CIRCULATION OF UNCONJUGATED ESTRADIOL-17β DURING MID-GESTATION IN BOVINE FETAL BLOOD, AMNIOTIC AND ALLANTOIC FLUIDS

By

HAIHUI HUANG

Bachelor of Medical Science

Hunan Medical University

Changsha, Hunan

People Republic of China

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Thesis Approved:

Thesis Adviser

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Estrogens belong to the family of sex steroid hormones. They play vital roles in many physiological processes with emphasis on reproductive-related actions, such as cell division and proliferation, tissue differentiation and organ growth. Those effects are mediated by specific receptors localized in the cell nucleus (Welshons et al, 1984), which cause alteration of specific gene expression. Estrogens are metabolites of cholesterol, converted from androgens by the aromatase enzyme. Estrone (E_1), estradiol (E_2) and estriol (E_3) are three major naturally occurring estrogens. Various forms of estrogens may be present in the fetal-placental compartment, although unconjugated estradiol-17β has the highest estrogenic potency (Sloss & Duffy, 1980). In this study, we will only focus on the analysis of unconjugated estradiol-17β.

Estrogens are secreted mainly by the ovaries in nonpregnant females, while during pregnancy the major site of production is transferred to the placenta. Within the placentomes are specific populations of steroidogenic cells. In the fetal-placental compartment, estrogens were measurable as early as day 27 of gestation in cattle (Eley et al, 1979). While mRNA of estrogen receptors could be first detected in the blastocysts of mouse embryos at the preimplantation stage (Hou & Gorski, 1993), it is unknown when estrogen receptors first appear in bovine fetuses, but there was evidence that estrogen

receptors appeared in the bovine fetal uterus near the end of mid-gestation (Malayer & Woods, 1998). Day 46 of gestation is the time that the bovine embryo completes organogenesis and begins fetal development (Noakes, 1997, p.30). Though Lubahn et al (1993) stated that after disrupting the estrogen receptor gene by gene targeting in the fetal mice could make them survive to adulthood with normal gross external body characteristic, those mice still demonstrated defects in the reproductive performances. In conclusion, the appearance of estrogens and the expression of estrogen receptors during this crucial period suggest potential functions of estrogens in fetal development, especially in the formation of the reproductive tract.

The average bovine gestation length is 281 days (Bearden & Fuquay, 1997, p.89). Throughout most time of pregnancy, bovine fetuses are surrounded and protected by amniotic and allantoic fluids. Those fluids arise from the secretion and excretion of the fetus. Besides water, these fluids contain metabolic products, proteins, electrolytes, hormones, and other structures (Baetz et al, 1976). Those constituents circulate through the fetal-placental compartment so as to participate in the processes of oxygen, nutrient and waste exchange, as well as endocrine communication with the mother.

Steroid hormones in the extracellular circulation are likely to be bound to proteins. Due to different protein binding characteristics, this binding may be either high affinity binding such as a specific steroid-binding globulin, or low affinity but high capacity binding to an abundant protein such as albumin. For example, alpha-fetoprotein (AFP) produced by fetal liver and present in fetal blood circulation has been well-studied and its specific estrogen binding properties were characterized in the rat (Aussel et al, 1973; Benassayag et al, 1975; Savu et al, 1975; Lai et al, 1976; Payne &

Katzenellenbogen, 1979; Keel & Abney, 1983). Relative amounts of estrogen available to influence target tissues might be dependent to some extent on the concentrations of steroid-binding proteins in the blood or fetal fluids. Estrogen binding proteins in the circulation serve to transport the hydrophobic steroids in aqueous media and may adjust the concentration of biologically available estrogens. Only free estrogens that are not bound to proteins have the ability to enter tissue cells, probably by simple diffusion (Clark & Peck, 1977). Then they are able to exert their crucial functions in the target cell nucleus mediated by specific receptors (Welshons et al, 1984). This estrogen-receptor complex is able to influence specific gene expression, altering the expression of mRNA transferred to the cytoplasm and assembled into the proteins at the ribosomes.

The total estrogen concentrations in maternal and fetal circulations throughout pregnancy in several farm animal species have been reported (Robertson et al, 1973, 1974, 1978, 1979, 1985; Challis et al, 1974, 1981; Lyngset & Lunaas, 1972; Carnegie & Robertson, 1978; Eley et al, 1979). The concentrations of free unconjugated estradiol-17β in the circulation, not bound to proteins, were not determined in any of these studies however. The free unconjugated estradiol-17β concentrations should more accurately reflect potential biological activity at the target cells. The objectives were as follows: 1) to measure the unconjugated total estradiol-17β concentrations in bovine fetal-placental compartment around mid-gestation; 2) to determine the free concentrations of unconjugated estradiol-17β in bovine fetal-placental compartment around mid-gestation; 3) to determine whether specific protein(s) is(are) present for transportation or sequestration of unconjugated estradiol-17β in bovine fetal circulation.

CHAPTER II

LITERATURE REVIEW

Bovine Fetal-Placental Compartment

Gestation is the course of pregnancy initiated from fertilization and completed with parturition (Bearden & Fuquay, 1997, p.89). Length of gestation averages 281 days in cattle. Variation in gestation length may arise due to breed as well as individual conditions from maternal, fetal and environmental aspects (Jainudeen & Hafez, 1993, p.214).

A fertilized ovum becomes a blastocyst at day 6 of gestation. The blastocyst has a single spherical outer layer of cells called the trophoblast and a group of cells at one polar end called the inner cell mass. The interior of the blastocyst is a fluid-filled chamber, called the blastocoele. Beginning at day 14, the blastocyst starts to elongate longitudinally and gradually transforms to threadlike appearance by day 18 of gestation (Peters & Ball, 1987, p.94). This process is called blastocyst elongation and it is also the period for maternal recognition of pregnancy.

Ectoderm, mesoderm and endoderm are the three germ layers that develop from the inner cell mass of blastocyst (Peters & Ball, 1987, p.94). Each germ layer gives rise to the formation of different organs and tissues of the developing fetus. From these

layers, extraembryonic membranes, the yolk sac, amnion and allantois, also start to form. These membranes help the embryo to attach the maternal endometrium so as to exchange gas, nutrients and hormones with the maternal system, following the process of bovine fetal implantation around day 30 of gestation (Melton et al, 1951). Implantation involves intimate connection of trophectoderm of the embryo and the uterine epithelial cells for oxygen, nutrient and waste exchange, as well as endocrine communication.

The yolk sac is a transient cavity only present at the early embryo stage before day 20 of gestation. It is the combination of endoderm and part of the separated mesoderm. Its blood circulation provides nutrients for the fetus and it also provides protection for the fetus. This function is soon replaced by the allantois after day 20 of gestation (Peters & Ball, 1987, p.94).

The amnion is an outgrowth from the mesoderm and ectoderm of the embryo and forms at days 13 to 16 of gestation (Noakes, 1997, p.29). It wraps over the whole fetus except a limited area around the umbilical cord. The amniotic unit is a double-walled sac with a transparent membrane. This sac is filled with fluid derived from the secretion and excretion of the fetus. Thus, it bathes the fetus for the major purpose of support and protection. The umbilical cord is one part of amnion wrapping about the yolk stalk and it encloses allantoic vessels to exchange nutrients, oxygen and wastes between the mother and the fetus.

The allantois, which outfolds from the embryonic hindgut around days 14 to 21 of gestation (Noakes, 1997, p.29), is also a double-walled cavity with watery fluid inside. It forms completely about days 24 to 28 of gestation and functions as a temporary storage

site for fetal excretory products and also helps to maintain fetal osmotic pressure of plasma (Jainudeen & Hafez, 1993, p.228).

Fetal fluids originate mainly from the fetal respiratory, urinary and digestive systems, and some from the fetal skin. The volume and composition of the fluids are also influenced by these sources (Jainudeen & Hafez, 1993, p.228). Both the amniotic and allantoic fluids contain metabolites of the fetus, electrolytes, enzymes, hormones, cells, and other constituents (Baetz et al, 1976). The total volume of amniotic and allantoic fluids increases progressively throughout pregnancy. Particularly there is a sharp volume rise from 400ml (days 61 to 90) to over 1,000ml (days 91 to 120) in bovine amniotic and allantoic fluids (Noakes, 1997, p.31). In general, these fluids function to protect the fetus from mechanical injury and from environmental infection.

The outermost layer of the conceptus, the trophectoderm, gives rise to the chorionic membrane. The allantois fuses completely with the chorion to form the chorioallantois by day 23 of gestation (Peters & Ball, 1997, p.94). The chorioallantois is a highly vascular structure with the vessels branching away from the umbilical cord. It eventually surrounds the whole entity of fetal-placental compartment. The chorioallantois attaches closely with the endometrium and serves in transferring of nutrients and oxygen from the maternal uterus to the fetus.

Ruminant placentas exhibit epithelial-chorial and endothelial-chorial barriers with diffuse and cotyledonary chorionic villous patterns (Jainudeen & Hafez, 1993, p.219). Structures called fetal cotyledons are actually restricted circular areas of chorioallantois. They are in close contact with specialized areas of maternal uterine endometrium called the caruncles. The placentome is a specialized structure in the ruminant placenta

consisting of fetal cotyledon and maternal caruncle. As illustrated in sheep (Makowski et al, 1968), 84% of uterine blood flow goes through maternal caruncles, while 94% of umbilical blood flow goes through fetal cotyledons. This provides maximal transportation of oxygen and nutrients to fetal circulation. Placental size and blood flow, genetics, environment and fetal hormones are major factors influencing fetal growth (Jainudeen & Hafez, 1993, p.225). A greater than 1100% increase in bovine fetal size appears between days 61 to 120 of gestation (Bearden & Fuquary, 1997, p.98). The placenta is a transitory endocrine organ during pregnancy like the corpus luteum. During the second and third trimesters of pregnancy, the cotyledon is a major site of production for estrogens (Evans & Wagner, 1981; Gross & Williams, 1988; Hoffmann et al, 1979). In addition, Shemesh (1980) found that estradiol-17β could be synthesized by fetal ovary as early as days 42 to 48 of gestation. Those hormones could be absorbed by the fetus or the mother and transformed to other conjugated forms such as sulfated estrogens, or they could be accumulated in amniotic and allantoic fluids throughout pregnancy.

Understanding Estrogens

Estrogens are involved in many critical physiological processes, including growth and differentiation of mammary and reproductive tissues and also brain development, by the way of affecting estrogen-responsive gene expression. Estrogens may be produced in ovary, adrenal cortex, placenta, and the testes in a variety of species including cattle (Mellin & Erb, 1965). The initial precursor of estrogens is acetate, which is converted to cholesterol. Then by continuing conversion from cholesterol to progesterone through a

complex but well organized process of synthesis, estrogens are finally formed by aromatization of androgens. In general, the sequence of this pathway is acetate → → → cholesterol → 20 α-hydroxycholesterol → pregnenolone → progesterone → 17-hydroxyprogesterone → androstenedione (or testosterone) → estrogens (Mellin & Erb, 1965). Estrone (E_1), estradiol (E_2) and estriol (E_3) are the three major naturally occurring estrogens. Various estrogens may be present in the fetal-placental compartment, although unconjugated estradiol-17β has the highest estrogenic potency (Sloss & Dufty, 1980). Clark & Peck (1979) found that estrogens penetrate into target cells by simple diffusion across plasma membranes. Those diffused hormones will bind to their specific receptors in cell nuclei (Welshons et al, 1984), which cause alteration of specific gene expression. The direct effects of estrogens are mediated by specific receptors. These effects may be to up-regulate or down-regulate target gene expression. On the other hand, those tissues that have few or no functional receptors will fail to respond to the normal circulating concentrations of these hormones. In general, four essential steps in this signal pathway of estrogen action have been demonstrated (Bearden & Fuquay, 1997, p.50): 1) the diffused estrogen binds to their receptors which are localized in the cell nucleus; 2) specific mRNA synthesis is modified by the estrogen-receptor complex; 3) specific protein synthesis is directed in the cytoplasm by the translocated mRNA from the nucleus; and 4) estrogen action on the target cells is controlled by those new proteins. This pathway could also be simply described as follows: steroid → (steroid-receptor) → (steroid-receptor-DNA) → mRNA → protein → functional response.

As a result of metabolism, steroids are cleared from the body by enzymes mostly located in the liver designed to inactivate them and to reverse their solubility in aqueous

media, i.e., by sulfation or hydroxylation. Those sulfated and hydroxylated steroids are called conjugated steroids and they help to keep a dynamic equilibrium with unconjugated steroids for normal body needs and facilitate excretion. The urinary system is considered as the major excretory pathway of estrogens (Mellin & Erb, 1965).

Extracellular Interactions of Steroids with Proteins

Some steroid hormones in the extracellular circulation are readily bound with two high-affinity specific proteins, which are generally called sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) (Hammond, 1993, p.1). Albumin also plays a role in steroid-protein interaction but with low affinity and limited specificity. However, though the association constant of steroids is low with albumin, the large amount of this protein present in circulation still attracts a large portion of hormones to form complexes (Westphal, 1986). Those extracellular proteins also function as hormone carriers and transporters in body circulation. In short, they regulate the concentrations of biologically available hormones in body fluids and influence the rates of metabolic clearance of those active steroids.

Some steroid-binding proteins have been studied extensively in various species, such as androgen (or testosterone) binding protein (Corvol & Bardin, 1973; Hagenas et al, 1975 and Cheng et al, 1985). Estrogens have a high affinity binding protein named alpha-fetoprotein (AFP) detected in pregnant rats and some abnormal adult rats, according to numerous reports (Aussel, et al, 1973; Benassayag et al, 1975; Savu et al, 1975; Lai et al, 1976; Payne et al, 1979; Keel & Abney, 1983). As reported by Keel &

Abney (1983), the Ka (equilibrium association constant) value determined by Scatchard analysis for the rat AFP-estradiol complex was $(2.83 \pm 0.78) \times 10^8\ M^{-1}$. It was about 50-200 fold lower than the affinity of estrogen receptor for estradiol-17β.

However, so far, the literature contains contradictory and somewhat perplexing reports with respect to estrogen-binding proteins in other species. No article has explicitly declared that there was any specific estradiol binding protein or group of proteins present in fetal-placental fluids of domestic animals, including cattle. One study (Nunez et al, 1974) determined that the amount of a specific estradiol-binding protein in human fetuses and their umbilical cord blood was extremely low up to the 5th month of pregnancy, and then increased progressively. Murphy (1967) was able to measure the specific radiolabeled estradiol binding to proteins in bovine plasma generally termed plasma globulin, but ignored the effects of background non-specific binding counts, resulting in an overestimation of the degree of specific binding. Fridlansky & Milgrom (1982) made a general conclusion that the binding of estradiol with proteins was nonspecific and implied that the important protein-estrogen interactions occurred as a result of low-affinity binding. It appeared that natural estrogens bound weakly with uteroglobin and [^{3}H]estradiol binding was comparatively about 500-fold lower than that of progesterone.

Protein Patterns in Fetal-Placental Compartment during Pregnancy

There are various reports in relation to protein patterns in fetal blood and fluids during pregnancy, but all are somewhat incomplete for covering the whole duration of

pregnancy in cattle. The functions of many proteins produced in the fetal-placental compartment during pregnancy remain unclear.

Uteroglobulin, also called blastokinin, is a low-molecular-weight protein (MW 15,000). It was usually secreted in the uterus of early pregnant rabbits, composing up to 50% of the total proteins present, although in other species it was present in much lower amounts than in the rabbits (Arthur & Daniel, 1972).

Uteroferrin, a protein providing a major source of iron in endometrial secretion, was examined in the fetal pig by Ducsay et al (1986). They found that this protein could be stored in placental and endometrial tissues as well as fetal fluids. The MW of this protein was not stated in their report.

Roberts and Parker (1974) reported the similarity of the macromolecules in bovine uterine fluids to those present in serum, and were able to detect some minor components defined as uterine-specific proteins. With further investigation published in 1976, they concluded that these minor but specific proteins might have the power of controlling luteolysis during pregnancy. Those minor proteins varied from MW of 11,000 to 48,000 daltons and were present at different stages of pregnancy. They found several of these proteins also appeared in amniotic and allantoic fluids, suggesting that there was a pathway from the uterine lumen to those fluids.

A chorionic gonadotropin-like protein from bovine cotyledons was classified by Ailenberg & Shemesh (1983) by the assessment of luteotrophic activity in the placenta. This may serve to maintain the corpus luteum of pregnancy. Another protein with a MW estimated at 68,000 daltons was identified in bovine allantoic fluid around days 24 to 37 of gestation. It was shown to have a vitro luteotrophic activity (Hickey et al, 1989).

Since the period of maternal recognition of pregnancy occurs before day 20 of gestation, these luteotrophic proteins may be the conceptus-derived stimuli to help maintain progesterone production by the corpus luteum of cows throughout pregnancy.

Progesterone-induced proteins called uterine milk proteins (MW = 55,000 and 57,000 daltons), were found and purified in the uterine secretion of sheep (Moffatt et al, 1987) and were present only during pregnancy. Extending this result, Newton (1989) demonstrated that these proteins also existed in fetal fluids and may be functional in those fluids after crossing the placenta from endometrium.

Rat AFP was first confirmed by Aussel and his associates (1973) as the fetus-specific serum protein with MW around 72,000 daltons. Smith et al (1979) then demonstrated its distribution in fetal plasma, allantoic and amniotic fluids, and maternal plasma of cows, with maximal concentration of AFP obtained by RIA between the 3rd and 4th month of gestation. This protein was further analyzed in pre- and post-implantation periods of bovine embryo by Janzen et al (1982). Here they found the concentration was much higher in embryonic tissues (first detected in day 14 of trophoblasts) and fluids (detected early as day 16 of gestation in the allantoic fluid) than in the maternal serum. The high level in the uterine fluid led to the conclusion that there was significant transfer of this protein from conceptus to uterine lumen by transudation across embryonic membranes. He & Keel (1994) detected differences in both the charge and lectin microheterogeneity of bovine and human AFP, suggesting the possibility of different binding properties. Baker (1988) analyzed the sequences of AFP and albumin and found the two proteins were related. Hsia et al (1986) suggested that AFP might increase the fetal uptake of diethylstibestrol (DES) in human and bovine species. But in

rat DES interacted weakly with AFP (Sheehan & Young, 1979). This suggested that bovine might be a better model to study hormone metabolism concerning human diseases than rodents. Nevertheless, no data in their study addressed binding property of bovine AFP with natural estrogens. Carlsson et al (1980) suggested that AFP might participate in the fetal metabolism of the long-chain polyunsaturated fatty acids by its property of high affinity binding to these fatty acids. This data suggested that AFP might exhibit specific binding with the steroid hormones in some species since steroids were lipid molecules.

Fetuin is another fetal protein discovered by Lai et al (1981). Later Suzuki et al (1994) found fetuin in bovine fetal serum could bind to calcium. This protein also participated in lipid transport during fetal development (Kumbla et al, 1991). Thus, fetuin seems to play roles in lipid metabolism during fetal development.

Albumin, fetuin, transferrin, AFP, alpha 1-acid glycoprotein, alpha 1-antitrypsin and alpha 2-macroglobulin were all identified in the porcine fetal fluids (McKenna, 1984). However, the functions of those proteins in the fetus need to be further explored.

Estrogen Concentrations throughout Pregnancy

Although considerable attention has been devoted to the study of estrogens in farm animals during pregnancy, most reports concentrated on maternal level (Robertson & Smeaton, 1973; Dobson & Dean, 1974; Robertson, 1974; Robertson & King, 1974; Robertson, et al, 1978). As indicated in the report of Dobson & Dean (1974), the unconjugated estradiol-17β concentration in non-pregnant cows is from 5 pg/ml to 14

pg/ml at estrus from early to late cycle. In the last stage of pregnancy, estradiol-17β concentrations range from 400 pg/ml 14 days before parturition to 1000 pg/ml one day before parturition, and 300 pg/ml on the day of parturition.

Most of the published data did not clarify whether the estrogen concentrations were present in a free or protein-bound state, a result of the limitation of the assays they chose. Challis et al (1974) reported the concentrations of total unconjugated estradiol from early to late gestation in the serum of fetal calves taken from mixed umbilical cord blood ranged from 184 pg/ml (month 8 of fetal age) to 1022 pg/ml (month 6 of fetal age). Robertson & King (1979) measured the unconjugated estradiol level in bovine amniotic and allantoic fluids. Due to the sensitivity limit of 40 pg/ml in their radioligand assay, they failed to obtain any significant changes in estradiol concentration since most values were less than the limit of sensitivity with just one or two individual exceptions as high to 400 pg/ml (around days 170 to 200 of gestation). Maternal plasma estradiol concentration was almost steady throughout pregnancy. Tsumagari et al (1993) found that estrone concentrations in bovine cotyledons and caruncles during gestation and parturition exhibited a biphasic pattern, which is, the maximum synthesis occurred at month 5 of gestation and immediately after parturition.

Carnegie & Robertson (1978) concluded that sulfoconjugated estradiols levels in fetal-placental fluids of the pregnant ewe ranged from 20 pg/ml (day 130 of gestation) to 1.6 ng/ml (day 60 of gestation). The highest concentration appeared early around day 60 of gestation in both fluids, declined gradually as fetal age advanced.

In swine, the estrogen level was characterized in both maternal uterine blood and amniotic and allantoic fluids (Knight et al, 1977). From the available samples (day 20 to

day 100 of gestation), the range of estradiol was from 1.4 pg/ml (day 40 of gestaion) to 34.8 pg/ml (day 100 of gestation) in venous blood, the range of estrone was from 0.5 ng/ml (day 40) to 537.7 ng/ml (day 100) in the allantoic fluid, and the range of estrone in amniotic fluid was from 73.8 pg/ml (day 40) to 31029.7 pg/ml (day 100).

Robertson et al (1985) found that the concentration of estrogens in fetal allantoic fluid reflected the concentration in maternal plasma in pig. This contrasted the result found in the ewe (Carnegie & Robertson, 1978) and in the cow (Robertson & King, 1979), in which the concentrations in fetal compartments of these two species were not related to maternal plasma levels.

Montano et al (1995) were able to measure the free fraction of fetal serum estradiol-17β using a method called centrifugal ultrafiltration. This method separated the protein bound and free fraction across a dialysis membrane. The range of this free estradiol portion was around 0.54 (day 19) - 2.17 (4 hours after birth) pg/ml during the fetal and early neonatal period of sexual differentiation in female rats. They also found the [^{3}H]estradiol injected into blood could be detected in fetal rat brain later. This finding demonstrated the potential biologically active role of circulating estradiol-17β in sexual differentiation of the brain, which was contrary to common hypotheses that "alpha-fetoprotein effectively sequesters the circulating estrogen in a biologically inactive form" (MacLusky, 1988, p.249).

CHAPTER III

EXPERIMENTAL OBJECTIVES

The main hypothesis of this project was to determine whether the free fraction of unconjugated estradiol-17β circulating in fetal blood during bovine mid-gestation is sufficient to activate estrogen-responsive gene expression.

To test this hypothesis, we divided the project into three portions:

(1) To verify previous results, we measured total concentrations of unconjugated estradiol-17β by radioimmunoassay in bovine fetal blood, amniotic and allantoic fluids collected around mid-gestation.

(2) To test the hypothesis that significant fractions of non-protein-bound unconjugated estradiol-17β differ in those fetal fluids, we determined by centrifugal ultrafiltration the portion of unconjugated estradiol-17β free to transverse a dialysis membrane with a molecular weight cut-off of 30,000 daltons.

(3) To test the hypothesis that difference in the ratio of unconjugated free estradiol-17β among those fetal fluids may be caused by shifts in the expression of binding protein(s) present in those fluids, we examined the array of proteins in these fluids by one dimensional polyacrylamide gel electrophoresis under both SDS and native

conditions. Ligand blotting was then used to test [^{125}I]estradiol binding to specific proteins. Dot blotting was used to determine whether total proteins isolated from those fetal fluids would bind [^{3}H]estradiol specifically.

CHAPTER IV

MATERIALS AND METHODS

Sample Sources

The fetal fluids were collected from 39 crossbred beef cows ranging from day 31 to day 190 of gestation at slaughter. The uterus was removed and aseptic collection of allantoic fluid was accomplished via needle puncture through the wall of contralateral uterine horn. The chorioallantois was then opened to drain off excess allantoic fluid and to expose the amniotic sac. The amniotic fluid was aspirated through the amniotic membrane carefully with minimum contamination. Following the aspiration of the amniotic fluids, heparinized fetal blood samples were obtained by heart puncture rather than from the umbilical cord. All the sample tubes were put on ice and transferred to the lab within the same collection day. Then the samples were centrifuged at 10,000-g force and the supernatants were stored at –20°C until analysis.

Fluids from both male and female fetuses were collected. Fetal estrogen concentrations were previously determined by Robertson et al (1979) and Tsumagari et al (1993) that no differences present between males and females during mid-gestation of pregnancy. Gestation age was estimated by measuring fetal crown-rump length (CRL) according to a standard formula (Rexroad et al, 1973), which is: "day of gestation = 8.4

+ 0.087 CROWN-RUMP + 5.46$\sqrt{\text{CROWN-RUMP}}$". The crown-rump length (CRL) is the fetal measurement from the vertex of the skull to the root of the tail.

Unconjugated Estradiol-17β Radioimmunoassay

Unconjugated estradiol-17β radioimmunoassay was performed by a modified procedure according to the manufacturer's recommended protocol (DPC, Los Angeles, CA). Sample volumes were arranged 200 ul per test tube and quantification was done with a sensitive double antibody [^{125}I]estradiol-17β RIA kit. All samples were measured in duplicates. Sensitivity of this assay was 3.125 pg/ml, and the linear range extended to 200 pg/ml. Intraassay coefficient of variation (CV) was 2.7%; interassay CV was 6.8%. The standard curve (Figure 1) was prepared in charcoal-extracted adult cow plasma (B_0 plasma). Samples were diluted from 5- to 40- fold with B_0 plasma, as needed, to obtain measurements of unconjugated estradiol-17β concentration within the linear portion of the standard curves below 200 pg/ml. If applied, concentrations were determined by multiplying the appropriate dilution factors.

For the validation of this radioimmunoassay in the fetal blood, amniotic and allantoic fluids, serial dilution was performed by preparing serial dilutions in B_0 plasma (Figures 2, 3 & 4) and arranging to determine the appearance of parallel displacement curves. Pure unlabeled unconjugated estradiol-17β was added to the fetal blood, amniotic and allantoic fluids to determine percent recovery rate (Figures 4, 5 & 6) of the unconjugated estradiol-17β in these fluids.

Centrifugal Ultrafiltration

Centrifugal ultrafiltration was performed for the purpose of measuring the free fraction of unconjugated estradiol-17β more accurately from undiluted samples. First, those fluid samples were assayed by RIA to determine unconjugated estradiol-17β concentrations as described above. Then, a modified procedure was adapted by using the MPS micropartition system (Amicon, Beverly, MA), to separate free from protein-bound microsolutes. Briefly, aliquots of samples (500 ul) were incubated with 2 ul highly purified radiolabeled [^{3}H]estradiol (440,000 dpm; specific activity is 1mCi/ml), at 37°C for 30 minutes and then at room temperature for another 30 minutes to equilibrate the radiolabeled estradiol with the total sample pools. Aliquots of 20 ul each were then transferred to scintillation vials to determine the representative total [^{3}H]estradiol values in those biological fluids by scintillation spectroscopy (Beckman, Fullerton, CA). Aliquots of 200 ul were then loaded onto the YMT membranes having a molecular weight cutoff of 30,000 daltons. They were then centrifuged in a fixed-angle rotor at 2000 x g for 10 min. The centrifuge (Eppendorf, Westbury, NY) was prewarmed to mimic in vivo temperature. Aliquots of 20 ul ultrafiltrate each were then transferred to scintillation vials, and [^{3}H]estradiol was measured the same as described above. The ratio of free versus total unconjugated estradiol-17β concentration was calculated from the relative counts of [^{3}H]estradiol in the ultrafiltrates and the original samples, after adjusting the background counts.

% free [^{3}H]estradiol = cpm from ultrafiltrates / cpm from the original samples

The actual concentrations of free unconjugated estradiol-17β were then determined from the % free [^{3}H]estradiol using the above formula. All the samples were run in duplicates.

Whether the membranes would be blocked by any sample impurities or particles during the process of centrifugation is a determining factor for validation of this method. This was tested by serial dilution of a pool of fetal blood (neat, 1:2, 1:4, 1:8) with PBS buffer and measuring the percentage of free unconjugated estradiol-17β (Figure 10). It was expected that a linear increase in the free unconjugated estradiol-17β percentage from PBS diluted samples would indicate the lack of interference from components of undiluted samples. Similarly, with the addition of increasing amounts of bovine serum albumin (BSA) to distilled water, it was expected that corresponding decreased amounts of free unconjugated estradiol-17β percentage would indicate a linear relationship between protein and free unconjugated estradiol-17β concentration (Figure 11).

Conversely, in order to address the possibility of any saturation effect of [^{3}H] estradiol to the accurate determination of dramatically different unconjugated estradiol-17β concentrations from various samples, pooled fluids were incubated with increasing concentrations of unlabeled estradiol-17β and a fixed mass of [^{3}H]estradiol. Addition of nonradioactive unconjugated estradiol-17β was expected to result in a similar percentage transfer of the free unconjugated estradiol-17β present under nonsaturating conditions.

Protein Determination

The frozen samples were thawed at room temperature and concentrated by using microconcentrators (Microcon 10) with MW cut-off of 10,000 daltons (Amicon, Beverly, MA). All the small molecules including free unconjugated estradiol-17β were regarded as having been centrifuged out of the samples. The protein concentrations of these samples were then determined by the method of Lowry (1951) with bovine serum albumin as a standard.

One Dimensional Gel Electrophoresis

One dimensional polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS-PAGE) or in the absence of SDS (native PAGE) by using a Mini-Protean®II Electrophoresis Cell (Bio-Rad, Hercules, CA). The procedures were described by Laemmli (1970). One microgram of each protein sample was loaded in each lane of gels. Following the PAGE, some SDS-gels were stained by silver staining. Briefly, the steps were: 1) gels were submerged in 10% glacial acetic acid for 20 minutes; 2) gels were rinsed three times with distilled water, each wash for two minutes; 3) gels were soaked for 30 minutes in staining solution (silver nitrate, 10 mg/10 ml dH_2O and 37% formaldehyde 15 ul/10 ml dH_2O); 4) gels were rinsed with distilled water for 20 seconds; 5) gels were developed in developer solution (sodium carbonate, 0.3 mg/10 ml dH_2O; 37% formaldehyde 15 ul/10 ml dH_2O and sodium thiosulfate, 20 ug/10 ml dH_2O) which has been chilled to 10-12°C in an ice bath, until the bands became visible; 6) developing reaction was terminated by adding an equal volume of 10% glacial

acetic acid and incubating for 2 to 3 minutes; 7) gels were rinsed for 5 minutes in distilled water; 8) gels were dried under vacuum. For further study, other SDS and native gels were directly transferred on acid-etched glass filter fibers (GFF) after PAGE as described follows.

[^{125}I]Estradiol Ligand Blotting

Following SDS and native PAGE, the gels, the acid-etched GFF, and filter paper were equilibrated in 1.0% acetic acid for 30 minutes at room temperature. They were then assembled into a blotting sandwich apparatus as described by Aebersold (1985). The blotting solution was 1.0% acetic acid equilibrated to 0-4°C. Electroblotting was performed at 80 V and 300 mA for approximately 12 hours. The GFF membranes were then equilibrated with 1.0% acetic acid again for 30 min and incubated overnight at 4°C with 2.25 x 10^6 dpm [^{125} I]estradiol (specific activity: 2,200 Ci/mmol) in 30 ml TN buffer solution (50 mM Tris, pH 7.3 and 200 mM NaCl). No blocking procedure was necessary for this specific activated GFF membrane.

In the next morning the incubated membrane was washed with TN buffer six times with 5 minutes each time, as recommended by Jefferies (1989), and dried under 37°C. Membranes were then assembled in an cassette with an intensifying screen in it and exposed to Kodak XAR film at –70°C for at least 3 days to 15 days for detection.

[^{3}H]Estradiol Dot Blotting

A dot blotting procedure for protein binding was adapted by the manufacturer's recommendation (Bio-Dot Microfiltration Apparatus, Bio-Rad, Hercules, CA). GFF membrane was dehydrated in distilled H_2O for 5 min and dried under open air before assembly. Each 100-ul of triplicate samples (approximately 10-20 ug proteins) was dot-blotted and dried on the membrane before incubating with various concentrations ranging from 0.2 nM to 30 nM of [^{3}H]estradiol (specific activity: 1mCi/ml) for 30 minutes at 37°C and another 30 minutes at room temperature consecutively. Only single animals from each interval were tested due to the limited sample sources. Specific binding of estradiol-17β to proteins was measured by subtracting the counts of estradiol-17β bound after incubation in [^{3}H]estradiol solution plus 200-fold excess unlabeled estradiol-17β (cold) from the values of samples incubated in [^{3}H]estradiol (hot) alone.

This method was validated by applying serial concentrations of fetal blood proteins (Figure 17) or bovine serum albumin (data not shown) on a GFF. It was expected that more specific estradiol-17β binding counts would be observed as protein concentrations were increased.

Statistical Analysis

The data obtained from radioimmunoassay and centrifugal ultrafiltration were analyzed with the general linear model procedure (GLM) of the Statistical Analysis System (SAS, 1985), after the natural logarithmic (LN) transformation of the original

data in order to get the equal variances among different groups. Starting from day 31 of gestation, twenty-day periods were grouped together as the independent intervals. Samples within the same period were collected together to calculate the mean value and standard error of this group. Analysis of variance (ANOVA) procedure and least significant difference (LSD) tests were chosen to compare whether the statistically significant difference existed at the α level of 0.05 among the mean values of different intervals within each fluid respectively.

CHAPTER V

RESULTS

Comparison of Total Unconjugated Estradiol-17β Concentrations

The mean concentrations of total unconjugated estradiol-17β in fetal blood, amniotic and allantoic fluids were determined by radioimmunoassay. The standard curve (Figure 1) of this assay was prepared in adult charcoal-extracted cow plasma (B_0 plasma). Sensitivity range of this assay was 3.125 pg/ml, and the linear range extended to 200 pg/ml. The intraassay coefficient of variation (CV) was 2.7%, and the interassay CV was 6.8%. As shown in Figures 2 to 7, the radioimmunoassay was validated in each of the different fluids through demonstration of the parallel displacement curves in each fluid, and through the high recovery rate (98% in the fetal blood, 98% in the amniotic fluid, and 99% in the allantoic fluid) of actually measured total unconjugated estradiol-17β concentrations. The correlation coefficient (R^2) of those linear trendlines were all within the acceptable 0.90 range. Therefore, there were no significant matrix effects in fetal blood, amniotic and allantoic fluids.

As shown in Table I, unconjugated total estradiol-17β was detectable in our earliest samples from all of these three fluids, around days 51 to 70 in the fetal blood, days 31 to 50 in the amniotic and the allantoic fluids. There was considerable variation

among individual animals throughout this duration in total unconjugated estradiol-17β concentrations. In the fetal blood, the highest concentration appeared during the period from day 111 to day 130 of gestation, when the mean value was 130.17 pg/ml. The lowest concentration was 49.59 pg/ml measured during the period between days 151 to 170. In the amniotic fluid, the highest concentration was also observed between days 111 to 130 when the mean concentration was 584.14 pg/ml, followed by a decline in the next twenty days. A second sharp rise was observed about day 151 to day 170 as shown by the mean value of 535.21 pg/ml corresponding to the low concentration in the fetal blood. The lowest mean total unconjugated estradiol-17β concentration in the amniotic fluid was 9.47 pg/ml during the early gestation period around days 31 to 50. The allantoic fluid contained the greatest concentration of total unconjugated estradiol-17β at the same time as in the fetal blood and amniotic fluid (days 111 to 130) when the mean value was 1916.17 pg/ml. A second period of elevated total unconjugated estradiol-17β concentration in the allantoic fluid appeared earlier, from day 71 to day 90 when the mean value was 1317.13 pg/ml. The lowest mean value in the allantoic fluid was also present at the earliest measured time, around days 31 to 50, when the mean value was 11.67 pg/ml. All of the above results from bovine fetal fluids were in the range reported by the others (Challis et al, 1974; Eley et al, 1979; Robertson & King, 1979). Fetal blood from the interval of day 31 to day 50 of gestation was unavailable.

Figures 8 & 9 showed the mean concentrations of total unconjugated estradiol-17β in the fetal blood, amniotic and allantoic fluids after natural logarithmic (LN) transformation of those values. The ln mean concentrations in the amniotic and allantoic fluids demonstrated significant differences among different periods ($P<0.05$) within each

fluid respectively. In the amniotic fluid, the ln mean concentrations of total unconjugated estradiol-17β from days 71 to 190 were significantly greater ($P<0.05$) than the previous period. The ln mean value of total unconjugated estradiol-17β from days 51 to 70 was also significantly higher ($P<0.05$) than the ln mean concentration from days 31 to 50. In the allantoic fluid, the ln mean concentrations of total unconjugated estradiol-17β during the periods from day 71 to day 90 and day 111 to day 170 were significantly greater ($P<0.05$) than the other periods. The ln mean values of total unconjugated estradiol-17β from days 91 to 110 and from days 171 to 190 were also significantly higher ($P<0.05$) than the ln mean concentration from days 31 to 70. There was no significant difference for the ln mean concentrations of total unconjugated estradiol-17β present in the fetal blood.

Comparison of Free Unconjugated Estradiol-17β Concentrations

The centrifugal ultrafiltration method for separating free unconjugated estradiol-17β from protein bound concentration was validated by testing the fractionation of unconjugated estradiol-17β in the presence of decreasing concentration of fetal blood proteins and BSA. As shown in Figures 10 and 11, there was a linear relationship between protein concentration (after LN transformation) and estradiol binding, through the range of 1.82 mg/ml to 14.56 mg/ml (fetal blood samples), and 0 mg/ml to 1mg/ml (BSA) respectively. R-squares were above 0.97. This type of validation was similar to the procedure done by Montano, et al (1995).

To determine whether unconjugated estradiol-17β concentration resulted in the saturation of estradiol-17β binding sites, non-radioactive estradiol-17β was added to determine whether the dynamics of labeled estradiol movement across the dialysis membrane would be altered. Since addition of nonradioactive estradiol-17β resulted in similar percentage recovery rate of labeled estradiol (Table II), it suggested that no saturation effect occurred.

Free unconjugated estradiol-17β concentration in the amniotic and allantoic fluids averaged 70% of the total. In the fetal blood, it was from 6% to 37%. Free unconjugated estradiol-17β concentration followed a similar change pattern as total unconjugated estradiol-17β concentration, shown in Table I and Figures 12 & 13. During mid-gestation, the mean concentrations ranged from 7.46 (days 31 to 50) to 447.44 (days 111 to 130) pg/ml in the amniotic fluid and from 10.19 (days 31 to 50) to 1280.73 (days 111 to 130) pg/ml in the allantoic fluid. While in the fetal blood, the highest mean concentration (23.91 pg/ml) appeared during the period from days 91 to 110. The lowest mean concentration was 4.08 pg/ml in the interval of days 131 to 150. The overall variation in the fetal blood was not as dramatic as in the amniotic and allantoic fluids, suggesting tighter regulation of free unconjugated estradiol-17β in blood circulation. After LN transformation of those data, the mean values of free unconjugated estradiol-17β in the amniotic and allantoic fluids also demonstrated significant differences among different periods ($P<0.05$) within each fluid respectively. In the amniotic fluid, the ln mean concentrations of free unconjugated estradiol-17β from days 71 to 170 were significantly greater ($P<0.05$) than other periods. The ln mean values of free unconjugated estradiol-17β from days 51 to 70 and from days 171 to 190 were also

significantly higher ($P<0.05$) than the ln mean concentration from days 31 to 50. In the allantoic fluid, the ln mean concentrations of free unconjugated estradiol-17β during the periods from day 71 to day 90 and day 111 to day 170 were significantly greater ($P<0.05$) than other periods. The ln mean values of free estradiol-17β from days 91 to 110 and from days 171 to 190 were also significantly higher ($P<0.05$) than the mean concentration from days 31 to 70. There was no significant difference for the ln mean concentrations of free unconjugated estradiol-17β present in the fetal blood.

Protein Profiles in the Fetal Blood, Amniotic and Allantoic Fluids

Figures 14, 15 & 16 represented the typical protein profile at each period in fetal blood, amniotic and allantoic fluids respectively. These profiles were limited to proteins from MW 10,000 to 200,000 daltons. Major protein bands of approximately 70,000, 66,200 (BSA), and 50,000 daltons appeared in the fetal blood and amniotic fluid (Figures 14 and 15). The band at 70,000 daltons corresponded to the reported size of AFP (Aussel et al, 1973). The amounts of protein bands of MW 70,000 and 50,000 daltons appeared to be decreased as the gestation time prolonged. The albumin band seemed to keep constant. The concentrations of protein bands in the amniotic fluid were much less than those in the corresponding fetal blood.

In the allantoic fluid, several proteins could be seen below MW 45,000 daltons and were present with low amounts (Figure 16). And at the early two periods (day 51 to day 70 and day 71 to day 90), major products around 200,000 daltons were clearly present. Interestingly, this high MW band disappeared as the gestation age advanced

beyond 80 days of gestation. Similar protein profiles in the fetal blood and fluids were also demonstrated by Newton et al (1989) in sheep.

[^{3}H]Estradiol Dot Blotting

Binding of [^{3}H]estradiol to proteins from bovine fetal serum on the membrane Glass Filter Fiber (GFF), using the dot blot procedure was shown in Figure 17 as the validation procedure. Increasing concentrations of serum proteins resulted in a linear increase in [^{3}H]estradiol binding. Likewise, linear increments in binding were observed with increasing concentrations of BSA placed in dot blots (data not shown). Non-specific binding counts remained almost constant with increasing concentration of protein. Therefore, the GFF was efficient for binding proteins to do the dot blotting procedure.

[^{3}H]estradiol binding assay was performed in equal volumes (100 ul) and approximately equal concentraions (10-20 ug) of proteins from the fetal blood and fluids by dot blotting (Figures 6, 7 & 8) under the same conditions. No significant variations with fetal age were observed in any of the fluids. Total binding was low, around 70-cpm in the fetal blood (except for one point at day 111 of gestation), around 60-cpm in the amniotic fluid and around 100-cpm in the allantoic fluid (except one point at day 60 of gestation). Comparatively high non-specific counts were measured in all these fluids.

The high non-specific binding counts suggested that in these three fluids, there existed a high amount of unconjugated estradiol-17β binding protein(s) with low affinity but high capacity binding characteristics. This data could be compared to the results of Rebuffe-Scrive et al (1990) and Mizutani et al (1994), in which the adipose tissue

samples they used exhibited a high nonspecific binding of estradiol-17β in the lipid environment. The slight higher binding counts present in the allantoic fluid suggested that there might exist an estradiol binding protein with higher specific binding characteristics in this fluid.

[^{125}I]Estradiol Ligand Blotting

Using a modification of the method of Jefferies (1989), binding protein(s) were tested by blotting with radiolabeled hormone. Proteins were resolved on 12% SDS or native polyacrylamide gels and electrophoretically transferred to GFF. The problem of high background binding of estradiol was avoided by using the GFF. The transferred protein bands could be seen on the GFF membrane by coomassie blue staining. Radiolabeled [^{125}I]estradiol (specific activity: 2200Ci/ml) was used as the probe for specific estradiol-17β binding. Using [^{125}I]estradiol concentrations of 20nM and prolonged incubation times, no specific bands were evident on autoradiographs after 15 days exposure to X-ray films.

Taken together, these results suggest that a low affinity, high capacity binding to one or more proteins which may include AFP or albumin was present in the fetal blood, amniotic and allantoic fluids.

CHAPTER VI

DISCUSSION

Cattle are important animals all around the world for providing many products including milk, meat, clothing, fertilizer and fuel. The economic importance of cattle underscores the value of detailed investigations to maximize the potential production of these animals. Among these is the improved understanding of the hormonal impact upon bovine fetal growth and development.

Our results showed that between 31 and 190 days of gestation, the mean values of total unconjugated estradiol-17β concentrations ranged from 49.59 (around days 151 to 170) to 130.17 pg/ml (around days 111 to 130) in bovine fetal blood, from 9.47 (around days 31 to 50) to 584.14 (around days 111 to 130) pg/ml in the amniotic fluid, and from 11.67 (around days 31 to 50) to 1906.17 (around days 111 to 130) pg/ml in the allantoic fluid. These mean values were within the ranges reported by others (Challis et al, 1973; Eley et al, 1979; Robertson & King, 1979), and exhibited patterns of increased and decreased concentrations similar to those reports. The total unconjugated estradiol-17β concentrations obtained from fetal blood circulation were lower than the mean values reported by Challis et al (1973). One explanation for this was that our samples were taken from direct fetal heart puncture, while Challis et al collected a mixture of umbilical blood. Samples from heart puncture should represent more precisely the hormone

concentration in the fetal post-hepatic circulation. The total volumes of bovine amniotic and allantoic fluids increase sharply from 400 ml (around days 61 to 90) to over 1,000 ml (around days 91 to 120) (Noakes, 1997, p.31). A greater than 1100% increase in bovine fetal size also occurs during this period (Bearden & Fuquay, 1997, p.98). The significantly higher ($P<0.05$) ln mean concentrations of unconjugated estradiol-17β starting from day 71 of gestation in the amniotic and allantoic fluids suggested that the unconjugated estradiol-17β might be a key factor accounting for this dramatic volume change and related rapid fetal growth at the beginning of bovine mid-gestation. Rice et al (1993) also concluded from their data that estrogens in the amniotic fluid was the major endocrine factor to influence the electrolyte permeability of the porcine placenta and fetal membranes to sodium and choloride. Decreased electrolyte permeability caused by estrogens would accumulate more fluids in the fetal-placental compartment. Our data also showed that the concentrations of unconjugated total estradiol-17β were in excess of those found during much of adult life. This results from the significant production by the placenta during pregnancy (Evans & Wagner, 1981). In early gestation, the fetal ovary might be a site for production of unconjugated estradiol-17β. As gestation advanced, this site is transferred to the placentome resulting in an even higher amount of unconjugated estradiol-17β being present in the fetal-placental compartment.

The range of mean unconjugated free estradiol-17β concentration in bovine fetal blood was 4.08 (around days 131 to 150) to 23.91 (around days 91 to 110) pg/ml. This concentration was within the range of unconjugated estradiol-17β concentration, which is 0.01 to 0.1 nM or about 2.5 to 25 pg/ml, required to activate intranuclear estrogen receptors. Thus, circulating unconjugated free estradiol-17β concentrations are sufficient

to elicit hormone responses in target cells. Even greater mean unconjugated free estradiol-17β mean concentrations were present in the amniotic (7.46 to 447.44 pg/ml) and allantoic (10.19 to 1280.73 pg/ml) fluids. It is unclear whether the fetus would be exposed to the highly elevated unconjugated free estradiol-17β concentration in the amniotic and allantoic fluids. Unconjugated free estradiol-17β in the amniotic fluid might be swallowed by the fetus and recirculated. Unconjugated free estradiol-17β in the allantoic fluid is more likely to be transported to the maternal circulation for excretion or maternal usage. For instance, those high concentrations of unconjugated free estradiol-17β might be transformed to sulfated estradiol-17β by sulfatase enzyme in bovine placenta to facilitate transportation to the maternal circulation for excretion. A dermatological condition in human neonates known as X-linked ichthyosis is associated with insufficient exposure to estrogens during pregnancy (Zalel et al, 1996). It is unknown whether a similar condition occurs in domestic animals.

Our protein profile gels (limited to 1 ug per lane) indicated that bovine fetal blood and amniotic fluid contained similar abundant protein bands. This suggested that they were in an exchangeable circulation loop to maintain normal fetal environment. The protein bands of MW 70,000 and 50,000 daltons were similar to two reported bovine pregnancy-specific protein bands, which were bovine α_1-fetoprotein (AFP) and protein B, detected in the extracts of endometrium and extraembryonic fluids by Butler et al (1982). The amounts of these two bands appeared to decrease as the gestation prolonged, suggesting the different protein needs for different time. Specifically, the band at MW approximately 70,000 daltons corresponded to the reported size range of rat AFP of MW 72,000 daltons (Aussel et al, 1973) and to a protein of MW 68,000 daltons identified

around days 24 to 37 of bovine gestation which was shown to have in vitro luteotrophic activity (Hickey et al, 1989). If it were the luteotrophic protein, it would be interesting to observe its presence after the time of maternal recognition of pregnancy that is before day 20 of gestation. Further confirmation of this protein band could be done by western blotting using specific antibodies. The major protein bands shown up in the allantoic fluid were different from the amniotic fluid and fetal blood, and varied by stage of pregnancy. This was similar to the finding of Newton et al (1989) in sheep. Those protein bands may have been either secreted directly by bovine placentomes or transudated from bovine uterine fluids as uterine-milk proteins (UTM-proteins). Clearly, they should have some specific functions for maintenance of pregnancy since they only appeared during pregnancy (Newton et al, 1989).

In addition, previous work demonstrated that AFP was a specific estradiol binding protein in rats (Benassayag et al, 1975; Savu et al, 1975; Lai et al., 1976; Payne et al, 1979; Keel & Abney, 1983). This protein was also shown to exist in the bovine embryo and its fluids by Janzen et al (1982). Our results from [^{125}]estradiol ligand blotting did not reveal a high-affinity estradiol binding activity to this protein, however.

Rice et al (1993) emphasized that in pig there was a significant effect of estrogens in the amniotic fluid on the permeability of its membranes, but no significant correlation of estrogens in the alllantoic fluid with the permeability of its adjacent membranes. Our results showing different protein profiles in the amniotic and allantoic fluids, tend to explain their results by saying differential sequestering of proteins might cause differential permeability on fetal membranes.

The low ratio of free unconjugated estradiol-17β in fetal blood suggested that one function of plasma estradiol binding protein in fetuses was to regulate the extremely high concentrations of total unconjugated estradiol-17β. The free unconjugated estradiol-17β concentration was within the range of physiological requirements to saturate 5% to 50% of estrogen receptors in target cells. Probably this binding protein was albumin in pregnant cows since albumin occupied a high amount of total protein concentration. On the other hand, our data also agreed with the conclusion made by Montano et al (1995), who stated that free unconjugated estradiol-17β concentration was not negligible during fetal development in the rat. The above data contradicted a generally accepted hypothesis that no biologically available estrogens were present in the fetal circulation (MacLusky, 1988).

Our results in the dot-blotting assay showed a low total [^{3}H]estradiol binding counts, with a comparatively high non-specific binding. Our efforts in [^{125}I]estradiol ligand blotting study also indicated that there were few specific estradiol binding proteins. The difficulty in detecting any specific bovine estradiol binding protein might be caused by the low amounts of high-affinity estradiol binding proteins compared with the high amounts of low-affinity binding protein(s) present in those biological fluids, or by the poor stability of the specific binding proteins with estradiol in bovine fetal circulation, or by the low availability of binding sites of those specific binding proteins under physiological conditions of pregnancy. All these indicated that a number of proteins involved in bovine fetal circulation, including AFP and albumin, might act in transportation and binding of estradiol with a low affinity, high capacity model. But

those above results were limited by the fact that only single animals from each interval were tested in our experiment due to limited sample sources.

What remains obscure is the role of the surprisingly high proportion of free unconjugated estradiol-17β concentration present in bovine amniotic and allantoic fluids. A variety of studies could be done in light of this discovery, relating to membrane permeability, placentome function, and estrogen transportation as well as metabolism, such as the transformation of unconjugated estradiol-17β to its sulfated form in bovine placentomes.

APPENDICES

REFERENCES

Aebersold, R., Teplow, D., Hood, L. & Kent, S. (1986). Electroblotting onto activated glass. *J. Biol. Chem.* 261, 4229-4338.

Ailenberg, M. & Shemesh, M. (1983). Partial purification of a chorionic gonadotropin-like protein from bovine cotyledons. *Biol. of Reprod.* 28, 517-522.

Arthur, A. & Daniel, J. J. (1972). Progesterone regulation of blastokinin production and maintenance of rabbit blastocysts transferred into uteri of castrate recipients. *Fertil. Steril.* 23, 115-122.

Aussel, C., Uriel, J. & Mercier-Bodard, C. (1973). Rat alpha-fetoprotein: isolation, characterization and estrogen-binding properties. *Biochime*. 55, 1431-1437.

Baetz, A., Hubert, W. & Graham, C. (1976). Changes of biochemical constituents in bovine fetal fluids with gestational age. *Am. J. Vet. Res.* 37, 1047-1052.

Baker, M. (1988). Evolution of alpha-fetoprotein: sequence comparisons among AFP species and with albumin species. *Tumor. Biol.* 9, 123-126.

Bazer, F., Geisert, R. & Zavy, M. (1993). Fertilization, cleavage and implantation. In Reproduction in Farm Animals, pp. 210-228. Edited by E. Hafez. Philadelphia: Lea & Febiger.

Bearden, H. & Fuquay, J. (1997). Gestation. In Applied Animal Reproduction, pp. 8, 88-104. New Jersey: Prentice Hall.

Benassayag, C., Vallette, G., Cittanova, N., Nunez, E. & Jayle, M. (1975). Isolation of two forms of rat alpha-fetoprotein and comparison of their binding parameters with estradiol-17beta. *Biochim. Biophys. Acta* 412, 295-305.

Butler, J., Hamilton, W., Sasser, R., Ruder, C., Hass, G. & Williams, R. (1982). Detection and partial characterization of two bovine pregnancy-specific proteins. *Biol. of Reprod.* 26, 925-933.

Carlsson R., Estes T., Degroot J., Holden, J. and Ruoslahti, E. (1980). High affinity of alpha-fetoprotein for arachidonate and other fatty acids. *Biochem. J.* 190(2), 301 -305.

Carnegie, J. & Robertson, H. (1978). Conjugated and unconjugated estrogens in fetal and maternal fluids of the pregnant ewe: a possible role for estrone sulfate during early pregnancy. *Biol. of Reprod.* 19, 202-211.

Challis, J., Kim, C., Naftolin, F., Judd, H., Yen, S. & Benirschke (1974). The concentrations of androgens, oestrogens, progesterone and luteinizing hormone in the serum of foetal calves throughout the course of gestaion. *J. Endocr.* 60, 107-115.

Challis, J. & Patrick, J. (1981). Fetal and maternal estrogen concentrations throughout pregancy in the sheep. *Can. J. Physiol. Pharmacol.* 59, 970-978.

Cheng, C., Musto, N., Gunsalus, G., Frick, J. & Bardin, C. (1985). There are two forms of androgen binding protein in human testis. Comparison of their protomeric variants with serum testosterone-estradiol binding globulin. *J. Biol. Chem.* 260, 5631-5640.

Clark, J. & Peck, E. J. (1979). Female sex steroids: receptors and function. Monogr. Endocrinol. 14, I-XII.

Corvol, P. & Bardin, C. (1973). Species distribution of testerone-binding globulin. *Biol. of Reprod.* 8, 277-282.

Dobson, H. & Dean, P. (1974). Radioimmunoassay of oestrone, oestradiol-17alpha and –17beta in bovine plasma during the oestrous cycle and last stages of pregnancy. *J. Endocrinol.* 61, 479-486.

Ducsay, C., Buhi, W., Bazer, F. & Roberts, R. (1986). Role of uteroferrin in placental iron transport in swine: relationship between uteroferrin levels and iron deposition in the conceptus during gestaion.

Eley, R., Thatcher, W. & Bazer, F. (1979). Hormonal and physical changes associated with bovine conceptus development. *J. Reprod. Fertil.* 55, 181-190.

Evans, G. & Wagner, W. (1981). In vitro oestrogen synthesis by bovine placental during pregnancy and induced parturition. *Acta Endocrinol. (Copenh)* 98, 119-125.

Fridlansky, F. & Milgrom, E. (1982). Steroid binding to uteroglobin. In Proteins and Steroids in Early Prgnancy, pp. 141-145. Edited by H. Beier & P. Karlson. Berlin: Springer-Verlag.

Gross, T. & Williams, W. (1988). In-vitro steroid synthesis by the placenta of cows in late gestaion and at parturition. *J. Reprod. Fertil.* 83, 565-573.

Hagenas, Li, Ritzen, E., Plooen, L., Hansson, V., French, F. & Nayfeh, S. (1975). Sertoli

cell origin of testicular androgen-binding protein (ABP). *Mol. Cell Endocrinol.* 2, 339-350.

Hammond, G. (1993). Extracellular steroid-binding proteins. In Steroid Hormone Action, pp. 1-17. Edited by M. Parker. Oxford: Irl Press.

He, Y. & Keel, B. (1994). Biochemical characterization of bovine alpha-fetoprotein and comparisons with human alpha-fetoprotein. *Comp. Biochem. Physiol. Biochem. Mol. Biol.* 108, 327-336.

Hickey, G., Walton, J. & Hansel, W. (1989). Identification of a luterotrophic protein in bovine allantoic fluid. *J. Reprod. Fert. Suppl.* 37, 29-35.

Hoffmann, B., Wagner, W., Hixon, J. & Bahr, J. (1979). Observations concerning the functional status of the corpus luteum and the placenta around parturition in the cow. *Anim. Reprod. Sci.* 2, 253-266.

Hou, Q., & Gorski, J. (1993). Estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryos during preimplantation development. *Proc. Natl. Acad. Sci. U S A* 90, 9460-9464.

Hsia, J., Wong, L. & Deutsch, H. (1986). Determination of the distribution of fatty acids and diethylstilbestrol between serum albumin and alpha-fetoprotein by concanavalin A affinity chromatography. *Biochim. Biophys. Acta* 880, 117-122.

Jainudeen, M. & Hafez, E. (1993). Gestation, prenatal physiology and parturition. In Reproduction in Farm Animals, pp. 213-236, 6th edition. Edited by E. Hafez. Philadelphia: Lea & Febiger.

Janzen, R., Mably, E., Tamaoki, T., Church, R. & Lorscheider, F. (1982). Synthesis of alpha-fetoprotein by the pre-implantation and post-implantation bovine embryo. *J. Reprod. Fert.* 65, 105-110.

Jefferies, L. & Roberts, P. (1989). A new method of detecting hormone-binding proteins electroblottted onto glass fiber filter: juvenile hormone-binding proteins from grasshopper hemolymph. *J. Steroid Biochem.* 35, 449-455.

Keel, B. & Abney, T. (1983). The kinetics of estrogen binding to rat alpha-fetoprotein. *Experientia* 40, 503-505.

Knight, J., Bazer, F., Thatcher, W., Franke, D. & Wallace, H. (1977). Conceptus development in intact and unilaterally hysterectomized-ovariectomized gilts: interrelations among hormonal status, placental development, fetal fluids and fetal growth. *J. Anim. Sci.* 44, 620-637.

Kumbla, L., Bhadra, S. & Subbiah, M. (1991). Mutifunctional role for fetuin (fetal

protien) in lipid transport. *FASEB J.* 5, 2971-2975.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lai, P., Forrester, P., Hancock, R., Hay, D. & Lorscheider, F. (1976). Rat alpha-fetoprotein: isolation, radioimmunoassay and fetal-maternal distribution during pregnancy. *J. Reprod. Fertil.* 48, 1-8.

Lai, P., Huang, L., Panrucker, D., Church, R. & Lorscheider, F. (1981). Distribution of bovine fetuin and albumin in plasma, allantoic and amniotic fluids during development. *J. Reprod. Fertil.* 63, 53-60.

Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Lubahn, D., Moyer, J., Golding, T., Couse, J., Korach, K. & Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. USA* 90(23), 11162-11166.

Lyngset, O. & Lunass, T. (1972). Amniotic and allantoic fluid levels of oestrogens at different stages of development in the goat. *Acta Vet. Scand.* 13, 269-271.

MacLusky, N. (1988). Developmental actions of gonadal steroids. *Prog. Clin. Biol. Res.* 281, 243-263.

Makowski, E., Meschia, G., Droegemueller, W. & Battaglia, F. (1968). Measurement of umbilical arterial blood flow to the sheep placenta and fetus in utero. *Circ. Res.* 23(5), 623-631.

Makowski, E., Meschia, G., Drogegemueller, W. & Battaglia, F. (1968). Distribution of uterine blood flow in the pregnant sheep. *Am. J. Ob. Gy.* 101, 409-412.

Malayer, J. & Woods, V. (1998). Development of oestrogen receptor expression and hormone response in the foetal bovine uterus. *J. Reprod. Fertil.* 112, 289-300.

McKenna, P. (1984). Proteins in the amniotic and allantoic fluids of fetal pigs. *Comp. Biochem. Physiol. [B]* 78, 663-667.

Mellin, T. & Erb, R. (1965). Estrogens in the bovine – a review. *J. Dairy Sci.* 48, 687-700.

Melton, A., Berry, R. & Butler, O. (1951). The interval between the time of ovulation and attachment of the bovine embryo. *J. Anim. Sci.* 10, 993-1005.

Mizutani, T., Nishikawa, Y., Adachi, H., Enomoto, T., Ikegami, H., Kurachi, H., Nomura, T. & Miyake, A. (1994). Identification of estrogen receptor in human adipose tissue and adipocytes. *J. Clin. Endocrinol. Metab.* 78, 950-954.

Moffatt, J., Bazer, F., Hansen, P., Chun, P. & Roberts, R. (1987). Purification, secretion and immunocytochemical localization of the uterine milk proteins, major progesterone-induced proteins in uterine secretions of the sheep. *Biol. of Reprod.* 36, 419-430.

Montano, M., Welshons, W. & Saal, F. (1995). Free estradiol in serum and brain uptake of estradiol during fetal and neonatal differentiation in female rats. *Biol. of Reprod.* 53, 1198-1207.

Murphy, B. (1967). Binding of testosterone and estradiol in plasma. *Can. J. Biochem.* 46, 299-302.

Newton, G., Hansen, P., Bazer, F., Leslie, M., Stephenson, D. & Low, B. (1989). Presence of the major progesterone-induced protiens of the sheep endometrium in fetal fluids. *Biol. of Reprod.* 40, 417-424.

Noakes, D. (1997). Normal pregnancy. In: Fertility and Obstetrics in Cattle, 2nd edition, pp. 28-33. Oxford: Blackwell Science Ltd.

Nunez, E., Vallette, G., Benassayag, C. & Jayle, M. (1974). Comparative study on the binding of estrogens by human and rat serum proteins in development. *Biochem. Biophys. Res. Commun.* 57, 126-133.

Payne, D. & Katzenellenbogen, J. (1979). Binding specificity of rat alpha-fetoprotein for a series of estrogen derivatives: studies using equilibrium and nonequilibrium binding techniques. *Endocrinology* 105, 743-753.

Peters, A. & Ball, P. (1987). Fertilization, conception, pregnancy and its detection. In: Reproduction in Cattle, 1st edition, pp. 91-98. London: Butterworths.

Rebuffe-Scrive, K., Bronnegard, M., Nilsson, A., Eldh, J., Gustafsson, J. & Bjorntorp, P. (1990). Steroid hormone receptors in human adipose tissues. *J. Clin. Endocrinol. Metab.* 71, 1215-1219.

Rexroad, C., Casida, L. & WJ, T. (1973). Crown-rump length of fetuses in purebred holstein-friesian cows. J. Dairy Sci., 346-347.

Rice, G., Hoier, R., Christensen, P., Dantzer, V. & Skadhauge, E. (1993). Endocrine correlates of placental permeability during gestation in the pig. *Comp. Biochem. Physiol.* 105A, 111-117.

Roberts, G. & Parker, J. (1974). Macromolecular components of the luminal fluid from

the bovine uterus. *J. Reprod. Fertil.* 40, 291-303.

Roberts, G. & Parker, J. (1976). Fractionation and comparison of proteins from bovine uterine fluid and bovine allantoic fluid. *Biochim. Biophys. Acta* 446, 69-76.

Robertson, H. & Smeaton, T. (1973). The concentration of unconjugated oestrone, oestradiol-17alpha and oestradiol-17beta in the maternal plasma of the pregnant ewe in relation to the initiation of praturition and lactation. *J. Reprod. Fertil.* 35, 461-468.

Robertson, H. (1974). Changes in the concentration of unconjugated oestrone, oestradiol-17alpha and oestradiol-17beta in the maternal plasma of the pregnant cow in relation to the initiation of parturition and lactation. *J. Reprod. Fertil.* 36, 1-7.

Robertson, H. & King, G. (1974). Plasma concentrations of progesterone, oestrone, oestradiol-17beta and of oestrone sulphate in the pig at implantaion, during pregnancy and at parturition. *J. Reprod. Fertil.* 40, 133-141.

Robertson, H. King, G. & Dyck, G. (1978). The appearance of oestrone sulphate in the peripheral plasma of the pig early in pregnancy. *J. Reprod. Fertil.* 52, 337-338.

Robertson, H. & King, G. (1979). Conjugated and unconjugated oestrogens in fetal and maternal fluids of the cow throughout pjregnancy. *J. Reprod. Fertil.* 55, 463-470.

Robertson, H., Dwyer, R. & King, G. (1985). Oestrogens in fetal and maternal fluids throughout pregnancy in the pig and comparisons with the ewe and cow. *J. Endocrinol.* 106, 355-360.

SAS (1982). SAS User's Guide: statistics. N.C: SAS Institute.

Savu, L., Nunez, E. & Jayle, M. (1975). Plasma diethylstilbestrol binding proteins of rat, mouse and man in the course of development: relations with the binding of estradiol. *Steroids* 25, 717-728.

Sheehan, D. & Young, M. (1979). Diethylstilbestrol and estradiol binding to serum albumin and pregnancy plasma of rat and human. Endocrinology 104(5), 1442-1446.

Shemesh, M. (1980). Estradiol-17β biosynthesis by the early bovine fetal ovary during the active and refractory phases. *Biol. of Reprod.* 23, 577-582.

Sloss, V. & Dufty, J. (1980). Pregnancy and gestation. In: Handbook of Bovine Obstetrics. Baltimore/London: Williams & Wilkins.

Smith, K., Lai, P., Robertson, H., Church, R. & Lorscheider, F. (1979). Distribution of

alpha 1-fetoprotein in fetal plasma, allantoic fluid, amniotic fluid and maternal plasma of cows. *J. Reprod. Fertil.* 57, 235-238.

Suzuki, M., Shimokawa, H., Takagi, Y. & Sasaki, S. (1994). Calcium-binding properties fetuin in fetal bovine serum. *J. Exp. Zool.* 270(6), 501-507.

Tsumagari, S., Kamata, J., Takagi, K., Tanemura, K., Yosai, A. & Takeishi, M. (1993). Aromatase activity and oestrogen concentrations in bovine cotyledons and caruncles during gestation and parturition. *J. Reprod. Fertil.* 98, 631-636.

Welshons, W., Lieberman, M. & Gorski, J. (1984). Nuclear localization of unoccupied oestrogen receptors. *Nature* 307, 747-749.

Westphal, U. (1986). Steroid-protein interactions revisited. In: Steroid-Portein Interaction II, pp. 1-8. Berlin: Springer-Verlag.

Zalel, Y., Kedar, I., Tepper, R., Chen, R., Drugan, A., Fejgin, M. & Beyth, Y. (1996). Differential diagnosis and management of very low second trimester maternal serum unconjugated estriol levels, with special emphasis on the diagnosis of X-linked ichthyosis. *Obstet. Gynecol. Surv.* 51(3), 200-203.

APPENDIX A

TABLES

TABLE I

Circulating unconjugated total and free E_2 concentrations in bovine fetal blood, amniotic and allantoic fluids

Fetal age (days)	Unconjugated total E_2 concentrations (pg/ml)			Unconjugated free E_2 concentrations (pg/ml)		
	No. of animals	Mean ± S.E.M.	Range	No. of animals	Mean ± S.E.M.	Range
(a) in Fetal Blood						
31 - 50	NA	NA	NA	NA	NA	NA
51 - 70	2	63.28 ± 61.05	20.11 - 106.45	NA	NA	NA
71 - 90	2	60.47 ± 18.24	47.57 - 73.37	2	9.45 ± 6.21	5.06 - 13.84
91 -110	8	94.25 ± 84.53	12.33 - 160.57	3	23.91 ± 30.89	3.13 - 59.41
111 - 130	6	130.17 ± 97.02	47.59 - 311.14	5	18.67 ± 23.81	5.02 - 61.09
131 - 150	2	50.25 ± 19.18	36.69 - 63.81	1	4.08	NA
151 - 170	5	49.59 ± 28.38	19.20 - 90.76	4	5.77 ± 2.36	3.65 - 8.22
171 - 190	3	77.66 ± 42.15	40.04 - 123.21	2	18.54 ± 20.07	4.34 - 32.73
(b) in Amniotic Fluid						
31 - 50	3	[a] 9.47 ± 13.88	0.22 - 25.43	3	[a] 7.46 ± 10.98	0.19 - 20.09
51 - 70	10	[b] 40.63 ± 26.19	4.03 - 88.75	10	[b] 31.06 ± 19.00	3.26 - 59.73
71 - 90	2	[c] 235.84 ± 201.48	93.37 - 378.30	2	[c] 181.68 ± 165.72	64.50 - 298.86
91 -110	8	[c] 237.39 ± 119.22	109.62 - 499.51	5	[c] 211.92 ± 105.34	125.26 - 389.62
111 - 130	4	[c] 584.14 ± 599.90	162.74 - 1458.20	4	[c] 447.44 ± 476.26	122.20 - 1148.47
131 - 150	2	[c] 183.78 ± 161.11	69.85 - 297.70	2	[c] 130.16 ± 116.07	48.08 - 212.23
151 - 170	4	[c] 535.21 ± 680.32	69.08 - 1517.96	4	[c] 390.24 ± 484.00	53.12 - 1086.25
171 - 190	3	[c] 100.44 ± 44.42	50.41 - 135.24	2	[b] 94.57 ± 9.81	87.63 - 101.51
(c) in Allantoic Fluid						
31 - 50	3	[a] 11.67 ± 7.61	5.30 - 20.10	3	[a] 10.19 ± 7.32	4.03 - 18.29
51 - 70	9	[a] 24.97 ± 15.74	6.24 - 53.16	9	[a] 18.95 ± 11.54	5.18 - 37.34
71 - 90	2	[b] 1371.13 ± 811.53	797.29 - 1944.96	2	[b] 878.97 ± 318.47	653.78 - 1104.16
91 -110	7	[c] 257.10 ± 306.12	34.77 - 865.95	6	[b] 255.81 ± 244.16	47.08 - 701.42
111 - 130	5	[b] 1906.17 ± 2729.92	334.63 - 6743.23	5	[b] 1280.73 ± 1807.23	214.36 - 4460.65
131 - 150	2	[b] 419.60 ± 38.44	392.42 - 446.78	2	[b] 260.55 ± 20.95	245.73 - 275.36
151 - 170	5	[b] 1220.86 ± 1391.02	98.21 - 3585.83	5	[b] 898.15 ± 1084.85	90.35 - 918.19
171 - 190	2	[c] 180.45 ± 41.45	151.14 - 209.76	1	[c] 123.76	NA

"**a, b, c**": Mean values for each fluid with the same superscript were not significantly different ($P<0.05$) between intervals, after natural logarithmic (LN) transformation of the above data.

TABLE II

Saturation effect test of [^{3}H]E_2 in centrifugal ultrafiltration

Spiked unconjugated E_2 in 200 ul pooled samples	**FB** (ratio of U_{avg}/T_{avg})	**AM** (ratio of U_{avg}/T_{avg})	**AL** (ratio of U_{avg}/T_{avg})
+ 0 pg in 2ul	29%	72%	76%
+ 50 pg in 2 ul	30%	73%	76%
+ 100 pg in 2 ul	32%	72%	73%
+ 200 pg in 2 ul	32%	70%	74%

FB: fetal blood

AM: amniotic fluid

AL: allantoic fluid

Uavg: average of [^{3}H]E_2 counts from the ultrafiltrates of samples run in duplicates

Tavg: average of [^{3}H]E_2 counts from the original samples run in duplicates

APPENDIX B

FIGURES

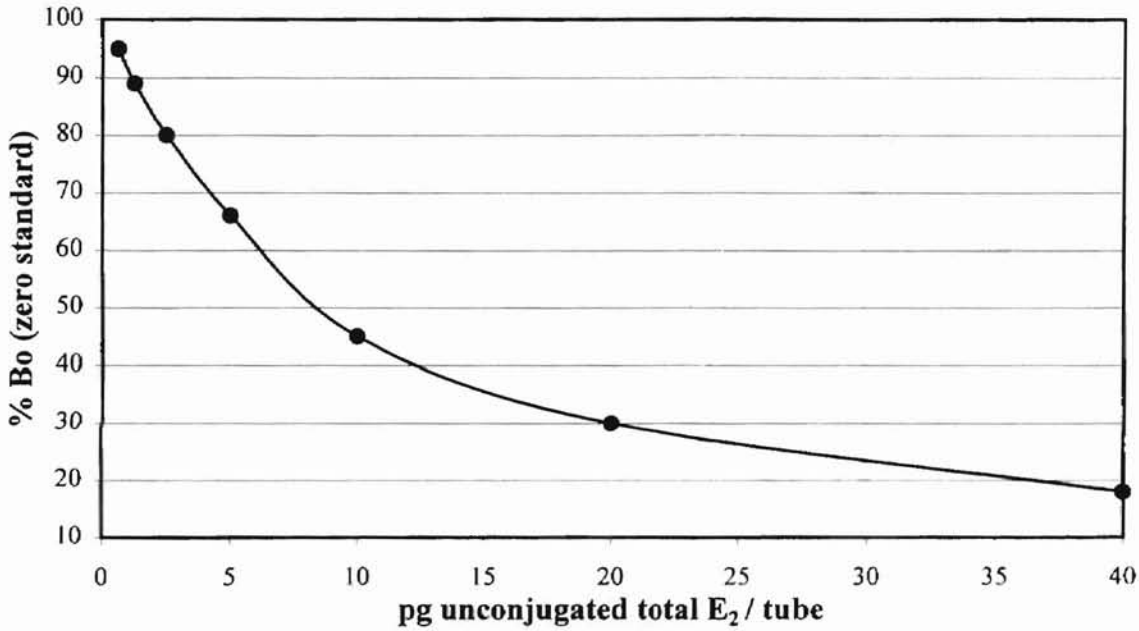

Figure 1. **Standard curve based on the charcoal-extracted adult cow plasma.**

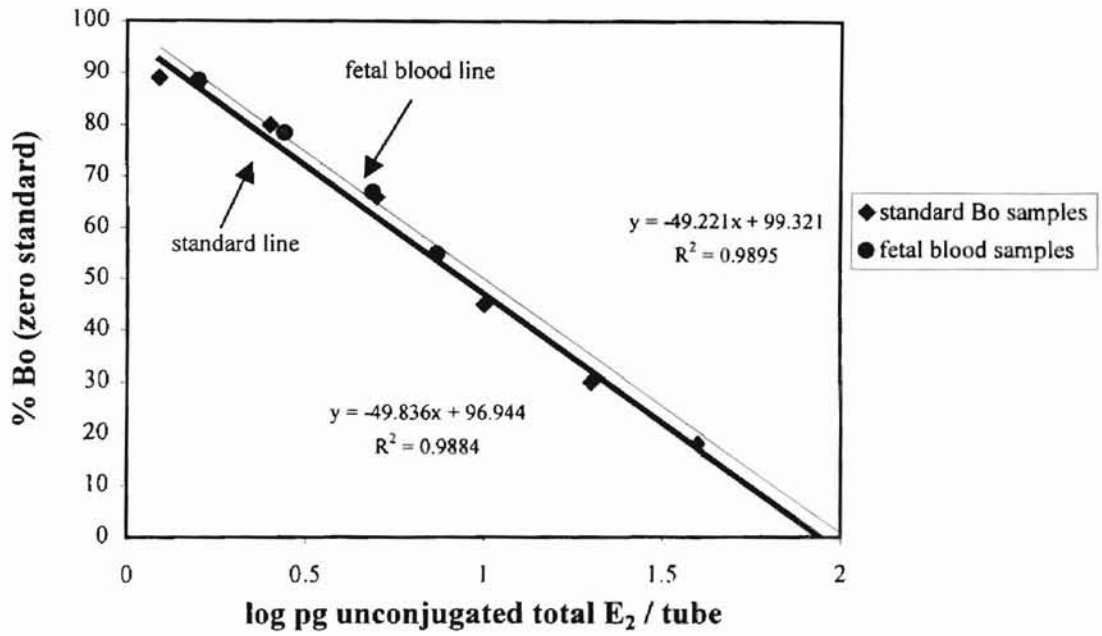

Figure 2. **Inhibition curve of unconjugated total E_2 in the fetal blood.** The displacement line drawn from the data of four fetal blood samples was parallel to the standard line drawn from the data of Bo plasma, after logarithmic transformation of those values. The R-squares of both lines were above 0.98. Therefore, there was no significant matrix effect in the fetal blood.

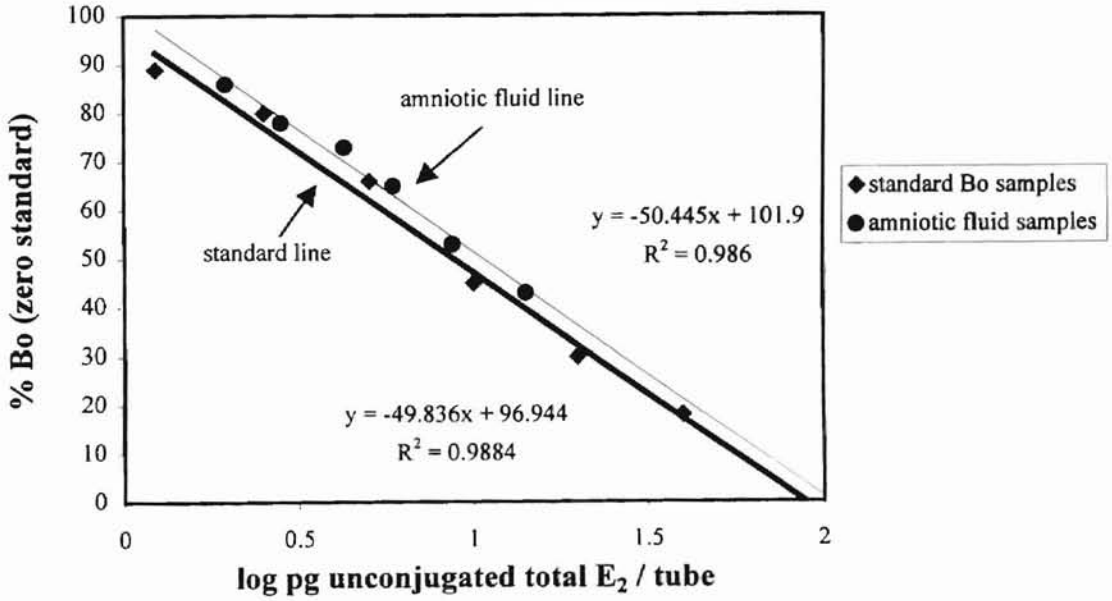

Figure 3. **Inhibition curve of unconjugated total E_2 in the amniotic fluid.** The displacement line drawn from the data of six amniotic fluid samples was parallel to the standard line drawn from the data of Bo plasma, after logarithmic transformation of those values. The R-squares of both lines were above 0.98. Therefore, there was no significant matrix effect in the amniotic fluid.

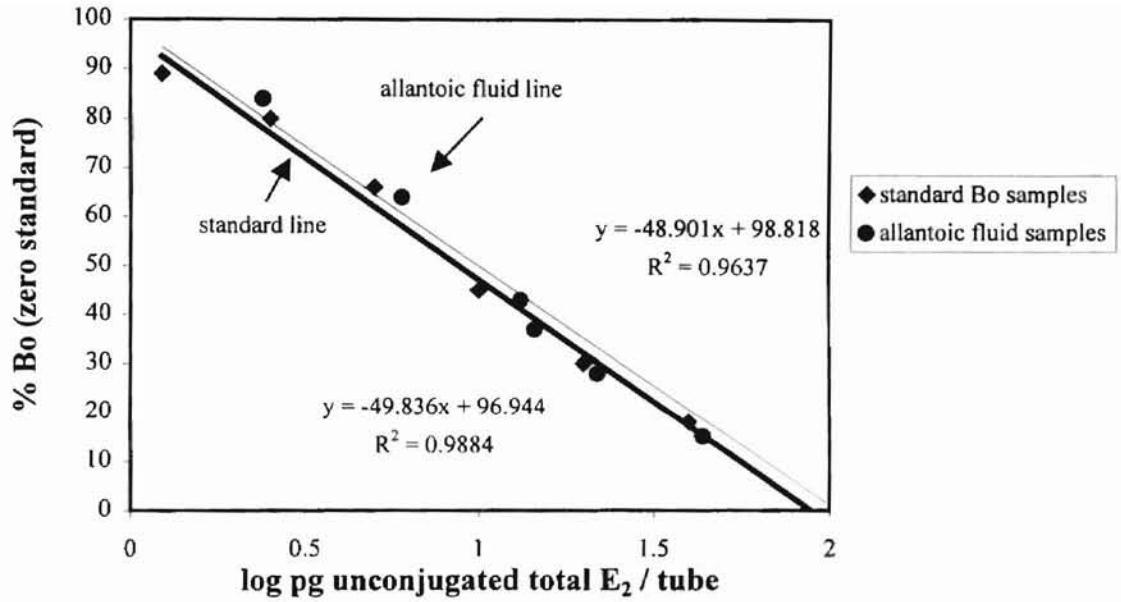

Figure 4. **Inhibition curve of unconjugated total E_2 in the allantoic fluid.** The displacement line drawn from the data of six allantoic fluid samples was parallel to the standard line drawn from the data of Bo plasma, after logarithmic transformation of those values. The R-squares of both lines were above 0.96. Therefore, there was no significant matrix effect in the allantoic fluid.

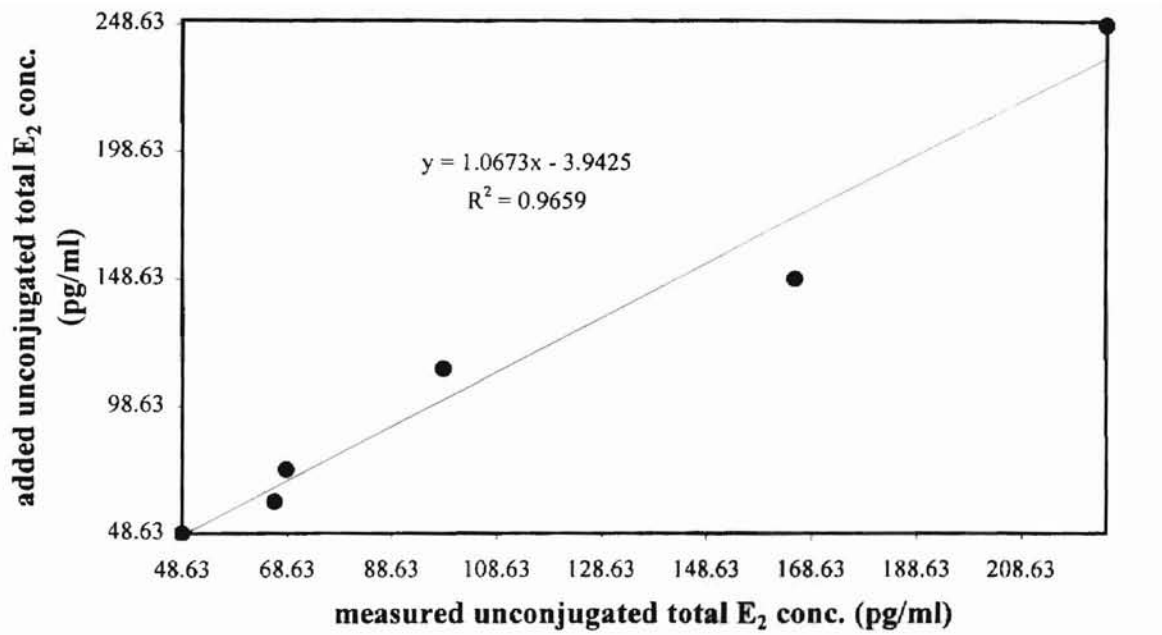

Figure 5. **Recovery of pure unconjugated E_2 added to the fetal blood.** There was a linear relationship between the measured actual E_2 concentrations and the added predicted E_2 concentrations. The R-square was above 0.96, suggesting a good recovery rate of pure unconjugated E_2 in the fetal blood.

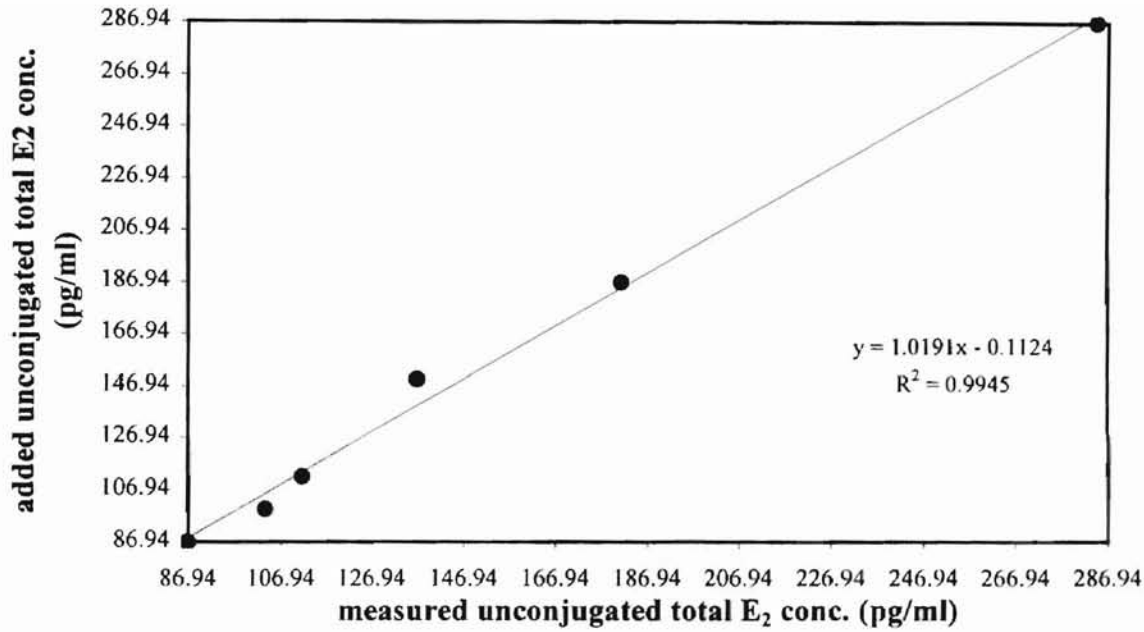

Figure 6. **Recovery of pure unconjugated E_2 added to the amniotic fluid**. There was a linear relationship between the measured actual E_2 concentrations and the added predicted E_2 concentrations. The R-square was above 0.99, suggesting a good recovery rate of pure unconjugated E_2 in the amniotic fluid.

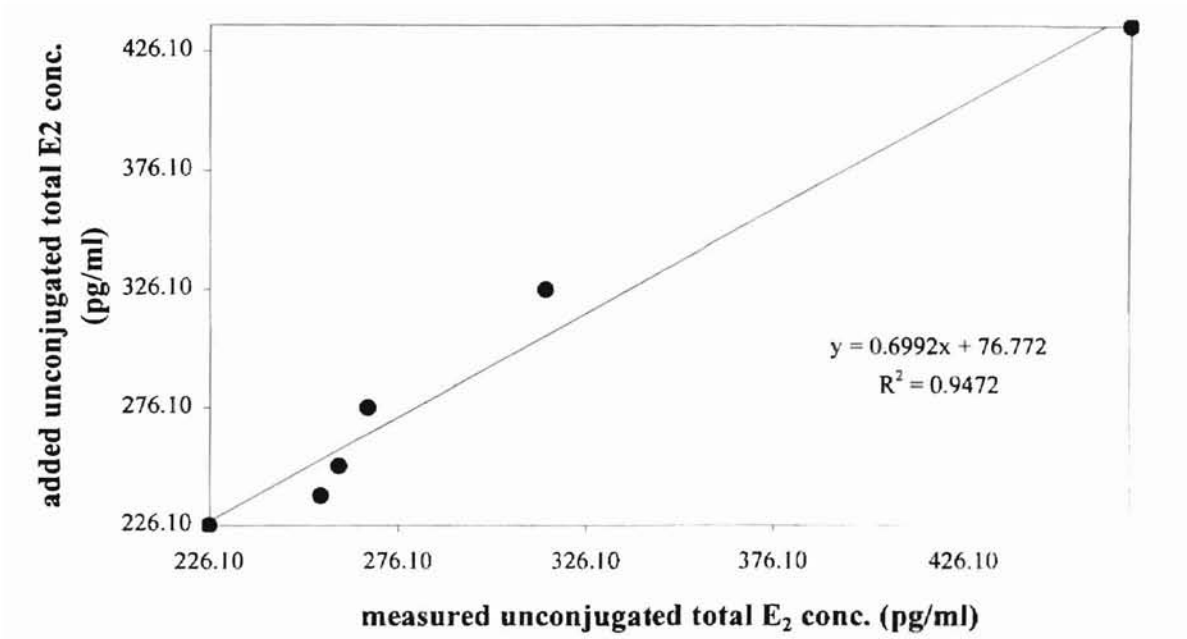

Figure 7. **Recovery of pure unconjugated E_2 added to the allantoic fluid.** There was a linear relationship between the measured actual E_2 concentrations and the added predicted E_2 concentrations. The R-square was above 0.94, suggesting a good recovery rate of pure unconjugated E_2 in the allantoic fluid.

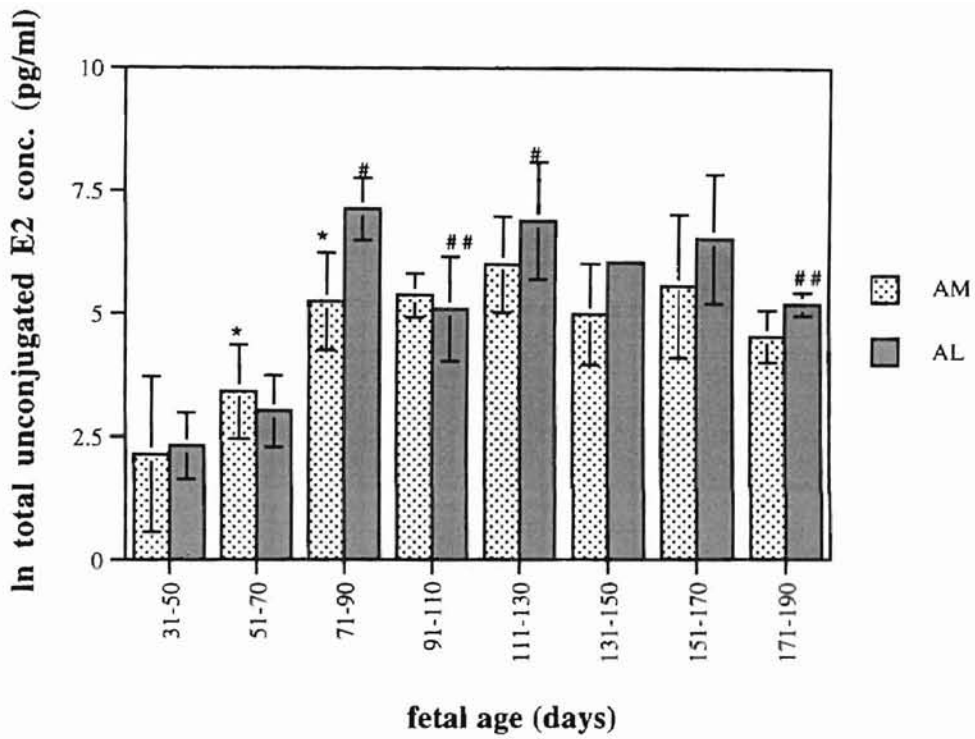

Figure 8. **Comparison of unconjugated total E_2 concentrations in bovine fetal fluids**. The results were presented as mean ± SEM. After natural logarithmic (LN) transformation of those unconjugated total E_2 concentrations, the mean values for the amniotic fluid indicated by "*" were significantly higher ($P<0.05$) than the previous interval. The mean values for the allantoic fluid indicated by "#" were significantly higher ($P<0.05$) than the previous interval; while those indicated by "##" were significantly lower ($P<0.05$) than the previous interval. Data were subjected to the analysis of variance (ANOVA). (AM: amniotic fluid; AL: allantoic fluid)

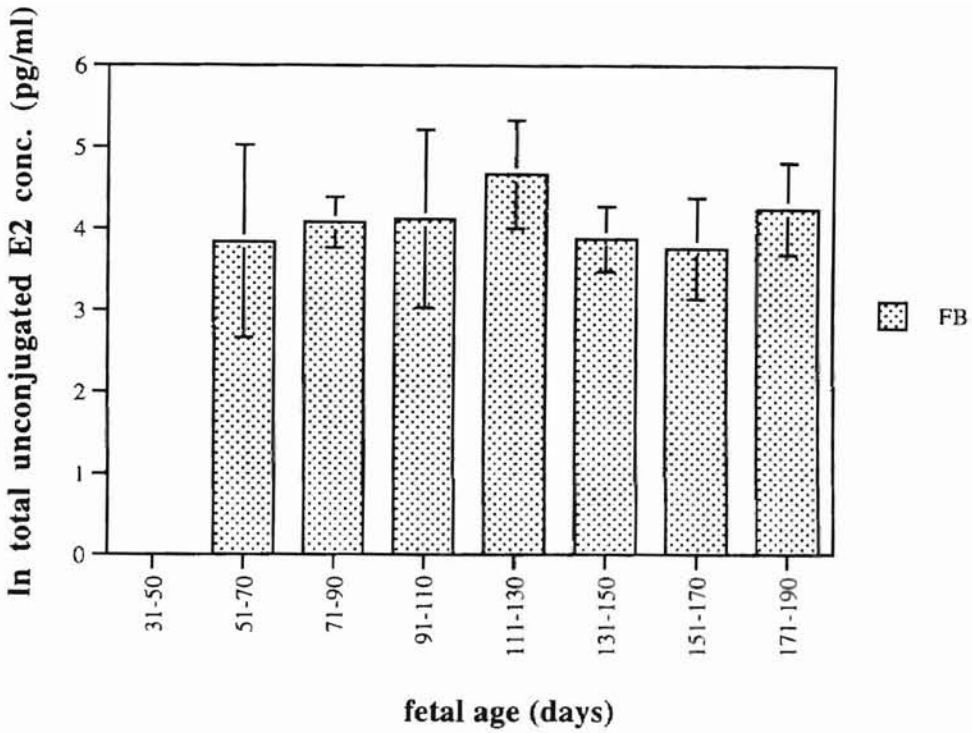

Figure 9. **Comparison of unconjugated total E_2 concentrations in bovine fetal blood**. The results were presented as mean ± SEM. No statistically significant differences ($P<0.05$) among those mean values, even after natural logarithmic (LN) transformation of the values of the unconjugated total E_2 concentrations. Data were subjected to the analysis of variance (ANOVA). (FB: fetal blood)

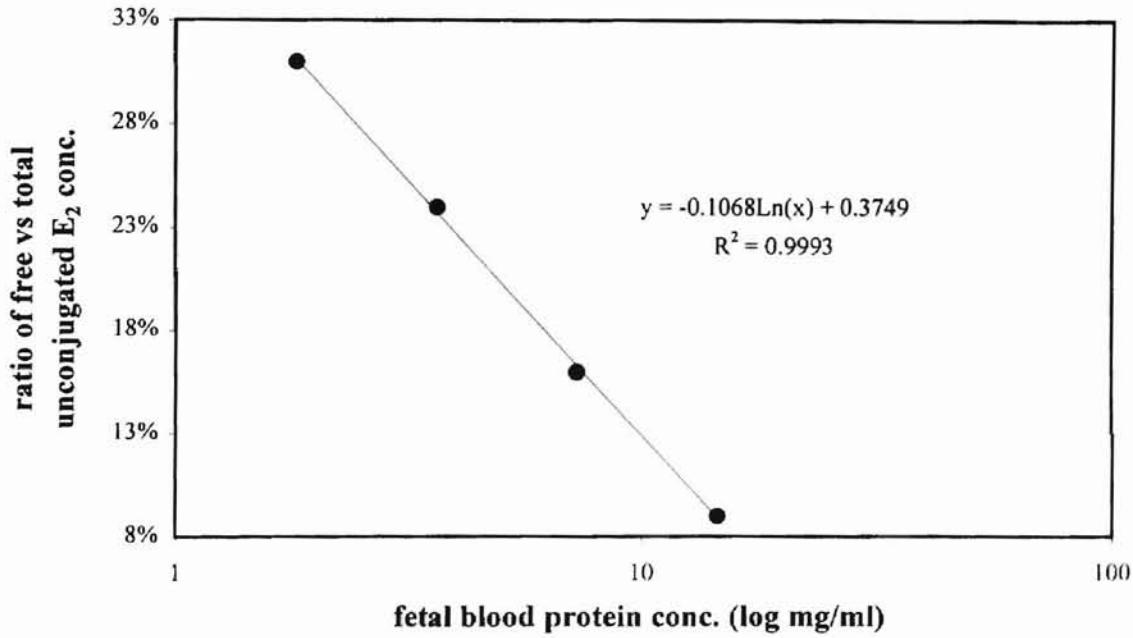

Figure 10. **Effects of fetal blood protein concentrations on unconjugated E_2 transfer across a dialysis (YMT) membrane**. There was a linear recovery rate of unconjugated free E_2 concentrations in the presence of reduced fetal blood protein concentrations (after natural logarithmic (LN) transformation) across the dialysis (YMT) membrane, through the range of 1.82 mg/ml to 14.56mg/ml. R^2=0.9993.

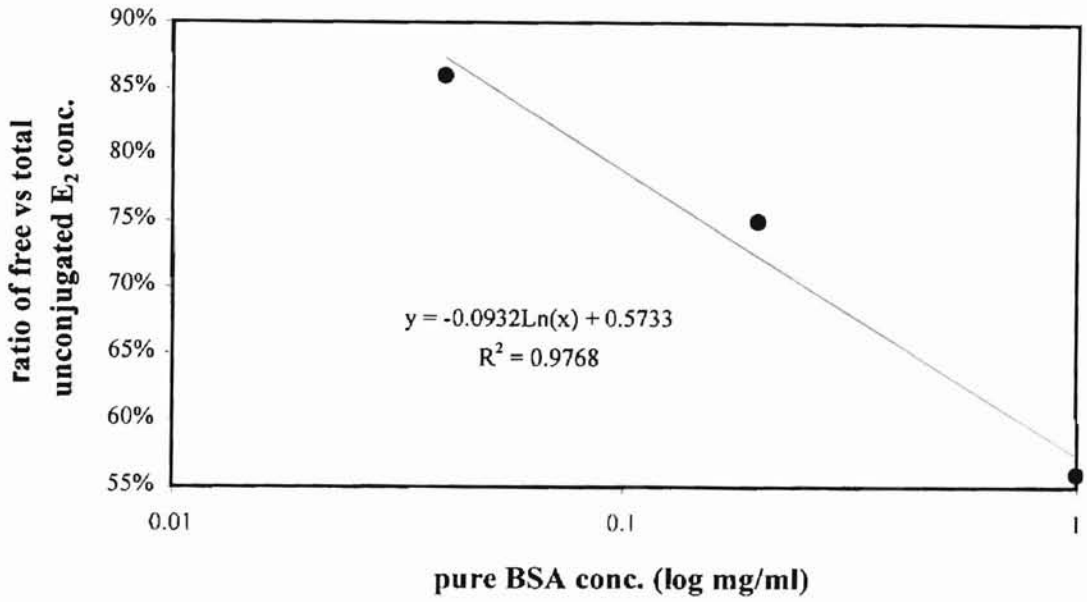

Figure 11. **Effects of pure BSA concentrations on unconjugated E_2 transfer across a dialysis (YMT) membrane**. There was a linear relationship of the reducing unconjugated free E_2 ratio in the presence of increased BSA protein concentrations (after natural logarithmic (LN) transformation) across the dialysis (YMT) membrane, through the range of 0 mg/ml to 1 mg/ml. R^2=0.9768.

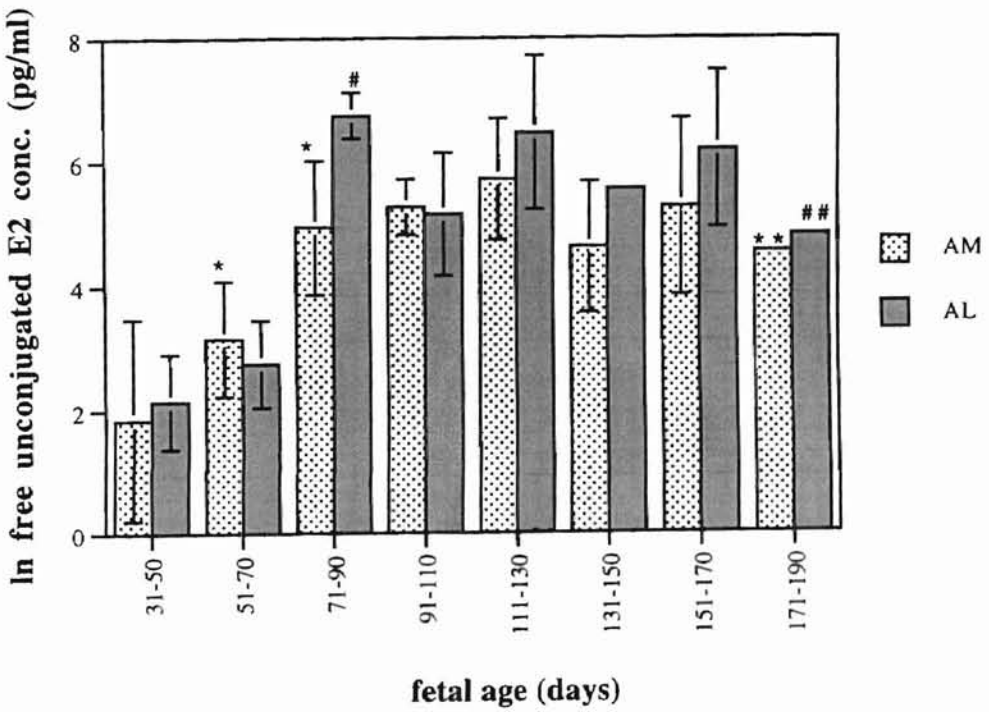

Figure 12. **Comparison of unconjugated free E_2 concentrations in bovine fetal fluids**. The results were presented as mean ± SEM. After natural logarithmic (LN) transformation of those unconjugated free E_2 concentrations, the mean values for the amniotic fluid indicated by "*" were significantly higher ($P<0.05$) than the previous interval; while those indicated by "**" were significantly lower ($P<0.05$) than the previous interval. The mean values for the allantoic fluid indicated by "#" were significantly higher ($P<0.05$) than the previous interval; while those indicated by "##" were significantly lower ($P<0.05$) than the previous interval. Data were subjected to the analysis of variance (ANOVA). (AM: amniotic fluid; AL: allantoic fluid)

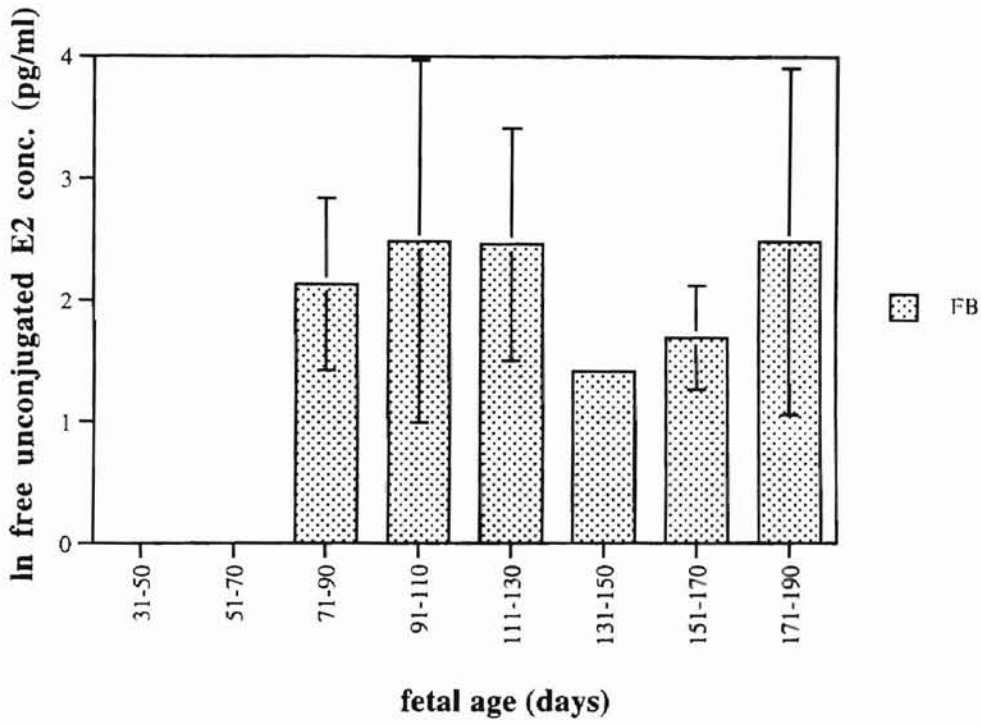

Figure 13. **Comparison of unconjugated free E_2 concentrations in bovine fetal blood**. The results were presented as mean ± SEM. No statistically significant differences ($P<0.05$) among those mean values, even after natural logarithmic (LN) transformation of the values of the unconjugated free E_2 concentrations. Data were subjected to the analysis of variance (ANOVA). (FB: fetal blood)

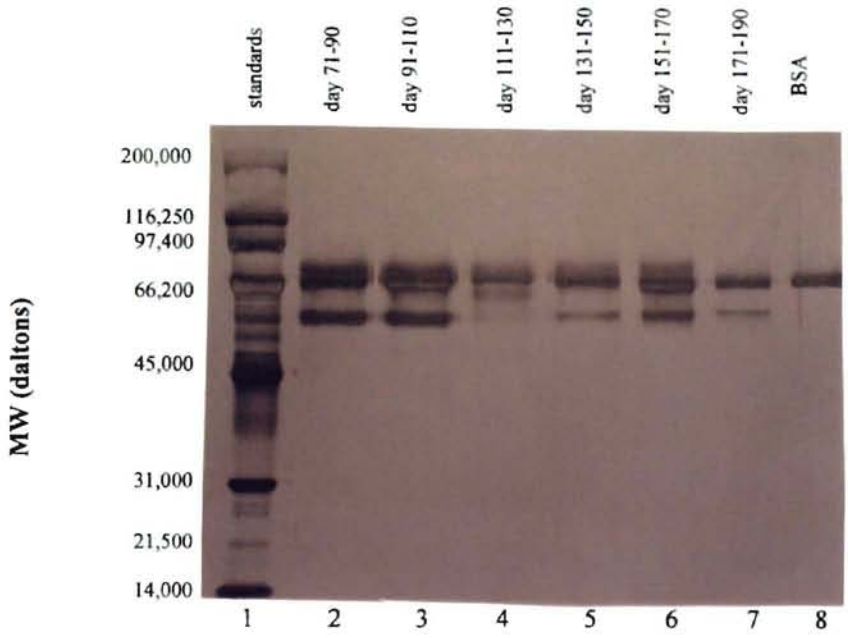

Figure 14. **Protein profile (limited to proteins from MW of 10,000 to 200,000 daltons) in bovine fetal blood.** A representative result of each period was shown from the individual samples by the 12% SDS-PAGE gel following the silver staining. Each lane was loaded with 1ug protein. Lane 1, SDS MW standards; lane 2, a sample from day 71-90 of gestation; lane 3, a sample from day 91-110; lane 4, a sample from day 111-130; lane 5, a sample from day 131-150; lane 6, a sample from day 151-170; lane 7, a sample from day 171-190; lane 8, a control sample from the commercial BSA. Major protein bands of approximately MW of 50,000, 66,200 (BSA) and 70,000 daltons appeared in all of the represented samples. The band of MW 70,000 daltons was within the reported MW range of alpha-fetoprotein (AFP). The band of MW 50,000 daltons was not identified. The amounts of two bands (MW of 50,000 and 70,000 daltons) decreased as the gestation advanced.

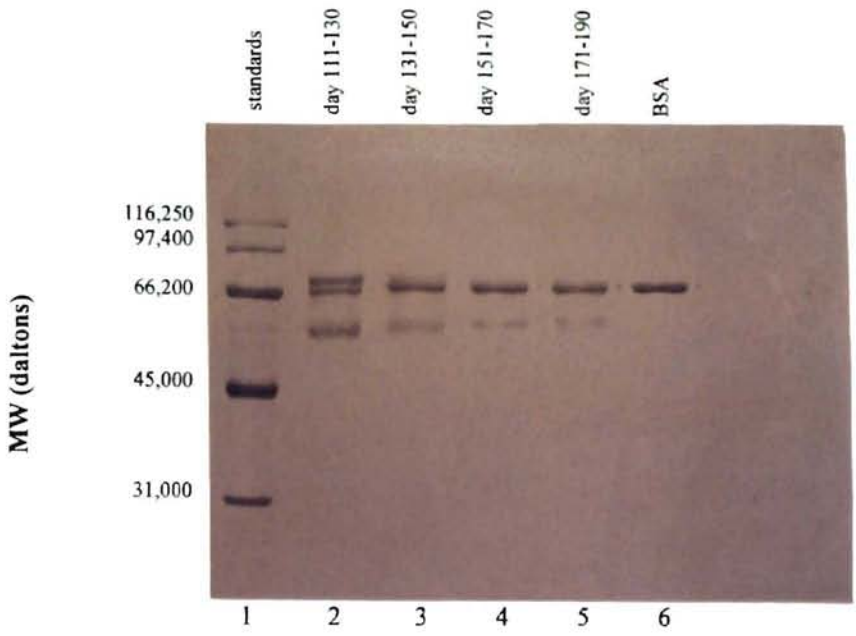

Figure 15. **Protein profile (limited to proteins from MW of 10,000 to 200,000 daltons) in bovine amniotic fluid.** A representative result of each period was shown from the individual samples by the 12% SDS-PAGE gel following the silver staining. Each lane was loaded with 1ug protein. Lane 1, SDS MW standards; lane 2, a sample from day 111-130 of gestation; lane 3, a sample from day 131-150; lane 4, a sample from day 151-170; lane 5, a sample from day 171-190; lane 6, a control sample from the commercial BSA. The bands of MW 50,000 and 66,200 daltons were the same as described in Figure 14, though the amounts were much less than those in bovine fetal blood samples. The amount of the albumin band (MW 66,200 daltons) kept constant throughout the whole tested periods; while the band of MW 50,000 daltons decreased as the gestation advanced. The protein band with MW 70,000 daltons corresponding to the reported size for alpha-fetoprotein (AFP). It appeared at the time of day 111-130, but disappeared after that period.

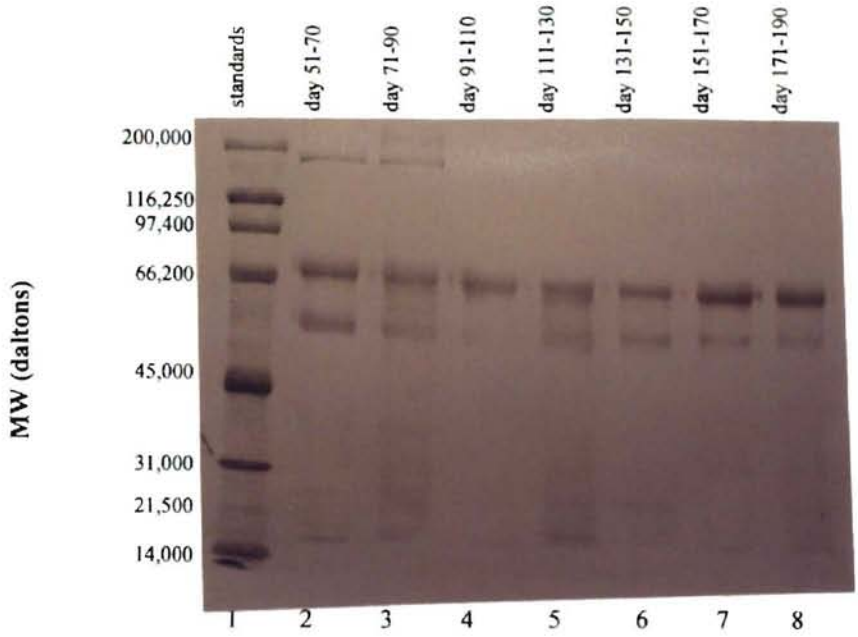

Figure 16. **Protein profile (limited to proteins from MW of 10,000 to 200,000 daltons) in bovine allantoic fluid.** A representative result of each period was shown from individual samples by the 12% SDS-PAGE gel following the silver staining. Each lane was loaded with 1ug protein. Lane 1, SDS MW standards; lane 2, a sample from day 51-70 of gestation; lane 3, a sample from day 71-90; lane 4, a sample from day 91-110; lane 5, a sample from day 111-130; lane 6, a sample from day 131-150; lane 7, a sample from day 151-170; lane 8, a sample from day 171-190. Besides the two major bands of MW 66,200 (BSA) and 50,000 daltons, major products around 200,000 daltons were clearly present at the two early periods (day 51 to day 70 and day 71 to day 90). Several protein bands could be seen below MW 45,000 daltons, and were present only in low amounts.

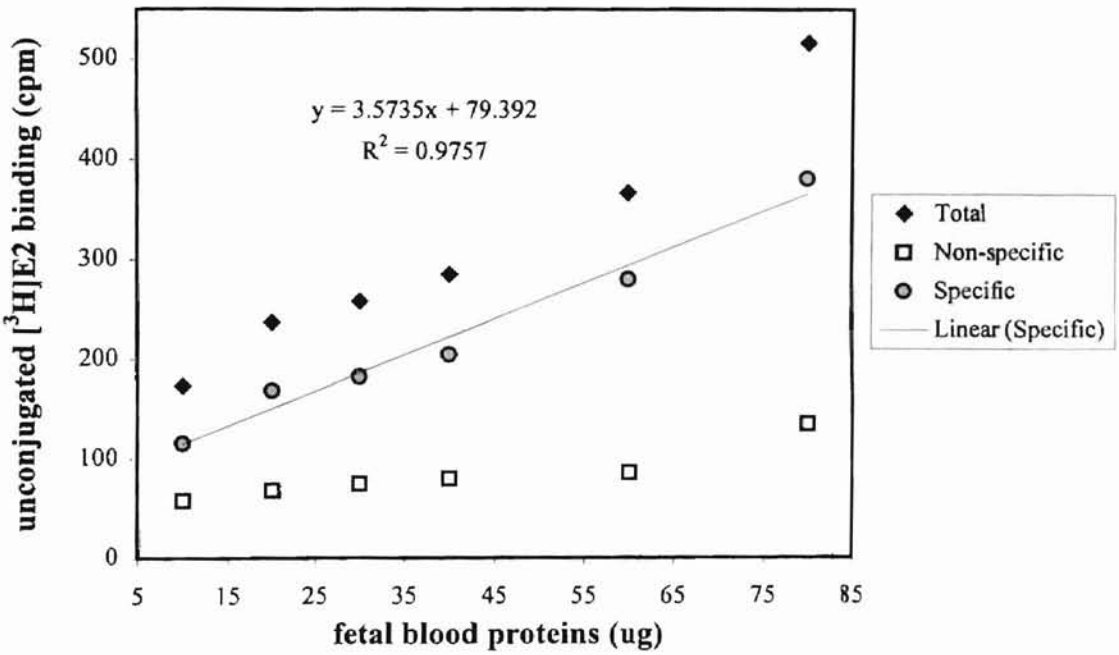

Figure 17. **Validation of glass fiber filter (GFF) for protein binding of $[^3H]E_2$.** The increasing concentrations of serum proteins resulted in a linear increase in $[^3H]E_2$ binding, through the range of 10ug to 80ug protein. Non-specific binding counts remained almost constant with the increasing concentrations of proteirs. Therefore, the GFF membrane was efficient for binding proteins to do the dot blotting procedure.

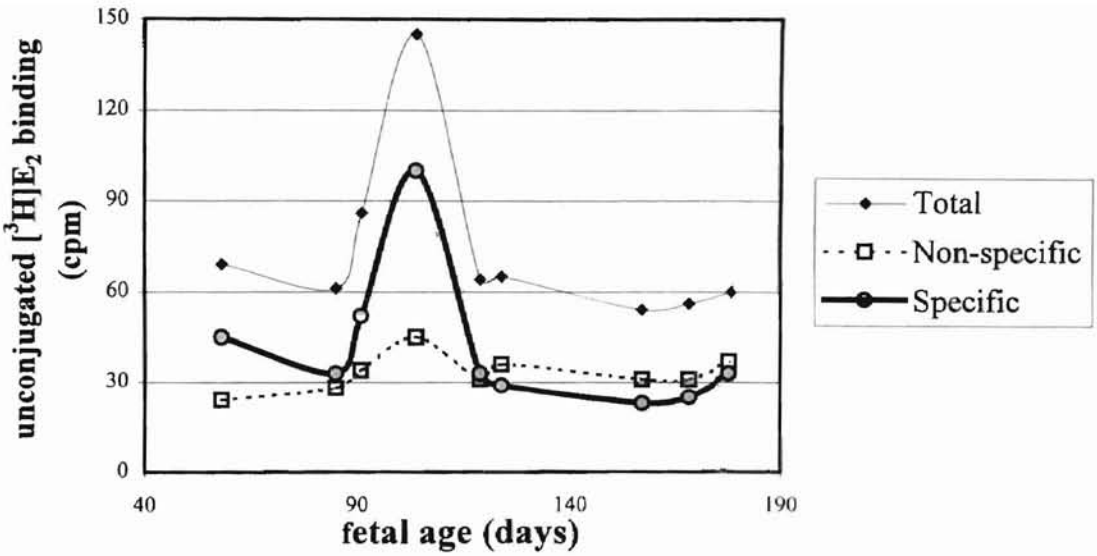

Figure 18. **Dot blot analysis of $[^3H]E_2$ binding in bovine fetal blood.** No dramatic variations of $[^3H]E_2$ binding present with the bovine fetal proteins (under fixed sample volumes) as fetal age changed. Total binding was low, around 70-cpm (except for one point at day 111 of gestation). Comparatively high non-specific counts were measured in the fetal blood.

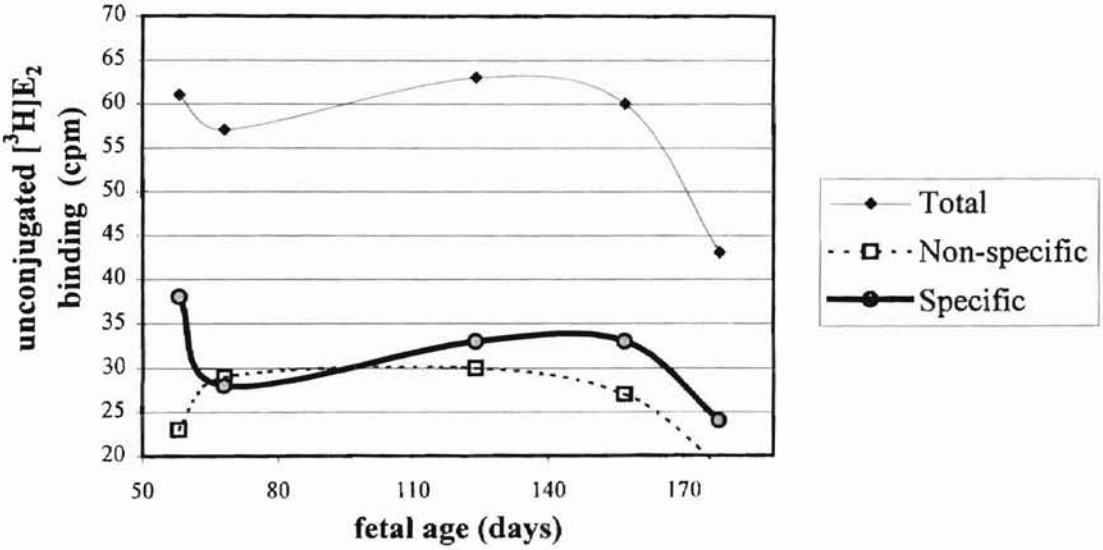

Figure 19. **Dot blot analysis of $[^3H]E_2$ binding in bovine amniotic fluid**. No dramatic variations of $[^3H]E_2$ binding present with the amniotic fluid proteins (under fixed sample volumes) as the fetal age changed. Total binding was low, around 60-cpm. Comparatively high non-specific counts were measured in the amniotic fluid.

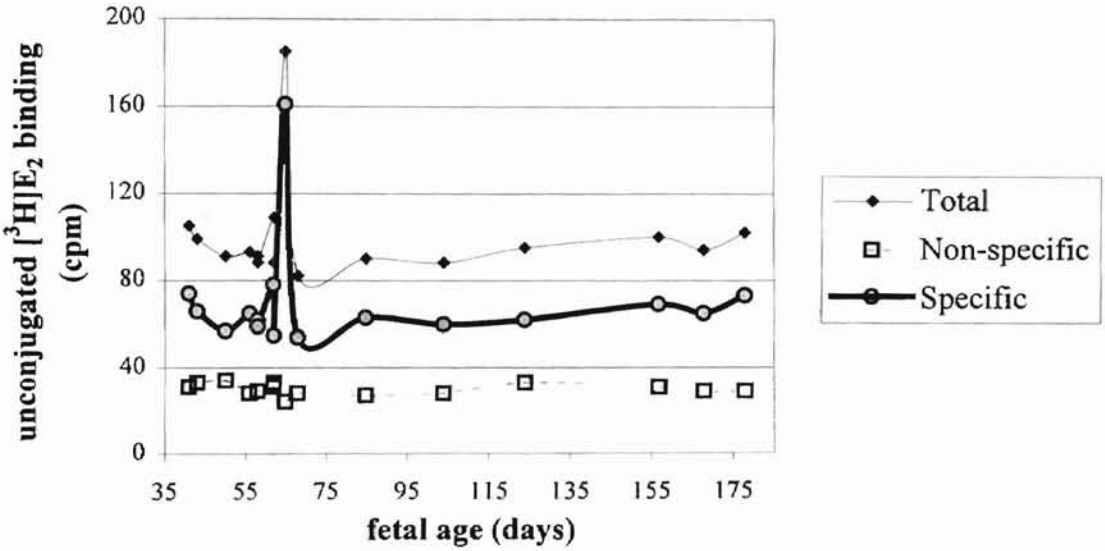

Figure 20. **Dot blot analysis of $[^3H]E_2$ binding in bovine allantoic fluid**. No dramatic variations of $[^3H]E_2$ binding present with the allantoic fluid proteins (under the fixed sample volumes) as the fetal age changed. Total binding was low, around 100-cpm (except one point at day 60 of gestation). Comparatively high non-specific counts were measured in the allantoic fluid.

VITA

Haihui Huang

Candidate for the Degree of

Master of Sciences

Thesis: CIRCULATION OF UNCONJUGATED ESTRADIOL-17β DURING MID-GESTATION IN BOVINE FETAL BLOOD, AMNIOTIC AND ALLANTOIC FLUIDS

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Hunan, P. R. China, on April 09, 1970, the daughter of Dongling Yin and Weixian Huang; married to Haobo Liu in 1995.

Education: Graduated from NO.5 Middle School, Changsha, Hunan, P. R. China in June, 1987. Received Bachelor of Medical Science degree from Hunan Medical University, Changsha, Hunan, and P. R. China in July, 1993. Completed the requirements for the Master of Science degree with a major in Physiological Sciences at Oklahoma State University in July, 1998.

Experience: Employed by the Baoan People's Hospital as a Doctor of Gynecology and Obstetrics, Shenzhen, Guangdong, P. R. China, 1993 - 1995. Employed by the Department of Physiological Sciences at Oklahoma State University in Stillwater, Oklahoma as a Teaching Assistant, 1996 - 1998.