COMPARATIVE STUDIES ON UTERINE AND FETAL FLUID PROTEINS IN PREGNANT AND NON PREGNANT BUFFALO *(BUBALUS BUBALIS)* USING ONE AND TWO DIMENSIONAL ELECTROPHORESIS

Thesis

SUBMITTED TO

UTTAR PRADESH PANDIT DEEN DAYAL UPADHYAYA PASHU-CHIKITSA VIGYAN VISHWAVIDYALAYA EVAM GO ANUSANDHAN SANSTHAN, MATHURA-281 001

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE

OF

Master of Veterinary Science

IN

Veterinary Biochemistry

2005

BY

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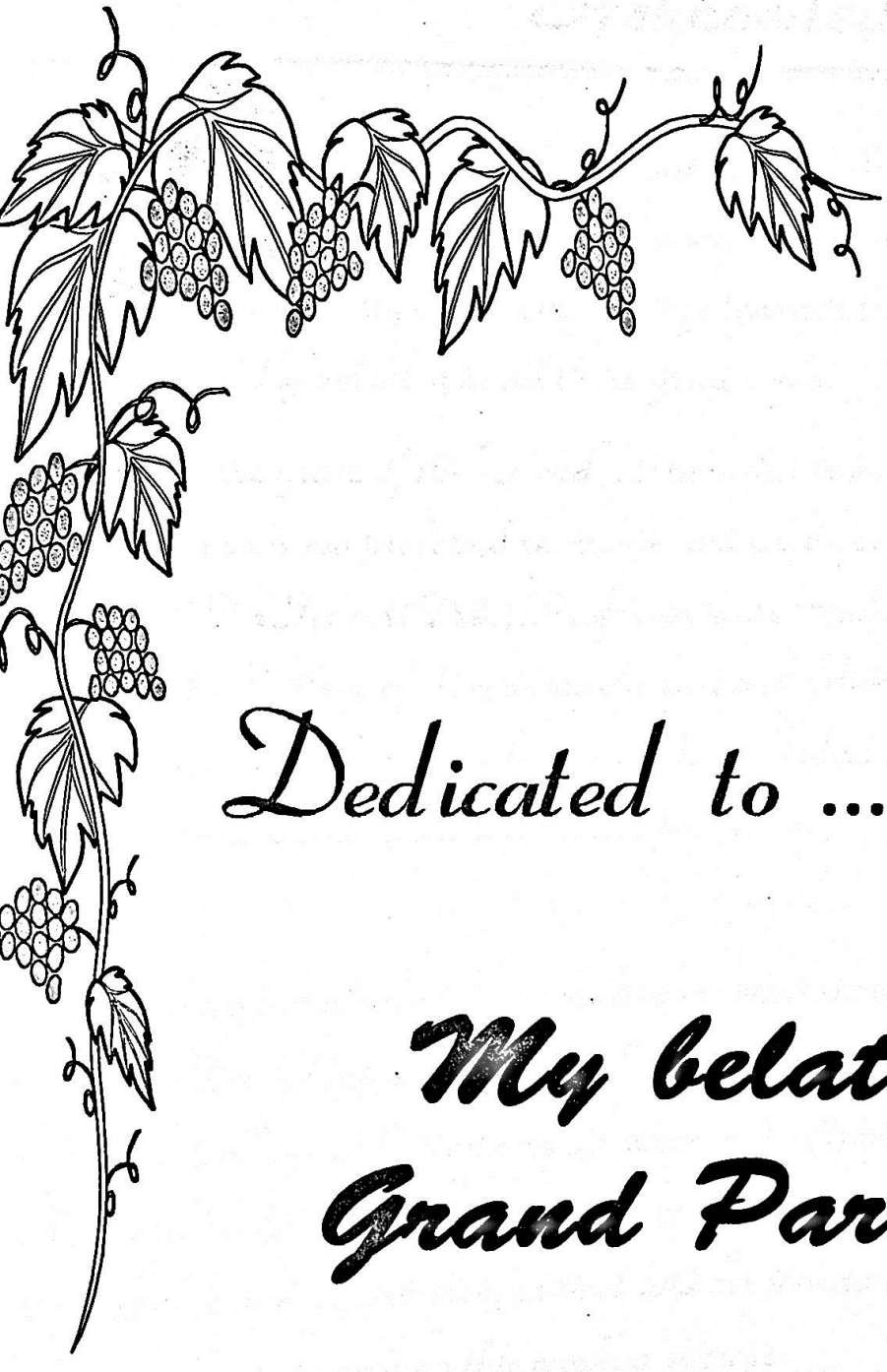
CERTIFICATE

It is certified that **DR.REETU RAJ GOGOI,** a candidate of **M.V.Sc. (final)** examination of **2005** in ***"Veterinary Biochemistry"*** has been working under my supervision during this session and that the accompanying thesis entitled, **"COMPARATIVE STUDIES ON UTERINE AND FETAL FLUID PROTEINS IN PREGNANT AND NON PREGNANT BUFFALO (*BUBALUS BUBALIS*) USING ONE AND TWO DIMENSIONAL ELECTROPHORESIS"** which he is submitting, is his genuine work.

(Kranti Dev)
(GUIDE)



Dedicated to .....

My belated Grand Parents

Acknowledgements

In the quest of life, many known and unknown learned souls enlighten the right path with their sheer knowledge and experience. No words would adequately express my feelings towards them. I shall ever remain thankfully indebted to all those great souls.

With the grace of the "Lord", I take this immense opportunity to put into words my profound reverence and subtle sense of gratitude to my guide **Dr Kranti Dev**, Professor and Head, Department of Biochemistry, College of Veterinary Science and Animal Husbandry, Mathura, U.P. for his valuable guidance, indispensable support, encouragement and constant supervision for the completion of this manuscript.

I take great pleasure in expressing my most sincere gratitude and regards to **Dr. Rajesh Nigam**, Professor, Department of Biochemistry, College of Veterinary Science and Animal Husbandry, Mathura, U.P. for his esteemed and precious guidance, constant encouragement and supervision, critical and constructive evaluation and generous efforts in making this work, a success.

Thanks are also due to **Dr. A.K.Jain**, Professor, Department of Biochemistry, College of Veterinary Science and Animal Husbandry, Mathura, U.P. for his necessary help and hearty cooperation during the course of research work.

I am deeply indebted to Dr. Jyotirmay Ghosh, Scientist, Department of Molecular Biology, National Institute of Animal Nutrition and Physiology (NIANP), Bangalore, for his active and keen interest, substantial and untiring help in laboratory work with his interpretation and analysis.

I extend my heartiest regards to Dr.H.S.Panwar, Dean, Post-Graduate Studies, Dr.S.D.Sharma,Dean, College of Veterinary Science and Animal Husbandry, Mathura, U.P. for allowing me to pursue a part of the research work at Bangalore.

I owe my sincere thanks and gratitude to all the supporting staff, Mr. Avesh Singh, Mr, Dutta Babu, Mr. Sanju Khan, Hukmi and Chote Lal , of the Department of Biochemistry for their constant help and benevolence throughout my study at this college.

Words would not suffice to expound my heartfelt gratitude to my "Beloved parents" for their love, sacrifice, encouragement, constant guidance and blessings showered upon me, throughout my every walk of life, without which it would be impossible in my part to accomplish such height.

I am greatly indebted to my fiancée Miss Lhamu Dolma, for her unconditional help, moral support and perseverance during my study at this college.

I take my greatest pride and pleasure to express my gratefulness to all my friends, Dr Pranjal Saikia, Dr. Alok Jain, Dr.Rahul Dubey, Dr.Ritesh Srivastava, Dr.Vivek

Srivastava, Dr.Vijay Jaiswal, my seniors, Dr.Sharad Singh and Dr.Vinod Verma, my juniors Dr.Ashish Singh, Dr.Swedevito Neikha, Dr.Sameer Pant and Dr.Sanjay Singh, for their immense help and moral boosting, during the research work. It is my privilege to thank Dr. Kumaravel, Ph.D (NDRI), Dr..Amarnath, SRF (NIANP), for the inspiration and support they gave me during my laboratory work.

At last but not the least, I pay my deep sense of obligation and gratitude to The Honorable Vice Chancellor Dr.S.K.Garg in providing me the necessary facilities and support.

Place: Mathura .

Date: 28th Sept '05

Reetu Raj Gogoi

(Reetu Raj Gogoi)

Abbreviations

%	-	Percentage
α	-	Alpha
τ	-	Tau
ω	-	Omega
°C	–	Degree Celsius
mg	-	Micro gram
µl	-	Micro liter
@	-	At the rate of
10x	–	Ten times dilution
1D	–	First dimension
2D	–	Second dimension
2x	–	Two times dilution
AL	–	Allantoic fluid
AM	–	Amniotic fluid
Approx.	–	Approximately
aqu	–	Aqueous
BSA	–	Bovine serum albumin
cDNA	–	Complementary DNA
DTT	–	Dithiothretol
EDTA	–	Ethylene Diamine Tetra Acetic Acid
EL	–	Early luteal phase
g	–	Gram
h	–	hour (s)
HCL	–	Hydro chloric acid
hUCRP	–	Human ubiquitin cross reactive protein
IEF	–	Iso-electric focusing
IFN	-	Interferon

kDa	–	Kilo Dalton
LL	–	Late luteal phase
M	–	Molar
mA	–	Milli ampere
mg	–	Milligram
Min	–	minute
ML	–	Mid luteal phase
mM	–	Milli mole
MM	–	Molecular marker
NaOH	–	Sodium Hydroxide
nm	–	Nanometer
pH	–	Concentration of hydrogen ion
pI	–	Iso-electric pH
PR	–	Pregnant
Rf	–	Relative front
SE	–	Serum
UCRP	–	Ubiquitin cross reactive protein
V	–	Volts
v/v	–	Volume by volume
w/v	–	Weight by volume

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INTRODUCTION

Introduction

Proteins in the uterine luminal fluid (ULF) of the mammals, such as sheep and pigs have been the subject of many previous studies that sought to understand how the biochemistry of the uterine environment adapts to the presence and supports the development of the conceptus. Since proteins in uterine secretion have been shown to serve as enzymes, carrier molecules for hormones, vitamins and minerals and as signal molecules of maternal recognition. There is direct relationship exists between the ovarian structure and the protein milieu of the uterine fluid.

There were three types of proteins detected in the uterine flushings of pregnant ewes and cattle, which were not found in serum. Developing antisera against bovine extra-embryonic membranes Butler *et al.,* (1981) reported the presence of pregnancy specific proteins in bovine extra-embryonic membranes. Most of the uterine fluid proteins that have been studied were highly abundant, and this had allowed the use of standard protein purification methods to enrich for and isolate sufficient material for identification by peptide sequencing or for use as immunogens to generate antibodies for the screening of cDNA expression libraries. Some proteins that have been identified by the use of the above approaches are the ovine uterine milk proteins. However, many more proteins have been detected and described in ULF but remain unidentified.

Poly acrylamide gel electrophoresis (PAGE) is a widely used technique to separate complex mixtures, to investigate subunit composition and to verify homogenecity of protein samples. Several workers used the electrophoretic mobility of protein as a criterion for detection of uterine specific proteins. Roberts

and Parker (1974) examined uterine luminal fluid by PAGE analysis at pH 3.5 and pH 8.9 and observed that some uterine-specific proteins migrated rapidly at pH 3.5 than any serum protein components and were therefore easily distinguishable. These uterine proteins were comprised not more than 2% of the total protein in uterine secretion. The mobility and amount of these varied according to the reproductive state. The remainders were identical immunologically with serum proteins probably derived from serum (Crutchfield and Kulangara, 1973). Studies in buffaloes (Ghosh *et al.,* 2002) during different stages of estrous cycle showed the major protein in uterine fluid had a relative molecular mass of 66 kDa and apparently was buffalo serum albumin since it ran similar to the bovine serum albumin molecular weight protein (approx. 93.5 kDa) was evident in buffalo blood serum but did not appear in uterine fluid, conversely, at least eleven uterus specific proteins (506, 470, 241, 114, 49, 38, 33, 26, 19.2, 16 and 14.3 kDa) have been detected comparing the proteins of serum. Out of these, 38-kDa polypeptide was found to be mid and late luteal stage specific, from the gel analysis it was evident that one protein (40 kDa), though present in the serum, its concentration is increasing during the mid and late luteal stage of the estrous cycle. Similarly, the 19.2 kDa polypeptide was found to be luteal stage specific. With the application of two-dimensional (2D) PAGE, Lee *et al.,* (1998) found 40 pregnancy-associated proteins in composition of ovine uterine luminal fluid (ULF) during early pregnancy. By 2D gel map comparison and western blotting they identified, transferrin which is secreted by the yolk sac from day 15, and cytoplasmic actin, one of the most abundant protein secreted by the trophoblast at day 17, apolipoprotein A1 and aldose reductase whose abundance were found markedly increased in pregnancy in ewes.

Gross composition and the changes of proteins during estrous cycle and early pregnancy have been studied in cattle (Roberts and Parker, 1974), horse (Zavy *et al.,* 1979), sheep (Iritani *et al.,* 1969) and pig (Murray *et al.,* 1972). However, no information is available on biochemical composition and

type of proteins present in uterine fluid of cycling and preimplantation buffaloes. Although buffalo is reported to low conception rate (40-45%) after artificial insemination as compared to cattle (60%). It is due to high embryonic death before and during final attachment. As in other ruminants, the buffalo embryo also lies free in the uterine space for relatively long period and derives nourishment from the fluid secreted in the uterus. Hence, a close interaction between the embryo and uterine environment is required for the growth, development and survival of embryo. Any alteration in such interaction may be the cause of most of the early embryonic losses. This area of reproduction is still unexplored in this species. During early pregnancy the conceptus growth, development and survival depends on several components of (i) serum transudation, (ii) uterine endometrium secretion under the influence of progesterone, (iii) secretion of trophoblast of developing embryo and (iv) conceptus induced endometrium secretion. Each of the components has their influence on embryonic development and study on all the components are essential to understand and devise a method to reduce the early demise of embryos before implantation or suggesting better management practice.

The allantoic is although regarded as the storage site for fetal nitrogenous waste Lee *et al.,* (1998) detected over 300 proteins by analyzing allantoic fluid protein using auto-radiography and showed that most of the conceptus-secreted proteins were rapidly transported and sequestered in the allantoic fluid.

Information on buffalo uterine fluid protein is very limiting and meager, as much work on this subject has not been done. Considering this, the present study was designed with the following objectives.

1. To detect uterine, cycle and pregnancy specific proteins.
2. To detect fetal fluid specific protein.

□□□

REVIEW OF LITERATURE

Review of Literature

2.1 UTERINE FLUID PROTEINS DURING ESTRUS CYCLE AND EARLY PREGNANCY

Proteins in uterine secretion have been shown to serve as enzymes, carrier molecules for hormones, vitamins and minerals (Zavy *et al.*, 1978) and as signal molecules of maternal recognition (Martal, *et. al.*, 1979). Proteins in the uterine luminal fluid (ULF) of the mammals, such as sheep (Wales, 1973; Moffatt *et al.*, 1987; Xie *et al.*, 1991), cattle (Old and Van Denmark, 1957), mare (Zavy *et al.*, 1978) and pigs (Basha *et al.*, 1980), have been the subject of many previous studies that sought to understand how the biochemistry of the uterine environment adapts to the presence, and supports the development, of the conceptus.

Available evidence (McRae, 1984) indicates that the uterine luminal milieu is dynamic in that the composition of uterine fluid exhibits marked differences between stages of estrous cycle and between the estrous cycle and pregnancy. Therefore the microenvironment of uterine lumen at estrous differs fundamentally from the period when implantation occur. In domestic animals, during early pregnancy till the final attachment the uterine fluid receives several biologically important proteins namely (i) serum transudation, (ii) uterine endometrium under the influence of progesterone secreted by corpus luteum, (iii) trophoblast of developing embryo and (iv) endometrium under the influence of trophoblast proteins those contribute to the endometrial physiology for the growth, development and survival of embryo. Uterine secretions of the pig have been suggested to act as complex culture medium for the loosely attached conceptus (Bazer *et al.*, 1982). A family of protease inhibitors in pig uterine

secretions have been described (Mullins *et al.*, 1980; Fazleabas *et al.*, 1982) that may protect the endometrium from protease released by the conceptus.

2.1.1 Serum derived proteins

Investigations on the macromolecular components of the luminal fluid at different stages of estrous cycle, from the bovine uterus (Roberts and Parker 1974), revealed that the components present were mainly serum proteins but small amount of uterine specific proteins were detected by electrophoresis. The mobility and the amount of the serum proteins varied according to the reproductive state. The proteins detected according to the electrophoretic band patterns, were albumin, postalbumin, pretransferrin, transferrin and γ-globulins. Current evidence provides indirect support for potentially important roles of some plasma proteins in endometrial physiology. Growth factors and growth factor-like substances perhaps of plasma origin might be required for optimal endometrial differentiation. This is based upon the findings of specific binding receptors for epidermal growth factor (EGF) in human (Hoffman *et al.*, 1984) and rat (Mukku and Stancel 1985) uterus. Binding of insulin was also observed in human (Sheet *et al.*, 1985) uterus. A marked deficiency of estrogen responses in diabetic rat perhaps might be due to absence of positive regulating interaction between estrogen and insulin (Frederick *et al.*, 1985). Extravasations of plasma proteins and extra vascular clotting might be important factors before modified tissue growth responses including tumor growth and angiogenesis (Dvorak *et al.*, 1985).

2.1.2 Endometrial Proteins

Evidences indicate that uterine endometrium synthesizes and secretes varieties of proteins although in the absence of histochemical localization it is not always clear whether epithelial and or stromal cells are primarily responsible. However, it appears that the majority of the uterine specific secretory products are synthesized by the luminal and glandular epithelium and secreted into the

luminal extracellular fluid (McRae, 1984). Uterine epithelial cells possess abundant Golgi apparatus and polyribosomes, well -developed and dilated rough endoplasmic reticulum and accumulated secretory granules or vacuoles in the apical region, all of which are suggestive of protein synthesis and secretion (Jamieson and Palade, 1971). These cells synthesize and secrete a variety of proteins (Roberts and Bazer, 1980) many of which are enzymatically active. For example, lysosomal-like enzymic activities, which resemble that of β-N-acetylglucosaminidase have been observed in luminal extracellular fluid of pigs, mare and ewes (Hansen *et al.,* 1985) and rabbits (Thie *et al.,* 1984, 1986). In the uterine fluid of rabbits, enzyme activities resembling that of α-fucosidase have also been observed (Thie *et al.,* 1984, 1986). Several anti-proteolytic enzymes have also been demonstrated, including a plasminogen activator inhibitor in western spotted skunk (Fazleabas *et al., 1*983) and protease inhibitor in pig (Fazleabas *et al.,* 1985). Proteins without enzyme activity such as prolactin (Ying *et al* .,1986) and relaxin (Larkin and Renegar, 1986) are also synthesized and secreted. It is indicated that the majority of these uterine specific secretory products are released into luminal extarcellular fluid as opposed to endometrial extracellular fluid. The exception to the rule is that human pregnancy- associated plasma protein-A and prolactin-inhibitory factor in rats. Pregnancy associated plasma protein-A is found in uterine fluid and plasma can be detected in glandular epithelium during proliferative phase of the menstrual cycle (Bischof *et al.,* 1984). Prolactin inhibitory factor in rat that synthesized in uterine epithelium appears to inhibit prolactin release from anterior pituitary *in vivo* (Gorospe and Freeman, 1985). It is suggested that stromal cells are capable of actively internalizing material form the endometrial extra cellular fluid by means of numerous plasmalemal-coat pits (Cornillie and Lauweryns, 1985). This uptake occurred either specifically into receptosomes via clathrin-coated pits and tubulvascular organelles or less specifically via the formation of macropinocytotic vacuoles. In addition, differentiation of phagocytes from the

endometrial stromal cells was described. These phagocytic cells appeared to accumulate ferritin and other stromal glycoproteins to result in a considerable storage of irons, in the form of haemosiderin complexes. They also appear to occupy a primarily perivascular location in the superficial endometrium. These cells are thought to compensate for the absence of a well-developed lymphatic drainage (Cornillie and Lauweryns, 1985).

Lee *et al.*, (1998) determined the maternal contribution to proteins in the ULF, by endometrial explants culture in the presence of methionine for 3 or 24 hours. They observed that compared to the conceptuses, the endometrial tissue was less active in protein synthesis and secretions. The only pregnancy associated, endometrial secretory proteins detected by Lee *et al.*, (1998) was a 14-kDa protein. They concluded that proteins available in ULF are most likely the progesterone-induced endometrial proteins as reported by Kazemi *et al.*, (1990) which were shown by immunochemistry and electron microscopy.

Krishnan and Daniel (1967) reported the presence of a specific protein in the uterine flushings of rabbit and designated as blastokinin or uteroglobin, that is secreted by the endometrium Beier and Maurer, (1975) and showed that the unfractionated uterine protein affect blastocyst expansion in the rabbit (Maurer and Beier, 1976).

2.1.3 Control of uterine protein secretion

A cause and effect relationship exists between the ovarian structure and the protein milieu of the uterine fluid. In pig, a luteolytic factor either protein or protein conjugate have been described in uterine flushing between 12 and 18 days of cycle by Schomberg (1969). Daniel (1972) observed that passively immunized pig with rabbit antisera against total porcine luteal phase proteins failed to produce offspring and didn't return to estrus for at least 6 months post mating. Murray et al., (1972) obtained a luteal phase specific proteins of low molecular weight range (45-13.7 kDa) by gel filtration using sephadex G200

column from uterine flushing of this species. Their observation revealed that the level of these proteins declines coinciding with the time of corpus luteum regression.

The gonadal steroids hormonally control much of the secretory function of the uterine endometrium with progesterone having the dominant role and a number of progesterone modulated secretory proteins have been reported in large domestic species (Bazer *et al.*, 1979 Hansen *et al.*, 1987; Moffatt *et al.*, 1987). Particular emphasis has been placed on the pig in which the uterus synthesizes and secretes large amount of protein in response to progesterone. In experiments with ovariectomized gilt (Chen *et al.*, 1975; Knight *et al.*, 1974; Roberts *et al.*, 1987) it has been found that progesterone causes a significant increase in the amount of protein that can be flushed from the uterus. By contrast estradiol alone has no effect relative to control however, it act synergistically with progesterone to promote protein production when it is administered at low concentration but is inhibitory as the dose increased (Knight *et al.*, 1974). Prolonged administration of progesterone or progesterone plus oestrogen over several weeks allows gram quantities of protein to be recovered from a single animal (Schlosnagle *et al.*, 1974; Roberts *et al.*, 1987). Knight *et al.*, (1974) reported that progesterone was the primary hormone responsible for the quantitative and qualitative changes in the intra luminal protein environment of the pig. Murray *et al.*, (1972) showed the amount of protein that can be flushed from the uterus of the pig changes as the estrous cycle proceeds. They observed uterine flushing of pig contained as less as 8 mg per horn in the initial 2 - 8 days of the cycle then begin to increase as progesterone comes to dominate the endometrium and by day 15, which marks the end of the luteal phase of the cycle, up to 50 mg could be recovered per horn and the flushings were colored purple due to presence of uteroferrin. The endocrine status of the pregnant female pig is not obviously distinguishable from that of the cyclic animal until day 15 (Bazer *et al.*, 1982). About that time progesterone level

drops by about two-thirds in the pregnant pig but do not fall to the basal values noted in the follicular phase. The amount of proteins in the uterine flushing also does not differ significantly between pregnant and non-pregnant animals until day 11 (Geisert *et al.*, 1982a). At that time the conceptus appears to induce the estrogen triggered exocytosis of the contents of the secretory vesicles from the glandular epithelial cells. Release of secretions into the non-pregnant uterus occurs more gradually but the total output of proteins over the next 4 days is about the same as in pregnancy (Geisert *et al.*, 1982b). However, whereas secretory activity of the pregnant endometrium is maintained beyond day 15, output of proteins from the endometrium of non-pregnant gilts falls to low levels as the end of the cycle approaches and as progesterone production by the corpus luteum ceases (Zavy *et al.*, 1984). Secretory activity of the endometrium increases markedly after day 30 and appears to reach a maximum between day 60 and 75 before declining as term approaches on day 115. Protein secretions by the endometrium are high when the ratio of progesterone to estrogen is at maximum, but is markedly reduced when estrogen concentration increases.

Synthesis of at least eight proteins is found controlled by progesterone and most of these proteins in bovine uterine washings are serum proteins with non-serum components accounting for less than 2% of the total protein (Roberts and Parker, 1974).

2.1.4 Specific proteins secreted under progesterone influence

2.1.4.1 Uteroferrin

A group of basic proteins dominate these uterine secretions after day 11 of pregnancy in pig and its best-characterized member is uteroferrin, an iron containing acid-phosphatase (Roberts and Bazer, 1984). The purple color of uteroferrin arises from the co-ordination of one of the iron atoms (two bounded iron atoms per polypeptide chain) with one or more tyrosine residues (Gaber

et al., 1979). In pig uterus the secretion of this protein was found under the influence of progesterone (Murray *et al.*, 1972). Uteroferrin-like acid phosphatases have also been purified from the uterine secretions of the horse (Mc Dowell *et al.*, 1982) and cow (Roberts and Bazer, 1988). The name uteroferrin was originally coined because of evidence that the protein is involved in iron metabolism within the pregnant uterus (Roberts and Bazer, 1980). Uteroferrin is synthesized and secreted by the epithelial cells of the uterine glands for subsequent transport to the fetal placental unit. It is taken by specialized absorptive cells of the placental areolae (Chen *et al.*, 1975; Renegar *et al.*, 1982;) and ultimately enters the fetal circulation (Renegar *et al.*, 1982) when the protein can be distributed in sites of iron metabolism such as liver and spleen. Based on the available data it is likely that uteroferrin serves not as an acid phosphatase, but as an intermediary in the transport of iron from the maternal uterine endometrium to the conceptus (Roberts *et al.*, 1986).

Two molecular weight classes of uteroferrin have long been recognized in crude uterine washings and in allantoic fluid (Chen *et al.*, 1973; Bazer *et al.*, 1975). The first is purple and having an apparent molecular weight of 35kDa; the second type of uteroferrin having MW of approximately 80kDa (Bazer *et al.*, 1975) which is pink and possesses the full enzymic activity characteristics of mercaptoethanol-reduced uteroferrin (Baumbach *et al.*, 1986). Baumbach *et al.*, (1986) concluded that this high molecular weight form of uteroferrin is a heterodimer in which one molecule of uteroferrin is associated non-covalently with a second polypeptide. The second peptide seems to maintain the acid phosphatase subunit in a stable form and the heterodimer is easily dissociable by pH condition below 5, by SDS-electrophoresis and by treatment with antibodies raised either against uteroferrin or the associated polypeptides. At least three molecular weight forms of uteroferrin-associated polypeptide have been identified (40,46 and 50 kDa). Each of these is recognizable by a single monoclonal antibody and thus anti-genically related molecules.

2.1.4.2 Proteases and proteases inhibitors

The first direct evidence for the presence of hormone-stimulated trypsin-like protease activity was obtained in the rat uterus. Lysozyme is another protease the activity of which is shown uterine secretions of pig in response to progesterone treatment (Roberts *et al.*, 1976) and is detectable in cervical secretions during the luteal phase of the human menstrual cycle (Schumacher, 1974). In both cases it is assumed that the role of the enzyme is antibacterial.

A group of plasmin/trypsin inhibitors has been identified in uterine secretions of pig during the luteal phase of estrous cycle, and during pregnancy (Mullins *et al.*, 1980; Fazleabas *et al.*, 1983) and pseudopregnancy (Fazleabas *et al.*, 1982). One of the inhibitor has been purified (Fazeleabas *et al.*, 1982). It is a low molecular weight (14kDa) basic polypeptide and appears to belong to a family of at least four immunologically related isoforms, which differ slightly in iso-electric point (pI). The form purified by them is shown synthesized predominantly by the surface epithelium of the uterus and not by the deeper glandular epithelium where uteroferrin is produced. It has been proposed that these uterine plasmin inhibitors of the pig serve to control proteolytic activity within the uterus (Fazleabas *et al.*, 1982, 1983).

2.1.4.3 Uterine milk proteins in ewes

In the ewes, the major proteins secreted by the endometrium in response to progesterone from day 30 to term, are a pair of basic glycoproteins with molecular weights of 57 kDa and 55 kDa (Moffatt *et al.*, 1987). These proteins called the uterine milk proteins (UTM-proteins), are secreted by the luminal and glandular epithelium through out most of gestation. While the function of the UTM-proteins has not been discovered, their abundance in uterine secretions suggests that they serve an important role during pregnancy. Gram quantities of the protein can be recovered from the uterus of unilaterally pregnant

ewes (Moffatt *et al.*, 1987). The two UTM-proteins have slightly similar molecular weights and isoelectric points, which suggest that they are structurally related (Hansen *et al.*, 1987) both the polypeptides are glycosylated as demonstrated by Moffatt *et al.*, (1987).

2.1.4.4 Other minor progesterone-responsive proteins

Besides the above-mentioned proteins, two low molecular weight acidic proteins have been known to exist. They have molecular weights of about 20kDa and isoelectric points of 6.3 and 6.1 respectively. Like uteroferrin they are progesterone-responsive and are relatively abundant components of flushings from pseudopregnant pigs (Roberts and Bazer, 1988). It has been suggested that these low molecular weight proteins play a role in vitamin A transport to the fetus, although it is not clear whether they correspond to the retinal and retinoic acid binding proteins described by Adams *et al.*, (1981).

In addition to this, certain minor progesterone-responsive proteins have been found in the uterine flushing of pig that is not components of the serum. They are found to be hydrolytic in nature. Most of these appear to be present in small amounts and their presence may be of little significance. However, two glycosidases, β-hexoseaminidase and β-galactosidase are found in appreciable quantities in flushing derived from pseudopregnant gilts (Hansen *et al.*, 1985). These enzymes have acid pH optima and appear to be of the typical lysosomal type of acid hydrolase and the function is unclear.

2.1.5 Conceptus derived proteins

2.1.5.1 Interferon-tau

For successful maintenance of pregnancy in sheep and cattle normal pulsatile release of prostaglandin $F_{2\alpha}$ from the uterine endometrium must be attenuated. There is evidence now that a type-I interferon, interferon-tau (IFNτ) is released by the trophoectoderm of peri-implantation conceptus in sheep

and cattle and is the primary signal responsible for preventing luteolysis in these and related species (Roberts , 1991). The interferon-τs are related to other type I IFN in primary structure, sharing 30%, 50% and 70% amino acid sequence identity with β-, α- and ω- IFNs, respectively (Leaman and Roberts, 1992). Like any other type-I IFNs, the IFN-τs are composed of five major alpha helices connected by a loop, with a long loop region between helix A and B (Senda, *et. al.,* 1995). One feature of the IFN-τs, which is also present in their structural relatives, the IFN-ωs, is the presence of six amino acid extension at the carboxyl terminus, which results in polypeptides that are 172 amino acids in length rather than the standard 166 amino acids for most IFN-αs (Stewart *et al.,* 1989; Klemann *et al.,* 1990a; Charlier *et al.,* 1991). These IFN-τ also possess biological properties that are typical of type-I IFN, including an ability to induce an antiviral state, to modulate the immune system, and to inhibit cellular proliferation (Klemann *et al.,* 1990b; Li and Roberts, 1994). Intra-uterine injection of IFN-τ in sheep (Godkin *et al.,* 1984; Ott *et al.,* 1993) and cattle (Meyer *et al.,* 1995) can extend length of the estrous cycle for up to several weeks. A primary response of the endometrium to IFN-τ is to prevent up-regulation in oxytocin receptor concentration during diestrous (Vallet and Lamming, 1991; Spencer *et al.,* 1995). Control of the oxytocin receptor expression may involve IFN-τ acting indirectly, through a pathway that first requires down-regulating estrogen receptor expression (Spencer *et al.,* 1995). The subsequent inability of the endometrium to respond to oxytocin presumably contributes to reduction in the pulsatile release of $PGF_{2\alpha}$ that occurs during early pregnancy (Mc Cracken *et al.,* 1984; Flint et *al.,* 1992). Failure of the endometrium to respond appropriately to IFN-τ or failure of the conceptus to produce adequate amount of IFN-τ at the required time have been implicated as cause for pregnancy loses in sheep and cattle (Roberts, 1991).

The production of IFN-τ was investigated in early pregnancy in red deer, *Cervus elaphus* (Demmer *et al.,* 1999). The study showed the presence of

significant anti-viral activity in uterine flushes from pregnant hinds due to the release of interferon in detectable quantities. However amount of antiviral activity in red deer are low than those measured in bovids. Spontaneous luteolysis in red deer has been shown to involve synchronous pulsatile secretion of oxytocin and $PGF_{2\alpha}$ and may be initiated by a similar positive feedback interaction between uterus and corpus luteum (Bainbridge and Jabbour, 1997). It is not yet known how IFN acts to prevent luteal regression in red deer, although IFNα treatment suppresses the pre-luteolytic increase in endometrial oxytocin sensitivity (Bainbridge *et al.*, 1996).

2.1.5.2 Plasminogen activator

The elongating pig conceptus, during the day 11 – 14 periods, releases high amount of plasminogen activator (PA), Mullins *et al.*, 1980; Fazleabas *et al.*, 1983). Moreover, plasminogen is present in uterine secretions, presumably as a serum transudes, so that there is the potential for the generation of a damaging cascade of proteolytic activity in the early pregnant uterus (Fazleabas *et al.*, 1983). Proteases, particularly PA, have been implicated in implantation (Strickland *et al.*, 1976; Denker, 1980) and out growth of embryos (Kubo *et al.*, 1981), in tissue remodeling and morphogenesis (Beer *et al.*, 1975; Ossowski *et al.*, 1979), as well in the growth of normal cells (Unkeless *et al.*, 1974).

2.1.5.3 Pregnancy associated proteins

Lee *et al.* (1998) reported 40 pregnancy-associated proteins in the uterine luminal fluid at Day 17 and found majority of the proteins were secreted by the conceptus. By 2-D gel comparison and western blotting they identified transferrin secreted by the yolk sac from day 15, cytoplasmic actin one of the most abundant proteins produced by the by the trophoblast at day 17, apolipoprotein-A1and aldose reductase whose abundance were markedly increased in pregnancy, were identified by protein micro sequencing. The allantoic is regarded as the storage site for fetal nitrogenous waste. By incubating whole

day 18 conceptuses in labeled methionine for 3h Lee *et al.*, (1998), detected over 300 proteins by analyzing allantoic fluid protein using auto-radiography and showed that most of the conceptus-secreted proteins were rapidly transported and sequestered in the allantoic fluid. Lee *et al.*, (1998)

Study was done using the high resolution afforded by large-format two-dimensional (2D) polyacrylamide gels (Voris & Young, 1980) in separating complex polypeptide mixtures to identify proteins in ULF that are associated with pregnancy. This allowed the study of the qualitative changes in serum protein composition in ULF during preimplantation development and, in addition, to identify the proteins of interest. The identification of six protein spots by peptide sequencing and two others by deduction from published 2D gel maps and verification by Western blotting was thus reported. The results have provided new insights into the biology of the early sheep conceptus. Comparison of 2-D gels of ULF proteins with gels of serum proteins enabled to determine which protein spots were serum proteins and which were derived from other sources. In ULF samples from Day 12 to Day 14, serum proteins made at least 50% of the total proteins .The identities of some of these proteins were deduced by comparison with published 2-DE maps of plasma proteins (Golaz *et al.*, 1993). A majority of the ULF proteins have been known to originate from the selective filtration of serum proteins through the endometrium (Salamonsen *et al.* 1984; Fischer and Beier, 1986). The most abundant serum proteins in ULF were albumin and the immunoglobulins , the latter was observed as a broad band at approximately 55kDa. It was observed that one serum protein in ULF whose abundance was altered in pregnancy, was more abundant in pregnant than in non-pregnant ULF at Day 17 and was also present in culture supernatants of endometrial tissue explants (Lee *et al.*, 1998). The majority of the pregnancy-associated proteins detected in day 17 and 18, were derived from the conceptus. At this stage of development, even when the yoke sac is rapidly regressing, it continues to synthesize large amount of serum proteins and one identified is

Transferrin, an iron binding and transport protein. It is likely that the transferrin in pregnant UTF is derived from both maternal serum and the conceptus (Meehan *et al.*, 1984).

2.1.6 Interferon induced uterine secreted proteins

It has been shown that interferon (IFN) that is secreted by the bovine conceptus from Day 15 to 30 of pregnancy (Godkin *et al.*, 1988; Roberts *et al.*, 1990) has got some relation with the secretion of proteins in the pregnant uterus. This IFN is a major product of the conceptus and may be involved in coordination of endocrine and / or paracrine events in the uterus to provide an environment that is favorable for maintenance of pregnancy in ruminants. It is generally accepted that IFN-τ is the maternal recognition of pregnancy signal in ruminants. In cattle, IFN-τ functions to limit the release of the luteolysin prostaglandin $F_{2\alpha}$ (PGF), thereby rescues the corpus luteum from regression (Thatcher *et al.*, 1995; Bazer *et al.*, 1997). In this way continuous exposure of the endometrium to progesterone supports the process of adhesion, implantation, placentation and embryogenesis and prevents the ensuing estrous cycle. Interferon-τ also induces the expression of numerous uterine proteins. One of these proteins is the ubiquitin homolog IFN-stimulated gene product 17 (ISG17). ISG17 becomes covalently linked to targeted intracellular proteins (Johnson, *et al.*, 1998), is released from endometrial cells (Austin *et al.*, 1996), and may function as a paracrine modulator (Pru, *et al.*, 2000). Through the evaluation of proteins secreted by the endometrium in response to IFNs in *vitro*, (Naivar *et al.*, 1995), have identified three molecular size classes of uterine proteins i.e. P8, P16 and P28 in terms of their molecular weight in kDa. Because this uterine protein was similar in size to a human ubiquitin cross-reactive protein (hUCRP) that also is regulated by IFN (Recht *et al.*, 1991), it was suspected that they were related. This hypothesis was tested using, uterine flushings, medium from cultured endometrium and endometrial tissues were examined for the presence of ubiquitin-immunoreactive proteins. A 16-kDa protein that

cross-reacted with ubiquitin and hUCRP antisera was released by the endometrium and was present in uterine flushings of 18 days old pregnant females. This 16-kDa protein was named as bovine-UCRP.

Other IFN-induced endometrial proteins although have been identified, the function in the endometrium during early pregnancy or in the event of implantation is unknown because most of the uterine proteins induced or up regulated by IFN have yet to be examined. These proteins may play an important role in establishing communication between mother and embryo and in preparing the uterus for implantation and include granulocyte chemotactic protein 2 (Teixeira *et al.*, 1997), the GTPase Mx (Ott *et al.*, 1998; Ellinwood *et. al.*, 1998) and β2 microglobulin (Vallet *et al.*, 1991), IFN regulatory factor 1 and 2 (Spencer *et al.*, 1998).

Several studies (Rueda *et al.*, 1993; Sharif *et al.*, 1989) had shown that the total secretion of endometrial proteins is not effected by IFN treatment or by pregnancy status. Secretions of proteins in response to IFN were investigated during several stages of the estrous cycle and early pregnancy. For all proteins examined, day of estrous or pregnancy had no effect on secretions.

❑❑❑

MATERIALS

& METHODS

Material & Methods

3.1 CHEMICALS

The following chemicals were used for protein estimations, and poly acrylamide gel electrophoresis analysis, of buffalo uterine fluid protein. Copper sulfate, glacial acetic acid, formaldehyde was purchased from Ranbaxy Chemicals, Bangalore; urea from S.D. Fine Chemicals, Mumbai,; Sodium carbonate, sodium hydroxide, silver nitrate, , potasium dichromate, isobutanol, , ethylene diamine tetra acetic acid (EDTA), methanol, ortho-phosphoric acid, citric acid and hydrochloric acid from Glaxo Laboratories, Mumbai; Folin-Ciocalteau's phenol reagent, acrylamide, glycerol, glycine, tris(hydroxymethyl) aminomethane, tris (hydroxymethyl) aminomethane hydrochloride, sodium thiosulfate and bovine serum albumin from SISCO Research laboratory Bombay; trichloroacetic acid and sodium azide from Central drug house, Mumbai; TritonX100 from Hi-Media Laboratories, Mumbai; azocasein, 50% glutaraldehyde, dithiothretol, ammonium persulfate, ampholine (pH 3-10), sodium lauryl sulfate, N,N,N',N',-Tetra methyl ethylene diamine (TEMED), bis-acrylamide, , bromophenol blue and comassie brilliant blue R-250 from Sigma chemicals Co., USA; agarose (low melting) and SDS-PAGE broad range protein molecular weight marker from Bangalore Genei, Bangalore.

3.2 EQUIPMENTS, GLASS AND PLASTIC WARES

The equipments used for this study were pH meter (APX-175 C ® Control Dynamics, India), Weighing balance (Sartorius, Germany), Spectrophotometer (Cinatra-10e®, GBC, Australia), Vacuum concentrator (Maxi-Dry Lyo® Heto-Holten, Denmark); Electrophoresis system including Power Pac and Gel

Documentation system (Bio-Rad, USA), micropipettes (Finnpipette®, Labsystems, Finland), Shaker Waterbath (Culture Instruments, Bangalore), Table top centrifuge (Rota 4R-V/Fm® Plasto Craft, Bombay).

All glass wares and plastic wares used were obtained from Borosil®, India and Tarson®, India.

3.3 COLLECTION OF BUFFALO UTERINE FLUID, FETAL FLUID AND SERUM

Whole genitalia of early, mid, late luteal stage and 18-22 days old (pre-implantation stage) pregnant buffaloes were collected from Corporation Slaughter House Bangalore. Immediately after slaughter they were packed in sterile plastic zip bags and brought to the laboratory in icebox.

The stage of cycle was categorized based on the corpus luteum morphology as described in cattle by Noakes, (2001).

1. **Early luteal stage** : The diameters of the corpus luteum, in the range of 1.2 - 1.4 cm, corpus luteum soft on palpation having dull cream colour.
2. **Mid luteal stage** : The corpus luteum of diameters measuring 2.0 – 2.5 cm having a yellow to orange-yellow in color, , firm and more compact on palpation .
3. **Late luteal stage** : The diameters measured, were 0.5- 1.5 cm with its protrusion from the ovarian surface much smaller and less distinct. The colour observed was bright yellow, which strikingly contrasts the previous two stages.

3.3.1 Collection of uterine fluid

The extra tissues from the uterus of 10 each, pregnant and non-pregnant buffaloes were removed. The opening towards the cervical end and one of the openings towards the oviductal end were clamped. 20ml normal saline (0.33 M NaCl) containing 0.02% Sodium Azide was injected through the other uterine horn and then clamped. The uterine musculature was gently massaged for

proper mixing of the fluid inside the uterine lumen. One of the uterine horns towards the oviductal end was unclamped and the fluid was collected in a beaker on an ice tray. The fluid was centrifuged at 10,000 rpm for 30 minutes at 4°C to remove the cells and cell debris. The samples of the respective uterine tract were then pooled and stored in deep freeze (-70°C) for later use.

3.3.2 Collection of foetal fluid

The allantoic fluid samples from the respective conceptuses were aspirated from the allantoic sacs using a sterile needle and syringe. After complete withdrawal of the fluid, the chorio-allantoic membrane was removed. The aspirated samples were collected in dry test-tube, pooled and stored in deep freeze (-70°C). Similarly the amniotic fluids of the respective conceptuses were aspirated , pooled and stored at the same refrigerated condition, samples were later used for electrophoretic comparison of luteal and non-luteal phase uterine proteins.

3.3.3 Collection of blood

Blood was collected from the jugular vein of the respective animals before slaughter, using a sterile needle and a syringe. The blood was allowed to flow slowly along the wall of the test-tube in slanting position to avoid hemolysis.

3.3.4 Collection of serum

The blood was kept in a test-tube in slanting position, undisturbed at room temperature for at least 4 hours, was centrifuged at 3500 rpm for 15 minutes and the supernatant was decanted and stored in deep freeze (-70°C) for protein analysis.

3.4 METHOD OF TOTAL PROTEIN ESTIMATION IN DIFFERENT UTERINE FLUID SERUM AND FETAL FLUID SAMPLE:

Lowry's method as modified by Peterson (1977) was used for estimation of total protein. Sodium-dodecyl-sulfate (SDS) in the estimation was used to

avoid interferences of non-ionic and cationic detergents, lipid and to promote rapid solubilisation and de-naturation of proteins.

3.4.1 *Reagents*

1. CTC *Reagent*

Copper sulfate (0.1%w/v), sodium-potassium tartrate (0.2%w/v) was mixed in one half the final volume initially. The sodium carbonate (10%w/v) was dissolved in about one half the final volume in a similar way. The equal amount of sodium carbonate solution was added slowly to the solution of copper sulfate-tartrate while stirring.

2. *Reagent*–A

One part of CTC reagent was mixed with two parts 5%SDS and one part 0.8M sodium hydroxide. The reagent was kept at 4°C temperature. The white precipitate that appeared at cooler temperatures re-dissolved upon warming.

3. *Reagent*–B

One part 2N Folin-Ciocalteu's phenol reagent was mixed with 5 parts distilled water just before estimation.

3.4.2 Procedure

Aliquots of Bovine serum albumin (BSA) standard (1mg/ml) and samples (25 µl) were taken in duplicates and the volume was made to 1ml with distilled water. 1ml of Reagent A was added and incubated for 10minutes at room temperature. Then 0.5ml of Reagent B was added and incubated in dark at room temperature for 30minutes. Amount of protein present in unknown sample was calculated by extrapolating the graph obtained by regression analysis using the optical density values of known standard. The color was read at 750nm wavelength in spectrophotometer.

3.5 ANALYSIS OF UTERINE FLUID AND SERUM PROTEINS BY ONE DIMENSION DENATURING POLY ACRYLAMIDE GEL ELECTROPHORESIS.(PAGE)

Separation of uterine and serum proteins for comparison were achieved by denaturing SDS-PAGE as described by Laemmli, (1970).

3.5.1 *Reagents*

1. Acrylamide solution (30%T / 2.6% or 0.8%C): 100 ml

Acrylamide-29.2g or 27.4g and bis-acrylamide-0.8g or 2.6g were made 100ml with distilled water, filtered and stored in a refrigerator at 4°C. For non-denaturing PAGE 30%T, 0.8%C was used whereas 30%T and 2.6%C solution was used for denaturing PAGE analysis.

2. 1.2% (w/v) SDS: 100ml

1.2g SDS was dissolved in about 90ml of distilled water and final volume was made to 100ml.

3. 2X Sample buffer: 10ml

0.5MTris-Cl (pH: 6.8)-2.5ml, glycerol-2ml, bromo-phenol blue-1mg and 0.4g SDS was added to make 10ml final volume, filtered and stored at 4°C.

4. Electrode buffer: 500ml

Tris-base (0.05M)-3.0285gm, glycine (0.384 M)-11.256g were dissolved in distilled water and made volume up to 500ml. SDS (0.1%)-0.5gm was added to the solution for denaturing PAGE.

5. Reagents for coomassie blue staining

i. Fixing solution: 50%methanol and 10%acetic acid as per requirement.

ii. Staining solution: 0.25%Comassie brilliant blue in 40%methanol and 7%glacial acetic acid as per requirement.

iii. Destaining solution: 25%methanol and 10%glacial acetic acid as per requirement.

6. Reagents for silver staining:

i. Rapid fixative 1: 40%methanol/10%acetic acid: 40ml methanol and 10 ml acetic acid was taken and made up to 100ml.

ii. Rapid fixative 2:10%methanol/5%acetic acid:5ml methanol ,2.5 ml acetic acid was taken and made upto 50 ml.

iii. Oxidizer (1x):

a. Oxidizer (10x): 5g potassium dichromate; 1 ml 70%(w/v) nitric acid made upto 500ml with distilled water

b. Working solution (1x):10x oxidizer diluted 1/10 times with H_2o (this is made fresh each time before use)

iv. Silver nitrate (0.17%, w/v): 0. 17g silver nitrate added to 100ml dist water.

v. Thiosulfate developing solution: 1.5g sodium carbonate; 50ml formaldehyde; 20ml 1%sodium thiosulfate made up to 50ml with distilled water.

vi. Acetic acid (5%, v/v): 5ml glacial acetic acid taken and made up to 100ml with distilled water.

3.5.2 Preparation of poly-acrylamide gel

Separating gel solution (10%T, 10 cm x 7cm x 0.1cm) was prepared as per the composition given in table-1. A layer of water-saturated isobutanol was added on the top of the solution to have a uniform gel surface. Once the gel solution got polymerized and solidified, isobutanol was removed from the top and washed with distilled water, separating gel buffer and stacking gel buffer sequentially and then the top of the gel was dried using filter paper. The stacking gel (4.5%T, 10cm x 2cm x 0.1cm) solution (table 1) was poured on top of the gel and comb was placed immediately to form the wells. The comb was removed before sample application.

Table 1 : Composition of separating and stacking gel solutions for denaturing-PAGE

Reagents	Final Acrylamide Concentration		
	Separating gel (10cmx7cmx0.1cm)		Stacking gel (10cmx2cmx0.1cm)
	10%T	12.5%T	4.5%T
30%Acry/, 2.6% bis-acry (ml)	2.000	2.500	0.300
1.5M Tris-HCl pH 8.8 (ml)	1.500	1.500	—
0.5m Tris-HCl pH 6.8 (ml)	—	—	0.500
1.2% SDS (ml)	0.500	0.500	0.167
Distilled water (ml)	1.950	1.450	1.020
TEMED (µl)	0.003	0.003	0.002
10%Ammoniumpersulfate (µl)	0.045	0.045	0.010
Total Volume (ml)	6.000	6.000	2.000

3.5.3 Electrophoresis of uterine and serum protein samples

Uterine fluid, buffalo blood serum and standard protein samples were mixed with 4X or 2X sample buffer depending upon concentration of protein present in solution. Samples were prepared with out mercapto-ethanol and were heated in boiling water for five minutes in a sealed cap micro centrifuge tube. Approximately 70µg proteins in about 30µl volume prepared were loaded on each well for subsequent Commassie blue staining and approximately 25 µg protein in about 30µl volume prepared for silver staining, were loaded onto each well. Electrophoresis was performed using continuous electrode buffer at 50V for about one hour till trekking dye entered the separating gel. Then running conditions were set fixed at 20mA, till the samples reaches to end of the separating gel.

The following proteins were used as SDS-PAGE electrophoresis molecular weight standards: Myosin, Rabbit Muscle (205 kDa); Phosphorylase b (97.4 kDa); BSA (68 kDa);Ovalbumin (43 kda); Carbonic anhydrase (29 kDa);

Soyabean trypsin inhibitor (20 kDa); Lysozyme (14.3 kDa); Aprotinin (6.5 kDa); Insulin (3 kDa). Molecular weight marker was dissolved in 1x Tris HCl buffer containing SDS, mercaptoethanol, glycerol and bromophenol blue and heated in boiling water for a minute in a sealed cap microcentrifuge tube.

3.5.4 Fixing and Coomassie blue staining of proteins in gel

Resulting gel was kept in fixing solution containing 50% methanol and 10% acetic acid for 30 minutes before they were stained over night with 0.25% coomassie brilliant blue R-250 in 50% methanol and 10% glacial acetic acid. A complete background de-staining of the gel was achieved by shacking the gel in de-staining solution that was changed at 2-to 3 hours interval.

3.5.5 Silver staining of proteins in gel

After electrophoresis, the gel was soaked in 5 gel volumes of rapid fixative 1 in a glass tray with a lid, for minimum of 60 minutes and poured off. Then the gel was kept similarly in rapid fixative-2 for 30 minutes. After decanting the solution 100ml of freshly prepared 1x oxidizer was added and kept for 30 minutes. Removing the oxidizer, the gels were thoroughly washed 4 times with five-gel volumes water for 15 min in each step. Then 100ml of 0.17% silver nitrate was added and kept for 45 minutes. After decanting the solution the gel was then washed for 2 minutes in running distilled water. For developing the bands in gel initially 1 gel volume of thiosulfate developing solution was added and discarded as soon as the precipitates developed, then 5 more gel volumes of freshly prepared developing solution was added immediately. Once the protein bands reached the desired intensity, the staining reaction was stopped by pouring off the developing solution and by replacing with 5 % acetic acid.

3.5.6 Analysis of gel

The gel was then stored in this 5%acetic acid for further analysis. Gel images were analyzed for comparison of bands and determination of molecular weights of unknown proteins using Quantity-one® 1D gel analysis software of Bio Rad, USA.

3.6 ISO-ELECTRIC FOCUSING (IEF) SEPARATION OF PREGNANT UTERINE FLUID PROTEIN, ALLANTOIC AND AMNIOTIC FLUID PROTEIN

3.6.1 *Reagents*

i. Acrylamide solution (30% T, 1% C): 100 ml

Acrylamide-29g and bis-acrylamide-1g were made upto100 ml with distilled water, filtered and stored in refrigerator at 4 °C up to 100 ml with distilled water, filtered and stored in refrigerator at 4 °C upto100 ml with distilled water, filtered and stored in refrigerator at 4 °C.

ii. 2X IEF sample buffer

40 mM Tris-HCl, 0.6% SDS, 2% (v/v) Triton X-100, 2% ampholyte, 50% (v/v) Glycerol, 8 M Urea where made up to a final volume of 10ml with distilled water.

iii. Electrode buffers

Cathode buffer—20 mM NaOH, anode buffer 25 mM H_3PO_4

iv. Fixing solution

10% Trichloroacetic acid, 1% Trichloroacetic acid.

v. Staining solution

0.25% (w/v), Comassie blue R-250 in 45% (V/V) methanol and 10% (v/v) glacial acetic acid prepared as per requirement.

vi Destaining solution

45% (v/v) methanol and 10% (v/v) glacial acetic acid prepared as per requirement.

3.6.2 Preparation of isoelectric focusing gel

A vertical (8x7 cm) denaturing isoelectric-focusing gel (5% final concentration) containing 8 M urea was cast with 1 mm thickness mixing the components as given in table 2. Comb was placed to form well and the gel was allowed to polymerize for 1 h. After polymerization, the comb was removed and the wells were rinsed with distilled water. The wells and upper chamber were filled with 25mM ortho-phosphoric acid (anode solution) and the lower chamber was filled with 20 mM NaOH (cathode solution). The electrodes were connected so that the polarity was reversed consistent with the electrode solutions.

Table 2: Composition of isoelectric-focusing gel

Reagents	Volume
30%Acrylamide 1% Bis-acrylamide	0.830 ml
Ampholyte (2%)	0.360 ml
Triton ×-100 (2%)	0.120 ml
Urea (8M)	2.88 g
Distilled water	1.5 ml
TEMED	10 µl
APS	40 µl

3.6.3 Electrophoretic run of gel

Electrophoresis was performed at room temperature with pre cooled buffer, pre-focusing of the gel was done for 1.5 h at a constant voltage of 150 V. The samples were mixed with half the volume of 2X sample buffer and loaded into the wells. Then the gel was run at a constant voltage of 150 V for 1.5 h followed by the run of 1.5 h at an increased voltage of 400 V. A vertical long slice of gel was kept for measuring gradient of pH in gel and rest portion kept for fixing.

3.6.4 Fixing, staining of protein bands in gel

After electro-focusing was complete the gels were placed into 10% (v/v) trichloroacetic acid, gently shaken for 10 min and then transferred into 1% trichloroacetic acid and kept overnight to remove ampholytes. After a brief rinse with distilled water the gels were stained with staining solution for 5 h. Excess stains were rinsed off with distil water and the gels were destained over night. The pH gradient in gel was measured and the pH of the protein band in gel was determined.

3.7 TWO DIMENSION ELECTROPHORESIS ANALYSIS OF IEF GELS

3.7.1 *Reagents*

i. 1%(w/v) agarose: 1g agarose in 100 ml distilled water

ii. IEF gel equilibration buffer: 3g SDS; 7.4 ml 2%(w/v) EDTA, pH 7.0; 10 ml glycerol; 2ml 1.0M Tris HCl, pH 8.6, 0.3ml bromophenol blue made volume up to 100 ml.

3.7.2 Procedure

Gel strips of respective samples were cut from the IEF gel and kept in gel equilibration buffer for 15 min before putting on top of the 10 % SDS separating gel of 1.5mm thickness prepared as per the composition described in table-1. Excess gel equilibration buffer was drained off and the gel strips were dried using dry filter paper strips before putting melted 1 % agarose solution to immobilize the gel strips on top of the separation gel. Once the agarose got solidified the casted gels were put into the electrophoresis apparatus with the same requisites of buffer as in the case of SDS-PAGE running described earlier. The gels were run at 50v for the first 1 h and 100v till the dye front reached the bottom of the gel slab.

3.7.3 Silver staining of protein spots in two dimension-PAGE gels

After electrophoresis the 2D-PAGE gels were taken out from the electrophoresis equipment, silver-staining procedure was followed according to Switzer et al. (1979).

3.7.3.1 Preparation of reagents

i. Fixing solution A: 50%methanol/10%acetic acid .50 ml methanol and 10 ml glacial acetic acid was taken and made up to 100ml with dist, water.

ii. Fixing solution B: 5%methanol/7%acetic acid: 5ml methanol, 7ml acetic acid and made up to 100 ml.

iii. Glutaraldehyde (10%, v/v): Glutaraldehyde (50% aqu. Sol^{n})- 10 ml made up to 50 ml with distilled water.

iv. Dithiothretol (DTT) (5mg/ml): Dithiothretol - 0. 25 mg made up to 50 ml with double distilled water.

v. Silver nitrate (0.1%, w/v): Silver nitrate- 0.1g made up to 100ml with double distilled water.

vi. Carbonate developing solution: Formaldehyde (37%, v/v) - 0.05ml and sodium carbonates: 3g, added and made up to 100 ml with double distilled water.

vii. Citric acid (2.3 M): Citric acid - 4.41g dissolved and made volume up to10 ml in double distilled water.

viii. Sodium carbonate (0.03 %, w/v): Sodium carbonate- 0.03g dissolved and made volume up to 100 ml in distilled water.

3.7.3.2 Procedure

The gels were removed from electrophoresis apparatus and placed in a glass tray equipped with a lid. 10 gel volumes of fixing solution A was then added and agitated for 30 minutes. Fixing solution A was then decanted and 10 gel volumes of fixing solution B was added and then again agitated for 30 minutes. Fixing solution was then removed and 50 ml of 10% glutaraldehyde was added, and agitated for 10 minutes in closed lid condition. Glutaraldehyde solution decanted .10 gel volumes of distilled water added and then agitated

for 15 minutes. This was repeated for eight times. Water was then decanted and 5 gel volumes of 5µg/ml dithiothretol was added. Agitated for 30 minutes. DTT was then poured off and without rinsing 5 gel volumes of 0.1%silver nitrate was added and agitated for 30 minutes. Silver nitrate was then decanted. Then gel was rinsed once for 15 seconds with water by filling the container with water and then pouring it out. The gel was rinsed quickly with few ml of carbonate developing solution. Finally add 100ml carbonate developing solution and agitated until bands appeared. When proper staining was achieved 5ml of 2.3 M Citric acid was directly added to carbonate developing solution to stop the staining reaction. Gels were then stored in 0.03%sodium carbonate for later analysis.

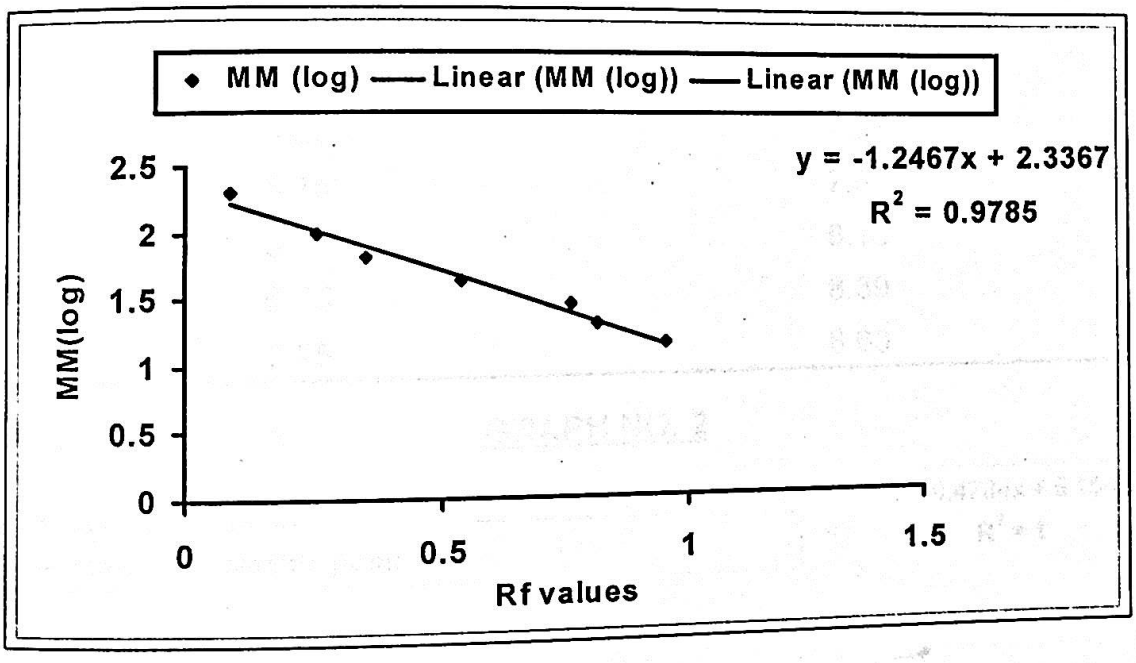
3.7.4 Analysis of gel

The spots observed in pregnant uterine sample were compared manually with non-pregnant samples and the spots in allantoic fluid proteins were compared with amniotic fluid sample. Calculation of molecular weights of protein spots in the 2DE gels was done by obtaining the logarithmic values of standard molecular weights and the respective Relative front (Rf) values, which were then plotted in a graph to obtain the derived line equation (graph no. 1). Extrapolation of the values of Rf (x) of the protein spots was then substituted into the line equation to determine the molecular weights of the protein spots. Similarly determination of pI values of protein spots IEF-gels was done by plotting the mean distance (breath) of the cut gel pieces and their respective iso-electric points to obtain the line equation (graph no.2). Extrapolation of the values of the distance migrated (x) of the protein spots were then substituted into the derived equation for determining the pI of the protein spots.

Table 3 : **Rf values and the logarithms of the standard mol. wt. markers plotted in graph no 1.**

MM (log)	Rf values
2.311754	0.0879
1.988559	0.2527
1.819544	0.3516
1.633468	0.5384
1.462398	0.7582
1.30103	0.8131
1.155336	0.956

GRAPH NO.1

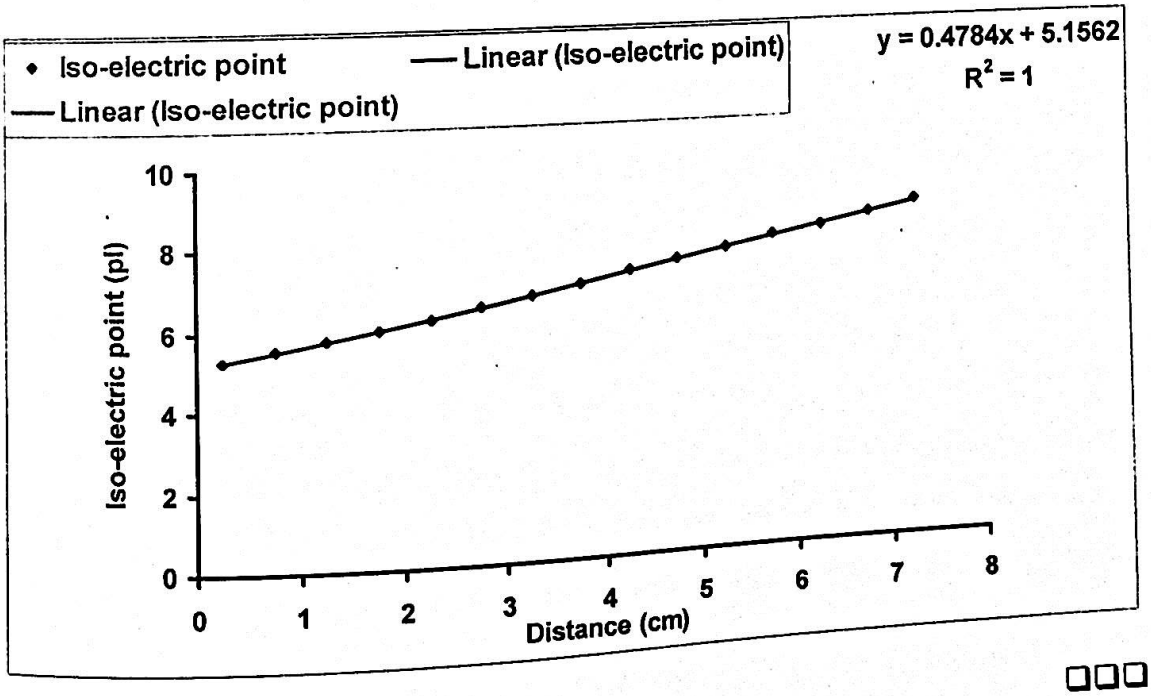


Rf : Relative front; Mol.wt: Molecular weight; MM: Standard Molecular Marker

Table 4 : **Mean distance (length) of the cut gel pieces and the pI values of the protein bands in the IEF-gel (Fig. no. 2) plotted in graph no. 2**

Distance (cm)	Iso-electric point (pI)
0.25	5.28
0.75	5.52
1.25	5.75
1.75	5.99
2.25	6.23
2.75	6.47
3.25	6.71
3.75	6.95
4.25	7.19
4.75	7.43
5.25	7.67
5.75	7.91
6.25	8.13
6.75	8.39
7.25	8.63

GRAPH NO. 2



RESULTS

Results

4.1 COMPARISON OF DIFFERENT CYCLING STAGE UTERINE PROTEIN, FETAL FLUID AND SERUM PROTEIN SAMPLES BY ONE DIMENSION NON-REDUCING SDS-PAGE

Comparison of protein bands in serum with different cycling stage revealed a 51.6 kDa protein band in serum that was not observed in any of the uterine fluid protein samples tested. Protein bands with approximate molecular weight of 40.7, 29.6 and 27.3 kDa were uterine specific since all were observed in early, mid and late luteal phase uterine fluid including anoestrus sample but not found in serum sample. All the higher molecular weights bands in serum were coinciding with the late luteal and mid luteal samples. A band of 11.5 kDa was found to be unique for mid luteal sample that was not observed in any of the other phases of cycle. Similarly 14.3 kDa band for late luteal and 9.9 kDa band were unique for early luteal phase samples those were not observed in other stages of the cycle and serum sample (Fig 1).

Comparison of pregnant and non-pregnant luteal uterine proteins samples revealed no difference in banding pattern both in coomassie blue and silver staining method of detection.

When the banding patterns were compared between allantoic and amniotic protein samples 12 bands were observed in allantoic fluid whereas 10 bands were present in amniotic fluid. A band of 6.8 kDa was observed in amniotic fluid that was not present in allantoic fluid. Similarly three bands of 20.6, 10 and 8.1 kDa were present in allantoic fluid those were not present in amniotic fluid (Fig 1).

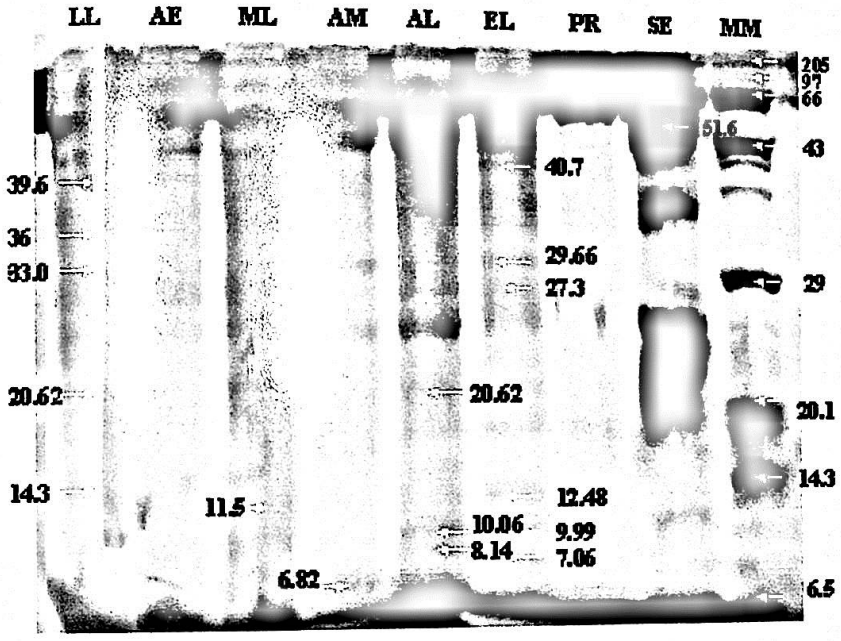


Figure 1 : 12.5% silver stained SDS-polyacrylamide gel showing unique bands in the following different protein samples: late luteal (LL), anoestrous (AE), mid luteal (ML), amniotic fluid (AM), allantoic fluid (AL), early luteal (EL), serum (SE). Lane MM is molecular marker

4.2 ISOELECTRIC POINTS OF PROTEINS IN PREGNANT UTERINE FLUID, LUTEAL PHASE, ALLANTOIC FLUID AND AMNIOTIC FLUID

Isoelectric-focusing of the uterine and fetal fluid proteins in a vertical polyacrylamide mini gel system provided rapid resolution of protein bands in respective pH range with reproducible results in the presence of 8 M urea with 3 to 10 pH range ampholine. The gel pH was in the range of 5.16 to 8.75 indicated that pH gradient had been established. In pregnant animals 18 protein bands were focused at different pH values of gel whereas 15 protein bands were observed in luteal phase uterine fluid (Table-10). Out of the 18 bands visible in pregnant animals 6 bands were not present in luteal phase proteins where as there were 4 bands present in luteal phase those were not present in pregnant protein sample. About 67 % of the bands were focused in 6 to 8 pH range in pregnant animals whereas 73% protein bands were focused in the same pH range in luteal phase proteins. Rest 33% in pregnant and 27% of luteal phase proteins was in the acidic range.Three unique proteins were present below 6.0 pH in the pregnant sample compared to luteal phase samples whereas only one unique band was found in luteal phase samples (Fig 2). There were six bands present in the range of 6.0 to 6.5 pH range and all were common for the pregnant and luteal phase samples. There were four more unique bands having pI values of 6.6, 6.7, 7.0 and 7.2 present in pregnant uterine fluid sample and three unique bands present with pI values 6.8, 7.4 and 7.5 in luteal phase sample.

Comparison of the protein bands in allantoic and amniotic fluid revealed a total 15 bands with different isoelectric points in allantoic fluid samples compared to only 7 bands in amniotic fluid protein samples (Table-11). There were no unique bands available in amniotic fluid those were not observed in allantoic fluid sample. Interestingly all the 8 unique bands in allantoic fluid

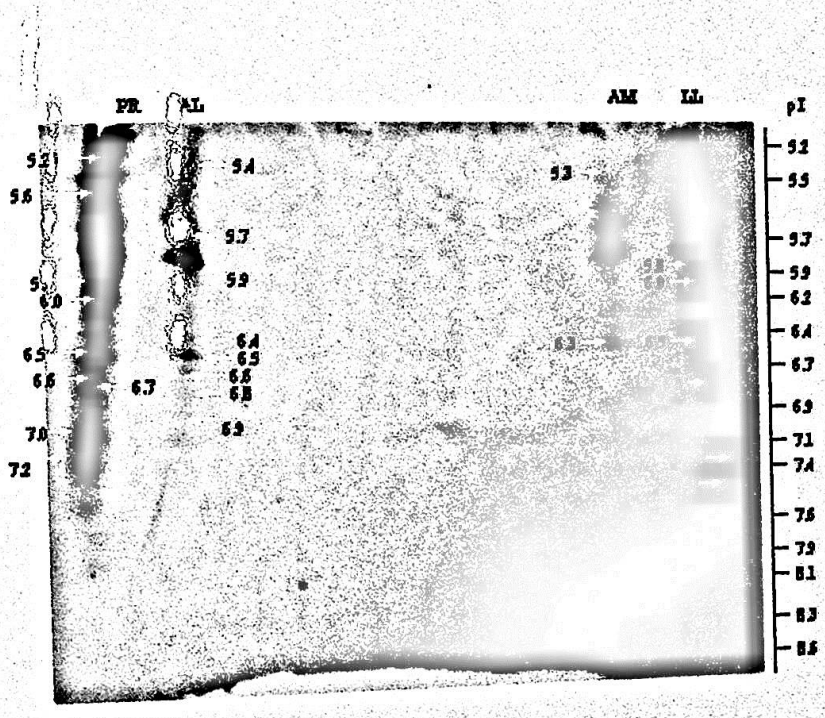


Figure 2 : **5% Iso-electric focusing gel showing the comparative difference of the iso-electric points of the different proteins present in pregnant (PR), luteal (LL), allantoic (AL) and amniotic (AM) fluid in buffalo**

were in the acidic pH range out of these 3 bands were having pI values of 5.4,5.7 and 5.9 rest 5 bands had pI values of 6.4,6.5,6.6,6.8 and 6:9. The soelectric pH of both allantoic and amniotic fluid ranged from 5.30 to 6.93 and 5.35 to 6.3 respectively indicated that proteins in both the fluids were acidic in nature but the proteins in amniotic fluid were more acidic in nature compared to allantoic fluid proteins.

Table 5 : Calculation of Logarithms of the Molecular weight markers (MM) and Relative front (Rf) values (graph no.1)

MM (kD)	Logarithm of MM	A/B	Rf values
205	2.3117	0.66/7.5	0.0879
97.4	1.9884	1.9/7.5	0.2527
66	1.8195	2.67/7.5	0.3516
43	1.6334	4.0/7.5	0.5384
29	1.4623	5.7/7.5	0.7582
20	1.3013	6.1/7.5	0.8131
14.3	1.1553	7.2/7.5	0.9560

Table 6 : Calculation of molecular weights of the proteins detected through 2DE in the pregnant uterine fluid sample of buffalo using the Rf values (x) of the respective protein spots and extrapolating the values of x in the derived line equation : y = -1.2467x + 2.3367 (graph no.1)

Protein spot no. (in figure 3)	A/B	Rf value (x)	Value of y (in equation)	Antilog of y (mol.wt in kDa)
a	0.8/7.5	0.1098	2.19	154.88
b	1.5/7.5	0.1978	2.09	123.02
c	1.6/7.5	0.2197	2.06	114.81
d	2.1/7.5	0.2747	1.99	97.7
e	1.4/7.5	0.1868	2.10	125.8
f	1.8/7.5	0.2527	2.02	104.7
g	2.2/7.5	0.2967	1.96	91.2
h	2.5/7.5	0.3406	1.91	81.2
i	2.4/7.5	0.3296	1.92	84.1
j	2.2/7.5	0.2967	1.96	91.2
k	1.8/7.5	0.2417	2.03	107.1
l	1.6/7.5	0.2197	2.06	115.3
m	1.4/7.5	0.1978	2.09	123.0
n	1.4/7.5	0.1978	2.09	123.0
o	4.6/7.5	0.6263	1.55	35.4
p	5.2/7.5	0.7032	1.46	28.8
q	5.4/7.5	0.7252	1.43	27.0
r	5.1/7.5	0.6923	1.47	29.7
s	4.7/7.5	0.6373	1.54	34.6
t	4.6/7.5	0.6153	1.56	37.0
u	4.7/7.5	0.6263	1.55	35.5
v	4.9/7.5	0.6593	1.51	32.6
w	4.9/7.5	0.6593	1.51	32.6

2DE: Two Dimensional Gel Electrophoresis; MM : Molecular Marker; kD : Kilodalton; A/B : Distance migrated by the protein/ Total distance migrated; Rf : Relative front; Mol.wt: Molecular *weight.*

Table 7: **Calculation of Molecular weight of protein detected through 2DE in allantoic and amniotic fluid sample of buffalo using the Rf values (x) of the respective protein spots and extrapolating the values of x in the derived line equation: y = -1.2467x + 2.3367 (refer graph no.1)**

Protein spot no. (in figure)	A/B	Rf value (x)	Value of y (in equation)	Antilog of y (mol.wt in kDa)
1	2.03/7.5	0.271	1.998	99.54
2	3.9/7.5	0.528	1.678	47.64
3	3.9/7.5	0.528	1.678	47.64
4	1.6/7.5	0.214	2.069	117.21
5	1.5/7.5	0.2	2.087	122.17
6	1.3/7.5	0.185	2.106	127.64
7	1.2/7.5	0.157	2.140	138.03

2DE : Two Dimensional Gel Electrophoresis; kD : Kilodalton; A/B : Distance migrated by the prtein / Total distance migrated; Rf : Relative front; Mol.wt: Molecular weight.

Table 8 : Calculation of Iso-electric point (pI) of the proteins detected in pregnant and late luteal (non-pregnant) uterine fluid sample in buffalo, through Iso-electric focusing by extrapolating the distance migrated by the protein bands (x) in the derived line equation, y = 0.4784x + 5.1562 (graph no.2)

Distance migrated (x) in cm	Pregnant (pI value)	Non-pregnant (pI value)
0.2	5.2	-
0.5	5.4	5.4
0.7	-	5.5
0.8	5.5	-
1.0	5.6	-
1.1	-	5.6
1.2	5.7	-
1.4	-	5.8
1.5	5.8	-
1.7	-	6.0
1.8	6.0	-
2.0	6.1	-
2.1	-	6.1
2.2	6.2	6.2
2.4	6.3	6.3
2.6	-	6.4
2.7	6.4	-
2.9	6.5	6.5
3.0	6.6	-
3.3	6.7	-
3.4	-	6.7
3.6	6.9	6.9
3.9	7.0	-
4.2	7.2	-
4.6	-	7.3
4.8	7.4	7.4
5.0	-	7.5

Table 9 : **Calculation of Iso-electric point (pI) of the proteins detected in Allantoic and Amniotic fluid sample in buffalo, through Iso-electric focusing by extrapolating the distance migrated by the protein bands (x) in the derived line equation, y = 0.4784x + 5.1562 (graph no.2)**

Distance migrated (x) in cm	pI (Allantoic fluid)	pI (Amniotic fluid)
0.3	5.3	-
0.4	-	5.3
0.5	5.4	-
0.7	5.5	5.5
1.0	5.6	5.6
1.2	5.7	-
1.3	-	5.8
1.4	5.8	-
1.6	6.0	-
2.0	6.1	6.1
2.2	6.2	6.2
2.4	6.3	6.3
2.6	6.4	-
2.9	6.5	-
3.1	6.6	-
3.4	6.8	-
3.7	6.9	-

Table 10 : Comparison of different isoelectric point of pregnant and luteal phase uterine fluid proteins

Pregnant	Luteal phase
5.25	
5.4	5.4
5.5	5.5
5.6	
5.7	5.7
	5.8
5.9	
6.0	6.0
6.1	6.1
6.2	6.2
6.3	6.3
6.4	6.4
6.5	6.5
6.6	
6.7	
	6.8
	6.9
6.9	
7.00	
7.2	
	7.4
	7.45
7.45	
	7.5

Table 11: Comparison of different isoelectric point protein bands of allantoic and amniotic fluid

Allantoic fluid	Amniotic fluid
5.3	5.3
5.4	
5.5	5.5
5.6	5.6
5.7	
5.8	5.8
5.9	
6.1	6.1
6.2	6.2
6.3	6.3
6.4	
6.5	
6.6	
6.8	
6.9	

4.3 TWO DIMENSION GEL PROTEIN SPOT COMPARISON OF PREGNANT AND NON-PREGNANT LUTEAL PHASE UTERINE PROTEINS

Comparisons of protein spots between pregnant and non-pregnant uterine secretion revealed about 23 spots in pregnant samples those were different than the comparable spots in non-pregnant luteal phase samples. The molecular weight as appeared in gel is shown in table 12. Pregnant sample contained four high molecular weight proteins spots (154.8, 123, 114.8 and 97.7 kDa) between 5 to 5.5 pI those were not present in luteal phase sample. Similarly, two clusters of different protein spots were observed in pregnant sample after 6.7 to 7.8 pI region with one cluster having 10 spots (125.8, 123, 123, 115.3, 107.1,104.7, 91.2, 91.2, 84.1 and 81.2 kDa) in relatively high molecular weight region and other one with 9 different spots in the low molecular weight region (35.4, 28.8, 27, 29.7, 34.6, 37, 35.4, 32.6, 32.6 kDa) but not in non pregnant luteal phase sample (Fig 3).

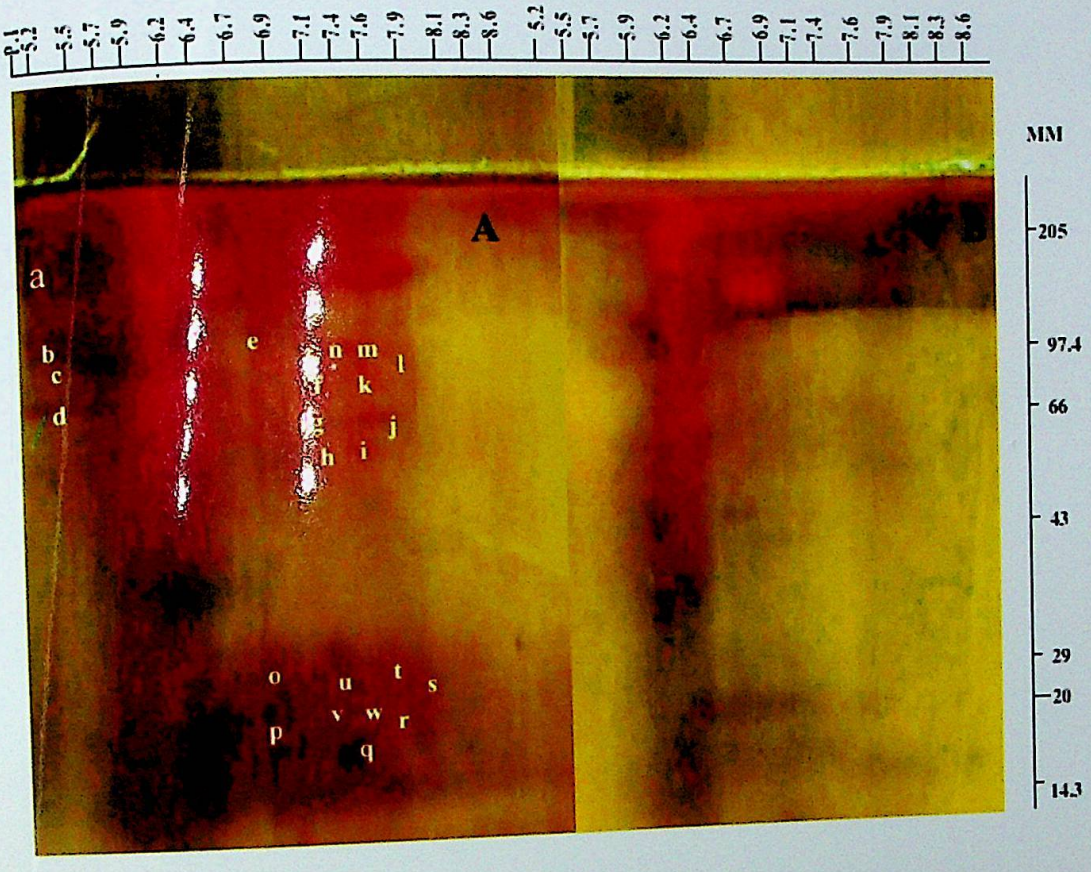


Figure 3 : **Two-dimensional silver stain gel of pregnant (A) and the non- pregnant luteal phase (B) buffalo uterine proteins. The difference in protein spots between pregnant and non-pregnant has been marked with alphabetic letters. Spot a,b,c,d in the pregnant sample indicates the presence of higher acidic proteins with pI values ranging from 5.2 to 5.5, than the non pregnant luteal sample.**

Table 12 : **Rf values and molecular weights of the different protein spots detected in pregnant which were not present in the non-pregnant samples**

Spot no.	Relative Fronts	Molecular weight (kDa)
a	0.11	154.8
e	0.187	125.8
b	0.197	123.0
m	0.198	123.0
n	0.198	123.0
c	0.22	114.8
l	0.219	115.3
k	0.241	107.1
f	0.252	104.7
d	0.274	97.7
g	0.296	91.2
j	0.296	91.2
i	0.329	84.1
h	0.340	81.2
t	0.615	37.0
o	0.626	35.4
u	0.626	35.4
s	0.637	34.6
v	0.659	32.6
w	0.659	32.6
r	0.692	29.7
p	0.703	28.8
q	0.725	27.0

4.4 TWO DIMENSION GEL COMPARISON OF PROTEIN SPOTS IN ALLANTOIC AND AMNIOTIC FLUID PROTEINS

The comparison of protein spots between allantoic and amniotic fluid revealed about 6 different spots in allantoic fluid proteins those were not

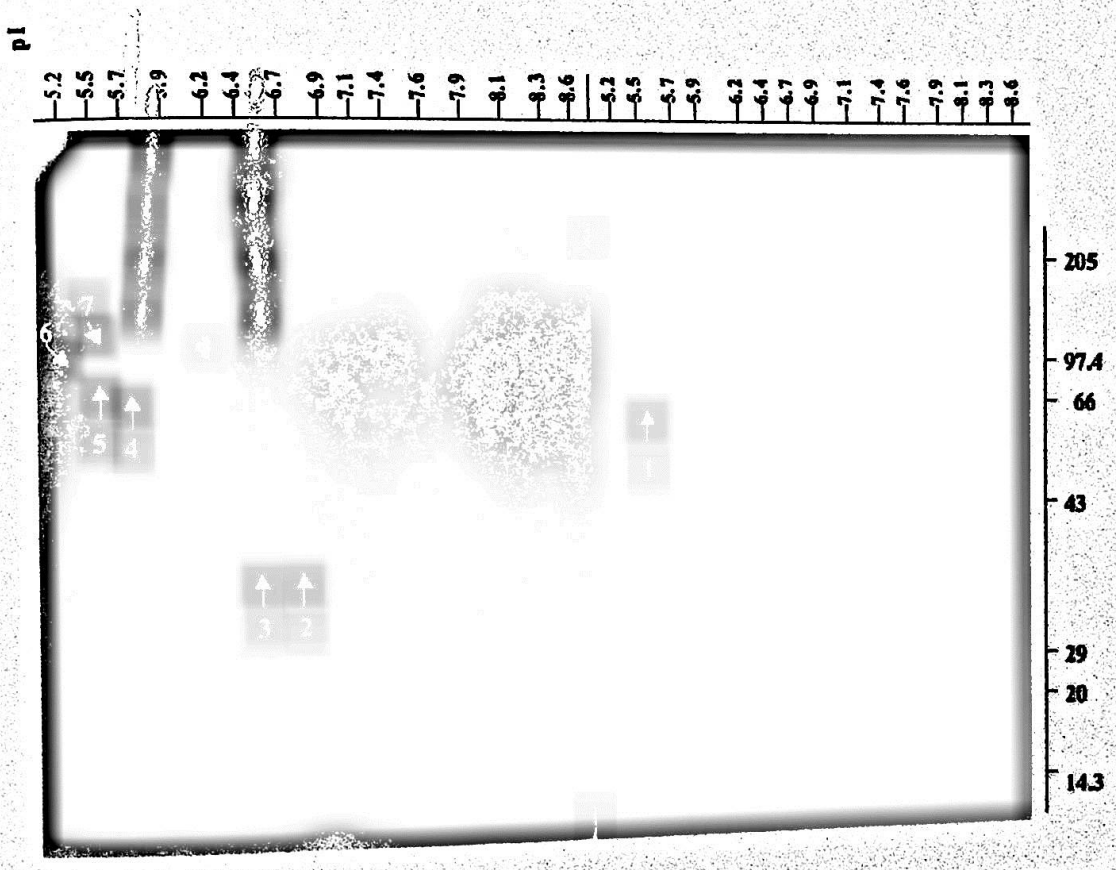


Figure 4 : Two-dimensional silver stained gel of allantoic (A) and amniotic (B) fluid proteins. The difference in protein spots has been marked with arrows. Presence of six different protein from spot no. 2 to 7 out of which four spots 4, 5, 6 and 7 were seen to be highly acidic, not seen in the amniotic sample

observed in amniotic fluid. However, only one spot (99.5 kDa) was found unique for the amniotic fluid protein that was not present in allantoic fluid. Out of the six unique bands present in allantoic fluid four were in the higher molecular weight (138, 127.6, 122.1 and 117.2 kDa) in the pH region of 5.2 to 5.8 and two had lower molecular weight (47.6 and 47.6 kDa), and were in the pH range of 6.5 to 6.8. (Table.13; Fig.4).

Table 13 : Rf values and molecular weights of the different protein spots detected in the allantoic fluid which were not detected in the amniotic fluid and vice versa

Spot no.	Relative front	Amniotic fluid (kDa)	Allantoic fluid (kDa)
7	0.157		138.0
6	0.185		127.6
5	0.2		122.1
4	0.214		117.2
1	0.271	99.5	
2	0.528		47.6
3	0.528		47.6

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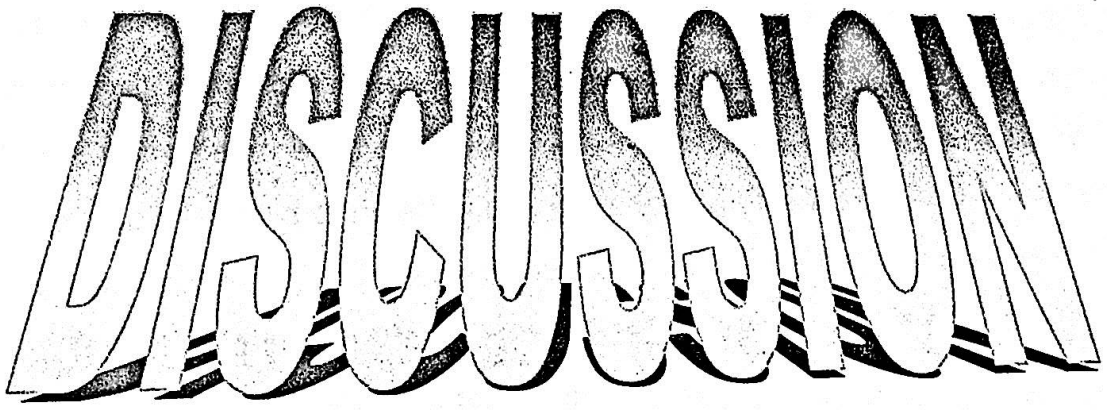
DISCUSSION

Discussion

Throughout the experiment including two-dimension denaturing PAGE analysis, the proteins in crude sample were not reduced to keep the subunits structure of the proteins intact. A unique protein band in serum protein indicated selection of serum proteins in the uterine secretion. The permeability and transport properties of endothelial cell of the uterine microvasculature and the epithelial cell lining the lumen determine the rate-limiting boundaries between the extra cellular fluid compartments of the endometrium and lumen. There are free access of the low molecular weight proteinous molecules like different growth factors and insulin (Hoffmann *et al.*, 1984; Sheet *et al.*, 1985). On the other hand there are restriction for the molecules in higher molecular weight range depending upon the hormonal status that controls the vascular permeability and formation of gap junction in luminal epithelium. Since microvasculature are less permeable to hydrophilic molecules than lipophilic one (Verheugen *et al.*, 1984), lipid solubility and the uterine blood flow might be important factor influencing which protein molecules of serum be available to the uterine lumen and to what extent although some of the components are transfered to the lumen by cellular uptake.

The presence of the uterine specific proteins as observed in this study has been reported by others in different species. In cattle most of these proteins in bovine uterine washings are reported to be serum proteins with non-serum components accounting for less than 2% of the total protein (Roberts and Parker, 1974). Evidences indicate that the uterine endometrium synthesizes and secretes varieties of proteins although in the absence of histochemical

localization it is not always clear whether epithelial and or stromal cells are primarily responsible. However, it appears that the majority of the uterine specific secretory products are synthesized by the luminal and glandular epithelium and secreted into the luminal extracellular fluid (McRae, 1984). Uterine epithelial cells possess abundant Golgi apparatus and polyribosomes, well -developed and dilated rough endoplasmic reticulum and accumulated secretory granules or vacuoles in the apical region, all of which are suggestive of protein synthesis and secretion (Jamienson and Palade, 1971). These cells synthesize and secrete a variety of proteins (Roberts and Bazer, 1980) many of which are enzymatically active. For example, lysosomal- like enzymic activities, which resemble that of β-N-acetylglucosaminidase have been observed in luminal extracellular fluid of pigs, mare and ewes (Hansen *et al.*, 1985) and rabbits (Thie *et al.*, 1984, 1986). In the uterine fluid of rabbits, enzyme activities resembling that of α-fucosidase have also been observed (Thie *et al.*, 1984, 1986). Several anti-proteolytic enzymes have also been demonstrated, including a plasminogen activator inhibitor in western spotted skunk (Fazleabas *et al.*, 1983) and protease inhibitor in pig (Fazleabas *et al.*, 1985). Proteins without enzyme activity such as prolactin (Ying *et al.*, 1986) and relaxin (Larkin and Renegar, 1986) are also synthesized and secreted. It is indicated that the majority of these uterine specific secretory products are released into luminal extarcellular fluid as opposed to the endometrial extracellular fluid.

At least three low molecular weight luteal phase specific bands could be detected in this study. Corpus luteum secreted progesterone is found to be the main hormone that influences the protein synthesis activity of the endometrium. In experiments with ovariectomized gilt (Chen *et al.*, 1975; Knight *et al.*, 1974; Roberts *et al.*, 1987) it has been found that progesterone causes a significant increase in the amount of protein that can be flushed from the uterus. By contrast, estradiol alone has no effect relative to control however, it

act synergistically with progesterone to promote protein production when it is administered at low concentration but is inhibitory as the dose increased (Knight *et al.*, 1974). Some specific proteins secreted under progesterone influence in the uterine lumen are reported in different species. Uteroferrin, an iron containing acid-phosphatase one of such proteins is found in pig (Murray *et al.*, 1972), horse (Mc Dowell *et al.*, 1982) and cow (Roberts and Bazer, 1988) uterine secretion. A group of plasmin / trypsin inhibitors has been identified in the uterine secretions of pig (Mullins *et al.*, 1980; Fazleabas *et al.*, 1983; Fazleabas *et al.*, 1982). One of the inhibitor interestingly had molecular weight of 14kDa which was a basic polypeptide and appears to belong to a family of at least four immunologically related isoforms, which differ slightly in pI (Fazeleabas *et al.*, 1982), is shown synthesized predominantly by the surface epithelium of the uterus and not by the deeper glandular epithelium where uteroferrin is produced. Although protease inhibitory activity was not detected in gel, a band of 14.3 kDa was observed in this study. Detection of protease inhibitory activity of electrophoretically separated protein bands in gel may be useful to know whether the same protein band had any such activity or not. Lysozyme is another protein that appears in the uterine secretions of the pig in response to progesterone treatment (Roberts *et al.*, 1976) and is detectable in cervical secretions during the luteal phase of the human menstrual cycle (Schumacher,1974). In the ewes, the major proteins secreted by the endomertium in response to progesterone from day 30 to term, are a pair of basic glycoproteins with molecular weights of 57 kDa and 55kDa (Moffatt *et al.*, 1987). These proteins called the uterine milk proteins (UTM-proteins) are secreted by the luminal and glandular epithelium through out most of gestation. While the function of the UTM-proteins has not been discovered, their abundance in the uterine secretions suggests that they serve an important role during pregnancy. Besides the above-mentioned proteins, two low molecular weight acidic proteins have been known to exist. They have molecular weights

of about 20kDa and isoelectric points of 6.3 and 6.1, respectively. Like uteroferrin they are progesterone-responsive and are relatively abundant components of flushings from pseudopregnant pigs (Roberts and Bazer, 1988). It has been suggested that these low molecular weight proteins play a role in vitamin A transport to the fetus, although it is not clear whether they correspond to the retinal and retinoic acid binding proteins described by Adams *et al.*, (1981).

In addition to this, certain minor progesterone-responsive proteins have been found in the uterine flushing of the pig that is not components of the serum. They are found to be hydrolytic in nature. Most of these appear to be present in small amounts and their presence may be of little significance. However, two glycosidases, β-hexoseaminidase and β-galactosidase are found in appreciable quantities in flushings derived from pseudopregnant gilts (Hansen *et al.*, 1985). These enzymes have acid pH optima and appear to be of the typical lysosomal type of acid hydrolase and the function is unclear. Both by coomassie blue and silver staining detection method failed to detect any difference in protein bands between pregnant and non-pregnant luteal phase sample in one dimension non-reducing SDS-PAGE. However, isoelctric focusing in a vertical gel and the second dimension PAGE in mini gel system proved to be effective for comparison and detection of difference in protein bands/spots between these two uterine fluid and amniotic and allantoic fluid proteins samples. One of the reason of non detection could be that the uterine secretion of early pregnancy constituted of about 50% with serum proteins which limit the amount of proteins that can be loaded for first dimension hence limited their detection (Lee *et al.*, 1998). In isoelctric focusing and two-dimension gel number of proteins bands/spots were detected those had pregnancy specificity and were not present in luteal phase uterine protein sample. Although, it is likely that with this study all the pregnancy specific proteins were not be detected since in presence of major serum proteins like immunoglobulins and albumins there will be low relative abundance of different proteins and they will interfere

the migration of other proteins. Using two-dimension electrophoresis, Lee *et al.*, (1998) reported 40 pregnancy-associated proteins in the ovine uterine luminal fluid at Day 17 and found majority of the proteins were secreted by the conceptus. Voris *et al.*, (1980) identified about eight protein spots in sheep associated with early pregnancy using large format two-dimension gel. In both the studies spots were compared with the serum spots to identify specific proteins. On the other hand comparison with luteal phase sample was made with the pregnant sample in this study to eliminate the proteins those will be available from serum and the uterine tissues in absence of conceptus.

The allantoic is although regarded as the storage site for fetal nitrogenous waste, Lee *et al.*, (1998) by incubating whole day 18 conceptuses in labeled methionine for 3h detected over 300 proteins by analyzing allantoic fluid protein using auto-radiography and showed that most of the conceptus-secreted proteins were rapidly transported and sequestered in the allantoic fluid. This study showed allantoic fluid proteins 12 different bands by one dimension non-reducing electrophoresis and 15 bands through isoelectric focusing. Comparison of banding/spot pattern in allantoic and amniotic fluid protein by one and two dimension gel electrophoresis study revealed absence of many bands/spots in and presence of one low molecular weight and a high molecular weight spot in amniotic fluid. Origin of these proteins in amniotic fluid is either fetus or the amniotic membrane is not ascertained.

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SUMMARY

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SUMMARY & CONCLUSION

Summary and Conclusion

Gross composition and the changes of proteins during estrous cycle and early pregnancy have been studied in cattle, horse, sheep and pig. However, no information is available on biochemical composition and type of proteins present in uterine fluid of cycling and pre-implantation buffaloes. Similarly, report is not available on protein constituents of any of the fetal fluids of pre-implantation buffaloes. Therefore, the experiment is designed with the objectives to detect the pregnancy or uterine specific proteins through one and two dimension electrophoresis analysis. Uterine washing of the early, mid, late luteal stages of the estrous cycle, pre-implantation stage of pregnancy and the fetal fluid were collected and compared using one and two dimensional eletrophoresis including isoelectric focusing techniques under non-reducing condition. Result of one dimension gel comparison indicated a band in serum proteins that was not available in uterine secretion and there were at least three uterus-specific protein bands of approx. 40.7, 29.6 and 27.3kDa molecular weight those were not present in serum. A band of 11.5 kDa was found to be unique for mid luteal sample that was not observed in any of the other phases of the cycle. Similarly 14.3 kDa band in late luteal and 9.9 kDa band in early luteal were not observed in other stages of the cycle and serum sample. Comparison of bands in pregnant and non-pregnant luteal phase sample did not show any difference in banding pattern in one dimension gel detected with coomassie blue or silver stain. However, comparison of bands in isoelectric focused gel showed four unique bands having pI values of 6.6, 6.7, 7.0 and 7.2 in pregnant uterine fluid sample and three unique bands with pI values 6.8, 7.4 and 7.5 in luteal phase sample. Two-dimension electrophoresis analysis of

pregnant and non-pregnant luteal sample revealed at least four high molecular weight proteins spots (154.8, 123, 114.8 and 97.7 kDa) between 5 to 5.5 pl those were not present in luteal phase sample. Similarly, two clusters of different protein spots were observed after 6.7 to 7.8 pl region with one cluster with 10 spots (125.8, 123, 123, 115.3, 107.1,104.7, 91.2, 91.2, 84.1 and 81.2 kDa) in relatively high molecular weight region and other one with nine different spots in the low molecular weight region (35.4, 28.8, 27, 29.7, 34.6, 37, 35.4, 32.6, kDa) in pregnant sample not present in non pregnant luteal phase sample.

Comparison of both allantoic and amniotic fluid protein band in one dimension non-reducing PAGE method showed three bands of 20.6, 10 and 8.1 kDa were present in allantoic fluid those were not present in amniotic fluid. Similarly a band of 6.8 kDa was observed in amniotic fluid that was not present in allantoic fluid. Isoelectric focusing of mixture of protein analysis showed 8 unique bands in allantoic fluid in the acidic pH range out of these 3 bands were having pl values of 5.4,5.7 and 5.9 rest 5 bands had pl values of 6.4,6.5,6.6,6.8 and 6.9. Two dimension comparison of protein spots between allantoic and amniotic fluid revealed about four higher (138, 127.6, 122.1 and 117.2 kDa) and two lower molecular weight bands (47.6 and 47.6 kDa), different spots in allantoic fluid proteins those were not observed in amniotic fluid. However, only one spot (99.5 kDa) was found unique for the amniotic fluid protein that was not present in allantoic fluid.

In conclusion this study revealed that analysis of the serum, uterine and fetal fluid proteins in different electrophoresis method was useful for identifying different proteins and successful in detecting serum, uterine, luteal phase, pregnancy and fetal fluid specific proteins. None of the single method may be ideal for separation and detection of different proteins available in a mixture of sample that contain varying nature of proteins protein. It requires combining different electrophoresis methods to find out actual difference. Therefore, 2-D electrophoresis is the best method that combines two principles

to get differences in protein spot where a spot represents a protein. Future studies combining the immuno detection of some of the characterized pregnancy specific proteins in different species and explant culture with study of protein synthesis may reveal the actual contribution of the endometrial and conceptus components to these protein mixture of the uterine luminal secretion and fetal fluid during early pregnancy.

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