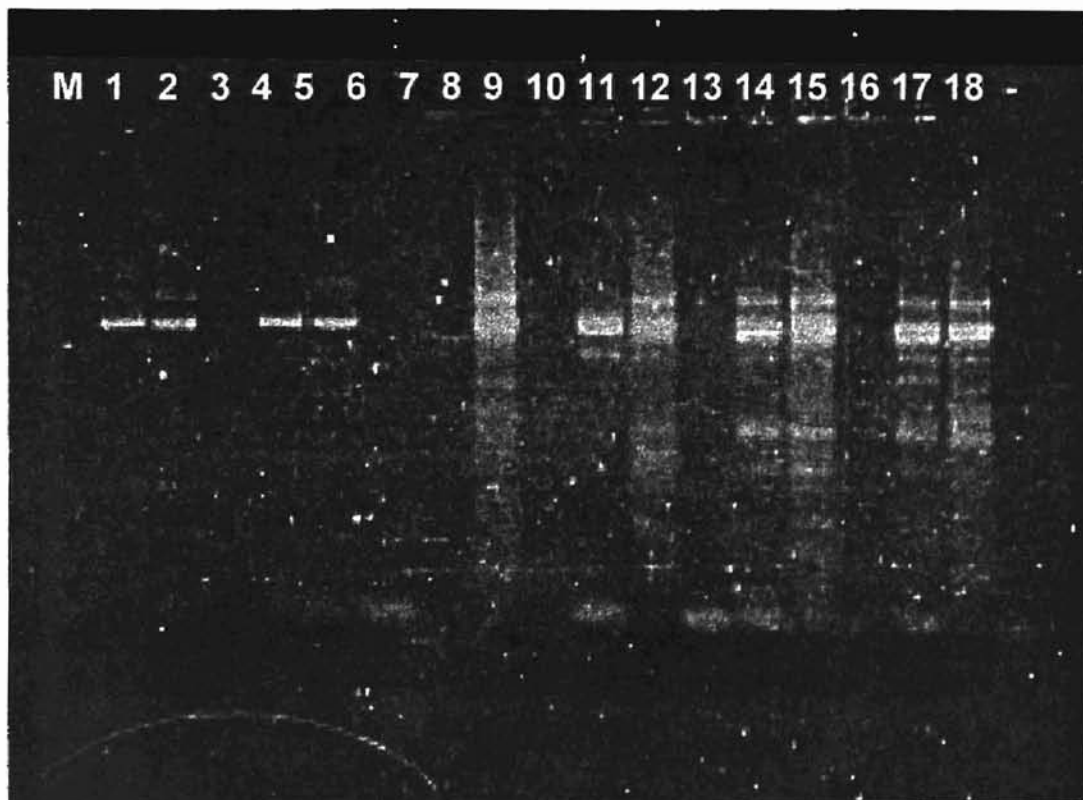


Figure 4.2. Nucleotide sequencing of the 400 bp PCR product is 83% homologous to bovine LMW kininogen. p indicates the porcine sequence; b indicated the bovine sequence with black color as homologous base pairs.

p6      cccagacctg gaggagcctc **taaac**-attc catcgccaag  
 b901    cccagacctg gaggagcctc **tgagcc**attc catcgcaaag  
  
 p46     cttaatgcag ag**aaca**atg**c** ag**tctt**ctat ttc--gattg  
 b941    cttaatgcag ag**catg**atg**g** ag**cctt**ctat ttc**aag**attg  
  
 p86     ac**ct** tgtg**ga** aaaagcaaca gtacaggtgg tggctgg**nnn**  
 b981    ac**actg**tg**aa** aaaagcaaca gtacaggtgg tagctgg**a t t**  
  
 p126    **nnnn**tattct attg**gt**gttc **acggcc**aggg aaaccacatg  
 b1011   **gaag**tattct attg**-gt**ttc **atagca**aggg aaaccacatg  
  
 p166    ttc**caag****caa** agtaatgaag ag**tgac****aga** **aag**ttgtgag  
 b1051   ttc**taag****gga** agtaatgaag ag**ctga****caa** **ga**gtttgtgag  
  
 p206    atcaag**aa****ac** **ct**gggtcaaatt tctaaagtgt **a**atgct**ct**g  
 b1091   atcaat**ata**c **at**gggtcaaatt tctacactgt **ga**tgct**aat**g  
  
 p246    tttac**gt**gggt **ac**cttggggag **nnnnnn**tt**ct** accctactgt  
 b1131   tctat**gt**gggt **g**ccttggggag **gaaaa**agttt accctactgt  
  
 p286    caaatgtcaa **tt**acttggac agacctcact **ga**tgaaaagg  
 b1171   caactgtcaa **cc**acttggac agacctcact **ca**tgaaaagg  
  
 p326    cctcc**ag**gtt tttcaccttt ccgatcagt**a** caagtg**gaga**  
 b1211   cctcc**g**gtt tttcaccttt ccgatcagt**t** caagtg**atga**  
  
 p366    aaact  
 b1261   aaact



Figure 4.3. Coomassie Blue staining (A) of explant culture media during the estrous cycle (C) and early pregnancy (P) and Western Blot analysis (B) using human LMW kininogen. M indicates marker lane. Arrow indicates 66 kD immunoreactive product.

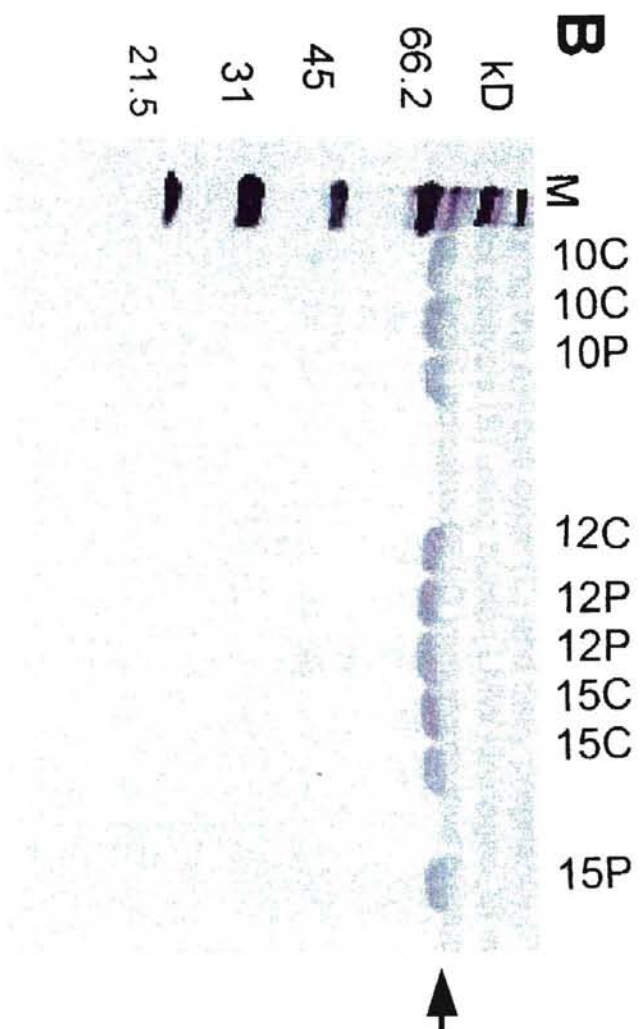
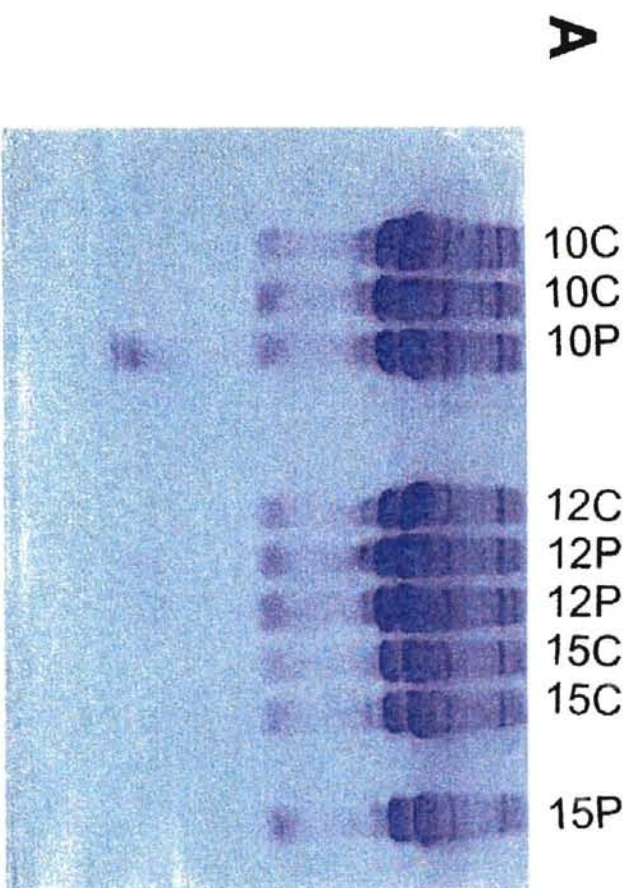


Figure 4.4. Representative Coomassie Blue staining (A) of uterine flushings during the estrous cycle (C) and early pregnancy (P) and Western Blot analysis (B) using human LMW kininogen. M indicates marker lane. Arrow indicates 66 kD immunoreactive product.

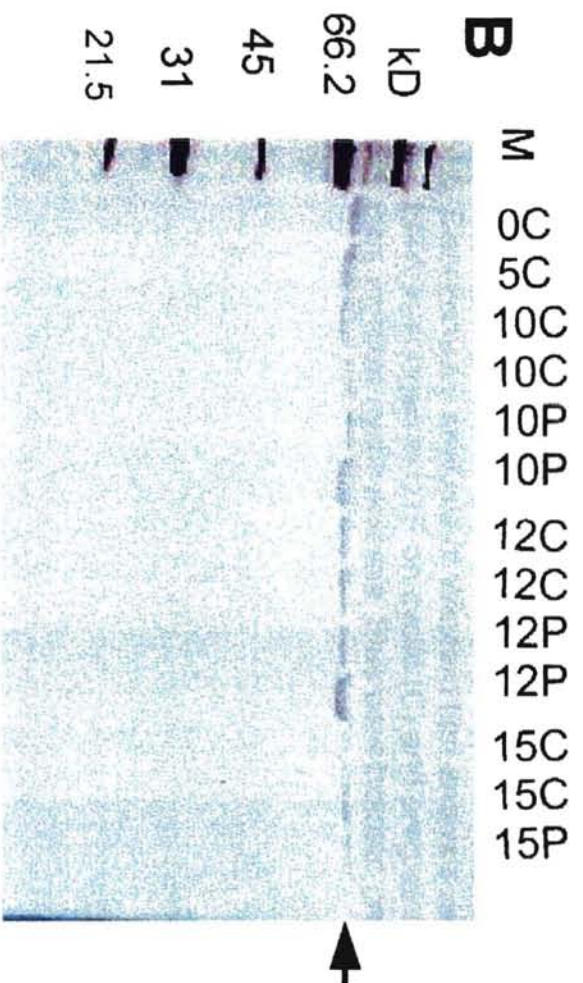
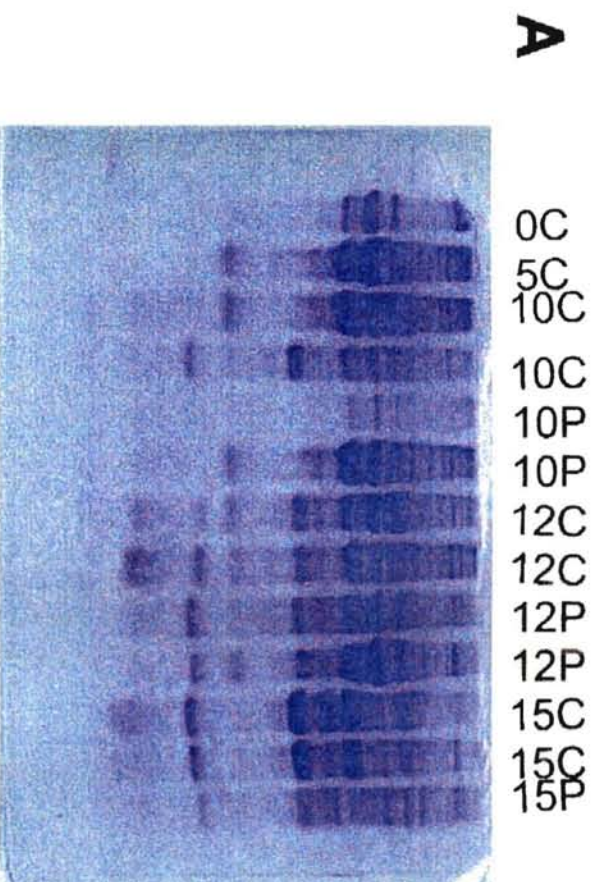


Figure 4.5. Densitometry analysis of Western blot analysis using antibody to LMW kininogen in UTF on cyclic (C) and pregnant (P) gilts. Yellow bars indicate cyclic animals. Blue bars indicate pregnant animals.



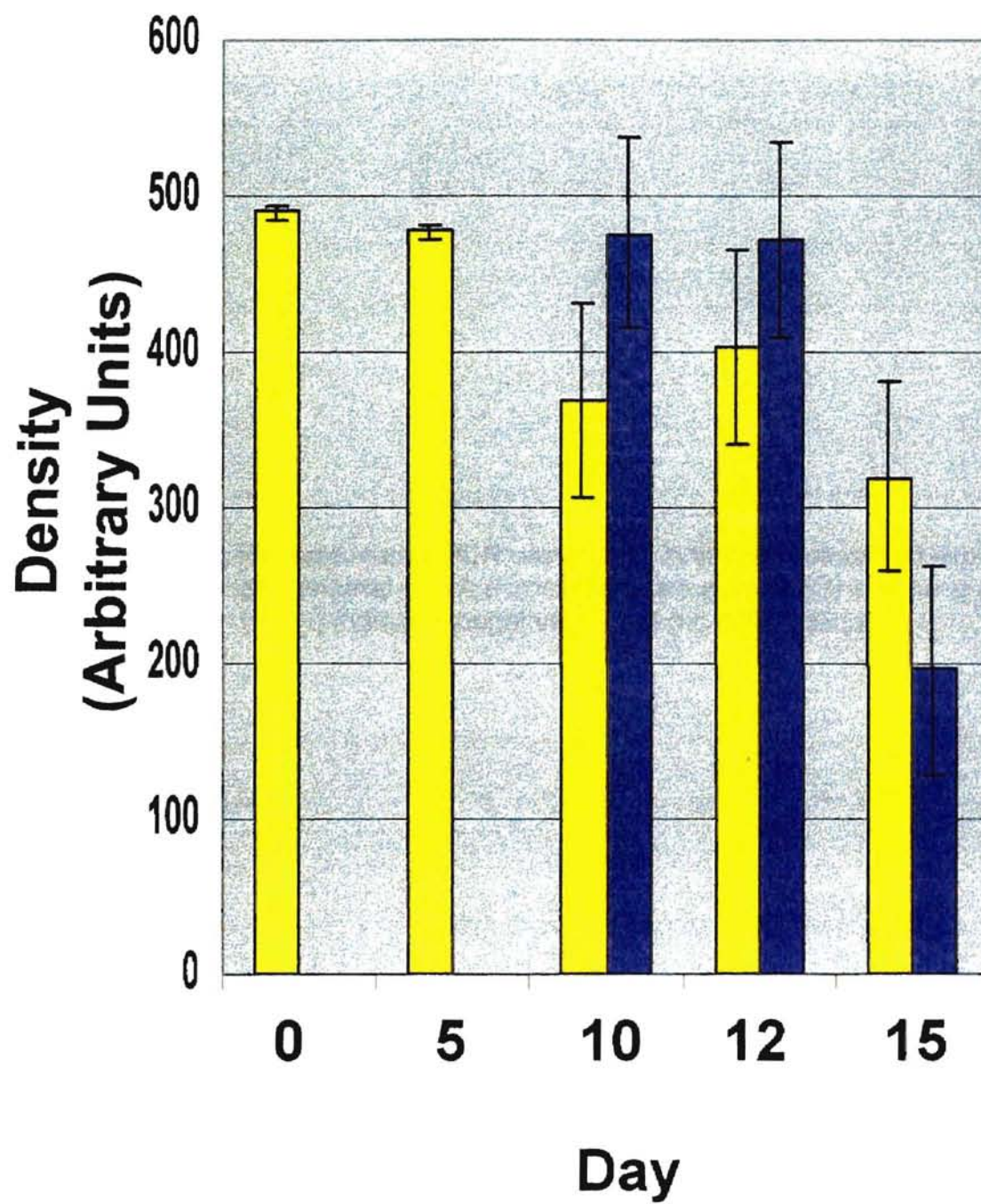


Figure 4.6. Representative PCR using LMW kininogen primers to express endometrial cDNA across the estrous cycle (C) and early pregnancy (P). (-) indicates negative control (no cDNA added).

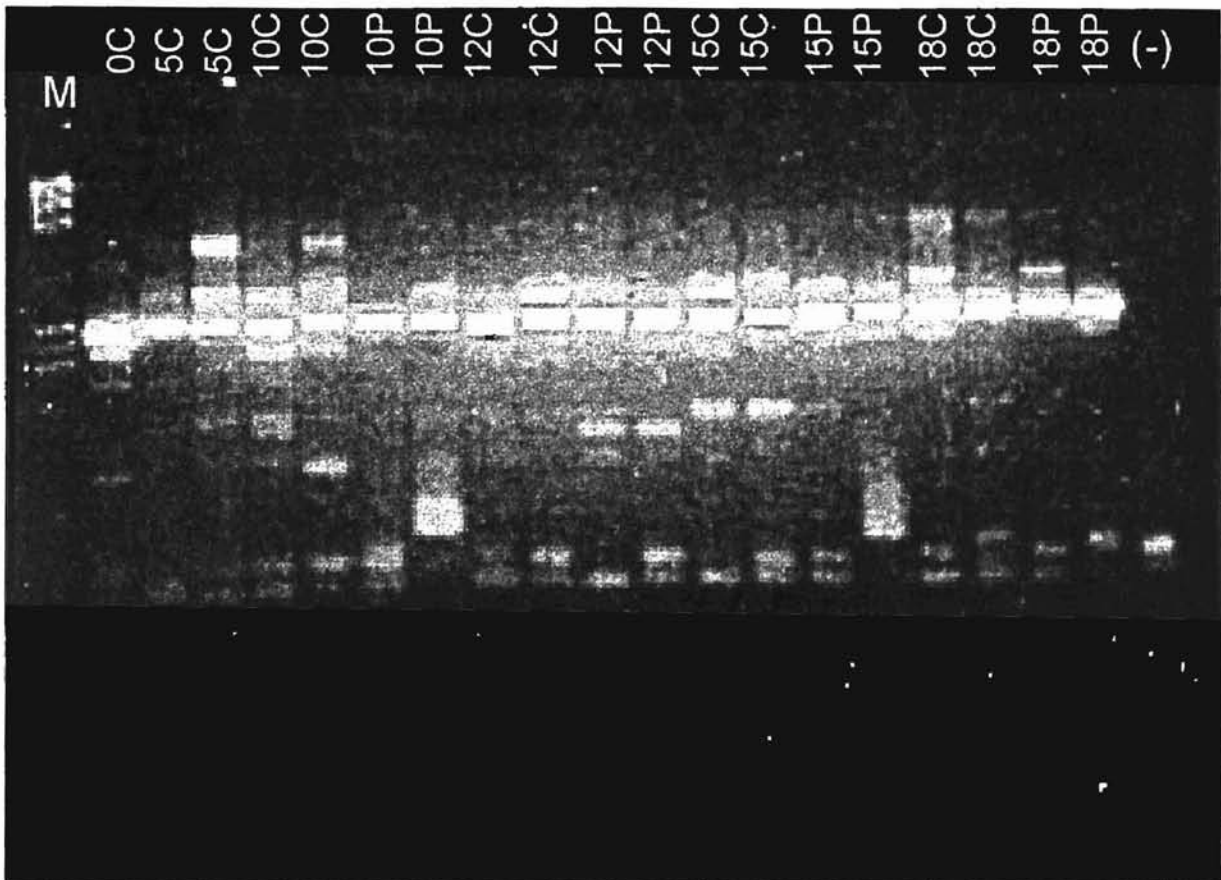
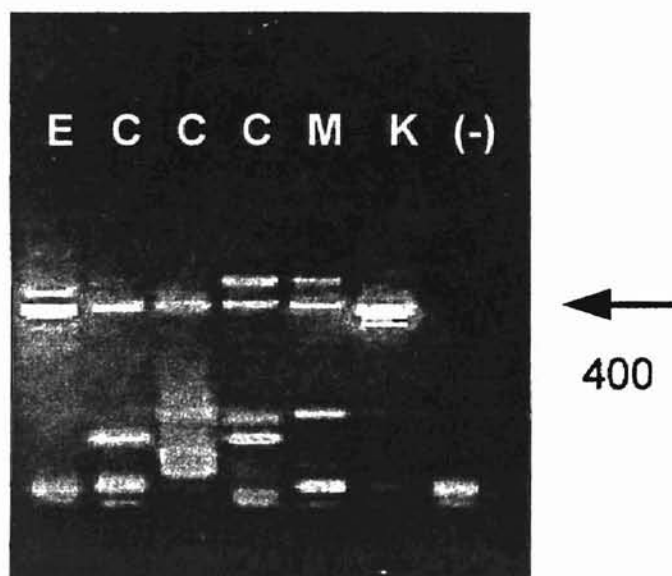




Figure 4.7. PCR using LMW kininogen primers on porcine endometrium (E), conceptus(C), muscle (M) and kidney (K) cDNA. (-) indicates negative control (no cDNA added).



## CHAPTER V

### General Discussion

In domestic species, such as the pig, cow, and ewe, implantation is a relatively late event when compared to rodents and humans. The early embryo is first nourished by uterine milk until its demands for nutrition drive it to seek other sources, nourishment from the maternal side. Establishment of placentation is a necessary event for a successful pregnancy. In order to establish its placenta, the porcine conceptus undergoes a rapid morphological transformation on day 12 (Anderson et al., 1978, Geisert et al., 1982b). After attaining ~10mm in diameter, the porcine conceptus elongates into a long, filamentous thread in the course of 2-4 hours without an increase in cell number (Geisert et al., 1982b; Pusateri et al., 1990). During this process, the conceptus releases estrogens to act as the maternal recognition of pregnancy across the endometrium in the pig (see Geisert et al., 1990). Conceptus estrogens influence uterine protein secretion (Roberts et al., 1993), redirect prostaglandin release from an endocrine to an exocrine direction (Bazer and Thatcher, 1977), increased uterine blood-flow (Ford et al., 1982) and altered uterine cellular morphology (Geisert et al., 1982b;

Keys and King, 1990). Estrogen is imperative for these events to occur, not only on day 12, but a second sustained conceptus estrogen release is essential on day 15 to maintain long term corpora lutea function. Knowledge of the effects that estrogens are having on the uterine dynamics remains unclear, i.e. estrogens may be directly or indirectly influencing a physiological process.

On day 13, the porcine conceptus begins to attach to uterine epithelium in order to establish its placenta. Unlike other species that actively invade through the uterine epithelium, implantation by the pig conceptus is non-invasive and forms the diffuse, epitheliochorial placenta (Keys and King, 1990). The nature of porcine conceptuses is proteolytic if the porcine conceptus is placed outside the uterine lumen, as it will actively invade into the surrounding tissue (Samuel and Perry, 1972). Therefore the porcine endometrium must produce proteolytic inhibitors. Trophoblastic attachment to the uterine surface epithelial glycocalyx present on the microvilli provides close contact between the maternal endometrial surface and the conceptus to establish placental attachment and attainment of nutrients (Keys and King, 1990). Before the conceptus can attach, the uterine surface epithelium must be altered to allow for close contact with the conceptus. The opening of the "implantation window" is necessary for successful placental establishment.

One of the major factors that hinder attachment in the pig is the glycoprotein, Muc-1 (Bowen et al., 1996, 1997). When Muc-1 is depleted from the glycocalyx, attachment can occur through extracellular matrix proteins binding to integrins on the uterine surface (Hynes, 1992). Other glycoproteins are

also present on the glycocalyx, including  $\alpha$ I family members (Geisert et al., 1998). After initial contact between conceptus and uterine epithelium, the extracellular matrix needs to be stabilized.  $\alpha$ I family members play an important role in extracellular matrix stabilization (Bost et al., 1998). Hyaluronic acid makes up a large majority of the extracellular matrix and is present on the trophoblastic surface.

Geisert et al. (1998) have found  $\alpha$ IH4 to be present in the porcine uterus. Similar to other  $\alpha$ I heavy chains,  $\alpha$ IH4 contains a von Willibrand domain which is known to bind to integrins and heparin (see Salier et al., 1992) and therefore has been proposed to be involved with attachment. However  $\alpha$ IH4 is unique as it does not bind the serine protease inhibitor bikunin (see Salier et al., 1992), therefore possesses no proteolytic activity. Even more interesting,  $\alpha$ IH4 contains a cleavage site for the serine protease, kallikrein (Nishimura et al., 1995). Kallikrein has been shown to cleave  $\alpha$ IH4 in porcine plasma to release a 100 and 30 kD fragments with further cleaving of the 100 kD to 70 kD (Hashimoto et al., 1996). Geisert et al. (1995) have detected a 30 kD fragment in the porcine uterus, therefore it is possible that kallikrein activity is present in the uterine lumen.

The present study detected kallikrein activity, protein and gene expression in the porcine lumen during the estrous cycle and early pregnancy in the pig. Kallikrein activity in pregnant UTF was elevated over cyclic animals on day 12, and a three-fold increase was measured on day 15 with cyclic animals having greater activity than pregnant gilts. Kallikrein levels have been known to be

regulated by ovarian hormones (Brann et al., 1995; Valdes et al., 1996), with progesterone hindering kallikrein activity and estrogen stimulating activity. On day 15 of the estrous cycle, the corpora lutea are regressing and follicles are beginning to grow, thus plasma concentrations of progesterone are declining with increasing concentrations of estrogen. This may be the reason for the increased kallikrein activity seen on day 15 of the estrous cycle. In order to better understand the role ovarian hormones play on kallikrein activity in the pig, ovariectomized gilts receiving estrogen, progesterone, and estrogen and progesterone treatments need to be evaluated. This will provide a clearer picture of how kallikrein is regulated under the influence of ovarian steroids. Besides hormonal regulation, another regulator of kallikrein is its binding protein, kallistatin (Chao et al., 1996). Protein and gene expression for kallistatin also needs to be investigated. It makes sense that the uterus would inhibit the serine protease, kallikrein, by producing a binding protein as seen with plasmin and metalloproteinases.

In ECM, there was a significant increase of kallikrein activity on Day 12 of pregnancy compared to the estrous cycle. Differences in endometrial activity suggest that the conceptus may regulate local kallikrein release. This is verified with the kallikrein activity detected in UTF containing different conceptus sizes. Kallikrein activity was related to the increase in estradiol secretion from the filamentous embryos. Although no difference was detected in estradiol concentration between tubular and filamentous conceptuses, there was a four

fold increase in kallikrein activity only when conceptuses elongated throughout the uterine horn.

Detection of gene expression in endometrium suggests that the luminal source of kallikrein is from the uterus. Kallikrein has been known to be stored in secretory vesicles in the rat (Valdes et al., 1996). Amount of kallikrein stored may dictate the amount of mRNA expressed for kallikrein. Once kallikrein is released from secretory vesicles, more mRNA needs to be translated into kallikrein. Kallikrein mRNA was also detected in conceptuses that were large spherical and filamentous. If the conceptus translates kallikrein mRNA, it may further regulate actions occurring on the uterine surface.

Presence of kallikrein may function to cleave  $\alpha\text{H4}$ , allowing for attachment of the porcine conceptus. Besides cleaving  $\alpha\text{H4}$ , kallikrein has been known to act on other substrates including IGFBPs (Cohen et al., 1992) and kininogen (Bhoola et al., 1992). Increase in kallikrein activity could also play a role in regulating IGF activity within the porcine uterine lumen. Increased amounts of IGF are present on day 12 of pregnancy (Simmen et al., 1989), and regulated by IGFBPs (see Simmen et al., 1995). Several investigators (Cohen et al., 1992; Rajah et al., 1996) have shown in other species and other tissues that kallikrein family members cleave IGFBP to increase local amounts of IGF. Recently, Lee et al. (1998) demonstrated that a serine protease in pregnant UTF was responsible for IGFBP cleavage in the uterine lumen. Aprotinin, a serine protease inhibitor, negated all IGFBP cleavage, but when a specific inhibitor for plasminogen activator was added, IGFBP cleavage remained (Lee et al., 1998).

There is a possibility that kallikrein acts to cleave IGFBP in the porcine uterine lumen. A straightforward experiment of measuring IGF (via RIA) and IGFBP (ligand blots subjected to [<sup>125</sup>I]-labeled IGF-I) prior to addition of kallikrein to cyclic and pregnant UTF, with IGF and IGFBP measured again after incubation would determine if this hypothesis is correct. IGF liberation is likely to add in further conceptus growth and development (see Simmen et al., 1995). Furthermore, addition of the kallikrein inhibitor, cyclohexylacetyl-phe-arg-ser-val-gln amide, would provide evidence that the cleavage detected above is specifically the proteolytic action of kallikrein.

Estrogen directed events that occur during the time of conceptus elongation and attachment, seem to mirror events that occur during a time of inflammation, trauma and tissue damage (Clements et al., 1997). An inflammatory response can be characterized by vasodilation, vascular permeability, edema, and pain. The kallikrein-kininogen-kinin (K-K-K) system is active at the site of inflammation (Bhoola et al., 1992). At the site of injury, it is typical to find mast cell activation, neutrophil invasion, as well as involvement of cytokines, prostaglandins and growth factors with the kallikrein-kininogen-kinin system (see Bhoola et al., 1992).

Present research detected LMW kininogen and LMW kininogen gene expression in porcine endometrium. Endometrial gene expression of LMW kininogen did not differ throughout the estrous cycle or pregnancy. However, there was a decrease in LMW kininogen on day 15 of pregnancy. Levels of kininogen on day 15 of pregnancy may be lower due to increase kallikrein



activity. With more kinins being liberated from kininogen, more biological events could occur. Increased kinin release results in increased blood-flow and vasopermeability (Bhoola et al., 1992). Ford and Christenson (1979) detected an increase in blood flow to the pregnant uterus in the pig on days 12 and 13. The association between uterine blood flow and pregnancy maintenance was further reported by Ford et al. (1982) where day 13 gravid horns had higher blood flow, as well as greater estrogen concentrations than to nonpregnant horns. It has been documented that with an increase in estrogen (Brann et al., 199; Valdes et al., 1996) there is an increase in kallikrein activity, thus increased kinin release in the rat uterus. The increase in blood flow that is seen on these critical days in the pig, may be due to or assisted by increased kinin release. As seen in the present study, kininogen gene expression does not change. Kallikrein may be the key regulator of LMW kininogen function to release kinins.

Kininogen isn't just playing a passive role in the porcine uterus. Kininogens contain a domain that inhibits the proteolytic factor, cathepsin L (Coleman, 1996; Schmaier, 1997). Geisert et al. (1997) demonstrated an increase of cathepsin L activity on day 15 of pregnancy, the same day LMW kininogen levels decline (this study). The increase that Geisert et al (1997) documented may be due to kallikrein depleting its inhibitor, LMW kininogen.

The kinins that are released from endometrial sources may be acting with the conceptus to further potentiate the physiologic processes occurring. Bradykinin has been shown to stimulate IL-6 and IL-8 secretion from human decidua derived cells (Rehbock et al., 1997). Mathialagan et al. (1992) indicated

that IL-6 is secreted from porcine conceptuses between day 13-17. Furthermore, IL-6 upregulates  $\alpha$ I heavy chains -1, -2, and -3 which contain a von Willibrand domain. Therefore, it is possible that kallikrein cleaves LMW kininogen, releasing bradykinin on day 12, which binds to kinin  $\beta_2$  receptors on the conceptus (Allen et al., 1998), to stimulate IL-6 release from porcine conceptuses to upregulate  $\alpha$ I members for conceptus attachment to the uterine epithelial surface.

What may have started as a simple inquiry to determine if kallikrein cleaves  $\alpha$ IH4, has turned out to be the discovery of a potent biological system present in the porcine uterus. The effects and potential of the K-K-K system are innumerable. The processes that are known to occur during this critical state of pregnancy only add strength to the argument that the K-K-K system is active in the porcine uterus and may serve to function during conceptus elongation and attachment.

## APPENDIX

### **Microconcentration Using Centricon 10**

1. Add 2 ml of sample to the sample reservoir of Centricon 10 microconcentrator inserted into a filter cup. Cover the sample reservoir with Parafilm.
2. Place the concentrators in the Sorvall RC-5B Refrigerated Superspeed Centrifuge SM-24 rotor (fixed angle  $28^{\circ}$ ) in either the inner or outer row. Spin for 1 hour at 6500 rpm for the inner row or 6000 rpm for the outer row (1000-5000 x g).
3. Repeat steps 1 and 2 (for total of 4 ml sample concentrated).
4. Place the retentate cup over the sample reservoir and discard the filtrate cup.
5. Invert the unit and spin for 2 minutes at 3000 rpm for the inner row or 2500 rpm for the inner row (300-1000 x g)
6. Cap the retentate and store at  $-80^{\circ}\text{C}$  or continue immediately with the Lowry Assay.

## Lowry Protein Assay

### Sample/ Standards Preparation

- Uterine flushings and explant culture media should be concentrated prior to doing the Lowry to ensure good readings.
- All samples and standards are prepared on ice in 13 x 100 mm disposable tubes.
- Samples should be done in triplicate with volumes at 1  $\mu$ l, 3  $\mu$ l and 5  $\mu$ l. Use Millipore water to bring to a final volume of 1 ml.
- Standards are prepared using BSA solution (1 mg/ml). 5 standard concentrations with 2 tubes each are prepared. The standard volume is brought to a final volume of 1 ml with Millipore water.

20 $\mu$ g BSA	20 $\mu$ l BSA + 980 $\mu$ l H <sub>2</sub> O
40 $\mu$ g BSA	40 $\mu$ l BSA + 960 $\mu$ l H <sub>2</sub> O
80 $\mu$ g BSA	80 $\mu$ l BSA + 920 $\mu$ l H <sub>2</sub> O
100 $\mu$ g BSA	100 $\mu$ l BSA + 900 $\mu$ l H <sub>2</sub> O
200 $\mu$ g BSA	200 $\mu$ l BSA + 800 $\mu$ l H <sub>2</sub> O

A blank is prepared using 1 ml Millipore water.

### Lowry C Solution Preparation

Lowry A = 2% Na<sub>2</sub>CO<sub>3</sub> in 0.1 M NaOH

Lowry B = 1% CuSO<sub>4</sub> + 2% NaKTartrate mixed 1:1

Lowry C = Lowry A + Lowry B mixed 50:1

Prepare Lowry C just prior to use and use at room temperature

### Procedure

Remove sample and standard tubes from the ice. Add 5 ml of Lowry C to each tube using a repeat pipetter. Allow tubes to sit at room temperature for 10 minutes.

Add 0.5 ml of 1 N Phenol Reagent to each tube using the Oxford bottle pipettor. Cover each tube with Parafilm and mix immediately after adding Phenol. Allow tubes to sit for 30 minutes at room temperature. Read absorbance using the Beckman DU-64 spectrophotometer at 750 nm visible light.

## **Solutions**

### 0.1N NaOH

16.0 g NaOH. QS to 4 liters with Millipore water.

### 2% Na<sub>2</sub>CO<sub>3</sub> in 0.1 N NaOH

81.63 g Na<sub>2</sub>CO<sub>3</sub>, QS to 4 liters with 0.1 N NaOH

### 1% CuSO<sub>4</sub>

1.01 g CuSO<sub>4</sub>, QS to 100 ml with Millipore water.

### 2% NKC<sub>4</sub>H<sub>4</sub>O<sub>6</sub>·4H<sub>2</sub>O

2.04 g NaKTartrate, QS to 100 ml with Millipore water.

### 1N Phenol Reagent

Dilute 2N stock solution 1:1 with Millipore water.

### BSA, 1mg/ml

100 mg BSA QS to 100 ml with Millipore water.

## 1D Acrylamide Gel Electrophoresis Mini-Gel

Gels can be poured up to 24 hours before needed. Remove solutions 7, 8, and 11 from the refrigerator and allow them to reach room temperature (aliquot out amount needed to save time). Assemble two gel sandwiches. Mark the glass with a Sharpie 5.5 cm from the bottom of the plate. Wear gloves when handling acrylamide.

For 2 gels

### Running gel

1. Mix the following in a 50 ml flask:

	<u>12.5% Gel</u>
Acrylamide stock – <b>Solution 7</b>	7.81 ml
Running Buffer B – <b>Solution 8</b>	4.72 ml
20% SDS – <b>Solution 10</b>	94.5 $\mu$ l
dH <sub>2</sub> O	3.66 ml
2. Add 2.5 ml ammonium persulfate (APS) (0.14 g/50 ml dH<sub>2</sub>O) quickly and swirl the flask to mix thoroughly. Pour the mixture into a 20 cc syringe.
3. Using a 20 gauge needle, pour to the 5.5 cm mark.
4. Overlay with 0.1% SDS.
5. Allow acrylamide solution to polymerize for ~30 minutes.

### Stacking Gel

1. Mix the following in a 25 ml flask:

Acrylamide stock – <b>Solution 7</b>	1.2 ml
Stacking Gel Buffer E – <b>Solution 11</b>	1.0 ml
20% SDS – <b>Solution 10</b>	40 $\mu$ l
dH <sub>2</sub> O	4.05 ml
2. Blot the running gel with Kimwipes until dry.
3. Quickly add 5  $\mu$ l TEMED and 1.75 ml APS to the flask and swirl to mix thoroughly. Pour mixture into a 10 cc syringe.
4. Using a 20 gauge needle, pour to 0.25 cm below the top of the plates.
5. Carefully insert the comb without introducing air bubbles.
6. Allow the gel solution to polymerize for ~30 minutes.
7. Prepare 500 ml electrophoresis buffer:  
1.5 g Tris; 7.2 g glycine ; 0.5 g SDS; QS to 500ml.
8. Slowly remove the combs from the gels by gently pulling the comb straight up.

9. Rinse the wells with dH<sub>2</sub>O using a tuberculin syringe. Suck out and replace with electrophoresis buffer.
10. Assemble the upper buffer chamber and lower it into the lower buffer chamber. Fill the upper buffer chamber with electrophoresis buffer and check for leaks. Pour the remaining solution into the lower chamber. Let gels sit for 2 hours prior to use. Gels can stand overnight.

### Sample Preparation

1. Prepare Sample Buffer (SDS Reducing Buffer) the same day of use.

Millipore water	4.0 ml
0.5 M Tris-HCl pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
β-mercaptoethanol	0.4 ml
0.1% (w/v) Bromophenol Blue	0.2 ml

2. Prepare samples. I loaded 50 µg of total protein (as determined by Lowry) 50 µg of sample is brought to a final volume of 15 µl and then 15 µl of sample buffer for a total volume of 30 µl to add to each well.
3. Using Low range biotinylated standard (from BioRad); add 7.5 µl standard + 22.5 µl sample buffer.
4. Denature the samples and standard for 5 minutes over steam.
5. Immediately place in ice after denaturing.
6. Pulse down samples. Load into wells using microcapillary pipet tips. Load 10 µl of standard to each gel. Load all 30 µl of sample \*\*Volume of sample added depends on comb size—consult BioRad book for more information.

### Electrophoresis

1. Attach the lid to the lower buffer chamber and connect it to the power supply (red to red; black to black in the same channel).
2. Set the power supply at constant current. Run at 30 mA for 5 minutes until the dye reaches the running gel. Then increase the mA to 40. Let migrate until the dye is ~1 cm from the bottom of the gel. Usually takes about 65 minutes.

### SOLUTIONS

#### Running Gel Acrylamide (solution 7)

- 150.0 g acrylamide
- 4.0 g bis acrylamide (N, N'-Methylene-bis-acrylamide)

Bring to 500ml with dH<sub>2</sub>O. Filter. Degas. Store in dark bottle in refrigerator. Warm to room temp before use.



Running Buffer B (solution 8) pH 8.8 – 9

90.5 g Tris

1.6 ml TEMED

Bring to 200 ml with dH<sub>2</sub>O. Adjust to pH to 8.8 with about 250 ml of 1N HCl.  
Adjust volume to 500ml. Filter. Degas. Store at 4°C.

20% SDS (solution 10)

10.0 g SDS

Bring volume to 50 ml with dH<sub>2</sub>O.

Stacking Gel Buffer pH6.6 – 6.8

29.9 g Tris

2.3 ml TEMED

Bring to 200 ml with dH<sub>2</sub>O. Adjust pH to 6.8 with about 240 ml of 1N HCl.  
Adjust volume to 500 ml. Filter. Degas. Store at 4°C.

## Western Blot Analysis

1. After electrophoresis, remove gel from glass plates by gently prying off the top glass with a spacer. Cut off the stacking gel and dispose. Nick the left hand corner (where standards are) and place gels into transfer buffer on a rocking platform for 15-20 minutes.
2. While the gel is in the transfer buffer, prepare the PVDF membrane by soaking it in:
  - a) Methanol, 1-3 seconds
  - b) Millipore water, 1-2 minutes
  - c) Transfer buffer, 2 minutesDo not touch the membrane with ungloved hands!! Do not allow the membrane to dry out at any time after it is hydrated!
3. Prepare the transfer unit on the semi-dry electroblotting apparatus. Place the sandwich together on the anode base as follows:
  - a) 1 sheet (6 cm x 8.5 cm) 3MM paper soaked in anode buffer 1
  - b) 2 sheets (6 cm x 8.5 cm) 3MM paper soaked in anode buffer 2
  - c) PVDF membrane (6 cm x 8.5 cm) soaked in transfer buffer
  - d) Gel soaked in transfer buffer
  - e) 3 sheets 3MM paper (6 cm x 8.5 cm) soaked in cathode buffera-e equals one trans-unit. Repeat for second gel. Place adjacent to first sandwich when electroblotting. The sandwich should be stacked evenly with no air bubbles!
4. Place on lid (cathode) on top of the sandwiches. Loosely attach screws to prevent desiccation.
5. Electroblot for 35 minutes at 150 mA (constant current) for 2 sandwiches.

## Immunostaining

\*Both kallikrein and kininogen antibodies were used in the same fashion, but with varying amounts of antibody added—see details below. All amounts are for 2 membranes.

1. After electroblotting, immerse the membranes in TBS for 2 minutes. Place gels in gel fixative overnight. Stain the next day with Coomassie blue for 2 hours then destain.
2. Pour off the TBs and add 60 ml of the first blocking solution (3% gelatin in TBS) Incubate for 1 hour.
3. Wash the membrane in TTBS one time for 10 minutes.
4. Pour off the TTBS and add the first antibody solution. (Kallikrein= 250  $\mu$ l Ab/50 ml Ab buffer; Kininogen= 50  $\mu$ l Ab in 50 ml Ab buffer). Let incubate overnight.
5. Wash the membrane in TTBS three times for five minutes each.

6. Pour off the TTBS and add the second antibody solution. Incubate for 2 hours.
7. During the 2<sup>nd</sup> Ab incubation, measure 66 ml of HRP color development buffer and allow it to reach room temperature. Thaw and measure 13.33 ml of HRP color reagent A and protect from light.
8. Wash the membranes in TTBS two times for 5 minutes each.
9. Wash the membranes in TBS two times for 5 minutes each.
10. Pour off the TBS and add the color development solution. Color development incubation should last about 15 minutes. Cover to prevent fading.
11. Pour color development solution into waste bottle. Wash with Millipore water.
12. Scan membranes. Store in water at 4°C and protect from light.

## SOLUTIONS

### Transfer Buffer

25mM Tris, 192 mM glycine in 15% (v/v) methanol; pH 8.2-8.3

For 2 membranes: 400 ml containing 1.21 g Tris, 5.77 g Glycine.

### Anode Buffer 1

0.3 M Tris in 10% (v/v) MeOH; pH 10.4

200 ml contains 7.27 g Tris

### Anode Buffer 2

0.025M Tris in 10% (v/v) MeOH; pH 10.4

200 ml contains 0.61 g Tris

### Cathode Buffer

0.025M Tris, 40 mM glycine in 20% (v/v) MeOH; pH 9.4

200 ml contains 0.61 g Tris , 0.60 g glycine

### Tris-buffered saline (TBS)

20 mM Tris, 500mM NaCl; pH 7.5

For 2 membranes, 800 ml contains 1.94 g Tris, 23.38 g NaCl

### Wash Solution (TTBS)

20mM Tris, 500 mM NaCl , 0.05% Tween-20, pH 7.5 .

For 2 membranes, 500ml TBS with 250 µl Tween-20

### First Blocking Solution

3% gelatin in TBS; heat to dissolve (not over 65°C)

For 2 membranes, 60 ml TBS contains 1.86 g gelatin

#### Antibody Buffer

1% gelatin in TTBS

For 2 membranes, 120 ml TTBS contains 1.21 g gelatin

#### First Antibody Solution

For 2 membranes:

Kallikrein—250  $\mu$ l Ab in 50 ml Antibody buffer

Kininogen—50  $\mu$ l Ab in 50 ml Antibody buffer

#### Second Antibody Solution

For 2 membranes:

20  $\mu$ l Goat Anti-Rabbit IgG HRP Conjugate (stored at 4°C),

20  $\mu$ l avidin HRP conjugate (stored at 4°C), in 60 ml antibody buffer

#### Color Development Solution for Horseradish Peroxidase

For 2 membranes:

66 ml HRP color development (or Tris) at room temp, 200  $\mu$ l HRP color reagent buffer B (3% H<sub>2</sub>O<sub>2</sub>), 13.33 ml HRP color reagent a (0.3% 4-chloro-1-naphthol in diethylene glycol) at rm temp. Prepare just before use.

#### Coomassie Staining Procedure

1. Fix gels in 7% acetic acid and 40% EtOH overnight.
2. Coomassie blue stain:
  - 1.25 g Coomassie blue in 400 ml 95% EtOH, stir several hours then add: 70 ml conc. acetic acid
  - 530 ml distilled water
  - Filter with filter paper.
3. Stain gel for minimum of 2 hours at rm temp.
4. Destain gel in 7% acetic acid and 10% EtOH. Will need several changes.

## Kallikrein Enzyme Assay

### Solutions:

#### 0.1M Tris-HCl buffer containing 0.15 M NaCl; pH 8

1.58 g Tris-HCl

0.88 g NaCl

pH to 8; QS to 100 ml \*\*\*Filter using 2  $\mu$ m filter.

### Substrate

Pro-Phe-Arg 7-Amido-4-Methyl-Coumarin

From Sigma Chemicals (P-9273)

Stock solution: ~5 mg/1 ml buffer= ~8.68 mM

Working solution: 10x dilution

50  $\mu$ l stock:450  $\mu$ l buffer

### Standard

7-Amino-4-methyl-Coumarin

From Sigma Chemicals (A-9881)

Stock solution: 1 mg/ 1 ml DMSO

Working solution: Dilute 50x

100  $\mu$ l stock:4.9 ml buffer

### Instructions to turn on Fluorescent Spectrophotometer

1. Turn on water bath
2. Turn on main power source
3. Make sure the fan is plugged in
4. Turn lamp on (button on power source)
5. Turn recorder on
6. Turn spec on
7. Set excitation wavelength at 370; slit width 5
8. Set emission wavelength at 460; slit width 5
9. Set on data; ratio mode

### Standard Curve:

Mix in microfuge tube and vortex for proper mixing

Standard curve consists of 5 points. Add 4  $\mu$ l, 9.8  $\mu$ l, 15  $\mu$ l, 20  $\mu$ l and 32  $\mu$ l separately to 250 ml buffer for each point. Record wavelength. After recorder wavelength for the 32  $\mu$ l point, set recorder range by dividing the wavelength by 8. Type this number into spec and then use chart recorder to record the

wavelength. This is needed to for further calculations (gives mM/mV) Record the setting of mV/min on chart recorder that you have selected.

### Samples

All samples and substrate are kept on ice until assayed.

Run each sample in duplicate. Add 10  $\mu$ l of substrate and 200  $\mu$ l of buffer in a 1.5  $\mu$ l bullet tube. Add 40  $\mu$ l of sample Vortex and immediately put into spec for reading. Record using chart recorder at 6 cm/min. Let assay run for at least one minute.

To calculate specific activity, divide mM/mV by the total protein concentration.

### Reminders of General Procedures for Polymerase Chain Reaction (PCR)

For each new primer that is designed, an optimization must be done. The amount of Taq polymerase stays constant for every reaction at 0.175  $\mu$ l / tube. The total volume in each tube is 25  $\mu$ l. 2.5  $\mu$ l of 10x Buffer is added to each tube. Add other ingredients as indicated in the below table. QS to 25  $\mu$ l with PCR water.

Lane	Primer (5 $\mu$ M stock)	dNTPs (10 mM stock)	10 x MgCl
1	0.5 $\mu$ l	0.5 $\mu$ l	1.25 $\mu$ l
2	0.5	0.5	2.50
3	0.5	0.5	3.75
4	0.5	1.0	1.25
5	0.5	1.0	2.50
6	0.5	1.0	3.75
7	1.5	0.5	1.25
8	1.5	0.5	2.50
9	1.5	0.5	3.75
10	1.5	1.0	1.25
11	1.5	1.0	2.50
12	1.5	1.0	3.75
13	2.5	0.5	1.25
14	2.5	0.5	2.50
15	2.5	0.5	3.75
16	2.5	1.0	1.25
17	2.5	1.0	2.50
18	2.5	1.0	3.75

After optimal conditions are found, proceed with "regular" PCR.

Fill out a PCR reaction sheet prior to each reaction.

Prepare Master Mix (MM). MM includes all ingredients except cDNA.

A negative control should be included in each PCR reaction to check for contamination. Negative control is prepared by adding PCR water in place of cDNA. A genomic sample can be added to test for DNA contamination in cDNA.

The work area, PCR pipettors, and tube opener should be cleaned with 10% Clorox prior to use.

Only tips and tubes designated for PCR use, should be used.

Throughout the entire procedure, all ingredients and tubes should be kept on ice. Taq should remain at  $-20^{\circ}\text{C}$  until use.

25 ml of PCR oil should be added to the top of every tube prior to placing tubes into thermal cycler to prevent desiccation.

PCR requires a hot start. Allow heat block to reach  $85^{\circ}\text{C}$  before placing tubes in thermal cycler.

Store PCR products at  $4^{\circ}\text{C}$  until run on agarose gel.



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## VITA

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