ISOLATION, PARTIAL PURIFICATION AND IMMUNO-BIOCHEMICAL CHARATERISATION OF FERTILITY ASSOCIATED PROTEIN OF FRESH AND FROZEN SEMINAL PLASMA- A COMPARATIVE STUDY BETWEEN GIR AND JERSEY BULL

A Thesis
Submitted to the
West Bengal University of Animal and Fishery Sciences
in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy
in
VETERINARY BIOCHEMISTRY

By

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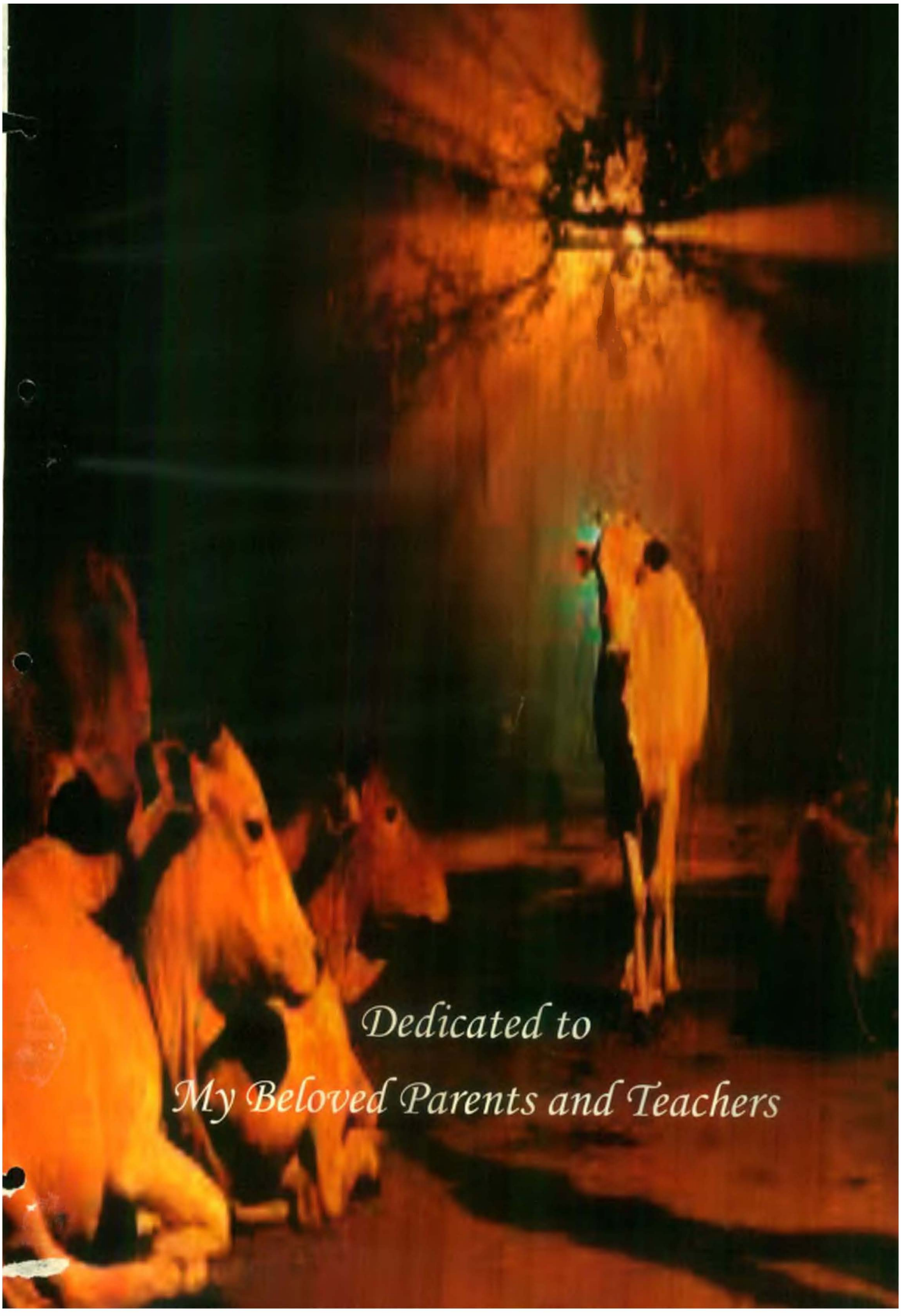
Dedicated to
My Beloved Parents and Teachers

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CERTIFICATE

This is to certify that the work recorded in the thesis entitled ***"Isolation, partial purification and immuno-biochemical characterization of fertility associated protein of fresh and frozen seminal plasma-A comparative study between Gir and Jersey bull"****, submitted by* ***Sanju Mandal,*** *in partial fulfillment of the requirements for the* ***Degree of Doctor of Philosophy in 'Veterinary Biochemistry'*** *of the West Bengal University of Animal and Fishery Sciences, is the faithful and bonafide research work carried out under my personal supervision and guidance. The results of the investigation reported in the thesis have not so far been submitted for any other Degree or Diploma.*

The assistance and help received during the course of investigation have been duly acknowledged.

Chattopadhyay 26/11/13

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APPROVAL OF EXAMINERS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY BIOCHEMISTRY

We, the undersigned, having been satisfied with the performance of **Sanju Mandal**, in the Viva-Voce Examination, conducted today, the 18th February, 2014, recommend that the thesis be accepted for the award of the Degree of Doctor of Philosophy in Veterinary Biochemistry.

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Sanjay Mandal

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List of Abbreviation

%	:	Percentage
µg	:	Microgram
µl	:	Microlitre
^{0}C	:	Degree Celsius
APS	:	Ammonium persulphate
BSA		Bovine serum albumin
Cm		Centimetre
CSP		Crude Seminal plasma
DAB	:	Diamino benzene
DEAE	:	Diethyl amino ethyl
DID	:	Double immuno-diffusion test
ELISA		Enzyme linked immunosorbent assay
FCA	:	Freund's complete adjuvant
FIA		Freund's incomplete adjuvant
Fig	:	Figure
GCSP		Gir crude seminal plasma protein
GFSP		Gir frozen seminal plasma protein
GPSP		Gir purified seminal plasma protein
GP		Gel filtrated part
gm	:	Gram
HIS		Hyper immune serum
HRPO		Horse radish peroxidase
Hrs		Hours
JCSP		Jersey crude seminal plasma protein
JFSP`		Jersey frozen seminal plasma protein
JPSP		Jersey purified seminal plasma protein
kDa	:	Kilo Dalton

M	**:**	**Molar**
mA	**:**	**Milliampere**
mg	**:**	**Milligram**
min	**:**	**Minute**
ml	**:**	**Millilitre**
mM		**Millimolar**
MW		**Molecular weight**
N		**Normal**
NCP	**:**	**Nitro cellulose paper**
nm		**Nanometer**
no.		**Number**
NSS		**Normal saline solution**
O. D.	**:**	**Optical density**
OPD	**:**	**O- phenylene diamine**
PAGE	**:**	**Polyacrylamide gel electrophoresis**
PBS	**:**	**Phosphate buffer saline**
PBS-T	**:**	**PBS Tween- 20**
PMSF	**:**	**Phenyl methyl sulfonyl fluoride**
PPCSP	**:**	**Partially Purified Crude Seminal Plasma**
R_f	**:**	**Relative mobility**
rpm	**:**	**Revolution per minute**
SDS	**:**	**Sodium dodecyl sulfate**
sec	**:**	**Second**
TCA	**:**	**Trichloro acetic acid**
TEMED	**:**	**N,N,N',N'- tetramethyl ethylene diamine**

Chapter - I

INTRODUCTION

INTRODUCTION

Reproductive capacity has tremendous impact on the animal industry. Reproductive merit is several time more important economically, than growth performance, selecting males with highest fertility potential is one way of improving reproductive efficiency in the livestock and offsetting large economic losses that are incurred as a result of poor fertility. Regrettably criteria to accurately selecting males exhibiting highest fertility are poorly defined and the physiological importance of parameters as reliable indicators of fertility potential still poorly understood.

Morphological examination of sperm cell and semen alone is insufficient to predict a male reproductive success. Variations in fertility between bulls that produce similarly accessed semen contribute greatly to the problem of identifying bulls with the highest fertility potential. Fertility in a bull is greatly defined as the ability to impregnate females. It is often said that a bull can contributes half production in a calf crop. A good bull offers both high fertility & high genetic breeding rate for one or more economically important characteristic before 1990 quality semen are characterized by sperm count, motility live & dead sperm ratio, volume etc; but effort soon followed to determined dramatic difference in the fertility among bulls with similar sperm quality.

This observation forced the scientists to reveal a protein from the prostate, cowper's gland and seminal vesicle that released into the semen in ejaculation. This protein referred to as heparin binding protein commonly known as fertility associated

antigen (Nass *et al.*, 1990). Further research showed the degree of attachment of fertility associated antigen varies greatly among bulls suggesting a reason why fertility differs so much among bulls when they passed Bull Soundness Examination (BSE) (Bellin *et al.*, 1994; 1996).

Research conducted in bovine seminal plasma showed that the abundance of specific protein in semen is related to fertility. Two seminal plasma protein 26 kDa and 55 kDa were more abundant in semen of high fertility bulls and two different proteins 16 kDa (pI 6.7) and 16 kDa (pI 4.1) were more abundant in lower fertility bulls (Killian *et al.*, 1993).

The fertility of bull depends on a series of factors which range from animal behavior and physical condition to features link directly to the semen such as spermatozoa motility and physio-biochemical composition and most important proteins (Austin & Short, 1990).

Evaluation of sperm concentration and motility is frequently used to assess semen quality, but provides limited information about the potential fertility of sires (Correa *et al.*, 1997; Brahmkshtri *et al.*, 1999). Other criteria such as computerized analysis of motility and acrosome integrity also help to estimate semen quality and have been related to non-return rates of bulls, but correlations are not high or even consistent (Farrell *et al.*, 1998; Januskauskas *et al.*, 2000a, b). There is obvious evidence that motility is important for fertilization (Mortimer, 1997) but, within groups of bulls that have met a certain threshold for sperm motility, this parameter has only a limited contribution to detect differences in fertility scores (Flowers, 1997). Bulls from artificial insemination centers still show differences in non-return rates by

as much as 20 to 25 %, but these results are not explained by routine semen analysis (Killian *et al.*, 1993; Larson and Miller, 2000). Thus, the existence of sub fertile sires that appear to show normal semen quality is an important observation and has stimulated the study of other markers of fertility, such as molecular components of the seminal plasma (Braudmeyer and Miller, 2001).

The potential influence of seminal proteins on male reproduction came to attention because of the studies showing that their expression is associated with breeding scores of dairy bulls (Killian *et al.*, 1993; Cancel *et al.*, 1997), beef bulls (Bellin *et al.*, 1994, 1996; Parent *et al.*, 1999) and horses (Brandon *et al.*, 1999). Despite the relevance of those proteins, few have been identified (Cancel *et al.*, 1997; Gerena *et al.*, 1998; McCauley *et al.*, 2001) and in most cases our understanding of their function and relationships with fertility indexes is incomplete.

The success of AI also depends upon the ability to screen for semen with high fertilization potential (Holt *et al.*, 2007). Thus, given the biological and economic importance of knowing with certainty the potential fertility of the semen for AI before insemination, it becomes essential to explore aspects that relate to fertility. Although the most conclusive evidence of fertility from freeze-thawed semen is made on the basis of pregnancy rate in the females served, semen evaluation offers predictive information on expected performance of the male.

However, semen during cryopreservation, most seminal plasma is replaced with semen extenders, mainly egg yolk or milk proteins. Screening of the semen at initial stages allows elimination of poor quality semen while the proper assessment of

the post-thaw quality of spermatozoa can provide insights into the fertilizing capacity of the cryo-preserved spermatozoa. A number of laboratory evaluation tests measuring the physical and functional integrity of spermatozoa *in-vitro* have been devised over the past few decades. Keeping in view the economic and time constraints in field conditions, routine semen evaluation tests like sperm viability, progressive motility, hypo-osmotic swelling tests, acrosomal integrity and morphological abnormalities have been evaluated that are rapid, relatively inexpensive and easily executable. Success of AI (Artificial Insemination) is dependent on the quality of fresh semen and its capacity for dilution and storage with minimum loss of fertilizing ability.

Andrological investigation of breeding bulls periodically is a boon for sound and successful breeding programmes nation-wide, particularly with use of frozen semen and artificial insemination technique. Jersey (exotic) and Gir (indigenous) are two important milch breeds in India, makes a valuable contribution to the livelihood of economic section. More than 40% of A.I. sires have one or the other problems affecting its semen quality, lower freezability and fertility rates in respect to fertility associated proteins. A good deal of research has been carried out on frozen semen technology in advanced countries, but its application in developing countries is facing serious challenges of shortage of sound proven sires and lack of their thorough investigation.

Therefore, the present studies were undertaken with the following objectives to overcome from above problems.

Objectives:

- To evaluate the physico-morphological and biochemical characteristic of fresh and frozen seminal plasma.
- Isolation and partial purification of the fertility associated protein of Gir and Jersey bull fresh and frozen seminal plasma.
- Immuno-biochemical characterization of above partially purified proteins.

Chapter - II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The seminal plasma contains secretions from testicles, epididymus and accessory sexual gland source (Evans and Maxwell, 1987) from which the sperm acquire many proteins during the epididymal transit and at ejaculation. The present study was taken parameter wise under the major heads of:

A. Physio-biochemical semen characteristics

B. Isolation and purification of fertility associated protein in seminal plasma of fresh and frozen semen.

C. Immunobiochemical characterization of purified fertility associated protein.

A. Physio-biochemical semen characteristics:

Seminal plasma was purified from bull seminal plasma and seminal vesicle extract and investigated its tissue localization, structure, physicochemical and biological characteristics (Reddy *et al.*, 1983).

Physio-biochemical semen characteristics of good and poor freezability of crossbred bulls studied by Belorker *et al.*, 1988.

Comparative appraisal of blood and seminal plasma biochemistry of Gir and Jafrabadi breeding bulls reported by Shelke and Dhami (2002).

B. Isolation and purification of fertility associated protein in seminal plasma:

The seminal plasma proteins are originally from plasma blood and partially synthesized and secreted by testicles, epididymus and vesicular gland (Manjunath *et al.*, 1994).

Many protein components from seminal plasma were related to fertility in male bovine. Acott and Hoskins (1978) showed forward motility protein in bovine seminal plasma could be partially purified by heat treatment, gel chromatography in urea followed by affinity chromatography on agarose or concavalin A and their enzymatic treatment suggested a glycoprotein nature of it.

Srivastava and Farooqui, (1979) noted bull seminal-plasma hyaluronidase was purified 180-fold by chromatography on concanvalin A-Sepharose, heparin Sepharose, Sephadex G-200 and Sephacryl S-200.

Edwards *et al.*, 1981 observed mammalian seminal plasma contains many distinct components which are important for spermatozoa function and survival.

Bovine seminal plasma gelatin binding proteins (BSP-A1, BSP-A2, BSP-30kDa) were purified by affinity chromatography. Chromatography of bull seminal vesicular fluid also exhibited an elusion pattern similar to that obtained from bull seminal plasma (Manjunath *et al.*, 1987).

Chandonnet *et al.*, 1990 observed chromatography of alcohol precipitate of bovine seminal fluid on a heparin-sepharose column resolved these proteins into three peaks. Peak 1 and 2

(Retarded proteins) were eluted upon extensive washing of the column with 0.05 M phosphate buffer (pH 7.4). Proteins in peak 3 represented absorbed proteins and were eluted with phosphate buffer containing 1M NaCl.

San Agustin and Lardy (1990) showed caltrin of the bull changes from an inhibitor of calcium transport to an enhancer when non-covalently bound anions are removed. Inhibitory activity is restored by the addition of phosphotidyl serine, but other phospholipids tested were ineffective. In guinea pig caltrins I and II, the transition form inhibitor to enhancer is accomplished by the removal of covalently bound carbohydrate residues. Bull caltrin binds specifically over the sperm acrosome and on the tail but not on the posterior portion of the sperm head or on the midpiece. In one guinea pig caltrins binds only over the acrosome and prevents calcium-induced release of hyaluronidase from the acrosome but does not prevent hyperactivation. The second guinea pig caltrin binds only to the tail and prevents calcium-induced hyperactivation but does not block the release of acrosomal hyaluronidase.

Gerena *et al.*, 1998 observed isolation of SP26 was done by cutting the spots corresponding to SP26 from 2D SDS gels and electroeluted in 50mM ammonium bicarbonate, 0.1%SDS buffer at 10 mA for 4 hour. Two procedures ware developed to purify SP26 from seminal plasma. In first procedure fraction containing SP26, collected from phenyl Sepharose column (1.5×10cm) were pulled and applied to a DE52 column (2 × 10 cm). In the second procedure, SP26 containing fractions were purified by using the phenyl Sepharose column followed by DE52 column. Then the purified fractions were applied to a CM 52 column (1.5 × 7 cm).

McCauliy *et al.*, 1999 noted 31 kDa heparin binding protein known as fertility associated antigen (FAA) was isolated by heparin affinity chromatography and reverse phase high performance liquid chromatography near homogenecity.

Osteopontin was recovered and electroeluted from spots (55kDa, 4.5PI) excised from 2D SDS-PAGE gels by Cancel *et al.*, 1999.

The sperm rich fraction was centrifuged at 10,000 ×g and the seminal plasma aspirated from the pelleted sperm cells (Brandon *et al.*, 1999).

Nauc and Manjunath, 2000 observed BSP-A1/A2, BSP-A3 and BSP-30kDa proteins of bovine seminal plasma were isolated using gelatin agarose affinity chromatography. The adsorbed fractions were resolved on Sephadex G-75 and G-200 columns.

Gerena *et al.*, 2000 showed the fusion protein of bovine lipocalin type PGD synthase (26kDa) was purified by affinity chromatography with GSH Sepharose 4B. The recombinant bovine lipocalin type prostaglandin synthase was recovered from the resin by incubation with thrombin at room temperatures overnight. It was further purified by mono-S column chromatography in 10mM sodium citrate (pH 4.5).

Seminal plasma was fractionated by affinity chromatography using lectin, concavalin A (con A), showing affinity for poly-mannose glycoprotein residues (Strzezek *et al.*, 2002)

Lactoferrin with a molecular mass of 80kDa was purified from equine seminal plasma by heparin-agarose affinity

chromatography and Sephacryl S-200 gel filtration. (Inagaki *et al.*, 2002)

Bison seminal vesicle secretory protein were precipitated by adding cold ethanol and recovered by centrifugation. The precipitates were resuspended in ammonium bicarbonate, dialyzed and lyophilized. Lyophilised protein were dissolved in 0.05 M phosphate buffer and loaded onto a gelatin-agarose column. The unadsorbed and adsorbed proteins were eluted with phosphate buffer and 5M urea in phosphate buffer respectively. A 16kDa protein was separated by heparin-Sepharose chromatography. (Boisvert *et al.*, 2004)

Precipitated proteins from Suffolk ram seminal plasma were loaded onto gelatin agarose column. The unadsorbed (fraction A) and retarded proteins (fraction B) were removed by washing the column with phosphate buffer saline and the adsorbed proteins (Fraction C) were eluted with 5 M urea. (Bergeron *et al.*, 2005)

Barrios *et al.*, 2005 observed whole seminal plasma protein was isolated by exclusion chromatography in sephacryl-100. After this, two major components of fraction six with an approximate relative molecular weight of 14 (P14) and (P20) kDa were recovered from the gel by cutting and mincing and obtained by electroelution in a 422 electroeluter under nondenaturing conditions for six hours.

Seminal plasma of four Saanen Bucck (*Capra hircus*) was pulled and dialyzed against distilled water and freeze dried. Lyophilized proteins (20mg) were dissolved in 0.02 M phosphate buffer (pH 7), and load onto an ion-exchange chromatography column (DEAE-sephacel, 1.2×20 cm). Dialyzed lyophilized

proteins from the main peak of DEAE- Sephacel were applied to a C2/C18 column (100×4.6 mm) coupled to a reverse phase high performance liquid chromatography (RP-HPLC) system observed by Teixeira *et al.*, 2006.

Interactions of boar, bull, and human seminal plasma proteins with heparin and phosphorylcholine were studied by affinity liquid chromatography using heparin immobilized to a Toyopearl support. A step gradient elution from 0.15 to 1.50 M NaCl was employed to elute the seminal plasma proteins. A capillary electrophoresis method was developed for separation of seminal plasma proteins (Varilova *et al.*, 2006).

Kumar *et al.*, 2008 showed purification of seven clinically important proteins from human seminal fluid (HBPS) through heparin affinity chromatography and RP-HPLC, were reported with higher yield.

C. Immunobiochemical characterization of purified fertility associated protein.

i) SDS PAGE:

Acott and Hoskins., 1978 showed forward motility protein in bovine seminal plasma exhibited multiple forms when fractionated on the basis of charge or mol. wt. and the mol. wt. of this monomeric protein was 37.5 kDa.This protein was involved in the development of the capacity for motility as sperm traverse the epididymis.

Polyacrylamide-gel electrophoresis indicated that the purified bull seminal-plasma hyaluronidase enzyme migrated as a

single band on 7.5 and 10% (w/v) gels at pH 4.3 and 5.3 (Srivastava and Farooqui., 1979).

An androgen–binding protein of 70-90 kDa was identified in the seminal plasma, rete-testis fluid, seminiferous tubular fluid, testes, epididymis and prostate of the ram,man,monkey and several other animals (Jegou *et al.*, 1979).

A highly potent anti-microbial, 504 kDa, basic protein in bull seminal plasma was reported and named as seminal plasmin. (Reddy and Bhargwa., 1979).

Shabanowitz and killian, 1987 showed two dimensional electrophoresis of cauda epididymal fluid protein profile revealed prominent band at 16, 23 and 34 kDa.

Manjunath *et al.*, 1987 observed affinity column purified bovine seminal plasma proteins such as BSP A-1, BSP A-2, BSP A-3 and BSP-30 kDa were found homogenous by SDS-PAGE.

Seminal plasma from seven stallions (two good, three variable, two poor sperm motility) were analyzed by 10% SDS gel electrophoresis (Coomassie blue stain) and a total of 27 proteins, of 13kDa-122kDa mol. wt., were detected in seminal plasma. There was difference ($P<0.05$) amongst stallions in the proportion of ejaculates containing 13 of the 27 proteins (Amann *et al.*, 1987).

The presence of a 15-30 kDa glycoprotein hormone, inhibin, was identified in seminal plasma and ovarian follicular fluid of various mammalian species (Miyamoto *et al.*, 1989).

Chandonnet *et al.*, 1990 showed bovine seminal fluid proteins in each peak were characterized by SDS-PAGE under

reducing conditions. Peak 1 contained proteins with molecular weight 8-350 kDa, a single protein with a molecular weight of 14 kDa identified in peak2 and peak 3 contained proteins with molecular weight of 15.5 kDa, 16 kDa, 25 kDa and 30 kDa.

Killian *et al.*, 1993 observed seminal plasma samples were subjected to one dimensional and two dimensional SDS-PAGE. Four different polypeptides appeared to occur in association with bull fertility level. Two proteins (26kDa, PI 6.2; 55kDa, PI 4.5) occurred with greater frequency and density in bulls of higher fertility and two proteins (16kDa PI 4.1; 16kDa, PI 6.7) were more prominent in bulls of lower fertility.

Desnoyers *et al.*, 1994 showed auto radiography visualization of reduced, denatured bovine seminal plasma samples separated by 2D PAGE, identified the presence of polypeptides migrating in the pH range of 3.5-7.8 and at molecular weight between 6 to 100 kDa. On 2D PAGE, the purified iodinated proteins revealed isoform BSP protein. BSP A-1 was found at a mol. wt. of 16.5 kDa (PI 4.7-5); BSP A-2 at 16 kDa (PI 4.9-5.2); BSP A-3 at 15 kDa (Pi 4.8-5.2) and BSP-30 at 28 kDa (PI 3.9-4.6). BSP proteins were among the major glycoproteins found in bovine seminal plasma.

Major proteins of cattle bull seminal plasma were of molecular wt. 25, 20, 15, 13 and 12 kDa. Twenty and seventeen protein bands of varying intensities and in the mol. wt. range of 11-96 kDa were observed in blood plasma and seminal plasma of buffalo bulls respectively (Kulkarni *et al.*, 1996).

Seminal plasma proteins were separated by two dimensional SDS-PAGE and purified 55kDa seminal plasma protein was assessed by one dimensional SDS-PAGE (Cancel *et al.*, 1997).

Romao *et al*, 1997 observed 12.9 kDa polypeptide of the spermadhesin family, almost identical to that of porcine spermadhesins PSP-I and PSP-II, was isolated from bovine seminal plasma.

One dimensional and two dimensional SDS-PAGE separation of seminal plasma and cauda epididymal fluid proteins of bull was performed under denaturing condition (Gerena *et al.*, 1998).

Flowers, 1998 showed seminal plasma protein profiles of each ejaculates of 10 mature boars were determined using 2D PAGE with iso-electrical focusing and densitometry.

Cancel *et al.*, 1999 observed after slaughter, bull male reproductive tract tissue protein extracted from accessory sex gland, testes and epididymis (caput, corpus, cauda) were separated by one dimensional SDS-PAGE, transferred to nitrocellulose and analyzed using enhanced chemiluminescence (ECL) Western Blot technique. The presence of Osteopontin in urine from live animals was confirmed by 1D SDS- PAGE ECL Western Blot analysis using antiserum for seminal plasma OPN (55kDa). The findings that male reproductive fluid contained Osteopontin (55kDa) were further confirmed by 2D SDS-PAGE of seminal plasma, accessory sex gland fluid and seminal vesicle fluid.

Two dimensional PAGE of the seminal plasma was performed under denaturing condition which revealed that 14

proteins were common all stallions in the research population. 4 of these proteins (SP-1, SP-2, SP-3 and SP-4) were found to be significantly correlated with the breeding score assigned for each stallion. SP-1(72kDa, PI 5.6) was positively correlated with fertility while SP-2 (75kDa, PI6), SP-3 (18 kDa, PI4.3) and SP-4(16 kDa, PI 6.5) were found to be negatively correlated with fertility (Brandon *et al.*, 1999).

Urade and Hayaishi, 2000 showed Lipocalin type prostaglandin D-synthase is an N-glycosylated monomeric protein with molecular weight of 20 kDa – 30 kDa depending on the size of the glycosyl moiety.

Tedeschi *et al.*, 2000 observed Z-13 is a new bovine seminal plasma protein made up of two disulfide linked 13 kDa subunit of the spermadhesin family whose members are thought to play a prominent role in different aspects of fertilization, was identified by two dimensional PAGE.

Gatti *et al.*, 2000 noted the cauda epididymal fluid was separated by 2D gel electrophoresis. 20 % SDS PAGE of cauda fluid isolated to main reactive bands at 17 kDa and 23 kDa.

One dimensional SDS-PAGE (15%) separation of purified recombinant bovine lipocalin type PGD synthase protein (26kDa) was performed under denaturing condition observed by Gerena *et al.*, 2000.

The lipid free purified BSP protein was found to be homogenous as judged by SDS-PAGE under reducing conditions. Electrophoresis revealed a doublet at 16-16.5 kDa for BSP-A1/A2, a single band at 15 kDa for BSP-A3 and a single band at 28kDa for BSP-30 kDa showed by Nauc and Manjunath, 2000.

Aliquots of seminal plasma from 59 adult males were fractionated by SDS-PAGE; the blots were stained and analyzed densiometrically (Leone *et al.*, 2001).

The seminal plasma protein profile of each ejaculate of 400 boars was determined using 1D and 2D polyacrylamide gel electrophoresis with iso-electrical focusing and densitometry observed by Flowers, 2001.

Teixeira *et al.*, 2002 showed 12.5 kDa protein was isolated and characterized from buck seminal plasma which was homologous to spermadhesins from boar and stallion.

The protein fraction of seminal plasma were subjected to gel electrophoresis under denaturing condition using the Tris-Tricine-SDS buffer and observed by densitometric analysis observed by Strzezek *et al.*, 2002.

La Falci *et al.*, 2002 showed heparin affinity proteins, identified from goat seminal plasma, were of mol. wt. 73 kDa to 104 kDa and also of 119 and 178 kDa.

Inagaki *et al.*, 2002 observed purified equine seminal plasma protein Lactoferrin (80 kDa) was found to be homogenous on the bases of its migration as a single brand on SDS-PAGE.

GSP-14, GSP-20, and GSP-22 kDa has been isolated from goat seminal plasma separated according to heparin affinity showed by Villemure *et al.*, 2003.

Manaskova *et al.*, 2003 observed boar seminal plasma contains various types of proteins. Most of them belong to spermadhesins and some of them contain fibronectin type II domain. Aggregated forms of boar seminal plasma proteins

separated by size exclusion chromatography were analyzed by SDS-PAGE.

Gelatin adsorbed fractions of Bison seminal vesicle protein was analyzed by SDS-PAGE and identified 16 kDa, 17 kDa, 18 kDa and 28kDa major bison seminal vesicle proteins showed by Boisvert *et al.*, 2004.

The major proteins of equine seminal plasma have been isolated and characterized regarding their expression along the male genital tract, protein structure and their function observed by Topfer-Peterson *et al.*, 2005.

Bergeron *et al.*, 2005 showed SDS-PAGE of gelatin-agarose column purified ram seminal plasma protein indicated the presence of a 15.5 kDa protein which is the major ram seminal plasma protein. SDS-PAGE also identified 4 proteins with molecular weight of 15 kDa, 16 kDa, 22 kDa and 24 kDa.

Ram seminal plasma samples were submitted to 2D SDS-PAGE using 12% acrylamide gels and reveals 21-protein spots on the air dried gel with molecular wt. ranging from 15-115kDa and PI 3.2-8.7 in the image analysis software. The most prominent spots were those less than 30 kDa. The most intensely stained spots were (18-19 kDa, PI 4.8-5), (17-18 kDa, PI 5-5.2) (15-16 kDa,PI 6.2-6.4) and (105-108 kDa,PI6.8-7) observed by Jobim *et al.*, 2005.

SDS-PAGE analysis of fraction six showed 2 major protein bands, 1 faint band of approximately 14 kDa (P14) and 1 prominent band with an apparent molecular weight of 20.5 kDa together with 1 fainter band of approximately 22 kDa observed by Barrios *et al.*, 2005.

Susan *et al.*, 2005 showed the seminal plasma proteins were separated using 7 cm gels, pH 3-10 in the first dimension. Forty-two proteins were compared across the two lowest fertility boars and two higher fertility boars. Fertility in vivo was negatively correlated ($P<0.05$) with 20 kDa, pI-6 and 60 kDa, pI-6.5 protein, and positively correlated with a 25 kDa, pI-5.9 protein.

Maura *et al.*, 2005 showed cauda epididymal fluids of dairy bulls were evaluated by 2D SDS-PAGE gels stained with Coomassie blue. An average of 118 spots was detected in 2D maps of Cauda epididymal fluid.

Maura *et al.*, 2006 observed proteins from accessory sex gland fluid of dairy bulls were separated by 2D SDS-PAGE followed by staining with Coomassie blue. High of fertility dairy bulls was significantly associated with lower expression of 14 kDa spermdhesin Z-13 isoform and higher amount of 55 kDa Osteopontin and 58 kDa phospholypase A-2 (PLA-2) isoform. There was also presence of BSP 30 kDa protein in the accessory sex gland fluid gels.

SDS-PAGE was performed on 12.5% polyacrylamide gels after seminal plasma protein fraction of goat (*Capra hircus*) was isolated by ion exchange chromatography and showed apparent molecular mass of 12 kDa which was homologous to spermadhesins from boar and stallion (Teixiera *et al.*, 2006).

ii) Western Blot:

Chandonnet *et al.*, 1990 showed electrophoretic transfer of gelatin-agarose column purified bovine seminal fluid protein in peak 3 and peak 5 after SDS-PAGE to nitrocellulose membrane and probed with antibodies against the well characterized BSP

proteins indicated the presence of BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa protein.

Manjunath *et al.*, 1994 observed bovine seminal vesicles synthesize a family of closely related proteins, namely BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa (collectively called BSP proteins).Polyclonal antibodies against purified BSP proteins raised in rabbits were used to detect these antigens in bovine epididymal and ejaculated spermatozoa as well as in bovine seminal plasma.

Using Specific antibodies, Western blot of bovine seminal plasma proteins detected spots at 16.5 kDa (BSP-A1); 16 kDa (BSP-A2); 15 kDa (BSP-A3) and 28 kDa (BSP -30 kDa) by Desnoyers *et al.*, 1994.

Siiteri *et al.*, 1995 observed osteopontin, a bone cell adhesion molecule, was identified in the rat testes and epididymis by western immuno-blot analysis. Immuno-blot analysis with a monoclonal antibody against OPN detects in the presence of immuno-reactive polypeptides in rat testes homogenates as well as in the epididymal fluid and sperm extracts.

The Western blot of electro-eluted protein containing only a single band at 55 kDa and the antiserum against the whole bovine seminal plasma did not detect any other seminal plasma protein by Cancel *et al.*, 1997.

Gerena *et al.*, 1998 observed Western blot analysis was performed after protein separation either 1D or 2D SDS-PAGE gels followed by electrophoretic transfer to 0.2 μm nitrocellulose membrane. Polyclonal antiserum against 26 kDa seminal plasma protein of bull detected only one spot at the same position as that

of purified 26 kDa on immunoblots of 2D SDS-PAGE gels of whole seminal plasma.

Cancel *et al.*, 1999 observed Western blot analysis of fluid from the male reproductive tract separated by 1D SDS-PAGE showed that polyclonal antiserum raised against bovine seminal plasma OPN(55 kDa) recognized a 55 kDa protein in seminal plasma, accessory sex gland fluid, seminal vesicle fluid and urine. ECL Western blot of all fluids probed with anti 55 kDa protein detected two other protein bands at 45 kDa and 14 kDa along with the 55 kDa protein. The 55 kDa Protein band and two additional proteins at 45 kDa and 14 kDa also were recognized by ECL Western blot analysis of 1D SDS-PAGE of seminal plasma, accessory sex gland fluid and urine samples probed with LF123 and LF 124 antibodies.

McCauley *et al.*, 1999 observed Immuno-blots of proteins probed with mono-clonal antibodies identified heparin binding proteins variance of approximately 31 kDa, 24 kDa and 21.5 kDa that were associated with increased bull fertility.

Western blot analysis of stallion SP1 indicated that there was an antigenic homology with a bovine 55 kDa fertility associated seminal plasma protein identified in a study by Brandon *et al.*, 1999.

Gerena *et al.*, 2000 Western blot analysis was performed after protein separation on 1D SDS-PAGE (15%) gels followed by electrophoretic transfer to 0.2 µm nitrocellulose membrane. Western blots of bull seminal plasma samples probed with anti recombinant bovine PGD synthase were immunoreactive at 26 kDa along with another band of 29 kDa.

Rabbit polyclonal antibody was raised against ram cauda epididymal fluid protein (anti-CEF) by Gatti *et.al.*, 2000. The anti-CEF polyclonal antibody reacted mainly with a 17 kDa and 23 kDa compound in the cauda sperm extract.

Immuno-blotting was performed to verify the specificity of the antigen-antibody interaction. A doublet was detected by anti-BSP- A1/A2 in the alcohol precipitate of crude and purified BSP-A1/A2. Anti-BSP-A3 and anti-BSP-30-kDa recognized only the BSP-A3 and the BSP-30-kDa proteins respectively (Nauc and Manjunath, 2000).

Leone *et al.*, 2001 observed Immuno-blotting of seminal plasma of 59 adult males was analyzed using non-specific polyclonal antibody directed against lipocalin type prostaglandin D-synthase (L-PGD-S).

Inagaki *et al.*, 2002 observed immuno-blotting of seminal plasma protein found to be highly homogenous on the bases of the mono-specificity of rabbit antibodies to the purified protein.

Garlo *et al.*, 2002 observed 70 kDa and 45 kDa forms of OPN protein were detected in cyclic and pregnant endometrium of pigs by Western blotting.

Immuno-blotting of bison seminal vesicle proteins (BiSV) revealed BiSV-16 kDa cross reacted with BSP-A3 antibodies, BiSV-17 kDa and BiSV-18 kDa cross reacted with BSP-A1/A2 antibodies and BiSV-28 kDa cross reacted with BSP-30 kDa antibodies by Boisvert *et al.*, 2004.

Jobim *et al.*, 2005 observed Western blot analysis of ram seminal plasma proteins indicated that (18-19 kDa, PI 4.8-5)and

(17-18 kDa, PI 5-5.2) were similar to BSP-A1/A2 (16.5 kDa, PI 4.7-5 and 16 kDa Pi 4.9-5.2) identified in Manjunath's study from bovine seminal plasma, based on the specific reaction of the polyclonal antibody to those proteins.

Bergeron *et al.*, 2005 showed 15 kDa and 16 kDa ram seminal plasma proteins cross reacted with antibodies against bovine plasma proteins.

For Western blot analysis, polyclonal antibodies were raised against the whole fraction 6 and the purified P14 and P20 proteins of ram seminal plasma in rabbit immunization observed by Barrios *et al.*, 2005.

iii) ELISA:

Cancel *et al.*, 1997 observed ELISA detection of 55 kDa protein was done in samples collected after Mono Q chromatography.

Gerena *et al.*, 1998 observed fractions containing SP26 were collected by column chromatography and aliquots of each were analyzed by ELISA.

Inagaki *et al.*, 2002 observed a sandwich ELISA was developed for quantifying lactoferrin (80 kDa) in equine seminal plasma.

Manaskova *et al.*, 2003 observed aggregated forms of boar seminal plasma proteins separated by size exclusion chromatography were analyzed by immunochemical methods (ELISA and immunoblotting).

iv) Molecular Characterization:

Siiteri *et al*, 1995 observed Osteopontin mRNA and protein is present in rat testes and epididymis and epididymal spermatozoa also contain osteopontin on its surface.

Amino acid sequence comparison were made when pure 55 kDa seminal plasma protein was transferred to a polyvinyl difluoride (PVDF) membrane and sequenced observed by Cancel *et al.*, 1997.

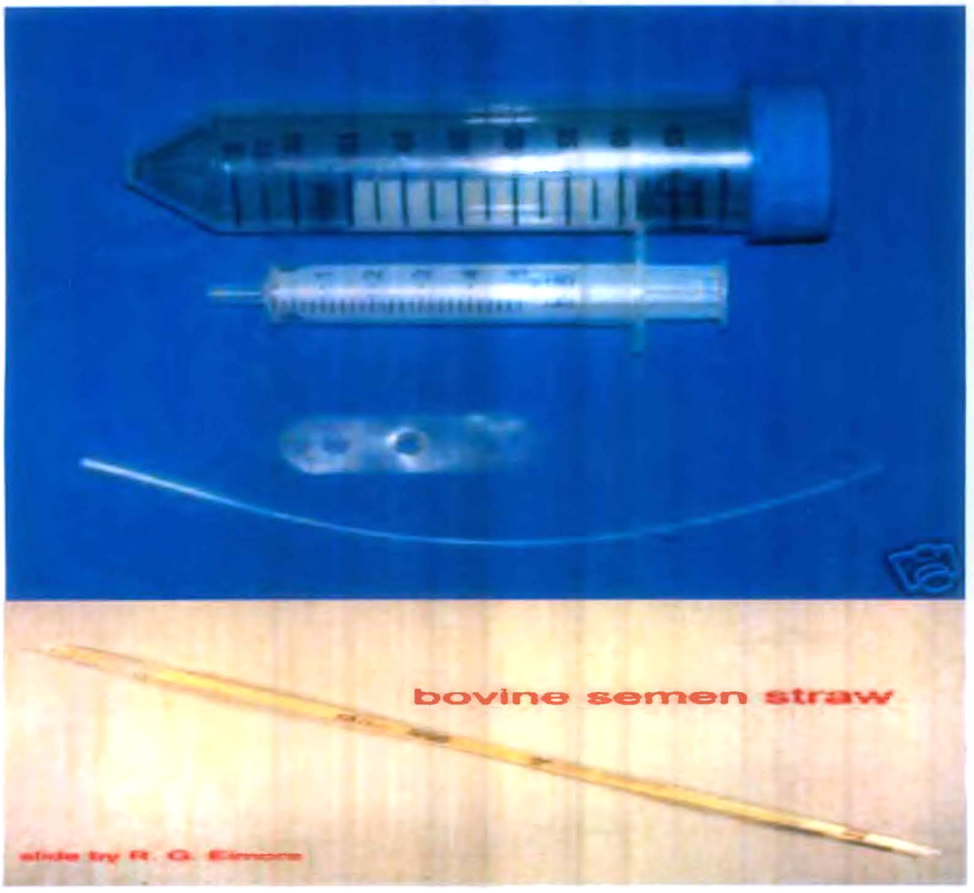
Gerena *et al.*, 1998 showed amino acid sequence comparison was made after pure protein was transferred to a polyvinylidine fluoride membrane and sequenced the full length cDNA (794 bp) for bovine 26 kDa lipocalin type PGD-synthase.

Boisvert *et al.*, 2004 studied the amino terminal sequencing of bison seminal vesicle protein share almost 100% sequence identity with bovine seminal plasma proteins.

Moura, A. A., 2005 observed that structure and expression of the osteopontin (55kDa) in the male supports the notion that osteopontin plays a role in sperm-egg binding and fertilization.

Teixeira *et al.*, 2006 observed that buck seminal fluid protein exhibited end terminal sequence homology to boar, stallion and bull spermadhesins.

Chapter - III



MATERIALS AND METHODS

MATERIALS AND METHODS

1. MATERIALS:

1.1 Glass wares

All the glass wares used in this study were procured from Borosil India and were sterilized by standard method before using.

1.2 Plastic wares

Plastic wares of M/S Tarson India were used in this study.

1.3 Laboratory animals: rabbits

Two healthy New Zealand White rabbits (1.5 kg each) used for the present Investigations were maintained in the Department of Veterinary Biochemistry, W.B.U.A.F.S., Kolkata-37.

1.4 Reagents used for Morphology

Reagents used for this method are presented in appendix.

1.5 Reagents used for gel filtration chromatography

Sephacryl S-200 obtained from Sigma-Aldrich Co., USA.

1.6 Reagents used for Lowry's method

Reagents used for this method are presented in appendix.

1.7 Reagents used for SDS-PAGE

The reagents used for this test are presented in appendix.

Protein Markers- Marker was obtained from Bangalore-GENEI, range: 14.3 – 97.4 kDa.

1.8 Reagents used for Double Immuno-diffusion

Reagents used for these tests are presented in appendix.

1.9 Reagents used for indirect ELISA

Reagents used for these tests are presented in appendix.

2.0 Reagents used for Western blotting

The reagents used for this test are presented in appendix.

Equipments

The following equipments were used during the study:

1. High speed centrifuge machine (Remi, India)
2. Low speed centrifuge machine (Remi, India)
3. Refrigerator 4^{0}C (Whirlpool)
4. Deep fridge -20^{0}C Vertical (Vestfrost)
5. Top pan digital balance sensitivity 0.01g (Roy Electronic)
6. Electronic analytical balance (Sartorius)
7. Magnetic stirrer (Remi, India)
8. Water bath (Digitech system)
9. pH meter (Systronics)
10. Micro oven (Samsung)
11. UV-VIS Spectrophotometer 119 (Systronics)
12. Gel filtration column (Bangalore-GENEI)
13. Vertical mPAGE (AE6530) and vertical slab gel electrophoresis chamber (AE8450) along with power supply and gel casting apparatus (ATTO Corporation, Japan)
14. Western blotting apparatus (Bangalore-GENEI)
15. Gel documentation systems (Bio-Rad)
16. Incubator (Semico)
17. Hot air oven (Semico)
18. Autoclave
19. ELISA reader (Multiskan EX, Thermo, Japan)
20. Double Distillation Plant (Borosil)

2 METHODS:

2.1 COLLECTION OF SEMEN FROM GIR AND JERSEY BULL:

Fresh semen samples were collected in the morning via artificial vagina from Gir and Jersey bulls of sound reproductive health maintained at "Frozen semen bull station Haringhata farm", Mohanpur, W.B. Then immediately after collection each ejaculate was transported to the lab in ice-cold condition by semen shipper for immediate evaluation of semen characteristics in terms of mass activity, motility and morphology of spermatozoa. The semen samples having poor quality of sperm were discarded. For frozen semen thawing of the semen was carried out by immersing straws in a water bath at 37°C for 15 seconds (Rasul *et al.*, 2000). The semen having apparently normal spermatozoa was taken into consideration for collection of seminal plasma.

2.2 EVALUATION OF SPERMATOZOA:

2.2.1 MASS ACTIVITY AND MOTILITY:

One drop of fresh semen was taken in a grease free clear glass slide. Then the mass activity was observed under low power microscope. A drop of fresh diluted semen was taken on a slide and covered with a cover slip and the motility was observed under microscope (45X) as per method described by Ganti. A. Sastri (1983).

2.2.2 MORPHOLOGY OF SPERMATOZOA:

One drop of semen was mixed with 10 drops of 3% sodium citrate buffer solution at room temperature in a watch glass. Then a smear was made from the diluted semen and dried. The dried smear was stained for 15 minutes in Rose Bengal Stain and the

slide was washed 2-3 times by distilled water and dried. It was then examined under microscope using high power (45X) as per method described by Ganti. A. Sastri (1983).

2.3 PREPARATION CRUDE SEMINAL PLASMA ANTIGEN:

The seminal plasma was separated by centrifugation at 3000 rpm for 20 min. at 4°C. The supernatant was collected very carefully and concentrations of the seminal plasma were determined (Lowry *et al.*, 1951). Then the samples were pulled and kept at -20° C until further use. 10% SDS-PAGE (Laemmli, 1970) were performed to detect the polypeptide bands of the seminal plasma.

2.4.A. PREPARATION OF PURIFIED SEMINAL PLASMA ANTIGENS OF THE GIR BULL BY GEL FILTRATION CHROMATOGRAPHY:

Fertility associated polypeptides of crude seminal plasma antigen were purified by gel filtration chromatography in a column on Sephacryl S-200 (2.1 cm diameter and 43 cm in length) in a buffer containing PBS (pH 7.2), PMSF (0.03mM) and 0.02% sodium azide at a flow rate of 20 ml per hour. The elutes were collected in 36 fractions of 4 ml each. The distribution of protein was monitored by taking the absorbance at 280 nm in a UV/VIS spectrophotometer (SYSTRONICS-119).

Protein fractions of seminal plasma were pooled together into 4 parts and named as F1 (fractions of test tube no. 12,13 & 14 of ascending loop first peak; test tube no.15 & 16 of descending loop first peak), F2 (fractions of test tube no.19,20&21 of second peak), F3 (fractions of test tube no. 24, 25 & 26 of third peak), F4 (fractions of test tube no. 28, 29 & 30 of fourth peak),

Then the 4 pooled fractions were concentrated by dialysis against sucrose using dialysis membrane (cut off value 12,000).

The protein concentrations of the 4 pooled fractions of seminal plasma were determined (Lowry *et al.*, 1951).The concentrated peak fractions were then preserved at –20°C in aliquots for further use.10% SDS-PAGE (Laemmli, 1970) were performed to detect the purified fertility associated protein.

2.4.B. PREPARATION OF PURIFIED SEMINAL PLASMA ANTIGENS OF THE JERSEY BULL BY GEL FILTRATION CHROMATOGRAPHY:

Fertility associated polypeptides of crude seminal plasma antigen were purified by gel filtration chromatography in a column on Sephacryl S-200 (2.1 cm diameter and 43 cm in length) in a buffer containing PBS (pH 7.2), PMSF (0.03mM) and 0.02% sodium azide at a flow rate of 20 ml per hour. The elutes were collected in 45 fractions of 3 ml each. The distribution of protein was monitored by taking the absorbance at 280 nm in a UV/VIS spectrophotometer (SYSTRONICS-119).

Protein fractions of seminal plasma were pooled together into 4 parts and named as F1 (fractions of test tube no. 13, 14 &15 of ascending loop of first peak), F2 (fractions of test tube no. 16, 17 & 18 of descending loop of first peak), F3 (fractions of test tube no. 24, 25 & 26 of second peak), F4 (fractions of test tube no. 27, 28 & 29 of third peak), Then the 4 pooled fractions were concentrated by dialysis against sucrose using dialysis membrane (cut off value 12,000).

The protein concentrations of the 4 pooled fractions of seminal plasma were determined (Lowry *et al.*, 1951).The

concentrated peak fractions were then preserved at –20^{0}C in aliquots for further use.10% SDS-PAGE (Laemmli, 1970) were performed to detect the purified fertility associated protein.

2.5 DIALYSIS OF PURIFIED FERTILITY ASSOCIATED SEMINAL PLASMA PROTEIN FRACTIONS:

The dialysis bag was initially activated by boiling in distilled water for 5 minutes. One end of the dialysis bag was tied with a cotton thread. Then the purified samples were poured into the bags for dialysis. The outer end was also closed in a similar fashion. To concentrate the proteins the dialysis bags (cut off value 12,000) were kept on a Petri dish and covered with sucrose for 1-2 hours depending upon the dilution. The dialyzed samples were preserved at –20° C until further use.

2.6 ESTIMATION OF TOTAL PROTEINS OF THE CRUDE AND PURIFIED FERTILITY ASSOCIATED PROTEIN OF FRESH AND FROZEN SEMINAL PLASMA PROTEIN:

2.6.1 Estimation by Lowry's method:

Protein estimation of crude seminal plasma samples and purified seminal plasma fractions were done by this method.

Procedure:

i) 100 µl of each of samples, BSA (annexure), and distilled water were taken in separate test tubes.

ii) 3 ml of Lowry's reagent A (annexure) was added in each of the test tubes.

iii) It was then mixed well and kept for 10 minutes at 37°C.

iv) 0.3 ml of diluted Folin and Ciocalteu's phenol reagent (annexure) was added to each of the test tubes.

v) It was then mixed thoroughly and kept for 30 minutes at 37°C.

vi) The reading of O.D. (optical density) was taken at 750 nm wavelength in UV-VIS Spectrophotometer.

Calculation:

Protein concentration in sample (mg/ml) = $\frac{\text{Optical density of sample X Conc. Of standard*}}{\text{Optical density of standard}}$

*** = 1 mg/ ml.**

2.7 IMMUNOBIOCHEMICAL CHARACTERIZATION OF PURIFIED FERTILITY ASSOCIATED SEMINAL PLASMA PROTEINS:

2.7.1 Determination of molecular weight of purified fertility associated seminal plasma proteins using Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE).

The crude and purified samples of Gir and Jersey bull of fresh and frozen seminal plasma were analyzed by One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in vertical mPAGE (AE-6530) and vertical slab gel electrophoresis chamber (AE-6200) along with power supply and gel casting apparatus (ATTO Corporation, Japan).

Under identical experimental condition according to the method described by Laemmli (1970), with some modification under denaturing and reducing condition using 10 % poly-

acrylamide slab gel of dimensions of 15 x 17 cm. The gel was then stained with Coomassie blue staining solution followed by destaining it with destaining solution after 6 hours.

Principle.

Electrophoresis of protein samples depends on the charge, size and shape of the protein molecules present in it. However, in SDS-PAGE proteins bind to SDS and become negatively charged with similar charge: weight ratio. Hence, when SDS-coated proteins were placed in an electric field their separation would depend only on their size and shape. By varying the conc. of polyacrylamide gel, used as a medium for the electrophoretic separation, different resolution ranges of molecular weight might be obtained .after a heating to 100°C in the presence of reducing agents and SDS, the proteins unfold and bind to SDS. The strong negative charge on the proteins thus meant that their electrophoretic mobility would be inversely proportional to the logarithm of their molecular weight.

Procedure for making gel.

i) The glass slabs were cleaned with soap water and was first rinsed in tap water followed by distilled water. Then was air dried and finally wiped with methanol soaked cotton to remove grease, if any.

ii) The slabs/plates were arranged with arranged with gasket in slab gel casting apparatus.

iii) The separating gel mixture (appendix) was prepared and immediately loaded in the space between the two glass slabs up to a level of 2 cm. from the top.

iv) This was overlaid gently with butan -1- al.

v) The gel was kept undisturbed until it has polymerized (about ½ hour).

vi) Butan-1-al was removed from the top by tilting the apparatus.

vii) It was then rinsed with a little amount of stacking gel mixture (before adding TEMED and APS) and removed by tilting.

viii) Then TEMED and APS were added to the rest of the stacking gel (annexure) and mixed thoroughly and loaded on the separating gel.

ix) The comb was inserted slowly and carefully avoiding any air bubble attached to the teeth of the comb and only up to 1/2 cm from the top.

x) Then the apparatus was left undisturbed for 30-45minutes for polymerization of stacking gel.

xi) The slabs containing gel were placed in the SDS-PAGE apparatus, keeping the square plate out ward.

Loading of proteins.

15 microlitre of samples and sample buffer (appendix) were mixed in 1:1 ratio and heated for 5 minutes by placing into a beaker containing water at 100°C.

i) Approximately 40 microgram of proteins was loaded in each lane.

ii) Molecular weight marker was also loaded parallelly.

iii) Then running buffer was poured on the apparatus for completing the electrical circuit.

iv) The apparatus was connected to power pack. The current was fixed at 22 volts and 300 mA.

v) The machine was made run until the dye front touched top to bottom of apparatus for about one and half hours.

vi) After completion of the run, the gel was removed carefully after separating the glass slabs.

vii) Gel was put in staining solution (appendix) for 8 hours.

viii) Gel was taken out and put into destaining solution (appendix) until clear background was obtained.

ix) Gel was finally preserved in solution containing 7% acetic acid.

2.7.2 Determination of molecular weight by SDS-PAGE:

Molecular weights were determined by SDS-PAGE by using protein markers (PMW –M, Bangalore Genei) containing Phosphorylase (97.4 kDa), BSA (66 kDa), Ova albumin (43 kDa), Carbonic anhydrase (29 kDa), Soabean trypsin inhibitor (20.1 kDa) and Lysozyme (14.3 kDa) by Gel Documentation systems (Bio-Rad).

2.8 IMMUNOCHEMICAL ANALYSIS:

2.8.1 Raising of Hyperimmune Serum:

Two healthy New Zealand white rabbit with an average weight of 1.5 kg of each (Purified seminal plasma proteins of Gir and Jersey bull) respectively were taken for this purpose. The purified seminal plasma was thoroughly mixed with equal volume of Freund's complete adjuvant (FCA) (1:1) and a total of 1 ml was injected intramuscularly at 3 sites into the thigh muscle and subcutaneously at 3 sites in the scapular region. Four booster

doses of the same antigen emulsified with Freund's incomplete adjuvant (FIA) (1:1) were given with subsequent increase in amount after Seven days interval following the first injection. Blood was collected from the rabbit 7 days after the last booster dose and serum was stored at –20°C in aliquots for further use with sodium azide (0.02%) as preservative.

2.8.2 Double Immuno Diffusion test (DID).

DID test was performed according to Ouchterlony (1963) with some modifications. 1.5% agarose solution was prepared with PBS (pH-7.4) and a small amount of sodium azide was added in it. After boiling 5 ml of melted agarose was poured on two different clean and grease free glass slides to give a thickness of 1.5 mm. The slides were then kept at room temperature for half an hour to solidify. Wells were then punched by gel puncher. The bottoms were again sealed with melted agarose solution. In left side of one slide, the wells were then filled with 20 µl of Gir purified seminal plasma antigen, 20 µl of normal serum (control) and 20 µl of hyper-immuno serum raised against purified seminal plasma protein of Gir bull. In right side of same slide 20 µl of Jersey bull purified seminal plasma antigen, 20 µl of normal serum (control) and 20 µl of hyper-immune serum raised against purified seminal plasma protein of Jersey bull in three consecutive wells. On the left side of another slide 20 µl of hyper-immune serum raised against purified seminal plasma of Gir bull, 20 µl of Gir purified seminal plasma antigen and 20 µl of Jersey bull purified seminal plasma antigen were taken in three consecutive wells to observe the cross-reactivity. On the right side of that slide 20 µl of hyper-immune serum raised against purified seminal plasma of Jersey bull, 20 µl of Gir purified seminal

plasma antigen and 20 µl of Jersey bull purified seminal plasma antigen were taken in three consecutive wells to observe the cross-reactivity. The slides were then placed in a humid chamber and incubated overnight at room temperature. The following day the slides were washed in PBS (pH 7.2) dried by blotting paper.

2.8.3 Immuno-blotting or Western blotting

The fertility associated proteins were characterized by Western blot technique according to Towbin *et al.* (1979) with some modifications to the labeled antibody and its substrate. The proteins were separated by SDS-PAGE (Laemmli, 1970) and then the resultant proteins were electroblotted to nitrocellulose filter paper (Immobilon- NC) from gel according to the method of Towbin *et al.* (1979), Svoboda *et al.* (1985) and Kataria *et al.* (2000).

Transformation of resultant proteins from the SDS-PAGE to solid support (Nitro cellulose filter paper)

i) The unstained slab gel following SDS-PAGE was placed in the electrode buffer for 20 mins.

ii) A piece of NCP was cut to the size of the acrylamide gel.

iii) This piece of NCP was immersed in distilled water and was soaked in electrode buffer for 5 mins.

iv) The blotting sandwich was assembled within the blotting cassette taking in order (from anode to cathode) –

   a) Perspex board drilled with holes to permit free passage of buffer.

   b) Scotch brite pad.

c) Three sheets of Whattman 3MM filter paper was cut to appropriate size and wetted with electrode buffer.

d) NCP

e) Polyacrylamide gel with one corner marked to define orientation.

f) Three sheets of Whattman 3 MM filter paper.

g) Scotch brite pad

h) Perspex board

As protein on gel migrated towards anode (positive) so, NCP should be placed towards anode.

v) The "sandwich" set was fixed with screws so as to hold the layers tightly.

vi) The cassette was submerged into the electrophoresis transfer chamber containing transfer buffer with NCP facing the anode.

vii) The blot apparatus was connected with power supply and current (50 mA) was applied for overnight at 4° C.

viii) After transfer, the sandwich was dismantled and the NCP was processed for immuno blotting.

Detection of immunodominant protein on Western blotting:

i) NCP was kept in blocking buffer (5% BSA) for two hours at 37°C in incubator.

ii) NCP was then washed with washing buffer (PBS-T) for four times.

iii) The NCP was incubated with hyperimmune serum raised against crude seminal plasma protein of Gir and Jersey bull of 1:80 dilutions in dilution buffer for two hours in a small Petri dish.

iv) NCP was washed with washing buffer (PBS-T) for four times.

v) After that NCP incubated with goat anti-rabbit horse radish peroxidase conjugate of 1:1000 dilutions in dilution buffer for two hrs. in a Petri dish.

vi) Then NCP was washed in washing buffer for four times.

vii) NCP was rinsed with substrate solution (Tris-HCl, H_2O_2 and DAB).

viii) Reactive protein bands appearing after few min. were observed.

ix) NCP was dipped into the distilled water to stop the reaction.

x) Lastly, it was dried up and preserved.

2.8.4 Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA):

Indirect ELISA for detection of seroreactivity crude and purified seminal plasma protein of Gir and Jersey bulls was

performed according to the method of Sarkar, et al., (2003) with some modifications.

The optimal concentration of ELISA reagents including the concentration of coating antigen, dilution of positive and negative reference sera as well as anti-rabbit IgG-horse radish peroxidase (HRPO) conjugate and optimal test conditions were determined by chequer board dilution assay. The dilution of the antigen showing highest maximum difference between the optimal density of the HIS and the corresponding dilution of the normal sera were used in the test. The optimum concentration of coating antigen was 5 µg. Protein per well and optimum dilution of HIS was 1:200, 1:400, 1:800 and 1:1600.

Procedure:

a) Crude seminal plasma (Gir bull), crude seminal plasma (Jersey bull), frozen seminal plasma of Gir bull, frozen seminal plasma of Jersey bull, purified Gir seminal plasma protein & purified Jersey seminal plasma protein were used as coating antigen for 84 wells of ELISA microtitre plate (100 µl of 5µg protein per well after diluting with coating buffer, pH 9.6) for the assay.

b) The plate was kept at 4°C over night.

c) On the next day three to four times washing done with washing buffer (0.05% Tween 20 in PBS).

d) The plate was blocked with 200 µl per well blocking buffer (2% BSA in PBS).

e) The plate was kept at 37°C in incubator for two hours.

f) Three to four times washing was done with washing buffer (0.05% Tween 20 in PBS).

g) 100 µl two fold serial dilution of hyper-immune and normal rabbit sera (1:200 1:400, 1:800 and 1:1600) were added accordingly and kept for 2 hours in incubator at 37°C.

h) The plate was washed for three to four times with washing buffer (0.05% Tween 20 in PBS).

i) 100 µl of conjugate solution containing goat anti-rabbit horse peroxidase conjugate (sigma) with the dilution of 1:1000 in PBS were added and kept for 2 hours.

j) Then it washed four times with washing buffer (0.05% Tween 20 in PBS).

k) 100 µl of substrate buffer (3 µl H_2O_2, 0.025 gm of O-Phenylene diamine in 25 ml. citrate buffer) was added and kept for 20 min. in dark at room temperature.

l) After the development of colour 2 N H_2SO_4 solution was added to each well to stop the reaction.

m) The absorption was taken at 492 nm. In an ELISA reader (Thermo).

Statistical analysis was done by t-test for Indirect ELISA using homologous and heterologous hyper immune serum (Snedecor and Cochran, 1967).

Chapter - IV

RESULTS

RESULTS

3.1 EVALUATION OF SPERMATOZOA:

Semen evaluation was done in terms of mass activity, motility and morphology from freshly collected semen of Gir and Jersey bulls. The mass activity in terms of moderate wave was noticed (++). The motility of individual spermatozoan was more than 60%. The morphology of spermatozoa was normal at the level of 80%, when stained with Rose Bengal (Fig-1 & 2).

3.2 ANALYSIS OF CRUDE SEMINAL PLASMA PROTEINS OF GIR AND JERSEY BULLS:

Crude seminal plasma protein of Gir and Jersey was prepared by centrifugation. The protein concentration of crude and frozen after thawing seminal plasma determined by Lowry *et al.* (1951) was 25.45 mg/ ml and 24.57 mg/ml respectively and after thawing Gir frozen and Jersey frozen 20.15 mg/ml and 19.64 mg/ml respectively. 10% SDS-PAGE of crude seminal plasma revealed several polypeptide bands when stained with Coomassie Brilliant R-250 blue (Fig-3 & 4).

3.3.A PURIFICATION OF FERTILITY ASSOCIATED PROTEINS FROM CRUDE SEMINAL PLASMA OF THE GIR BULL:

Purified seminal plasma proteins of Gir were prepared by gel filtration chromatography on Sephacryl S 200 with the flow rate of 20 ml/ hr in which the proteins were resolved into four major peaks (Graph No.-1). Protein fractions of seminal plasma were pooled together into 4 parts and named as F1 (fractions of test tube no. 12,13 & 14 of ascending loop first peak; test tube no.15 & 16 of descending loop first peak), F2 (fractions of test tube

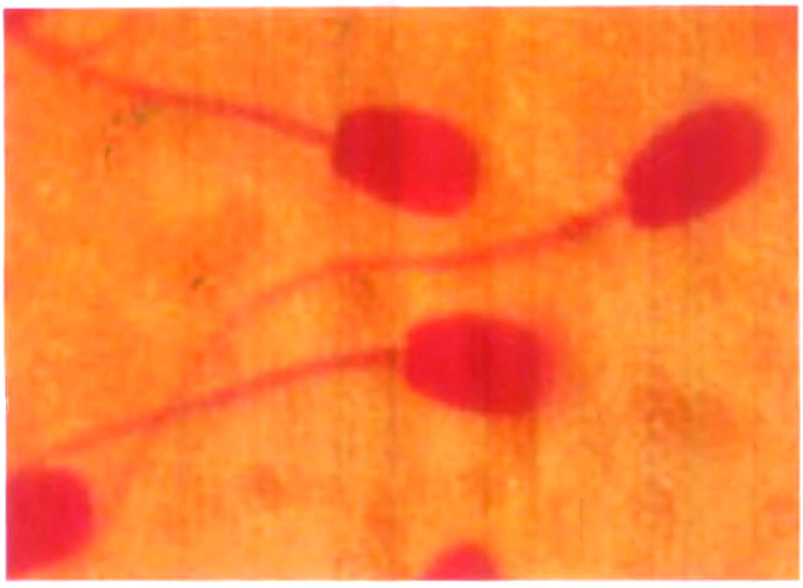


Fig-1 Morphology of spermatozoa of Gir bull
(Rose Bengal Stain-3%)

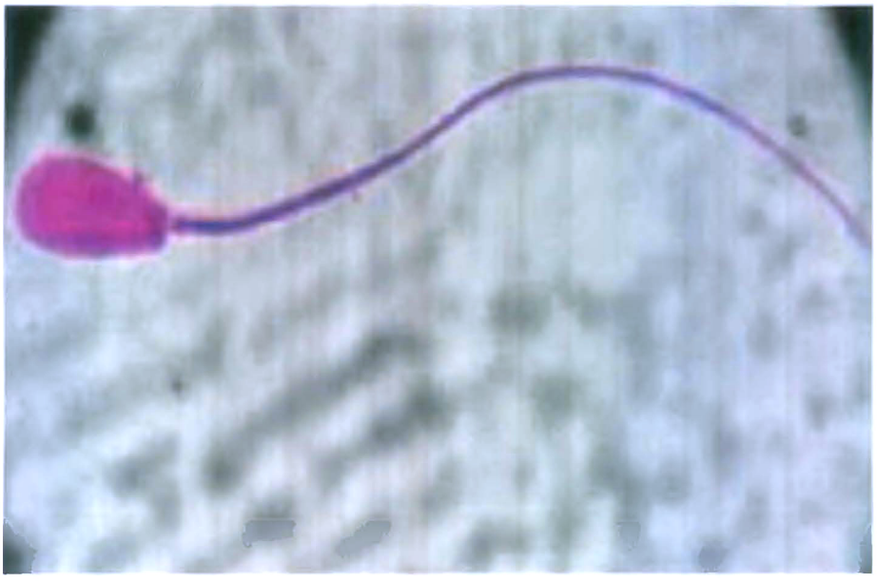


Fig-2 Morphology of spermatozoa of Jersey bull
(Rose Bengal Stain-3%)

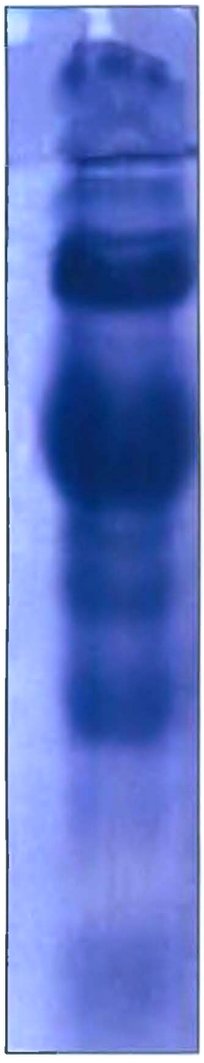


Fig-3

Fig-4

Fig-3. SDS-PAGE (10% gel) showing polypeptide profiles of crude seminal plasma of Gir bull.

Fig-4. SDS-PAGE (10% gel) showing polypeptide profiles of crude seminal plasma of Jersey bull.

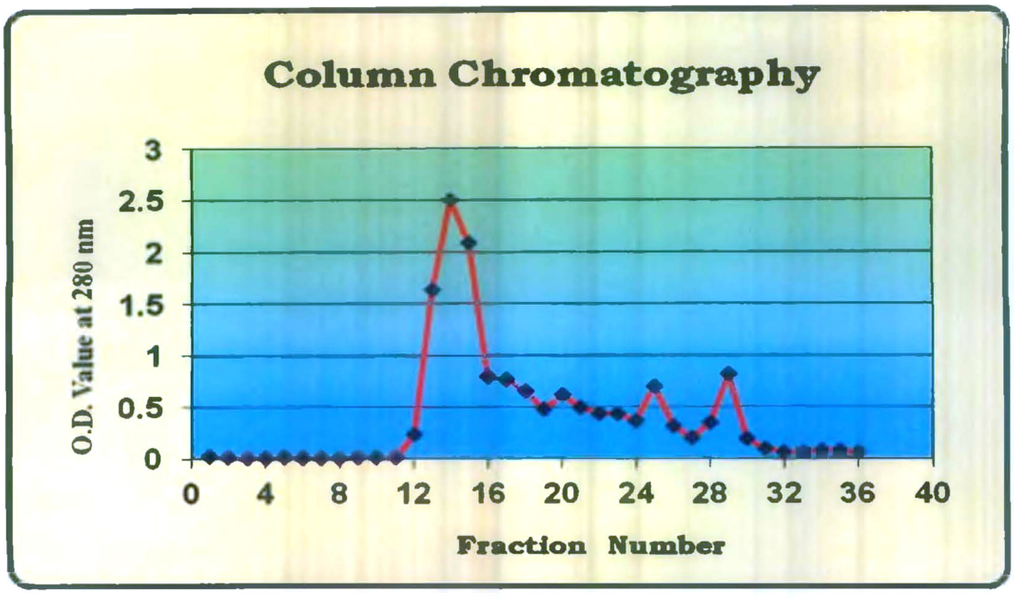
no.19, 20 & 21 of second peak), F3 (fractions of test tube no. 24, 25 & 26 of third peak), F4 (fractions of test tube no. 28, 29 & 30 of fourth peak), Then the 4 pooled fractions were concentrated by dialysis against sucrose using dialysis membrane (cut off value 12,000).

Then the four pooled fractions were dialyzed against sucrose using dialysis membrane (cut off value 12,000). The protein concentration of the four pooled fractions of seminal plasma were determined by Lowrey *et al.*, (1951) were 5.37 mg/ml, 1.05 mg/ml, 2.85 mg/ml and 3.69 mg/ml respectively.

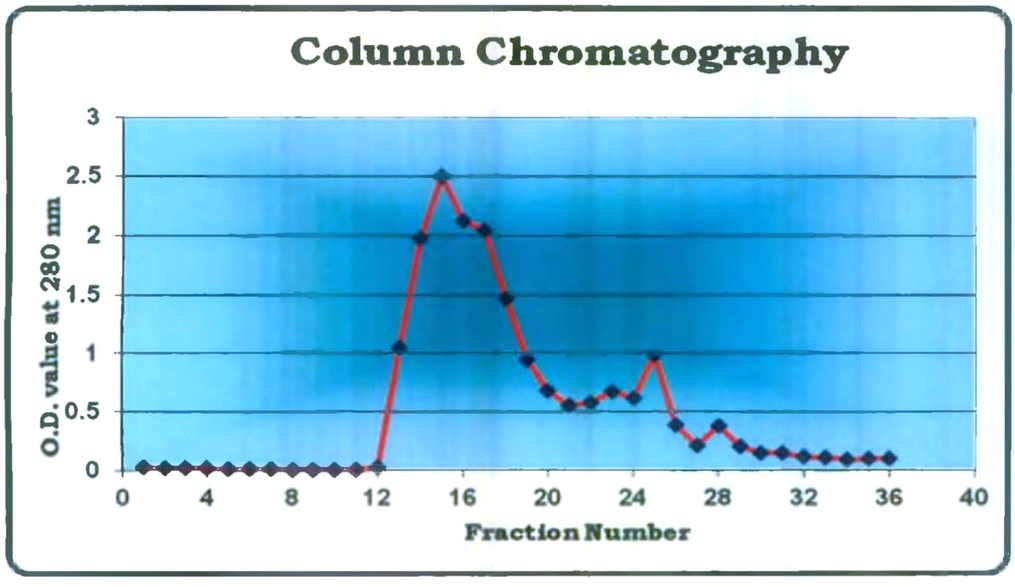
3.3.B. PURIFICATION OF FERTILITY ASSOCIATED PROTEINS FROM CRUDE SEMINAL PLASMA OF THE JERSEY BULL:

Purified seminal plasma proteins of Jersey were prepared by gel filtration chromatography on Sephacryl S 200 with the flow rate of 20 ml/ hr in which the proteins were resolved into four major peaks (Graph no.-2). Protein fractions of seminal plasma were pooled together into 4 parts and named as F1 (fractions of test tube no. 13, 14 &15 of ascending loop of first peak), F2 (fractions of test tube no. 16, 17&18 of descending loop of first peak), F3 (fractions of test tube no. 24, 25 & 26 of second peak), F4 (fractions of test tube no. 27,28&29 of third peak), Then the 4 pooled fractions were concentrated by dialysis against sucrose using dialysis membrane (cut off value 12,000).

Then the four pooled fractions were dialyzed against sucrose using dialysis membrane (cut off value 12,000). The protein concentration of the four pooled fractions of seminal plasma were determined by Lowry *et al.*, (1951) were 4.98 mg/ml, 3.57 mg/ml, 2.97 mg/ml and 1.76 mg/ml respectively.



Graph No.1. Line diagram showing O.D. values of purified fertility associated seminal plasma proteins of Gir bull by Gel Filtration Chromatography on Sephacryl S200



Graph No.2. Line diagram showing O.D. values of purified fertility associated seminal plasma proteins of Jersey bull by Gel Filtration Chromatography on Sephacryl S200

3.4 IMMUNOBIOCHEMICAL CHARACTERIZATION OF PURIFIED SEMINAL PLASMA FERTILITY ASSOCIATED PROTEINS:

3.4.A. Determination of molecular weight of purified seminal plasma fertility associated proteins using sodium dodecyl sulfate poyacrylamide gel electrophoresis (SDS-PAGE):

Purified proteins from Gir and Jersey bull seminal plasma were analyzed by Gel Documentation System (Bio-Rad) using standard molecular weight markers (range: 14.3-97.4 kDa) in 10% SDS-PAGE (Fig 5 & 6) and molecular weight of purified seminal plasma proteins were determined (Fig-5 &6).

The molecular weight of Gir bull fertility associated seminal plasma proteins were 55 kDa, 26 kDa and with minor polypeptide band 16 kDa (Fig-5).

The molecular weight of Jersey bull fertility associated seminal plasma proteins were 26 kDa and with minor polypeptide bands 12 kDa and 14.3 kDa respectively (Fig-6).

3.4.B. Raising of Hyperimmune Serum and Double Immuno-Diffusion Test:

Hyperimmune sera were raised in rabbit against purified seminal plasma protein from Gir and Jersey bull crude seminal plasma. Precipitin line was observed in double immunodiffusion test, when each protein was run their respective antibodies (Fig.-7). Purified seminal plasma protein of Gir and Jersey bull were also found cross reactive with heterogenous antibody raised in rabbit (Fig.-8).

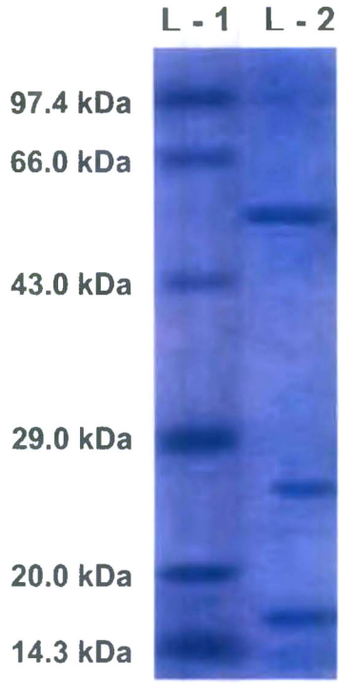


Fig-5. SDS-PAGE (10% gel) showing purified fertility associated seminal plasma proteins of Gir bull along with protein molecular weight marker (Coomassie blue stain).

Lane-1: Protein marker (range: 14.3-97.4 kDa).

Lane-2: Purified fertility associated seminal plasma proteins of Gir Bull.

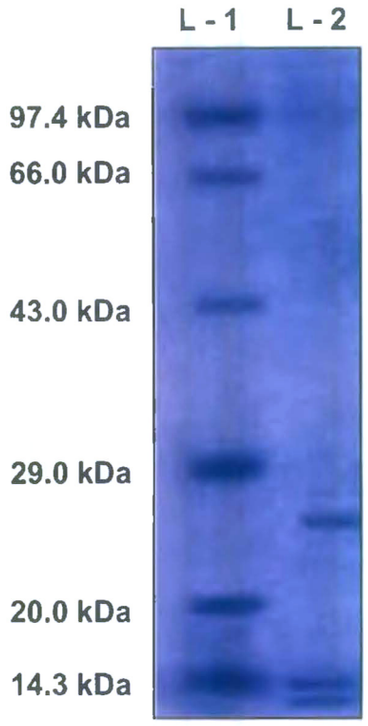


Fig-6. SDS-PAGE (10% gel) showing purified fertility associated seminal plasma proteins of Jersey bull along with protein molecular weight marker (Coomassie blue stain).

Lane-1: Protein marker (range: 14.3-97.4 kDa).

Lane-2: Purified fertility associated seminal plasma proteins of Jersey Bull.

Fig-7. Double Immuno Diffusion test showing the precipitin line of purified fertility associated seminal plasma proteins of Gir and Jersey bull with homologus antibody.
A=Antigen of Gir Purified Seminal Plasma.
B=Antibody raised against Gir Purified Seminal Plasma Protein.
C&F=Control Normal Seminal Plasma
D=Antigen of Jersey Purified Seminal Plasma.
E=Antibody raised against Jersey Purified Seminal Plasma Protein.

Fig-8. Double Immuno Diffusion test showing the crossreactivity of Gir and Jersey bull purified fertility associated seminal plasma proteins with the heterogenous antibody.
A= Antibody raised against Gir Purified Seminal Plasma Protein.
B=Antigen of Gir Purified Seminal Plasma.
C= Antigen of Jersey Purified Seminal Plasma.
E=Antibody raised against Jersey Purified Seminal Plasma Protein.
D=Antigen of Jersey Purified Seminal Plasma.
F= Antigen of Gir Purified Seminal Plasma.

3.4.C. Immuno-blotting or Western blotting:

Immunochemical analysis of purified Gir and Jersey bull seminal plasma protein were done by Western blot technique. These proteins were separated by SDS-PAGE (10% gel) and then the resultant proteins were electroblotted to nitrocellulose filter paper (Immunobilon-NC) from gel.

The seminal plasma protein purified from Gir bull crude seminal plasma was found immunoreactive against rabbit anti-goat antibody which was used as primary antibody in Western blot technique (Fig.-9).

The seminal plasma protein purified from Jersey bull crude seminal plasma was also found immune-reactive against rabbit anti-goat antibody which was used as primary antibody in Western blot technique (Fig.-10).

3.4.D.Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA):

Serological characterization of seminal plasma protein (crude, frozen & purified) of Gir and Jersey bull were done using hyper immune sera by indirect ELISA. The sero-reactivity is expressed as O.D. at 492 nm of crude, frozen and purified proteins that are shown (Table-1 and Graph no.-3). Using homologous hyper-immune serum the O.D. values recorded at 1:200 dilution were 1.425, 1.422, 1.416 and 1.395, 1.390, 1.312; 1.397, 1.389, 1.386 and 1.289, 1.278, 1.268 at 1:400 dilution; 1.327, 1.325, 1.312 and 1.276, 1.267, 1.245 at 1:800 dilution; 1.30, 1.26, 1.25 and 1.248, 1.236, 1.232 at 1:1600 dilution for crude, frozen and purified seminal plasma protein of both Gir & Jersey bull respectively.

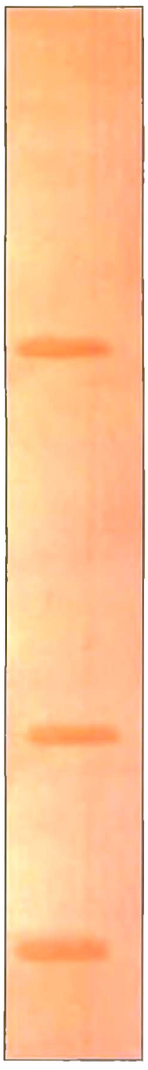


Fig-9

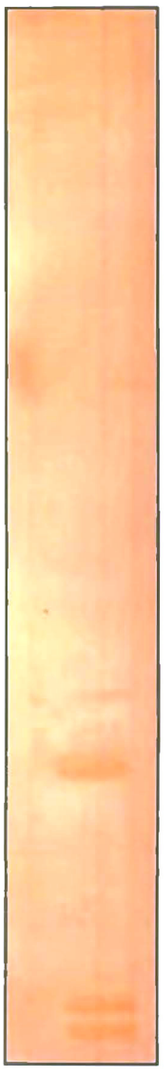


Fig-10

Fig-9. Western Blot analysis of purified fertility associated seminal plasma proteins of Gir bull.

Fig-10. Western Blot analysis of purified fertility associated seminal plasma proteins of Jersey bull.

Table 1: Comparative seroreactivity of crude, frozen and purified fertility associated seminal plasma proteins of Gir and Jersey bull by indirect ELISA using homologous hyperimmune sera raised in rabbit.

Antigen	Homologous HIS				P Value
	1:200	1:400	1:800	1:1600	
Gir Crude Seminal plasma	1.425^{xa}±0.001	1.397^{wb}±0.02	1.327^{wc}±0.001	1.30^{wd}±0.02	P<0.01
Gir Frozen Seminal plasma	1.422^{xa}±0.05	1.389^{wb}±0.07	1.325^{wc}±0.07	1.26^{wd}±0.04	P<0.01
Gir Purified seminal Plasma	1.416^{wa}±0.02	1.386^{xb}±0.04	1.312^{xc}±0.05	1.254^{xd}±0.05	P<0.01
Jersey Crude Seminal plasma	1.395^{ya}±0.05	1.289^{yb}±0.08	1.276^{yc}±0.02	1.248^{yd}±0.07	P<0.01
Jersey Frozen Seminal plasma	1.390^{ya}±0.02	1.278^{yb}±0.09	1.267^{yc}±0.08	1.236^{yd}±0.04	P<0.01
Jersey Purified seminal Plasma	1.312^{ya}±0.05	1.268^{yb}±0.04	1.245^{yc}±0.05	1.232^{zd}±0.07	P<0.01
P Value	P<0.01	P<0.01	P<0.01	P<0.01	

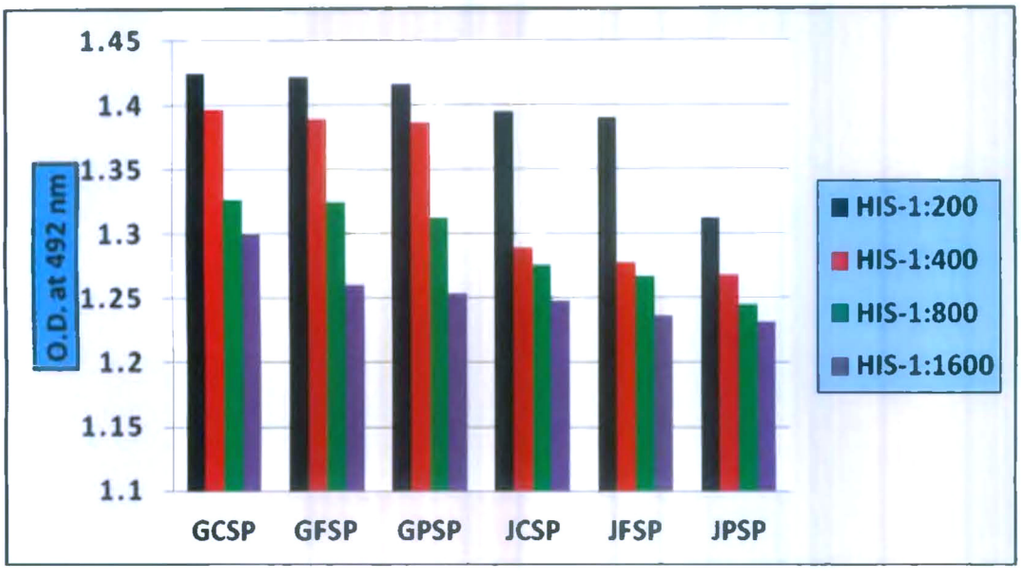
*a-d Mean and SE values bearing no common superscript in a row and w-z Mean and SE values bearing no common superscript in a column varies significantly (P<0.01).

Table 2: Comparative cross seroreactivity of crude, frozen and purified fertility associated seminal plasma proteins of Gir and Jersey bull by indirect ELISA using heterologous hyperimmune sera raised in rabbit.

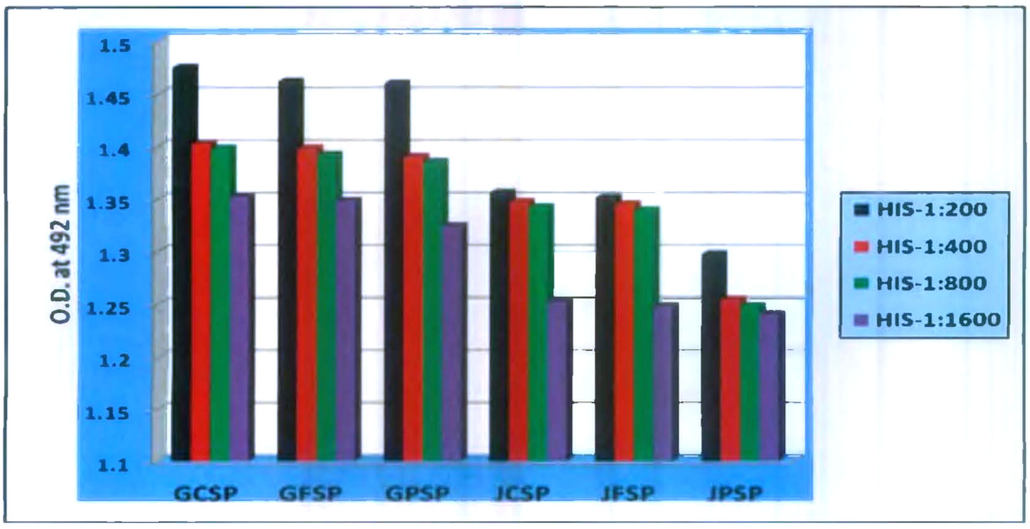
Antigen	Heterologous HIS				P Value
	1:200	1:400	1:800	1:1600	
Gir Crude Seminal plasma	1.475^{wa}±0.001	1.402^{wab}±0.07	1.398^{wb}±0.001	1.352^{wc}±0.05	P<0.01
Gir Frozen Seminal plasma	1.462^{wa}±0.001	1.398^{wb}±0.05	1.392^{xc}±0.001	1.348^{wd}±0.05	P<0.01
Gir Purified seminal Plasma	1.460^{xa}±0.02	1.390^{xb}±0.02	1.385^{zbc}±0.05	1.324^{zd}±0.04	P<0.01
Jersey Crude Seminal plasma	1.356^{wxa}±0.05	1.347^{wb}±0.08	1.342^{xc}±0.02	1.252^{wd}±0.07	P<0.01
Jersey Frozen Seminal plasma	1.351^{wxa}±0.02	1.345^{wab}±0.09	1.339^{xc}±0.08	1.248^{wd}±0.04	P<0.01
Jersey Purified seminal Plasma	1.297^{wxa}±0.05	1.254^{xb}±0.04	1.248^{zc}±0.05	1.240^{xd}±0.07	P<0.01
P Value	P<0.01	P<0.01	P<0.01	P<0.01	

*a-d Mean and SE values bearing no common superscript in a row and w-z Mean and SE values bearing no common superscript in a column varies significantly (P<0.01).

Graph No.3. Diagram showing seroreactivity of crude, frozen and purified fertility associated seminal plasma proteins (both Gir and Jersey origin) as assessed by indirect ELISA using homologous hyperimmune sera raised against purified seminal plasma protein(both Gir and Jersey origin) in rabbit.



Graph No.4. Diagram showing cross seroreactivity of crude, frozen and purified fertility associated seminal plasma proteins (both Gir and Jersey origin) as assessed by indirect ELISA using heterologous hyperimmune sera raised against purified seminal plasma protein(both Gir and Jersey origin) in rabbit.



GCSP= Antigen of Gir Crude seminal plasma protein.
GFSP= Antigen of Gir Frozen seminal plasma protein.
GPSP= Antigen of Gir Purified seminal plasma protein.
JCSP= Antigen of Jersey Crude seminal plasma protein.
JFSP= Antigen of Jersey Frozen seminal plasma protein.
JPSP= Antigen of Jersey purified seminal plasma protein.

The cross sero reactivity is expressed as O.D. at 492 nm of crude, frozen and purified protein that is shown (Table-2 and Graph no.-4). Using heterologous hyperimmune serum the O.D. values recorded at 1:200 dilution were 1.475, 1.462, 1.460 and 1.356, 1.351, 1.297; 1.402, 1.398, 1.390 and 1.347, 1.345, 1.254 at 1:400 dilution; 1.398, 1.392, 1.385 and 1.342, 1.339, 1.248 at 1:800 dilution; 1.352, 1.348, 1.324 and 1.252, 1.248, 1.240 at 1:1600 dilution for crude, frozen and purified seminal plasma protein of both Gir & Jersey bull respectively.

The O.D. values were quite similar when antigens of two separate bull species were used indicating presence of cross reactive epitopes in those antigens. However, crude and frozen seminal plasma protein of both Gir and Jersey showed significantly ($P>0.01$) more reactivity than the purified seminal plasma protein in all the dilutions as indicated in the O.D. values (Table.1).

From the Table.1 & 2 it was evident that the O.D. values decreased significantly ($P<0.01$) in case of all the four types of antigens are used with the increase dilution of homologous as well as heterologous both hyper immune serum.

There was significant ($P<0.01$) difference in O.D. values between crude, frozen and purified seminal plasma protein of Gir and Jersey bull in all the four dilutions of hyperimmune serum used in the assay (Table-1).

There was no significant difference in O.D. between seminal plasma protein of Gir and Jersey bull when reacted with heterologous hyper immune serum in 1:200, 1:400 but in 1:800 crude seminal plasma protein of Gir showed significantly ($P<0.01$)

higher O.D. value compared to Jersey bull and as in 1:800 and 1:1600 dilution purified seminal plasma of Gir bull showed significantly higher O.D. compared to Jersey bull (Table-2).

Chapter - V

DISCUSSION

DISCUSSION

The seminal plasma is a very complex mixture of secretions from the testes, duct system and accessory sex glands are mixed with sperm at ejaculation and contribute to the majority of semen volume and components (Thomas *et al.*, 2003). Seminal plasma contains a variety of biochemical components, some of which are relatively specific for the regulation of sperm function (Strzezek *et al.*, 1992). Seminal plasma protein has important effect on sperm function (Villemure *et al.*, 2003; Mortarino *et al.*, 1998 and Strezezek *et al.*, 2002). Seminal plasma protein composition varies among species and some of seminal plasma proteins have influence on sperm motility, viability and fertilization (Sanchez-Luengo *et al.*, 2004; Henricks *et al.*, 1998; Brandon *et al.*, 1999). Antifertility factors from seminal plasma have been described for several species and include decapacitation factors purified to various degrees (Shivaji *et al.*,1990), human antifertility factor1 (AF1) (Reddy *et al.*,1979; Audhya *et al*, 1987; Reddy *et al*, 1982), rabbit acrosome stabilizing factor (ASF) (Eng and Oliphant, 1978), and Bull Seminal Plasmin (SPLN) (Shivaji and Bhargava,1987). Generally, these factors are believed to inhibit sperm capacitation, the acrosome reaction, or acrosomal enzymes and ultimately to interfere with fertilization. Many seminal plasma proteins of bull (Killian *et al.*, 1993), swine (Flowers, 1998), horse (Brandon *et al.*, 1999) and buffalo (Harshan *et al.*, 2009) with freezibility in bulls (Asadpour *et al.*, 2007) were identified. Bovine seminal plasma proteins collectively called BSP are acidic in nature and secrete from seminal vesicles. The apperant molecular masses of BSP-A1, A2 and A3 ranging from 15 to 17 kDa (Manjunath *et al.*, 1994; Desnoyers *et al.*, 1994).

In this study, attempt has been made to purify and characterize this fertility associated protein from Gir and Jersey bull which give some valuable information to understand the status of these proteins. SDS-PAGE of crude seminal plasma was carried out and several polypeptide bands were identified which in correlation with the findings of the Amann *et al.* (1987). Molecular weight of the polypeptide bands could not be evaluated as the existence of seminal plasma protein might be aggregated forms in normal physiological condition. This is in support of the findings of Manaskova *et al.* (2003) who reported the aggregation of boar seminal plasma protein as they are mostly glycoprotein in nature. Crude fertility associated proteins were purified by gel filtration chromatography on sephacryl S-200, which resolved the proteins into three minor and one major peak. The fraction of major peak which were pooled and concentrated by dialysis against sucrose using dialysis membrane (cut off value 12,000) and revealed on 10% SDS-PAGE pure bands of 55 kDa, 26 kDa, 16 kDa, 14.3 kDa and 12 kDa in Gir and Jersey bull as analyzed by Gel Documentation system using Standard molecular protein marker (range: 14.3-97.4 kDa) in 10% SDS-PAGE. The presence of two additional fertility associated proteins of molecular weight 55 kDa and 26 kDa reported Killian *et al.* (1993), in Holstein bull seminal plasma. In the Gir and Jersey seminal plasma polypeptide bands of 55 kDa and 26 kDa along with 14.3 kDa were predominant in the both fraction number F1 and F2 respectively, when purified the crude seminal plasma through gel filtration. The result is supported by Killian *et* al. (1993), two-dimensional PAGE of seminal plasma samples indicated that two proteins (26 kDa and 55 kDa) predominated in higher-fertility bulls, and two proteins (12 kDa and 16 kDa) predominated in lower-fertility bulls. Densitometry data for these proteins in individual samples were

combined for bulls grouped by fertility level. Average density of the 26-kDa protein was significantly greater in seminal plasma of high-fertility bulls, and high-fertility seminal plasma also contained more of the 55-kDa protein than that of average and below average fertility bulls. Below average and low-fertility bull seminal plasma had significantly more of both 16-kDa proteins than that of average and high-fertility bulls. The available literature did not indicate any major bands of the valuable species like Gir and Jersey breed bull.

Antibodies were raised in rabbit against purified seminal plasma protein from Gir and Jersey bull crude seminal plasma. Single precipitin line was observed in double immune-diffusion test when each protein was run against their respective antibodies. Cross reactivity with heterologous antibody raised in rabbit by purified seminal plasma protein of Gir and Jersey bull.

The purified seminal plasma proteins were found immunoreactive against respective primary antibody in Western blot analysis. This may be due to antigenic similarity between the purified fertility associated proteins of Gir and Jersey bull. The hyper-immune serum raised against crude seminal plasma protein recognized the two partially purified polypeptides 55 kDa and 26 kDa in both species. This is in support of the findings of Cancel *et al.* (1997, 1999), who recognized the 55 kDa polypeptide in the bull seminal plasma fluids by western blot analysis using whole seminal plasma protein and recognized them as the accessory sex gland polypeptide and identified as Osteopontin. Gerena *et al.* (1998, 2000) analyzed a 26 kDa polypeptide from seminal plasma as Lipocalin type prostaglandin D synthase and identified its localization in epididymal tract of bull by western blot analysis and enhancement of sperm maturation. Desnoyers

et al. (1994) reported that using specific antibodies, western blot of bovine seminal plasma proteins detected spots at 15 kDa and 16 kDa of gel filtration.

In the present study the 26 kDa seminal plasma protein in Gir and Jersey bull is highly immunoreative even in higher dilution of hyperimmune serum. Gerena *et al.* (2000) demonstrated the presence of this Lipocalin type prostaglandin D synthase to the epical ridge of the acrosome on ejaculated spermatozoa. The high immunoreacivity of this 26 kDa Lipocalin type prostaglandin D synthase in higher percentage in the seminal plasma might be the presence of higher percentage of sperm having this lipocalin type PGD synthase. Gerena *et al.* (2000) demonstrated high percentage of immunofluorescent sperm in the seminal plasma having greater amount of lipocalin type PGD synthase.

In Indirect ELISA Gir Crude, Frozen and Purified seminal plasma protein showed higher ($P>0.01$) immunoreactive against homologous hyper immune serum compared to Jersey bull seminal plasma protein. There was no significant difference ($P<0.01$) observed in immunoreactive against heterologous hyper immune serum of crude and purified at the 1:400 dilution of Gir and Jersey bull seminal plasma, which indicated that there might be some share epitopes between two breed of bull. The finding is supported by Ghosh *et al.* (2008) who showed specific immunoreactivity in DID, Western Blot and Indirect ELISA in case of 55 kDa fertility associated protein in seminal plasma of sheep.

Chapter - VI

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

For the livestock and artificial insemination industry, production-tested sires with high fertilizing capacity are essential to assure optimal reproductive efficiency. Therefore, the search for indicators of male fertility has been the focus of several studies conducted in the past decades. The ability to select high fertility sires results in the production of semen samples with optimal quality that will, ultimately improve conception rates. Currently, routine semen analysis based on motility and morphology provides useful, but limited information about fertility indexes in the male. Proven, high-use bulls from artificial insemination centers still show differences in non-return rates by as much as 20 to 25 %. The existence of sub fertile sires with apparent normal semen quality is an important observation and has stimulated the search for other markers of fertility. Killian *et al.* (1993) reported the presence of four "fertility-associated proteins" in the Holstein seminal plasma.

In the present study an attempt has been made to throw some light on the reproductive performance of Gir and Jersey bull in terms of evaluation of polypeptide characteristics of seminal plasma as well as mass activity, motility and morphology of spermatozoa. Seminal plasma from apparently normal semen containing viable sperm was collected for isolation, purification and immunobiochemical characterization of fertility associated proteins as reported in boar and other breeds of bull (Killian et al., 1993; Flowers, 2001).

SDS-PAGE was performed to observe the polypeptide profiles of crude seminal plasma and different partially purified fractions separated by gel filtration chromatography and molecular weight was determined. The resolution of fraction for Gir bull, F1 (fractions of test tube no. 12,13 &14 of ascending loop of first peak), F2 (fractions of test tube no. 15,16&17 of descending loop of first peak) in 10% SDS-PAGE revealed major polypeptide bands of 55 kDa, 26 kDa and with minor polypeptide band 16 kDa of molecular weight. For Jersey bull fertility associated seminal plasma proteins as F1 (fractions of test tube no. 13,14 &15 of ascending loop of first peak), F2 (fractions of test tube no. 16,17&18 of descending loop of first peak) in 10% SDS-PAGE revealed molecular weight were 26 kDa and with minor polypeptide bands 14.3 kDa and 12 kDa.

Single precipitin line was observed in double immune-diffusion test, when each protein was run against homologous antibodies. Cross reactivity with heterologous antibody raised in rabbit by purified seminal plasma protein of Gir and Jersey bull were also found.

The purified antigens were then analyzed by western blot technique to verify immunoreactivity with hyperimmune serum raised in rabbit against crude seminal plasma of Gir and Jersey bull. The partially purified seminal plasma proteins with major bands 26 kDa, 55 kDa with minor bands of 12 kDa, 14.3 kDa and 16 kDa were found to be immunoreactive against hyperimmune serum.

In indirect ELISA test crude, frozen as well as purified seminal plasma protein of Gir bull showed higher ($P>0.01$) immunoreactivity against homologous hyper immune serum

compared to crude, frozen and purified seminal plasma of Jersey bull. There was no significant difference ($P<0.01$) observed in immunoreactivity against heterlogous hyper immune serum between crude, frozen and purified seminal plasma protein of Gir and Jersey bull indicating that two breeds might be shared immune reactive antigen.

It was evident that two different breeds of bull, Gir (Indigenous breed) and Jersey (Exotic breed) semen characteristics were almost similar. It could therefore be summarized that, semen of the Gir could be the good substitute of Jersey semen used for artificial insemination but the duration of preservation would be less.

CONCLUSION:

1. In the present study, Gir and Jersey bull seminal plasma was assessed for the detection of fertility associated protein like other breeds of bovine, boar, buck, stallion dog etc.

2. These proteins can be efficiently isolated and purified by gel filtration chromatography and purified fertility associated proteins were found immunoreactive.

3. The molecular weights of Gir bull purified seminal plasma fertility associated proteins were 55 kDa, 26 kDa and 16kDa.

4. The molecular weights of Jersey bull purified seminal plasma fertility associated proteins were 26 kDa, 12kDa and 14.3 kDa.

5. These polypeptides isolated from Gir and Jersey bull crude seminal plasma were highly seroreactive when studied by DID, Western Blot and ELISA.

6. Fertility associated proteins of Gir and Jersey bull were immunochemically identified.

Chapter - VII

FUTURE SCOPE OF RESEARCH

FUTURE SCOPE OF RESEARCH

On the basis of the present findings in Gir and Jersey bull seminal plasma, further investigation can be carried out on the following aspects.

1. Localization of 12kDa, 16kDa, 26 kDa and 55 kDa seminal plasma protein in the specific region of the male reproductive tract.

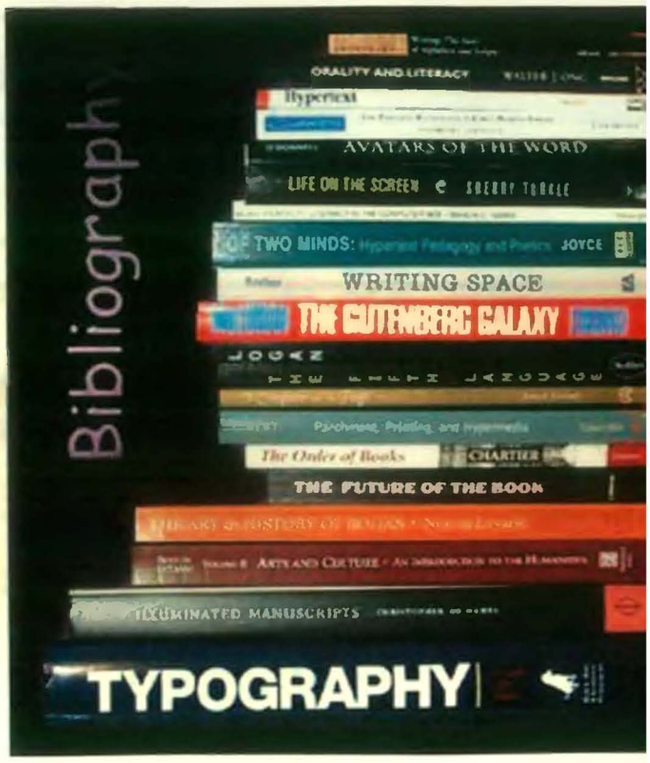
2. Amino acid sequencing of these polypeptides and their relationship with other fertility associated protein of different breeds of bull.

3. The identification and characterization of protein related with fertility helps to understanding further molecular basis of reproduction mechanism.

4. To justify the relationship development of fertility associated proteins in the seminal plasma of bull by evaluating the calving rate.

5. Monoclonal antibody based ELISA kit may be developed using bull seminal plasma purified antigen to detect etiology of infertility.

6. Useful for identifying potential biomarkers of fertility in the next generation.


Bibliography
ORALITY AND LITERACY
Hypertext
AVATARS OF THE WORD
LIFE ON THE SCREEN
TWO MINDS:
JOYCE
WRITING SPACE
THE GUTENBERG GALAXY
Psychotext, Printing, and Hypermedia
The Order of Books
CHARTIER
THE FUTURE OF THE BOOK
ILLUMINATED MANUSCRIPTS
TYPOGRAPHY

BIBLIOGRAPHY

BIBLIOGRAPHY

Acott, T. S. and Hoskins, D. D. (1978). Bovine sperm forward motility protein: Binding to epididymal spermatozoa. *Biology of reproduction,* **24**:234-240.

Amann, R. P., Cristanelli, M. J. and Squires, E. L. (1987). Proteins in stallion seminal plasma. *Journal of reproduction and fertility,* **35**:113-120.

Asadpour, R., Alavi-Shoushtari, S. Rezaii, S. and Ansari, M. (2007). SDS-PAGE of buffalo bulls seminal plasma proteins and their relation with semen freezibility. *Animal reproduction science,* **102**:308-313.

Audhya, T., Reddy, J. and Zaneveld, L. J. D. (1987). Purification and partial chemical characterization of a glycoprotein with antifertility activity from human seminal plasma. *Biology of reproduction,* **36**:511-521.

Austin, C. R. and Short, R. V. (1990). Reproduction in mammals-Germ cells and fertilization. New York: Cambridge University. 285p.

Barrios, B., Fernandez-Juan, M., Muino-Blanco, T. and Cebrian-Perez, J. A. (2005). Immunocytochemical localization and Biochemical characterization of two seminal plasma proteins that protects Ram spermatozoa against cold shock. *Journal of Andrology,* **26(4)**:539-549.

Belorkar, P. M., Dhami, A. and Kadagali, S. B. (1988). Pysico-biochemical semen characteristics of good and poor freezibility crossbred bulls. *Indian journal of animal sciences.* **58**:1419-1423.

Bellin, M. E., Hawkins, H. E. and Ax, R. L. (1994). Fertility of range beef bulls grouped according to presence or absence of heparin-binding proteins in sperm membranes and seminal fluid. *Journal of animal science,* **72**:2441-2448.

Bellin, M. E., Hawkins, H. E., Oyarzo, J. N., Vanderboom, R. J. and Ax, R. L. (1996). Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. *Journal of animal science,* **74**:173-182.

Bergeron, A., Villemure, M., Lazure, C. and Manjunath, P. (2005). Isolation and characterization of the major proteins of ram seminal plasma. *Molecular reproduction and development,* **71(4)**:461-470.

Boisvert, M., Bergeron, A., Lazure, C. and Manjunath, P. (2004). Isolation and characterization of gelatin binding bison seminal vesicle secretory proteins. *Biology of reproduction,* **70(3)**:656-661.

Brahmkshtri, B. P., Edwin, M. J., John, M. C., Nainar, A. M. and Krishnan, A. R. (1999). Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility in vitro. *Animal reproduction science,* **54**:159-68.

Brandon, C. I., Hausner, G. L., Caudle, A. B. and Fayrer-Hosken, R. A. (1999). Two dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. *Theriogenology,* **52(5)**:863-873.

Braundmeier, A. G. and Miller, D. J. (2001). The search is on finding accurate molecular markers of male fertility. *J Dairy Sci.* **84**:1915-1925.

Cancel, A. M., Chapman, D. A. and Killian, G. J. (1997). Osteopontin is the 55kDa fertility associated protein in Holstein bull seminal plasma. *Biology of reproduction,* **57**: 1293-1301.

Cancel, A. M., Chapman, D. A. and Killian, G. J. (1999). Osteopontin localization in the Holstein bull reproductive tract. *Biology of reproduction,* **60**:454-460.

Chandonnet, L., Roberts, K. D., Chapdelaine, A. and Manjunath, P. (1990). Identification of heparin binding proteins in bovine seminal plasma. *Molecular reproduction and development,* **26(4)**:313-318.

Correa, J. R., Pace, M. M. and Zavos, P. M. (1997). Relationships among frozen-thawed sperm characteristics accessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. *Theriogenology,* **48**:721-731.

Desnoyers, L., Therian, I., and Manjunath, P. (1994). Characterization of the major proteins of bovine seminal fluid by two dimensional polyacrylamide gel electrophoresis. *Molecular reproduction and development,* **37(4)**: 425-435.

Edwards, J. J., Tollaksen, S. L. and Anderson, N. G. (1981). Proteins of human semen. Two-dimentional mapping of human seminal fluid. *Clinical Chemistry,* **27**:1335-1340.

Eng, L. E. and Oliphant, G. (1978). Rabbit sperm reversible decapacitation by membrane stabilization with a highly purified glycoprotein from seminal plasma. *Biological Reproduction,* **19**:1083-1094.

Evans, G. and Maxwell, W. M. C. (1987). Salamon's artificial insemination of sheep and goats. Sydney: Butterworths.

Farrell, P. B., Presicce, G. A., Brocket, C. C. and Foote, R. H. (1998). Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology,* **49**:871-879.

Flowers, W. L. (1997). Management of boars for efficient semen production. *Journal of reproduction and fertility,* **52**:67–78.

Flowers, W. L. (1998). Relationships between seminal plasma proteins and boar fertility. Departmental Report, Department of Animal Science, *ANS Report No.**248**.*

Flowers, W. L. (2001). Relationship between seminal plasma proteins and boar fertility. *Swine News (USA),* 1-4.

Ganti, A. and Sastry. (1983). *Veterinary Pathology,* 6th ed.CBS Publishers and Distributors, New Delhi, India.

Gatti, J. L., Druart, X., Syntin, P., Guerin, Y., Dacheux, J. L. and Dacheux, F. (2000). Biochemical characterization of two rams cauda epididymal maturation dependent sperm glycoproteins. *Biology of reproduction,* **62**:950-958.

Gerena, R. L., Irikura, D., Urade, Y., Eguchi, N., Chapman, D. A. and Killian, G. J. (1998). Identification of fertility associated proteins in bull seminal plasma as lipocalin-type prostaglandin D-synthase. *Biology of reproduction,* **58**:826-833.

Gerena, R. L., Irikura, D., Eguchi, N., Urade, Y., and Killian, G. J. (2000). Immunocytochemical localization of Lipokalin-type prostaglandin D-synthase in bull testes and epididymis and on ejaculated sperm. *Biology of reproduction*, **62**:547-556.

Ghosh, P., Chattopadhyay, S. and Batabyal, S. (2008). Immunobiochemical characterization of 55 kDa fertility associated protein of Garole sheep (Ovis aries) seminal plasma. *Indian J. Vet Res.*, **17**:1-10.

Harshan, H. M., Sankar, S., Singh, L. P., Singh, M. K., Sudharani, S., Ansari, M. R., Singh, S. K., Majumdar, A. C. and Joshi, P. (2009). Identification of PDC-109 like protein in buffalo seminal plasma. *Animal reproduction science*, **115**:306-311.

Henricks, D. M., Kouba, A. J., Lackey, B. R., Boone, W. R. and Gray, S. L. (1998). Identification of insulin-like growth factor I in bovine seminal plasma and its receptor on spermatozoa: influence on sperm motility. *Biolical reproduction*, **59**:330-337.

Holt, W. V., O'Brien, J. and Abaigar, T. (2007). Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. *Reproduction fertility development*, **19:**709-718.

Inagaki, M., Kikuchi, A., Orino, K., Ohnami, Y. and Watanabe, K. (2002). Purification and quantification of Lactoferrin in equine seminal plasma. *The Journal of veterinary medical science*, **64 (1)**:75-77.

Januskauskas, A., Gil, J., Soderquist, L. and Rodriguez-Martinez, H. (2000a). Relationship between response to glycosaminoglycans in vitro and non-return rates of Swedish dairy AI bulls. *Reproduction domestic animals,* **35:**207-212.

Januskauskas, A., Johannisson, A., Soderquist, L. and Rodriguez-Martinez, H. (2000b). Assessment of sperm characteristics post-thaw and response to calcium ionophore in relation to fertility in Swedish dairy AI bulls. *Theriogenology,* **53**:859-875.

Jegou, B., Dacheux, J. L., Garnier, D. H., Terqui, M., Golas, G. and Courot, M. (1979). Bio-chemical and physiological studies of androgen-binding protein in reproductive tract of ram. *Journal of reproduction and fertility,* **57**:311-318.

Jobim, M. I., Oberst, E. R., Salbego, C. G., Wald, V. B., Horn, A. P. and Mattos, R. C. (2005). BSP A-1/A-2 like proteins in ram seminal plasma. *Theriogenology,* **63(7)**: 2053-2062.

Kataria, J. M., Singh, S. D., Dharma, K. and Verma, K. C. (2000). *Laboratory Manual on Poultry Disease Diagnosis,* Division of Avian Diseases, Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P., India. pp. 97-99.

Killian, G. J., Chapman, D. A. and Rogowski, L. A. (1993). Fertility associated proteins in Holstein bull seminal plasma. *Biology of reproduction,* **49**:1202-1207.

Kulkarni, B. A., Fernandes, J. E., Dhande, S. G. and Hegde, U. C. (1996). Characterisation of seminal plasma proteins of the Indian buffalo and cattle bulls by SDS-PAGE. *Buffalo Journal,* **12**: 147-56.

Kumar, V., Hassan, M. I. Kashav, T., Singh, T. P. and Yadav, S. (2008). Heparin-binding proteins of human seminal plasma: purification and characterization. *Molecular Reproduction & Development*, **75 (12)**:1767-1774.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, **227**: 680-685.

La falci, V. S., Tortorella, H., Rodrigues, J. L. and Brandelli, A. (2002). Seasonal variation of goat seminal plasma proteins. *Theriogenology*, **57**:1035-1048.

Larson, J. L. and Miller, D. J. (2000). Can relative spermatozoa galactosyltransferase activity be predictive of dairy bull fertility. *Journal dairy science*, **83**:2473-2479.

Leone, M. G., Abdel, H. H., Gennaro, G., Amici, S., Coute, D., Romanelli, F., Latini, M., Isidori, A., Saso, L. and Silvestrini, B. (2001). Changes of lipocalin type prostaglandin D-synthase in the seminal plasma of subfertile man. *Research communications in molecular pathology and pharmacology*. **110 (1-2)**:17-25.

Lowry, I. O. M., Rosenbrough, N. I., Farr. R. L., and Randall, R. J. (1951). Protein measurement with Folin phenol reagent. *Journal of biological Chemistry*, **193**:265-275.

Manaskova, P., Balinova, P., Kraus, M., Ticha, M. and Jonakova, V. (2003). Mutual interactions of boar seminal plasma proteins studied by immunological and chromatographic methods. *American journal of reproductive immunology*, **50(5)**; 399-410.

Manjunath, P., Sairam, M. R. and Uma, J. (1987). Purification of four gelatin binding proteins from bovine seminal plasma by affinity chromatography. *Bioscience reports*, **7(3)**:231-238.

Manjunath, P., Chondonnet, L., Leblond, E. and Desnoyers, L. (1994). The major proteins of bovine seminal vesicles bind to spermatozoa. *Biol Reprod.* **50**:27-37.

Maura, A. A. (2005). seminal plasma protiein and fertility indexes in the bull; the case for osteopontin. *Animal reproduction science,* V.**2**.n.1, 3-10.

Maura, A. A., Koc, H., Chapman, D. A. and Killian, G. J. (2006). Identification of proteins in the accessory sex gland fluid associated with fertility index of dairy bulls; a proteomic approach. *Journal of andrology*; **27(2)**:201-211.

McCauley, T. C., Zhang, H., Bellin, M. E. and Ax, R. L. (1999). Purification and characterization of fertility-associated antigen (FAA) in bovine seminal fluid. *Molecular reproduction and development,* **54**:145-153.

McCauley, T. C., Zhang, H., Bellin, M. E. and Ax, R. L. (2001). Identification of a heparin-binding protein in bovine seminal fluid as tissue inhibitor of metalloproteinases-2. *Molecular reproduction and development,* **58**:336-341.

Miyamato, R., Umezu, M., Ishi, S., Furusawa, Y. and Ohta, M. (1989). Serum inhibin, FSH, LH and testosterone levels and testicular inhibin contents of beef bulls from birth to puberty. *Animal reproduction science*, **89**:165-178.

Mortarino, M., Tedeschi, G., Negri, A., Ceciliani, F., Gottardi, L., Maffeo, G. (1998). Two dimentional polyacrylamide gel electrophoresis map of bull seminal plasma proteins Electrophorosis. **19**:797-801.

Mortimer, S. T. (1997). A critical review of the physiological importance and analysis of sperm movement in mammals. *Human reproduction Update,* 3:403-439.

Nass, S. J., Miller, D. J., Miller, M.A. and Ax, R. L. (1994).Male accessory sex glands produce heparin binding proteins that bind to caudal epididymal spermatozoa and are testosterone dependent. *Molecular Reproductive Development.* **25**:237.

Nauc, V. and Manjunath, P. (2000). Radioimmunoasdsays for Bull seminal plasma proteins (BSP-A1/-A2, BSP-A3 and BSP-30kDa) and their quantification in seminal plasma and sperm. *Biology of reproduction,* **63**:1058-1066.

Ouchterlony, O. (1963). Antigen-antibody reactions in gels types of reactions in coordinated system of diffusion. *Acta Pathol. Microbiol. Scand.*, **33**:231.

Parent, S., Lefievre, L., Brindle, Y. and Sullivan, R. (1999). Bull subfertility is associated with low levels of a sperm membrane antigen. *Molecular reproduction and development,* **52**:57-65.

Rasul, Z., Anzar, M., Jalali, S. and Ahmad, N. (2000). *Animal reproduction science,* **59(1-2)**: 28, 31-41.

Reddy, E. S. H. and Bhargava, P. M. (1979). Seminal plasmin: an anti-microbial protein from bovine seminal plasma which acts in *E. coli* by specific inhibition of RNA synthesis. *Nature (London),* **279**:725-728.

Reddy, J., Audhya, T., Goodpasture, J. C. and Zaneveld, L. J. D. (1982). Properties of a highly purified antifertility factor from human seminal plasma. *Biological reproduction,* **27**:1076-1083.

Reddy, S. P., Das, M. R., Reddy, E. P. and Bhargava, P. M. (1983). Inhibition of reverse transcriptase by seminal plasmin. *The Biochemical journal,* **209**:183-188.

Romao, M. J., Kolln, I., Dias, J. M., Karvalho, A. L., Romero, A., Varela, P. F., Topfer-Petersen, E. and Calvet, J. J. (1997). Crystal structure of acidic seminal fluid protein (aSFP) at 1.9 A resolution; a bovine polypeptide of the spermadhesin family. *Journal of molecular biology,* **274(4)**: 650-660.

San Agustin, J. and Lardy, H. A. (1990). Bovine seminal plasma constituents modulate the activity of caltrin, the calcium transport regulating protein of bovine spermatozoa. *Journal of biological chemistry,* **265**:6860-6867.

Sanchez-Luengo, S., Aumuller, G., Albrecht, M., Sen, P. C., Rohm, K. H. and Wilhelm, B. (2004). Interaction of PDC-109, the major secretory protein from bull seminal vescicles, with bovine sperm membrane Ca^{2+}- ATPase. *Journal of andrology,* **25**:234-244.

Sarkar, S., Mandal, S., Manna, A. K., Roy, S., Joardar, S. N. and Dasgupta, C. K. (2003). Detection of anti Fasciola antibodies in cattle and buffaloes sera using DID and ELISA. *Indian Journal of Animal Health.* **42 (1)**:26-30.

Shabanowitz, R. B. and Killian, G. J. (1987). Two dimensional electrophoresis of proteins in principal cells, spermatozoa, and fluid associated with the rat epididymis. *Biology of reproduction*, **36**:753-768.

Shelke, V. and Dhami, A. (2002). Comparative appraisal of blood and seminal plasma biochemistry of gir and jafrabadi breeding bulls. *Indian veterinary journal*, **79**:349-353.

Shivaji, S. and Bhargava P. M. (1987). Antifertility factors of mammalian seminal fluid. *BioAssay*, **7**:13-17.

Shivaji, S., Scheit, K. H. and Bhargava, P. J. (1990). Proteins of Seminal Plasma. New York: John Wiley and Sons, Inc.; 332-333.

Siiteri, J. E., Ensrud, K. M., Moore, A. and Hamilton, D. W. (1995). Identification of Osteopontin (OPN) mRNA and protein in the rat testes and epididymis and on sperm. *Molecular reproduction and development*, **40**:16-28.

Snedecor, G. W. and Cochran, W. G. (1967). Statistical Methods. 6th Edition. Oxford and IBH, New Delhi. 258-296.

Srivastava, P. N. and Farooqui, A. A. (1979). Heparin-sepharose affinity chromatography for purification of bull seminal plasma hyaluronidase. *The Biochemical journal*, **183**:531-537.

Strezezek, J., Kordan, W., Kostyra, H. and Zaborniak, A. (1992). Purification and partial characterization of a 5700 Da sperm motility inhibiting factor from seminal plasma of boar. *Animal reproduction science*, **29**:35-52.

Strezezek, J., Saiz-Cidoncha, F., Wysocki, P., Tyszkiewicz, A. and Jastrzebski, M. (2002). Seminal plasma proteins as markers of biological value of boar semen. *Animal Science Papers and Reports*, Institute of genetics and breeding, Jastrzebiec, Poland, **20(4)**:255-266.

Susan, N., Ana, R. S., John, C., Walter, D. and George, F. (2005). Seminal plasma proteins from fertile boars are related to differences *in vivo* fertility (W 540). *The society for the study of reproduction*, **38th** Annual meeting, Quebec City, Quebec, Canada. 24-27.

Svoboda, M., Meuris, S., Robyn, C. and Christophe, J. (1985). Rapid electrotransfer of proteins from polyacrylamide gel to nitrocellulose membrane using surface-conductive glass as anode. *Analytical biochemistry*, **151**: 16-23.

Tedeschi, G., Oungre, M., Mortarino, M., Negri, A., Maffeo, G. and Ronchi, S. (2000). Purification and Primary structure of a new bovine spermadhesin. *European journal of biochemistry*, **267(20)**: 6175-9).

Teixeira, D. I. A., Melo, L. M., Gadelha, C. A. D. A., Cunha, R. M. S. D., Jr., C. B., Radis-Bapitista, G., Cavada, B. S. and Freitas, V. J. D. F. (2006). Ion exchange chromatography used to isolate a Spermadhesin related protein from domestic goat (*Capra hircus*) seminal plasma. *Genetics and molecular research*, **5(1)**:79-87.

Thomas, C. J., Anbazhagan, V., Ramkrishnan, M., Sultan, N., Surolia, I. and Swamy, M. J. (2003). Mechanism of membrane binding by the bovine seminal plasma protein, PDC-109: a surface Plasmon resonance study. *Biophysical journal*, **84**:3037-3044.

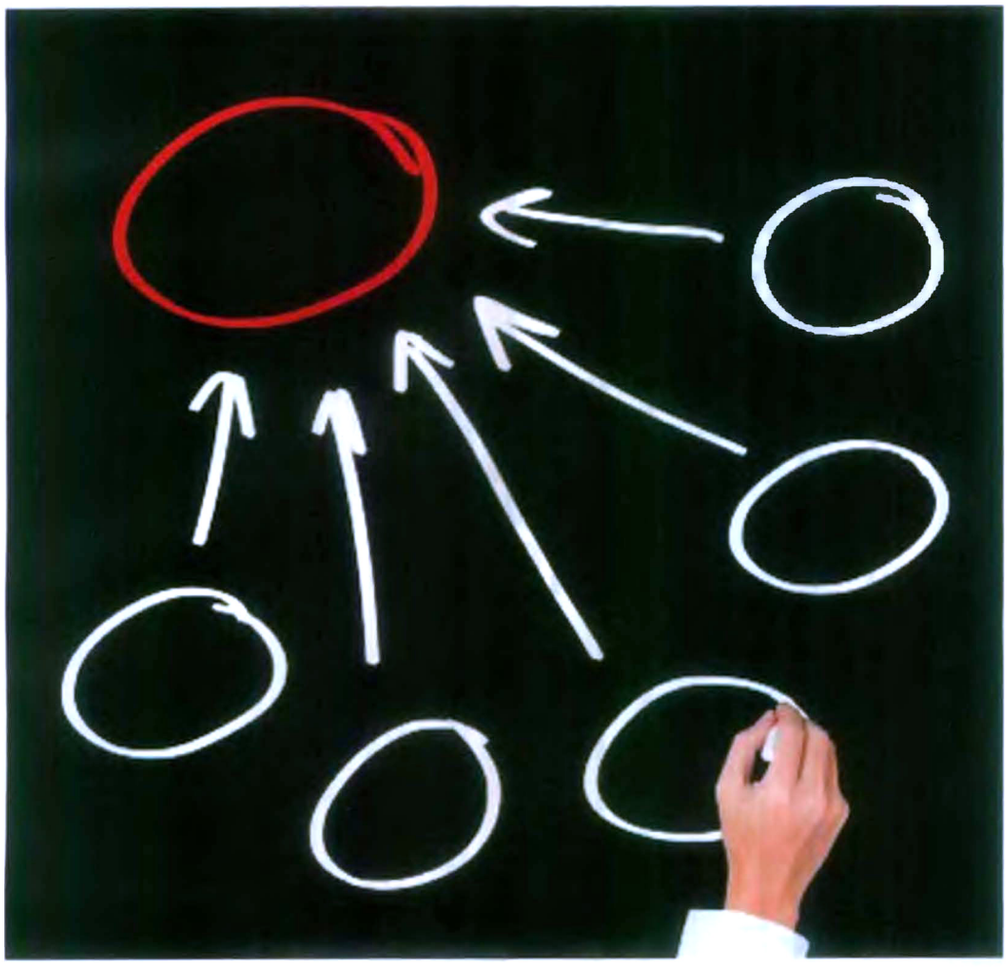
Topfer-Petersen, E., Ekhlasi-Hundricser, M., Kirchhoff, C., Leeb, T., and Sieme, H. (2005). The role of stallion seminal proteins in fertilization. *Animal reproduction science*, **89(1-2)**:159-170.

Towbin, H., Staehelin, T. and Gordan, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*, **76**: 4350-4354.

Urade, Y. and Hayaishi, O. (2000). Biochemical, Structural, genetic,physiological and pathophysiological features of lipocalin-type prostaglandin D-synthse. Biochemistry and Biophysics Acta, **1482(1-2)**:249-271.

Varilova, T., Semenkova, H., Horak, P., Madera, M., Pacakova, V., Ticha, M. and Stulik, K. (2006). Affinity liquid chromatography and capillary electrophoresis of seminal plasma proteins. *Journal of separation science*, **29(8):** 1110-1115.

Villemure, M., Lazure, C. and Manjunath, P. (2003). Isolation and characterization of gelatin-binding proteins from goat seminal plasma. *Reproductive biology and endocrinology*, **1**:39-49.



APPENDIX

APPENDIX

All the solutions were prepared in double glass distilled water and all the chemicals used were AR/ GR grade.

REAGENTS FOR BUFFERS AND SOLUTIONS:

1. **Normal Saline Solution (NSS)**

Sodium Chloride (NaCl)	0.85 gm
Distilled Water	100 ml

2. **0.15 M Phosphate Buffer Saline (PBS, pH 7.2)**

Sodium Chloride (NaCl)	8.0 gm
Potassium Chloride (KCl)	0.20 gm
Disodium hydrogen phosphate	1.15 gm
Potassium dihydrogen phosphate	0.20 gm
Distilled water	upto 1000 ml

3. **0.01 M Phosphate Buffer Saline (PBS, pH 7.4)**

Sodium Chloride (NaCl)	8.0 gm
Potassium Chloride (KCl)	0.20 gm
Disodium hydrogen phosphate	1.44 gm
Potassium dihydrogen phosphate	0.24 gm
Distilled water	upto 1000 ml

REAGENTS FOR ESTIMATION OP PROTEIN BY LOWRY'S METHOD:

Reagent A

2 % Na_2CO_3 in 0.1 N NaOH

Reagent B

0.5% $CuSO_4$, 5 H_2O in 1% Sodium potassium tartarate

Lowry's Reagent

Reagent A and Reagent B mixed in the ratio of 50:1

BSA	1.0 mg / ml

Folin reagent.

Folin reagent	1 ml
Distilled water	2 ml

REAGENTS AND BUFFERS FOR SDS-PAGE

1. Sealing gel (1% agar solution)

Agar	1.0 gm
Distilled water	100 ml

2. Solution A

Acrylamide	14.6 gm
Bisacrylamide	0.4 gm
Distilled water	50 ml

3. Solution B

Tris	9.1 gm
SDS	0.2 gm
HCl	1 ml
Distilled water	up to 50 ml

pH was adjusted to 8.8.

4. Solution C

Tris	3.05 gm
SDS	0.2 gm
HCl	2.1 ml
Distilled water	up to50 ml

pH was adjusted to 6.8

5. Solution D

APS	0.1 gm
Distilled water	1.0 ml

6. Running buffer

Tris	0.9 gm
Glycine	4.32 gm
SDS	0.3 gm
Distilled water	300 ml

7. Sample buffer

SDS	0.25 gm
Mercaptoethanol	0.25 ml
Solution C	2.5 ml
Glycerol	5.0 ml

Distilled water	2.0 ml
Bromophenol blue	10.0 mg

8. Stacking gel

Solution A	0.9 ml
Solution C	1.5 ml
Solution D	0.02 ml
TEMED	0.01 ml
Distilled water	3.6 ml

9. Separating gel- 10 %

Solution A	6.0 ml
Solution B	4.5 ml
Solution D	0.08 ml
TEMED	0.01 ml
Distilled water	7.5 ml

10 Coomassie brilliant blue staining solution

Coomassie brilliant blue	0.25 gm
Methanol	40 ml
TCA	20 gm
Distilled water	60 ml

11. Destaining solution

Methanol	30 ml
Acetic acid	10ml
Distilled water	60 ml

REAGENTS FOR DID TEST:

1. Normal saline solution (NSS)

Sodium chloride	8.5 gm
Distilled water	1000 ml

2. Agarose (1.5%)

Agarose	1.5 gm
Sodium azide	0.02 gm
NSS	100 ml

REAGENTS FOR INDIRECT ELISA

1. Coating buffer/Carbonate bicarbonate buffer (pH 9.5)

Na_2CO_3	0.159 gm
$NaHCO_3$	0.293 gm
Sodium azide	0.020 gm
Distilled water	100 ml

2. Blocking buffer

Skimmed milk powder	5 gm
PBS (0.01 M ph 7.4)	100 ml

3. Washing buffer

Tween -20	0.5 ml
PBS (0.01 M pH 7.4)	1000 ml

4. Phosphate-Citrate buffer (pH 5.0)

Solution A

Citric acid	2.1 gm
Distilled water	100 ml

Solution B

Sodium citrate	2.94 gm
Distilled water	100 ml

Stored at 4^0 C.

5. Substrate buffer

Citrate buffer	25 ml
H_2O_2	3.0 µl
OPD	0.025 gm

Always prepared fresh.

6. Stopping solution

H_2SO_4	5.5 ml
Distilled water	94.5 ml

REAGENTS FOR WESTERN BLOTTING

1. Phosphate buffer saline (PBS) (pH 7.4)

NaCl	8 gm
KCl	0.2 gm
Na_2HPO_4	1.15 gm

KH_2PO_4	0.2 gm
Distilled water	1000ml

2. Transfer buffer

Tris	12.1 gm
Glycine	14.4 gm
Methanol	50 ml
Distilled water	up to 1000 ml

3. Blocking buffer (pH 7.2)

BSA	3 gm
Tween- 20	0.1 ml
Negative rabbit serum	0.5 ml
Distilled water	up to 100 ml

4. Washing buffer (PBST) (pH 7.2)

Tween 20	50 µl
PBS	upto100 ml

5. Dilution buffer

BSA	1.0 gm
Tween 20	0.1 ml
Negative rabbit serum	0.5 ml
Distilled water	up to100 ml

6. Conjugate

Goat anti-rabbit horse radish peroxidase 1:1000 dilution.

7. Substrate solution

Tris HCl (50 mM, pH 7.5)	10 ml
H_2O_2	40 µl
DAB	2.5 mg

8. Phenyl Methyl Sulphonyl Fluoride (PMSF)

PMSF	174.2 gm
Isopropyl alcohol	1000 ml

ROSE BENGAL STAINING (3%):

Rose Bengal Dye	3 gm
Commercial Formalin	1ml
Distilled water	Up to 100 ml