IDENTIFICATION AND CHARACTERIZATION OF SPERM STIMULATING FACTORS FROM FOLLICULAR FLUID

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **IDENTIFICATION AND CHARACTERIZATION OF SPERM STIMULATING FACTORS FROM FOLLICULAR FLUID** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out by me during a period from July 2002 to June 2008 under the supervision of Dr. R. Prasad, Associate Professor Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(MANISH RANA)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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Date: June 4th, 2008

The Ph.D. Viva-Voce Examination of **Mr. Manish Rana**, Research Scholar, has been held on ...18.Oct.,2008

Signature of Supervisor

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Signature of External Examiner

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Date ١١ - ٠٦ - ٠٨

(MANSIH RANA)

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LIST OF ABBREVIATIONS

AC	Adenylyl Cyclase
cAMP	Cyclic Adenosine Mono-Phosphate
sAC	Soluble Adenylyl Cyclase
RNA	Ribonucleic acid
AR	Acrosome Reaction
mV	Milli Volt
ZP	Zona Pellucida
ATP	Adenosine Tri-Phosphate
SAP	Sperm Activating Peptide
cGMP	Cyclic Guanosine Mono-Phosphate
pM	Pico-Molar
µm	Micro-Meter
RGC	Receptor Guanylyl Cyclase
OR	Olfactory Receptor
mAC	Membrane Associated Adenylyl Cyclase
ANP	Atrial Natriuetic Peptide
fMLP	Formyl-Metheonine-Leucine-Phenylalanine
SSTA	Single Sperm Tracking Algorithm
RATTS	Real Time Automated Tracking and Trapping System
ADP	Adenosine Diphosphate
UTJ	Utero Tubal Junction
BWW	Biggers Whitten and Whittingham
µM	Micro-Molar
DMSO	Dimethyl Sulfoxide
HRP	Horseradish Peroxidase
PITC	Phenylisothiocynate
AA	Amino Acid
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography
TGF	Transforming Growth Factor

RANTES	Regulated on Activation, Normal T Expressed and Secreted Chemokine
PAS	Periodic Acid Schiff
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
FPLC	Fast Protein Liquid Chromatography
MALDI-ToF	Matrix Assisted Laser Desorption /Ionization- Time Of Flight
IEF	Iso-Electric Focusing
PVDF	Polyvinylidene Fluoride
CASA	Computer Assisted Sperm Analysis
BSA	Bovine Serum Albumin
PBS	Phosphate Buffer Saline
BBS	Brismark Brown Solution
RBS	Rose Bengal Solution
TMB	3,3',5,5'-Tetramethylbenzidine
IPG	Immobilized pH Gradient
TFA	Trifluoroacetic Acid
TEMED	Tetramethylethylenediamine
VSL	Mean Progressive Velocity
ALH	Mean
STR	Mean Straightness
BCF	Mean Beat Frequency
APS	Ammonium per Sulfate
DTT	Dithiothreitol
PMF	Protein Mass fingerprinting
FMSP	Forward Motility Stimulating Protein

Chapter-1

Introduction

CHAPTER 1: INTRODUCTION

Chemotaxis or movement towards the chemical attractant is important for diverse processes such as bacterial attraction to nutrients, morphogenesis in *Dictyostelium discodium*, neutrophil migration towards bacterial infections and neuronal growth cone projection. Chemotaxis of sperm towards an egg is yet another phenomenon which is vital during fertilization. During sperm chemotaxis, a chemoattractant source re-orients sperm motility towards the source, and hence towards the egg. This type of sperm behaviour is reported in various organisms, mostly marine invertebrates (such as sponges and sea urchins) and in some vertebrates (such as ascidians, amphibians, and possibly mammals). Inspite of the reports indicating occurrence of sperm chemotaxis in mammals (Villanueva-Diaz *et al.*, 1990; Ralt *et al.*, 1991) there are some basic differences between sperm chemotaxis of mammals and that of marine invertebrates, the most pronounced being the fractional chemotactic response in the mammals. Unlike spermatozoa of marine species, spermatozoa of mammals must undergo a process of maturation, termed as capacitation, for acquiring the ability to bind to the egg and penetrate it (Yanagimachi, 1994). At any given time, the percentage of capacitated spermatozoa is small due to both limited time span of the capacitated stage and the continuous replacement of the capacitated cells in the sperm population (Cohen-Dayag *et al.*, 1994). Chemotaxis as a form of sperm guidance in case of mammals was not easily accepted initially because of the inconsistent interpretations drawn from the initial studies, one of them being the failure to distinguish between chemotaxis and other causes of sperm accumulation (such as chemokinesis and trapping). This was largely on account of lack of fool proof assay methods available to study sperm chemotaxis and inconsistence results obtained during the studies (Eisenbach, 1999). Besides, in higher mammals, chemotaxis was believed to be unnecessary because fertilization is

internal and very large numbers of spermatozoa are ejaculated directly into the female reproductive tract of female (4-40 x 10^7 in humans) (WHO, 1992). This implied that sufficient number of spermatozoa would reach the egg coincidentally. In contrast to this view, it was found that the spermatozoa that actually reach the oviduct is extremely low, about six orders of magnitude less than the number of spermatozoa deposited in the female genital tract (Harper, 1982; Barrat and Cooke, 1991; Williams *et al.,* 1993) reducing the possibility of coincidental successful collision between a spermatozoa and the egg. It is therefore likely that additional mechanisms are involved in directing the spermatozoa to the egg.

Recent studies have revealed that chemoattractant signal triggering sperm chemotaxis are synthesized by the egg or, in some cases, by the somatic cells associated with the egg and are believed to exert influence over a short distance to enhance the efficiency of sperm-egg contact in higher animals (Oliveira *et al.,* 1999). The events of sperm chemotaxis are of interest for two main reasons. As this marks the first non-contact interaction of sperm with egg derived factors, the response can be considered to be the initial step towards fertilization. This process also enables the recruitment of selected population of sperm to fertilize the oocyte. Thus, a study on sperm chemotaxis could improve our understanding of the processes of natural fertilization. By identifying natural molecules that are involved it would be possible to get some insight into the signalling pathways of sperm chemotaxis. These molecules could also be exploited for sperm selection in assisted reproduction programs.

In the present study attempts were made to study migratory behaviour (chemotaxis) of spermatozoa using goat as animal model with the following objectives in mind:

a). To design, fabricate and validate improved techniques for measuring mammalian sperm chemotaxis *in vitro*.

b). To examine if capacitation of sperm improves its ability to respond chemotactically.

c). To ascertain the role of free amino acids, sugars and hormones (steroid and non-steroid), if any, on sperm migration

d). To establish if sperm responds chemotactically to ovarian follicular fluid collected at various stages of maturity.

e). To identify, isolate, purify and characterize factor(s) from ovarian follicular fluid that influence sperm motion characteristics.

Chapter-2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION:

Communication between spermatozoa and egg before contact by chemotaxis is prevalent throughout the animal kingdom. Chemotaxis was discovered as a form of sperm guidance in marine species where the gradient of the chemoattractant provides cues that guide sperm to the egg (Miller, 1985; Cosson, 1990). In non-mammalian species, sperm chemotaxis to factors secreted from the egg is well documented (Miller, 1985; Cosson, 1990; Eisenbach and Tur-Kaspa, 1994). Chemotaxis as a form of sperm guidance incase of mammals was not easily accepted initially because of the inconsistent results drawn from the initial studies, one of them being difficulty in measuring very low signal to noise ratio that was further compounded with suboptimal experimental condition and failure to distinguish between chemotaxis and other cause of sperm accumulation (Eisenbach, 1999). With recent advancement and development of assays combined with better understanding about mechanism of sperm chemotaxis it has been established that chemotaxis as a mechanism of sperm guidance occurs in mammals too. The first experiments in mammals showed that human sperm accumulated in the follicular fluid (Villanueva-Diaz *et al.,* 1990; Ralt *et al.,* 1991), and that there was a remarkable correlation between this *in-vitro* accumulation and egg fertilization (Ralt *et al.,* 1991). Subsequent experiments confirmed that this accumulation was indeed the consequences of chemotaxis (Ralt *et al.,* 1994). Sperm chemotaxis was later also demonstrated in frogs (Al-Anzi *et al.,* 1998), mice (Oliveira *et al.,* 1999; Giojalas *et al.,* 1998), and rabbits (Fabro *et al.,* 2002). So, sperm chemotaxis seems to be a widespread mechanism that guides spermatozoa to the egg, both when fertilization is internal or external.

2.2. NON-MAMMALIAN vs. MAMMALIAN SPERM CHEMOTAXIS:

Since the discovery of sperm attraction to female gametes over a century ago (Pfeffer, 1884) the process has been established in a large variety of species. Most of our knowledge on sperm chemotaxis originates from the study of marine invertebrates mainly sea urchin and star fish. In particular the genera *Arbacia, Strongylacentrotus* and *Asterias* have been used as a model system for the study of chemotaxis signaling.

In non-mammalian species where the fertilization takes place outside in an external aqueous environment that usually contains egg and spermatozoa from various species, the most important role of sperm chemotaxis is to guide large fraction of sperm population, towards the egg. Secondly it prevent cross species fertilization since the surrounding environment contains gametes from other species also. The chemoattractant released from one species is usually not recognized by the spermatozoa of other marine species (Miller, 1985; Cosson, 1990).The generalized view of sperm chemotaxis is shown in the figure 2.1 where sperm are immotile while they reside in the testis (for example in sea urchin). Upon spawning into seawater, flagellar beating commences and they begin to swim vigorously. Ionic changes that occur when sperm contact seawater are responsible for inducing the physiological changes required for the activation of motility. Within the gonad, high CO_2 tension keeps the intracellular pH (pHi) at, 7.2 (Johnson *et al.,* 1983). Below pH 7.3, the dynein ATPase that drives flagellar motility is inactive; with a pHi of 7.2, both respiration and motility are inhibited (Christen *et al.,* 1982, Lee *et al.,* 1983). When sperm are spawned into seawater, the CO_2 tension decreases, protons are released, and pHi increases to 7.5–7.6. The increase in pHi results in the activation of the dynein ATPase, which leads to the initiation of motility and an increase in ADP, the substrate for oxidative phosphorylation. In response to the rising concentration of ADP, mitochondrial

respiration is stimulated (Christen *et al.,* 1982). In addition to lowering the CO_2 tension, spawning may also cause a hyperpolarization of the sperm plasma membrane. Events leading to the hyperpolarization of sperm plasma membrane may also lead to the activation of adenylyl cyclase which increases the cAMP thereby activating cAMP-dependent protein kinase. Phosphorylation of proteins in the flagellar axoneme may be crucial for the initiation of motility (Garbers, 1989; Morisawa, 1994).When the sperm arrives in the vicinity of egg small peptides released from the egg jelly coat serves either to further activate motility and/ or to attract the sperm via chemotaxis.

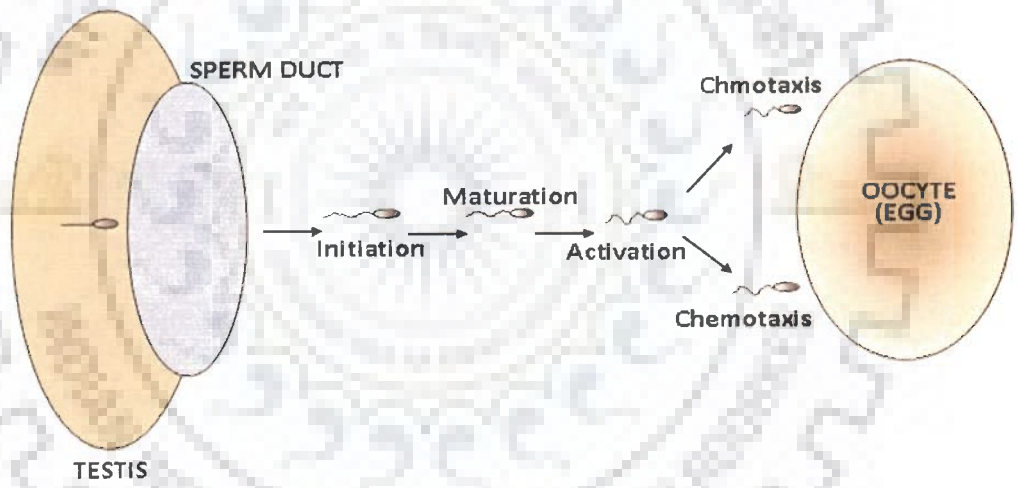


Figure 2.1: Generalized view of sperm chemotaxis in marine animals.

In contrast to the marine animals, in mammals, semen is placed inside the female genital tract of the female. Therefore, there is no real sperm competition, and if at all it exists, it is limited to semen from different individuals of the same species (Gomendio *et al.,* 1998). Species specificity in case of mammalian species was not detected in experiment conducted to show the

responsiveness of spermatozoa of different species to follicular fluid or egg conditioned media (Sun *et al.*, 2003). Two active mechanism of sperm guidance have been shown in mammals: i). chemotaxis, which is movement of sperm cell towards a concentration gradient of chemoattractant and ii). thermotaxis (Bahat *et al.*, 2003; Bahat and Eisenbach, 2006) the directed movement of the cells along a temperature gradient. Sperm from the caput epididymis of animals with internal fertilization show no motility just after spermiation. While passing through the epididymis, they undergo maturation and acquire motility by the time they reach the cauda epididymis. After ejaculation into the female reproductive tract, sperm undergo capacitation and attain hyperactivation especially with reference to motility. From now on, factors released from the oocyte and the surrounding cumulus cells cause the directional movement of sperm (Sun *et al.*, 2005).

2.3. MAMMALIAN SPERM GUIDANCE IN THE FEMALE GENITAL TRACT:

2.3.1. Site of Sperm Insemination and Distribution:

In case of most of the domestic species, sperm arrival in the female genital tract is associated with the timing of ovulation, either because ovulation is induced by coitus example cat, rabbit etc or because ovulation occurs shortly after estrus example cattle. The site of semen deposition (figure 2.2) defines the anatomical barriers that spermatozoa encounters during transit to the oviducts. These barriers restrict sperm passage, establish a gradient in sperm numbers along the tract, and may function as sperm reservoirs (Overstreet and Cooper, 1978b; Hunter, 1988). The establishment of a sperm gradient reduces the risk of polyspermy (Hunter, 1988). For species with uterine deposition of semen example dog, horse, pig, laboratory rodents , the uterotubal junction (UTJ) is the primary physical barrier to the oviducts, and a sharp gradient in sperm numbers occurs cranial to this barrier (Hunter, 1988). For species with vaginal

insemination (example ruminants, primates, rabbit, hare) the initial barrier is the cervix, and the UTJ serves to further restrict sperm access to the oviducts. In ruminants and primates, the cervical canal is filled with mucus, and the biophysical characteristics of this secretion are affected by the endocrine status of the female. Cervical mucus may block sperm passage during luteal phase and progesterone dominance. On the other hand, under estrogen dominance such as in the periovulatory period, the more hydrated mucus matrix forms channels through which spermatozoa can migrate (Harper, 1994).

Sperm distribution within the female tract occurs in phases, which are defined by their relationship in time to the event of insemination and the relative contribution of passive movement caused by visceral contractions of the female genital tract versus active sperm migration (Overstreet and Cooper, 1978a, b). The rapid transport phase is a pericoital event, characterized by the presence of sperm in the oviducts within minutes of mating or artificial insemination (Overstreet and Cooper, 1978a). This rate of transport is much faster than sperm swimming speeds; consequently, it is attributed to muscular contractility of the female tract and changes in intraluminal pressures (Drobnis and Overstreet, 1992; Harper, 1994). Rapidly transported spermatozoa do not contribute to the fertilizing population in the oviduct, because they are mostly moribund, dead or disrupted (Overstreet and Cooper, 1978a). The rapid transport phase is followed by a prolonged phase of sperm migration (Overstreet and Cooper, 1978b), during which the distribution of spermatozoa within the female tract continues and sperm reservoirs are established. It is during this phase that competent spermatozoa arrive in the oviducts. This journey takes 1–2 h in the pig (Hunter, 1988), 1.5–6 h in rabbits (Overstreet and Cooper, 1978b) and 6–8 h in sheep and cattle (Hunter, 1988).

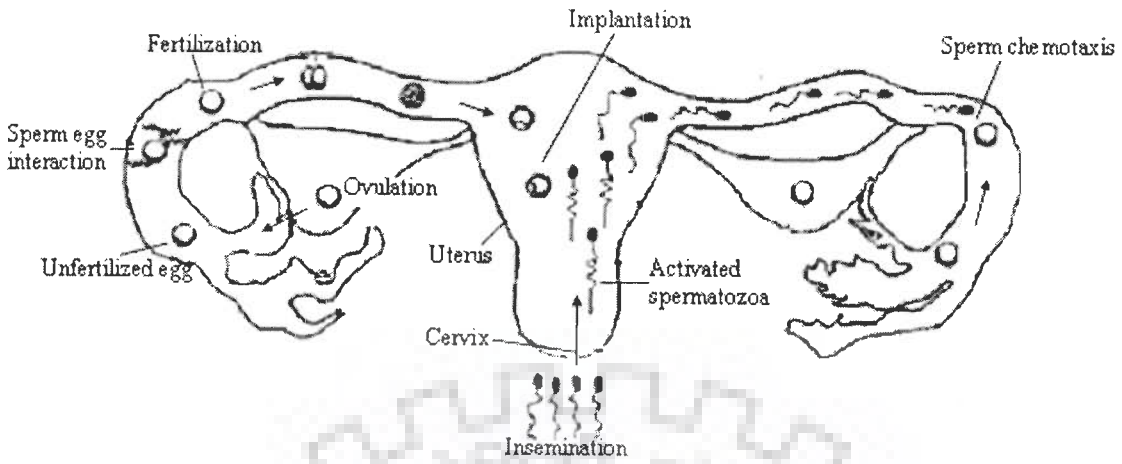


Figure 2.2: Mammalian female genital tract and possible guidance mechanism.

2.3.2. Sperm Motility and Transport:

Sperm motility is regarded as a manifestation of sperm functional competence which results in the active movement of flagellum resulting in the movement of the spermatozoa towards the egg. Within the female tract, sperm motility is modulated by the dynamic forces imposed on the flagellum by spatial constraints, epithelial surface characteristics, and the rheological characteristics of fluid secretions (Katz *et al.,* 1989). Motility is needed for spermatozoa to colonize and cross the cervix (Cooper *et al.,* 1979) and cervical mucus (Overstreet and Katz, 1990), and may also be requisite to traversing the UTJ. Spermatozoa that move progressively forward are more likely to penetrate the microstructure of cervical mucus (Katz *et al.,* 1989) or successfully cross the UTJ (Gaddum-Rosse, 1981). However, transport across the UTJ is significantly impaired for uterine spermatozoa that display the vigorous but non-progressive motility of hyperacti-vation, whether this motility is induced by *in vitro*

capacitation example hamster (Shalgi *et al.,* 1992) or preincubation of epididymal spermatozoa with calcium incase of mouse (Olds-Clarke and Wivell, 1992) prior to artificial insemination.

2.3.3. Potential Locations of Sperm Chemotaxis *in-vivo*:

The timing and site at which sperm chemotaxis takes place *in-vivo* is not known. A few potential possibilities seem reasonable: At least in mammals other than humans, a considerable fraction of the spermatozoa ejaculated into the female reproductive tract is retained with reduced motility in storage sites (usually the oviductal isthmus). Apparently, as the spermatozoa move up the oviductal isthmus, they encounter high mucus containing narrow lumen that impedes their forward progression. The spermatozoa frequently come in contact with the oviductal epithelium, where they can bind strongly to carbohydrate moieties on glycoprotein's or glycolipids on the surface of the oviductal epithelium, and consequently become stored there (Suarez, 1998). When ovulation occurs, some of the spermatozoa in the sperm reservoir resume high motility and travel the distance between the storage site and the fertilization site at the oviductal ampulla within minutes (Barratt and Cooke, 1991; Hunter, 1993; Overstreet and Drobnis, 1991). Only capacitated spermatozoa are detached from the epithelium and released from the storage site (Lefebvre and Suarez, 1996; Smith and Yanagimachi, 1991). One possibility is that chemotaxis is involved in directing the released capacitated spermatozoa toward the egg. This may be important in view of the relatively small number of spermatozoa released from the storage site (Hunter, 1993). However, because of the oviductal contractions a gradient of chemoattractant probably cannot be established over long distances in the oviduct; that is, the range of sperm chemotaxis in the oviduct may be relatively short. Therefore, if chemotaxis between the isthmic storage site and the ampulla occurs, it should be a multi-step

process, with each step sequentially directing the capacitated spermatozoa over a relatively short range, as was recently suggested for sperm chemotaxis in mice (Oliveira *et al.,* 1999).

In view of the observations suggesting that sperm chemotaxis in the oviduct may have a short range, it is possible that chemotaxis occurs only in close proximity to the cumulus-egg complex. The observation that the cumulus oophorus secretes a substance that alters the pattern of sperm movement (Bronson and Hamada, 1977) is consistent with this possibility. The cumulus oophorus, which surrounds the egg, provides a viscoelastic milieu that resists the stirring action of the oviduct (Cohen-Dayag *et al.,* 1994). Several findings are consistent with the possibility of sperm chemotaxis within the cumulus: (1) in mammals, the few first spermatozoa that enter the cumulus find the egg very effectively (Bedford and Kim, 1993), (2) only capacitated spermatozoa can penetrate the cumulus oophorus (Eisenbach, 1995; Jaiswal and Eisenbach, 1999), and (3) the initial distribution of spermatozoa at the site of fertilization usually tends to be approximately one spermatozoon per egg across most eggs in the cell mass of the cumulus (Hunter, 1993). The possibility of negative chemotaxis is also expected to occur within the cumulus. Determination of the cellular origin of the sperm chemoattractant(s) may distinguish between some of these possibilities.

2.3.4. Capacitation of Spermatozoa:

Testicular sperm in mammals are morphologically differentiated but are neither progressively motile nor able to fertilize egg. Although the ability to move forward is acquired during epididymal maturation, sperm are fertilization incompetent. Fertilization competence in spermatozoa is gained after a period of residence within the female tract. The physiological changes that occur to sperm during this stay in the female are collectively referred to as 'capacitation'. Mammalian sperm chemotaxis has some unique characteristics. First, the fraction

of chemotactic spermatozoa in a given sperm population is small (2-12% in humans) (Cohen-Dayag *et al.,* 1994) and approximately 10% in mice (Giojalas and Rovasio, 1998). Second, the chemotactic responsiveness of spermatozoon's is temporary and is acquired only once in the sperm's life time (Cohen-Dayag *et al.,* 1994). Third, there is a continuous replacement of chemo- tactically responsive spermatozoa within a sperm population due to changes taking place during capacitation. Some of the major changes taking place in sperm cell membrane during capacitation:

- Modification in surface glycoproteins caused by secretions of the female genital tract.
- Removed of cholesterol possibly leading to an increase fluidity of the sperm cell membrane
- Loss of glycoproteins which may expose the zona binding proteins
- Phosphorylation of proteins

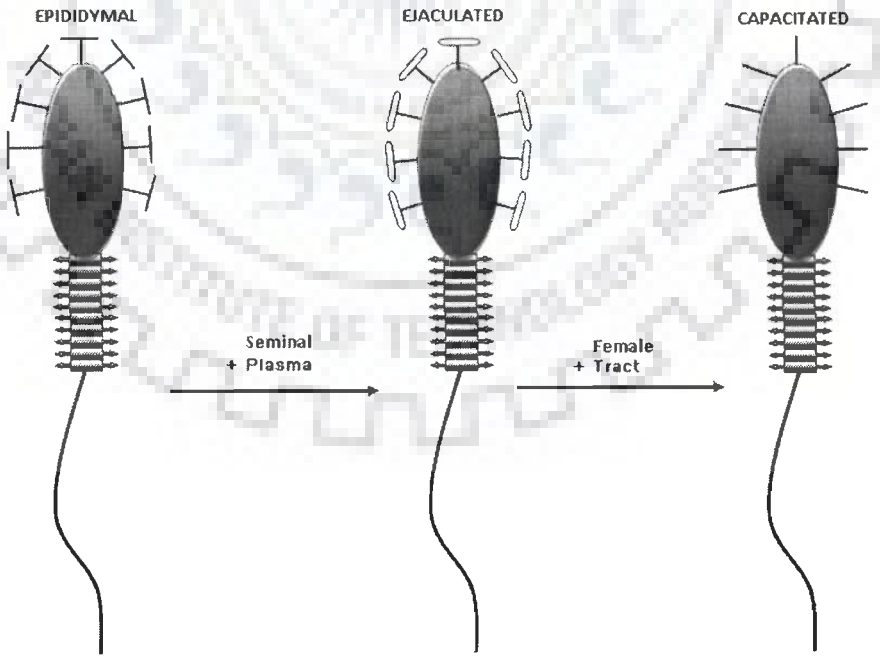


Figure 2.3: Changes taking place in sperm cell membrane during capacitation.

2.3.4.1. Effect of Capacitation on Spermatozoa:

The spermatozoa that has undergone capacitation reaction are endowed with number of abilities that are essential for fertilization (1) release of oviductal sperm from reservoir, (2) penetration of the cumulus layer that surrounds the egg, (3) binding to the sperm receptors on the egg coat, (4) Conversion of Pro-Acrosin (inactive) to acrosin (active) helping in penetration of egg coat, (5) Rapid beating of Flagellum; Result: Sperm are more motile, (6) Increase in the rate of metabolism (Jaiswal *et al.*, 1999).

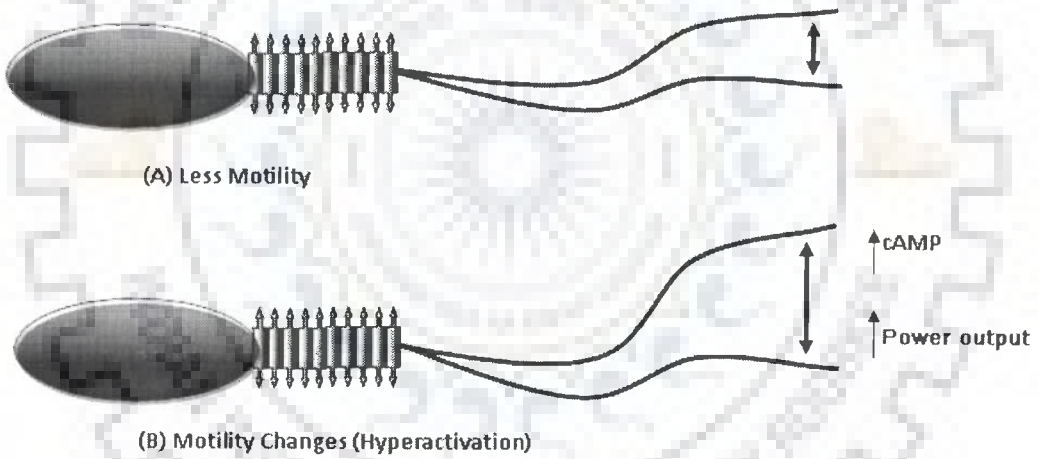


Figure 2.4: Effect of capacitation on spermatozoa.

2.3.4.2. Molecular Mechanism of Capacitation:

Mammalian sperms are morphologically differentiated after leaving the testis, but still have to acquire the ability to fertilize an egg. This occurs after completing in the female tract a

series of dynamic events called "capacitation" (Yanagimachi, 1994). This process primes sperm to respond to the zona pellucida (ZP), the extracellular egg coat that includes the acrosomal reaction (AR). The molecular mechanisms and the signal transduction pathways mediating capacitation are only partially known and appear to involve alterations in the levels of [Ca^{+2}] and other ions, plasma membrane lipid transfer, and remodeling, as well as protein phosporylation (Visconti *et al.*, 1999; Baldi *et al.*, 2000; Flesh and Gadella 2000). As in other cells, Na^+/Ca^{2+} and Ca^{2+}/H^+ exchangers, and Ca^{2+} ATPase regulate sperm [Ca^{2+}]i (Fraser, 1995). High amplitude and flagellar bends, known as, hyperactivation, accompany the [Ca^{2+}]i increases (Suarez, 1996). An increase in [cAMP]i is associated with sperm maturation through the epididymis and capacitation (White and Aitken, 1989; Visconti and Tezon, 1989; Visconti *et al.*, 1995).

In most somatic cells, extracellular signal modulates a transmembrane G-protein-regulated adenylyl cyclase (AC) that synthesizes cAMP. However, in sperm this may be carried out by a different form of AC that was recently cloned (Sinclair, *et al.*, 2000). This AC is directly regulated by bicarbonate but not by G-proteins or pHi (Okamura *et al.*, 1985; Chen *et al.*, 2000). A high level of RNA message for the soluble form of sAC is present in mature sperm. These finding suggest that this AC may play an important role in sperm maturation through the epididymis, capacitation, hypermotility, and /or the AR (Sinclair *et al.*, 2000).

During capacitation, the sperm membrane hyperpolarizes, at least in part due to an enhanced K^+ permeability (Zeng *et al.*, 1995; Arnoult *et al.*, 1999). It is unclear which of the biochemical modifications that occur during sperm capacitation affect K^+ channels. However, a pHi dependent inwardly rectifying K^+ channels has been identified very recently in spermatogenic cells (Munoz-Garay *et al.*, 2001). Before capacitation, sperm pHi is relatively

acidic and may impose a functionally quiescent state. An acidic pHi may negatively regulate sperm inwardly rectifying channels, thereby maintaining membrane potential depolarized and indirectly preventing unregulated Ca^{2+} entry, and thus AR (Vredenburgh-Wilberg and Parrish, 1995; Zeng *et al.*, 1996). The pHi changes that occur during capacitation could activate these channels, permitting K^+ efflux and consequently hyperpolarizing sperm. Membrane potential in sperm can become as negative as –80 mV, a value sufficient to relive steady state, voltage-dependent inactivation of voltage-gated Ca^{2+} T-channels (Arnoult *et al.*, 1996a; Santi *et al.*, 1996). Ca^{2+} influx through these channels, activated by ZP via an unknown mechanism, may drive AR. Though several types of voltage-gated Ca^{2+} are present in sperm, their role in capacitation is still unknown.

2.4. MOLECULAR MECHANISM OF SPERM CHEMOTAXIS:

In most animal species, the prospects of the spermatozoa coming in contact with eggs are very slim in the absence of some guidance mechanism, usually chemical in nature. Such chemical guidance, sperm chemotaxis, is now recognized in many marine invertebrates, fish, amphibians, and a few mammals (humans included) (Eisenbach, 2004). This suggests that sperm chemotaxis is a general guidance mechanism, irrespective of whether the fertilization is external, like in most marine species, or whether it is internal, as in mammals. In spite of this generality, there are some basic differences between sperm chemotaxis of mammals and that of marine invertebrates, the most pronounced one being the fractional chemotactic response in the former; namely, the restriction of the chemotactic response to a subpopulation of the spermatozoa. Unlike spermatozoa of marine species, spermatozoa of mammals must undergo a process of maturation, termed capacitation, for acquiring the ability to bind to the egg and penetrate it (Jaiswal and Eisenbach, 2002). At any given time, the percentage of capacitated

spermatozoa is small due to both the limited time for which spermatozoa remain in this stage and the continuous replacement of capacitated cells in the sperm population (Cohen-Dayag *et al.,* 1995). Since the role of sperm chemotaxis is to bring to the egg spermatozoa that are capable of fertilizing it, therefore, unlike in marine invertebrates where most, if not all, the spermatozoa appear to be chemotactically responsive, in mammals only the small fraction of capacitated spermatozoa are chemotactic (Cohen-Dayag *et al.,* 1994, 1995; Fabro *et al.,* 2002). Because of the massive chemotactic response in marine invertebrates and its much earlier discovery, most of our, limited, knowledge about the molecular mechanism of sperm chemotaxis is from these species.

2.4.1. Molecular Mechanism of Sperm Chemotaxis in Non-Mammals:

The events of sperm chemotaxis are of interest for a number of reasons. As this marks the first interaction of sperm with egg derived elements. Sperm chemotaxis signal transduction mechanism has been mostly studied in marine invertebrates therefore models, based primarily on studies with *S. purpuratus*, and *A. punctulata* were proposed for chemotactic signaling in sea urchin spermatozoa. *A. punctulata* egg release resact (CVTGAPGCVGGGRL), a peptide that stimulates the sperm respiration and motility and also functions as a chemoattractant. As sperm enters the resact gradient, which extends at least 1mm around the egg, they intermittently shift to highly asymmetric flagellar waveforms and, as a result swim in small diameter circles. This results in orientation of sperm motility towards the source of the resact gradient and hence swimming towards the egg (Garbers, 1989; Cook *et al.,* 1994). Resact belongs to a large class of sperm activating peptides (SAPs) that are released by a range of marine invertebrates. Another peptide speract (GFDLNGGGVG) is a related SAP that is released by egg of another sea urchin *Strongylocentrotus purpuratus* (Garbers, 1989). These peptides have similar effect on

intracellular signal transduction and in addition speract is chemoattractant in experimental conditions. Therefore, it was suggested that resact, speract and other SAPs functions through a common signal transduction pathway to stimulate and orient the sperm motility (Garbers, 1989; Darszon *et al.,* 2001; Cook *et al.,* 1994).

Cook *et al.* (1994) first proposed a model of SAP signal transduction in sea urchin subsequent it was refined (figure 2.5). It was based on experimental studies in which sperm plasma membranes were removed with detergent and motility was reactivated by addition of magnesium-ATP. Such experimental data showed that low levels of calcium generated symmetric flagellar waves and linear motility, where as higher levels of calcium generated asymmetric flagellar wave forms and turning behavior. Theses observations were assembled into a model in which the fine control of intracellular calcium ($Ca^{2+}{}_i$) accounts for switching between linear and curvilinear motility. The model shows the first step in chemoactivation and chemotaxis as binding of SAP to a cell surface ligand specific receptor, resulting in receptor activation. The resact receptor is a receptor guanylyl cyclase that is localized along the length of sperm flagellum (Garbers, 1989; Ward *et al.,* 1985) where as other receptors initiate the action of other SAPs. Figure 2.5 shows the generalized view of the chemotactic responses of sperm to resact. In this model, receptor stimulation results in the generation of cGMP, the opening of a potassium channel through direct or indirect action of cGMP and subsequent membrane potential hyperpolarization through potassium efflux. Membrane hyperpolarization then drives intracellular calcium below resting levels, probably through the activation of either a potassium-dependent or independent sodium-calcium exchanger (Su and Vacquier, 2002). These exchange molecules use the prevailing inward sodium gradient and the prevailing electrical gradient across the membrane (inwardly negative) to drive the efflux of calcium against its concentration

gradient. Calcium efflux by these exchangers is enhanced by hyper-polarization. The decreased levels of intracellular calcium produce symmetric flagellar waveforms and linear motility. The earlier model also proposes later events, in which sodium-calcium exchanger activation produces sodium entry, resulting in an eventual depolarization of membrane potential and the activation of a potential sensitive adenylyl cyclase. The resulting rise in cAMP results in elevated levels of intracellular calcium. On the basis of studies in demembranated sperm, it was inferred that high intracellular calcium initiates highly asymmetric flagellar wave and turning behavior. It is this change in turning behavior that underlies the re-orientation of sperm within a chemo-attractant field. Thus, the flagellum may be thought of as a sensor of intracellular calcium levels, shifting from linear motility to curvilinear motility in response to SAP-dependent alterations in intracellular calcium. This model accounts for the known responses of sperm when encountering peptide concentrations ranging from 0.001–1000 nM and, in most cases, for responses beginning after several seconds of peptide stimulation (Darszon *et al.*, 2001; Cook *et al.*, 1994). Those initial responses were studied by Kaupp *et al.*, (2003) who found that resact triggered a rapid elevation of cGMP levels (peak approximately 200 ms) followed by delayed rise in cAMP levels. However they were unable to detect the early decline in intracellular calcium that was predicted by the earlier models. Instead resact induced a biphasic increase in intracellular calcium. This early phase of the intracellular calcium response is very sensitive to resact: it showed a threshold response at 0.125 pM (a 10-20 fold lower concentration than previously reported resact response) and peak at 25 pM. By analysing the concentration- dependence of the delay times between resact uncaging and the early phase of intracellular calcium signaling, Kaupp *et al.* (2003) conclude that the early response represents the binding of single resact molecules to sperm, that binding is essentially irreversible and that

the early intracellular calcium response saturates when 50–100 molecules are bound per cell. This represents <1% receptor saturation (Bentley *et al.,* 1986). The early elevation of intracellular calcium levels can be fitted by proposed models in which each resact molecule is able to activate calcium channels within a 5-μm segment of the flagellum. At somewhat higher concentrations, resact also initiates an initial motor response marked by episodes of a highly asymmetric flagellar waveform and, consequently, by swimming in tight circles. Circular motility then serves as the basis for re-orientation during chemotaxis. It is presumed that the intracellular calcium responses are transduced into a motor response, based on the clue provided by the observation that caged-resact and caged -cGMP both trigger the same motor response. On the basis of a variety of experimental results, the authors suggest that the primary motor response is controlled by cGMP- evoked intracellular calcium transients and probably reflect conductance through a cGMP- gated calcium channel. This would then represent a new, early element of the sperm chemotaxis model (figure 2.5). Presumably, sperm register these early responses immediately after entering the resact gradient and are thereby informed at once of the proximity of an egg. However, primary motor responses are only observed at somewhat higher resact concentration than the early intracellular calcium transients. This early phase of signaling saturates at approximately 25 pM resact, although sperm respond with metabolic and motility changes to doses as high as 1 micromolar. It is therefore possible that the chemotactic signaling system adapts as sperm moves into the resact gradient, permitting them to continue orienting towards the source of the chemical gradient even after saturation of the primary response.

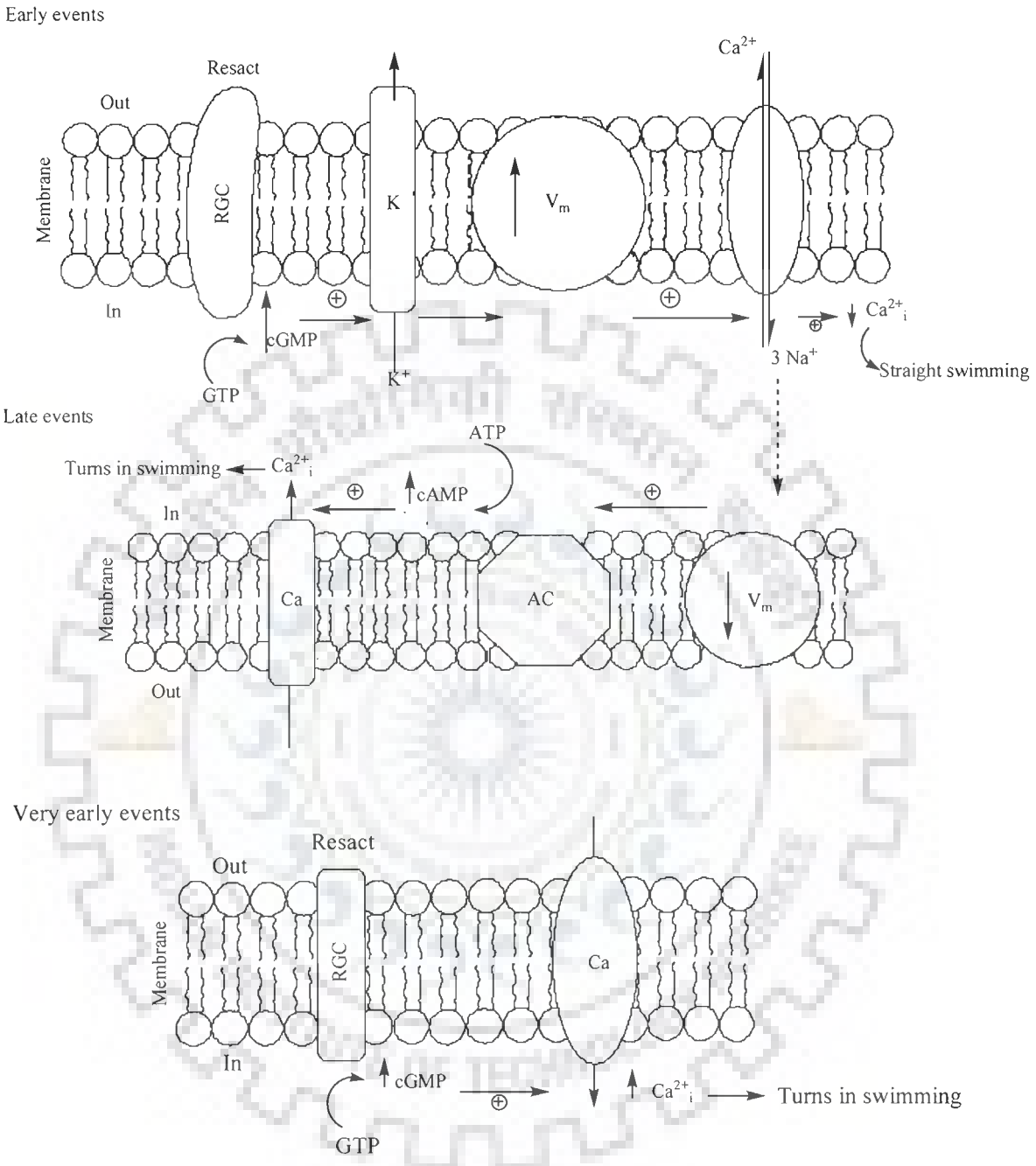


Figure 2.5. Generalized model of the chemotactic responses of sperm to resact. Early events of resact signaling include resact binding to a receptor guanylyl cyclase (RGC) on the flagellum (step 1), elevation of cGMP (step 2), activation of cGMP-dependent potassium channels (step 3),membrane potential (*V*m) hyper-polarization (step 4), activation of a membrane-potential-sensitive sodium calcium exchanger (step 5) and a decrease in intracellular calcium levels that

initiates straight swimming (step 6). Late events driven by sodium-calcium exchanger activation include sodium entry and membrane potential depolarization (step 7), activation of a membrane potential-adenylyl cyclase (AC; step 8), elevated cAMP (step 9), activation of a calcium entry channel by cAMP (step 10) and increased intracellular calcium levels that initiate turning behavior (step 11). This may describe many aspects of the adapted phase of resact signal transduction. c, A new model for the early events of signaling. Resact activates a receptor guanylyl cyclase, resulting in a rise in cGMP and inducing, either directly or indirectly, the opening of calcium (Ca) channel. Increased intracellular calcium levels then initiates turning behavior. These events occur at lower resact concentrations and at earlier times than described in the earlier model

2.4.2. Molecular Mechanism of Sperm Chemotaxis in Mammals:

The knowledge regarding molecular mechanism of sperm chemotaxis involved in case of mammals is very limited. It includes information regarding the identity of some receptors involved in the process of sperm chemotaxis and chemoattractant induced Ca^{2+}_{in} changes. The finding of G-protein coupled olfactory receptors in mammalian sperm (Walensky *et al.*, 1995; Asai *et al.*, 1996), and their localization to the midpiece of mature spermatozoa (Walensky *et al.*, 1995; Spher *et al.*, 2004), increased the likelihood that some of these proteins might be chemotaxis receptors (Parmentier *et al.*, 1992; Vanderhaeghen *et al.*, 1997). Recent studies have shown that two distinct olfactory receptors –OR17-4 (also known as OR1D2) present on the flagella of human spermatozoa (Spher *et al.*, 2003) and OR23 in mouse round spermatids (exact position not yet known) – were identified and their respective ligands, bourgeonal and lyral, were found to be sperm chemoattractants. The stimulation of human spermatozoa with bourgeonal results in the transient increase of Ca^{2+}_{in} (Spher *et al.*, 2003), originating at the flagellar midpiece and propagating to the sperm head (Spher *et al.*, 2004). This study was further substantiated by the experiments showing inhibition of chemotactic response by specific

antagonist of membrane associated adenylyl cyclase (Isoform of membrane associated adenylyl cyclase (mAC) present on the sperm flagellum). It is therefore possible that that the chemotactic stimulation of OR17-4 triggers a signaling pathway similar to that of olfactory system (Spher *et al.,* 2004). Many of the olfactory receptors are expressed in the testis also therefore it is quiet possible that many of them are present in the sperm increasing the possibility of multiplicity of chemoattractant.

Progesterone is known to act as sperm chemoattractant at a very low concentration in case humans and rabbit (Teves *et al.,* 2006). Two progesterone receptors have been identified on the cell surface of mammalian spermatozoa (Luconi *et al.,* 1998; Blackmore *et al.,* 1991; Meizel and Turner, 1991) and it is possible that at least one of them is a chemotaxis receptor. One of the progesterone receptors is located at the head of the sperm cell but it is not clear whether this one is the chemotaxis receptor or progesterone chemotaxis receptor is a different protein located on the flagellum, similar to OR17-4. The signaling pathway that takes place during chemotaxis to progesterone has not been studied in detail. However, Harper *et al.* (2004) have shown that very low concentrations of progesterone (10^{-8} M) stimulates Ca^{2+} oscillations at the junction of between the caudal head and the mid-piece of the spermatozoon. These Ca^{2+} oscillations are reminiscent of the faster oscillatory spikes observed in sea urchin *A. punctulata* on stimulation with the chemoattractant resact (Bohmer *et al.,* 2005) and are synchronous with the flagellar activity, indicating that they modulate the flagellar beat (Harper *et al.,* 2004). However, since progesterone stimulates a number of other sperm functions, such as hyperactivation, capacitation and acrosome reaction, it remains to be seen whether some of these intracellular events are also part of the progesterone induced chemotactic signaling pathway.

2.5. CHEMOATTRACTANT AND THEIR CELLULAR ORIGIN:

2.5.1. Chemoattractant of Non-Mammalian Origin:

In non-mammalian species the encounter between the gametes of marine species, in which fertilization is external and which occurs in a turbulent aqueous environment that contains both egg and sperm cells from various species is to recruit as many spermatozoa as possible to the egg but no sperm selection takes place. Most of the sperm chemoattractant that have been identified are peptide or proteins of low molecular weight (1-20 kDa), which are heat stable and sensitive to proteases (Miller, 1985; Cosson, 1990). But in exception to these there are sperm chemoattractant of corals, which are lipid like substances of 140-250 Da (Coll and Miller, 1992), and the attractants of ascidians *Ciona*, which are non-proteinaceous small molecules (Yoshida *et al.*, 1993). Examples of some of the chemoattractants and repellents of non-mammalian origin are given in Table 2.1.

SUBSTANCE	SPECIES	REFERENCES
A lipid like long fatty acid alcohol CH_3-$(CH_2)_8$-CH=CH-CH=CH-CH_2OH	Corals	Coll and Miller,1992
Resact: a 14 –mer peptide with the sequence CVTGAPGCVGGGRL-NH_2	Sea urchin	Ward *et.al.*,1985
SAAF	Ascidians	Yoshida *et.al.*,1994
Low molecular weight unstable pheromones of cyclic or linear structure	Algae	Maier and Muller, 1986; Cosson,1990
Dicaroxylic acids	Ferns	Brokaw, 1985
Startrak: a 13 kDa heat stable protein	Starfish	Miller and Vogt, 1996

Table 2.1. Sperm chemoattractants of non-mammalian species.

2.5.2. Chemoattractant of Mammalian Origin:

One of the first physiological sources to be investigated for chemoattractant activity was the mammalian follicular fluid (Eisenbach, 1999; 2004). Follicular fluid contains secretions of egg and its surrounding cells. Follicular fluid *per se* may have no physiological role after ovulation because in mammals only small quantities of this fluid are transported into the oviduct (Hansen *et al.,* 1991). Although large majority of scientists working on human sperm chemotaxis conclude that chemotaxis to follicular fluid occurs (table 2.2), but some others obtained different results. Sperm chemotaxis to follicular fluid was observed consistently in both in both humans (Cohen-Dayag *et al.,* 1994; Ralt *et al.,* 1994) and mice (Giojalas and Rovasio, 1998). An active fraction of follicular fluid that contains the chemoattractants, probably a heat-stable peptide (Manor, 1994) has been identified (Ralt *et al.,* 1994). However, it is unlikely that chemotaxis to follicular fluid also occurs *in-vivo* (Sun *et al.,* 2005) because the chemoattractant gradient in the oviduct is anticipated to be maintained for long as the egg survives in the female genital tract (approximately 24 hours post-ovulation in humans) (Harper, 1982). This would require a continuous supply of chemoattractant, where as the follicular fluid is only released as a single event at ovulation. Therefore, the physiological significance of the results obtained with follicular fluid is restricted to the implication that the egg and /or its surrounding cells secrets a chemoattractant within a follicle prior to ovulation. Recent studies have shown that the media conditioned either with individual, mature human egg, or with surrounding cumulus cells are chemotactically active (Sun *et al.,* 2005). This indicates that sperm chemoattractant are secreted, not only prior to ovulation within the follicle, but also after egg maturation outside the follicle, and there are two chemoattractant sources: the mature egg and the surrounding cumulus cells.

Villanueava-Diaz *et al.,* (1995) demonstrated in a choice assay that progesterone causes human sperm accumulation, that preincubation of spermatozoa with a progesterone receptor antagonist eliminates the accumulation, that dialysis of follicular fluid causes loss of this activity, that a lipid extract of follicular fluid causes sperm accumulation as does crude follicular fluid, and that heat or trypsin treatment does not affect the accumulation in follicular fluid. On the basis of these observations, they suggested that progesterone is the attractant in follicular fluid. However, this suggestion appeared to be in conflict with earlier results, demonstrating lack of correlation between sperm accumulation in follicular fluid and the level of progesterone in the fluid (Ralt *et al.,* 1991), as well as lack of correlation between the characteristics of the active fractions of follicular fluid and those of progesterone (Manor, 1994). This conflict was resolved by Jaiswal *et al.,* (1999) who demonstrated that progesterone indeed causes sperm accumulation, but this accumulation is caused primarily by physiologic trapping, not by chemotaxis. Chemotaxis was eliminated by track analysis and the finding that most of the spermatozoa present near the progesterone-containing well apparently reached there by coincidence, not by changing their swimming path toward the well. Physiologic trapping was apparently caused by acquiring motility patterns resembling hyperactivation (e.g. wide amplitude and marked lateral displacement of the head) (Yanagimachi, 1970; Burkman *et al.,* 1990). Progesterone is well known to cause sperm hyperactivation (Yanagimachi, 1970; Oehninger *et al.,* 1994). Accordingly, Jaiswal *et al.* (1999) found that, upon approaching a progesterone-containing well, about half of the spermatozoa acquired hyperactivation-like motility. Because one of the characteristics of hyperactivation is very small progressive motility despite the vigorous motion (Robertson *et al.,* 1998), the hyperactivated spermatozoa remained in the vicinity of the well. In this manner, some of the spermatozoa that happened to reach the

vicinity of the progesterone containing well by coincidences were essentially trapped there. Further experiments demonstrated that removal of progesterone from follicular fluid does not eliminate the chemotactic activity of the fluid but does eliminate its hyperactivation-causing activity (Jaiswal *et al.,* 1999). This provided additional evidence that progesterone is at least not the major attractant in follicular fluid.

Atrial natriuetic peptide (ANP) is a polypeptide hormone that is secreted from the atrial portion of the heart and from other mammalian cell types, and that activates particulate guanylyl cyclase (Brenner *et al.,* 1990; Ruskoaho, 1992). ANP is present in follicular fluid (Sundfjord *et al.,* 1989) and ANP specific receptors have been identified on human spermatozoa (Silvestroni *et al.,* 1992). Sperm chemotaxis to ANP was demonstrated but only in the presence of a neutral endopeptidase inhibitor, such as phosphoramidon, to avoid inactivation of ANP by the residual neutral endopeptidase containing seminal fluid (Zamir *et al.,* 1993; Anderson *et al.,* 1995). Therefore, it is not clear whether ANP is involved in sperm chemotaxis *in-vivo*.

Small synthetic peptides such as N-formyl-Met-Leu-Phe (fMLP) are attractants for neutrophils and macrophages (Schiffmann *et al.,* 1975). Such peptides bind to specific sites on human spermatozoa (Gnessi *et al.,* 1986; Ballesteros *et al.,* 1988) and cause accumulation of both human (Gnessi *et al.,* 1985) and bull (Iqbal *et al.,* 1980) spermatozoa (Makler *et al.,* 1992). At least in the case of bull spermatozoa, track analysis has ruled out chemotaxis as the cause of accumulation, since the accumulation was demonstrated to result from sperm adhesion to the glass surface inside the peptide-containing capillaries (Miller, 1982). These complications demonstrate the importance of carrying out assays that distinguish chemotaxis from other processes resulting in accumulation.

SUBSTANCE	SOURCE	SPECIES	REFERENCE
ANP	FF	Human	Zamir *et al.*, 1993; Anderson *et al.*, 1995
Bourgeonal	Unknown	Human	Spehr *et al.*, 2003
Lyral	Unknown	Mouse	Fukuda *et al.*, 2004
Peptides (<1.3 and 13kDa)	FF	Human	Manor, 1994
Progesterone	CO, FF, OF	Human, Rabbit	Teves *et al.*, 2006
RANTES	FF	Humans	Isobe *et al.*, 2002
*Antithrombin III	FF	Pig	Lee *et al.*, 1994
*Heparin	FF	Human, Mouse	Sliwa, 1993
*Peptide (8.6 kDa)	FF	Pig	Serrano *et al.*, 2001
*Substance P	FF	Mouse	Sliwa, 2001
*Hyaluronic Acid	CO	Human	Sliwa,1999
*Oxytocin	FF	Mouse	Sliwa, 1994
*Beta-Endorphin	FF	Mouse	Sliwa, 2001
*Calcitonin	FF	Mouse	Sliwa, 1995
*Acetylcholine	FF	Mouse	Sliwa, 1995

Table 2.2. Confirmed and putative chemoattractant for mammalian spermatozoa.

(* substance that have been reported to cause sperm accumulation but have not been confirmed as chemoattractant)

Sperm response to several other substances has been tested (Table 2.2). Tso *et al.* (1979) demonstrated by accumulation assays with ascending and descending chemical gradients that p-nitro-phenyl-glycerol is a repellent for rat spermatozoa, in addition to being an inhibitor of sperm motility. (Makler *et al.*, 1995) studied hydrochloric acid solution, sodium hydroxide, ethanol, and glutaraldehyde as potential repellents for human spermatozoa, but found no evidence for sperm repulsion.

2.6. ASSAYS FOR SPERM CHEMOTAXIS:

In general, assays that are used to measure sperm chemotaxis (*in-vitro* assays) are not foolproof since they may or may not have the ability to distinguish between chemotaxis and other processes that may cause sperm accumulation (example: chemokinesis or trapping of any kind). However, in a recent publication the authors claim to have developed a microfluidic device with a flow through design that overcomes difficulties associated with distinguishing chemotaxis from trapping (Koyama *et al.*, 2006). Assays that have been used to measure chemotaxis *in vitro* can be divided into four categories (Eisenbach, 1999). Figure 2.6 shows the various assay designs that are in use to study chemotaxis.

2.6.1. Sperm Accumulation- Ascending Gradient:

This technique is a macroscopic assay wherein the spermatozoa senses an ascending gradient of the chemo attractant and accumulates near or at its source. The chemo attractant gradient is established by diffusion. A number of variations of this type of assay, which uses different apparatuses, have been published (Eisenbach, 1999; Eisenbach and Tur- Kaspa, 1999). The main disadvantage with his kind of assay is that they cannot distinguish between chemotaxis and other causes of sperm movement and accumulation (table 2.3).

2.6.2. Sperm Accumulation-Descending Gradient:

This is an inverted capillary assay. In this technique the spermatozoa is suspended in a solution that already contains the presumed attractant, and the capillary contains either a control buffer or the attractant (Ralt *et al.*, 1994). When the capillary contains buffer (control), the spermatozoa senses a descending gradient of the attractant as they move from the well to the capillary. When capillary and the well (solution containing spermatozoa and attractant) both contains attractant, they sense no gradient at all. Thus, this assay measures the sperm tendency to leave the

attractant rather than to accumulate in it. By counting the spermatozoa accumulated in the capillary, it is possible to distinguish between chemotaxis, chemokinesis and trapping (Ralt *et al.,* 1994), as only chemotaxis is gradient dependent.

2.6.3. Choice Assay:

The spermatozoa in this technique choose between two wells one containing the attractant and the other containing buffer as control. A number of designs of this sort have been published: a sealed chamber for microscopic measurement and for macroscopic measurement apparatus with two or more wells or chambers, connected by a tube or a groove (Eisenbach, 1999). Such assay can distinguish between chemotaxis and chemokinesis, but cannot distinguish between chemotaxis and trapping.

ASSAYS FOR DETERMINING SPERM CHEMOTAXIS		
Assay	**Distinction Between**	
	Chemotaxis and Chemokinesis	**Chemotaxis and Trapping**
Sperm accumulation Ascending gradient	-	-
Sperm accumulation Descending gradient	+	+
Choice assay	+	-
Track analysis of swimming spermatozoa	+	-

Table 2.3. Assays used for determining human sperm chemotaxis.

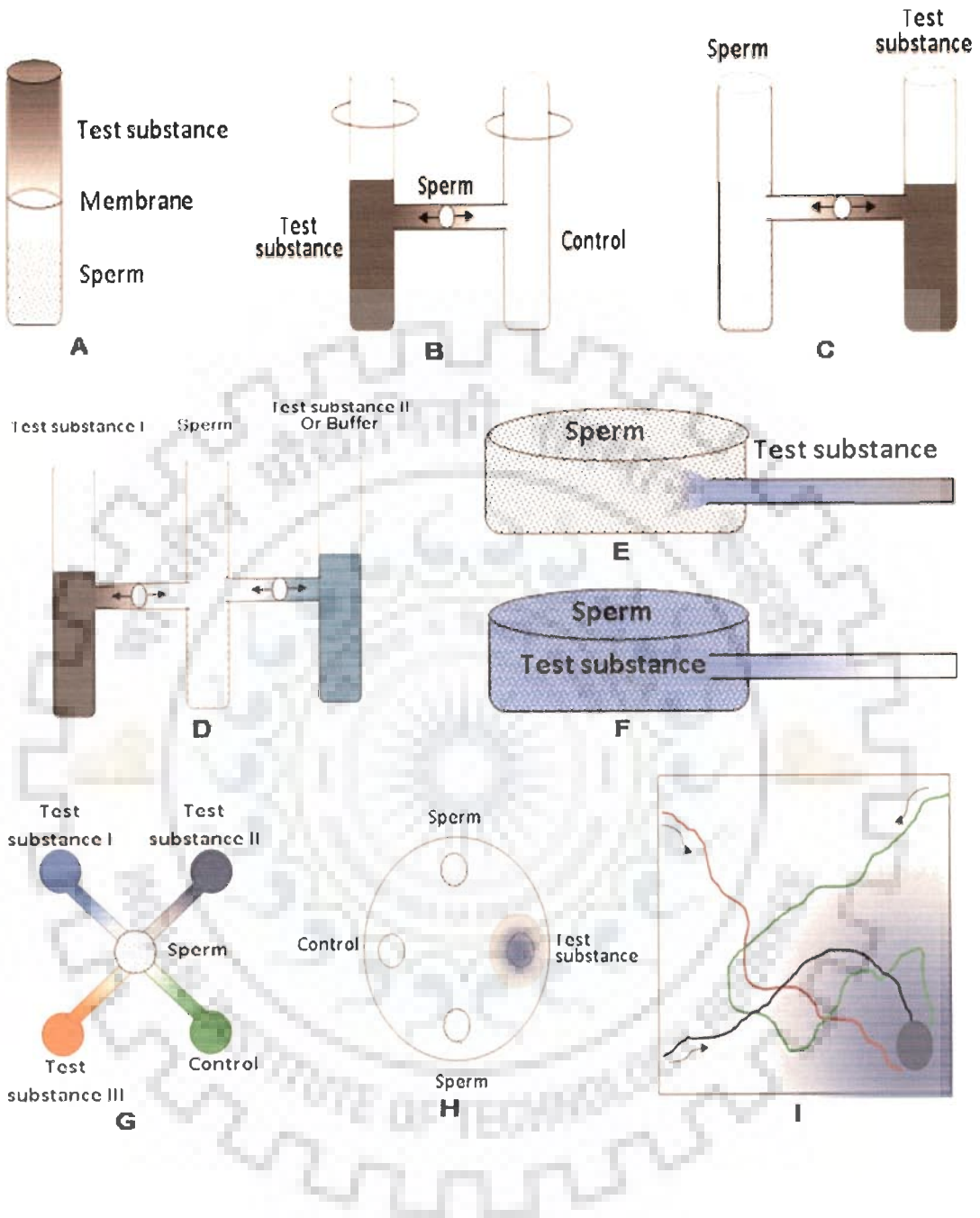


Figure 2.6: Various assays and designs used to study sperm accumulation and chemotaxis. A). Accumulation assay apparatus consisting of two wells separated by thin polycarbonate membrane (Gnessi *et al.*, 1985). B). Accumulation assay in an apparatus consisting of two wells connected via tube (Cohen-Dayag *et al.*, 1994). C). Choice assay in an apparatus consisting of two wells (Villanueva-Diaz *et al.*, 1992). D). Choice assay in an apparatus consisting of three wells. E). Sperm accumulation in a capillary assay (Ralt *et al.*, 1994). F). Inverted capillary

assay (Ralt *et al.,* 1994). G). Top view of an apparatus consisting of five wells or a choice assay (Villanueva-Diaz *et al.,* 1990; Sliwa, 1993a). H). Top view of a microscopic choice assay in sealed chamber (Makler *et al.,* 1992).

2.6.4. Track Analysis of Swimming Spermatozoa:

This is an essential assay, to distinguish between chemotaxis and other processes that may cause sperm accumulation, as tracks made by spermatozoa in gradient of attractant can be studied. This tracking is done by video-recorded tracks either manually or by a computerized motion analysis system (Ralt *et al.,* 1994). More recently, non-invasive microfluid-compatible automated motile cell capture and analysis with optical traps have been attempted (Shao *et al.,* 2007). Software such as Single sperm tracking algorithm (SSTA) and real-time automated tracking and trapping systems (RATTS) have also been developed to monitor the swimming trajectories of sperm. They are useful for high throughput sperm sorting based on motility and chemotaxis.

2.7. CONCLUSION AND FUTURE PERSPECTIVES:

In recent years, the concept of mammalian sperm guidance has received a balanced outlook, unlike the dogma that existed for a long time; it has become clear now that mammalian spermatozoa have the ability to be actively guided to the egg. Two of the mechanisms by which this guidance is achieved have been resolved: chemotaxis and thermotaxis. Yet, many important questions continue to be unanswered. For example all the physiological chemoattractants especially those present in the follicular fluid of mammals and the physiologically relevant sources of chemoattractant *in-vivo* remain to be identified. In mammals, several other aspects like specificity of sperm chemoattractants and the behavioral response mechanism of sperm together with the signal transduction pathways involved in sperm chemotaxis deserve special

attention. Whether these pathways function in concert or individually under different conditions needs to be studied in detail. Furthermore, all the experiments conducted till now to elucidate the function and location of sperm chemotaxis have been reached on the basis of *in-vitro* experiments only and they are yet to be confirmed *in-vivo*.

Further advancement in research and better techniques employed in studying sperm chemotaxis will not only increase our understanding of mammalian fertilization process but may also allow clinical application for obtaining enriched capacitated spermatozoa *in-vitro*. They can also be exploited as a diagnostic tool to assess sperm quality and in the long run can also be used in contraception by interfering with the normal process of fertilization.

Chapter-3

CHAPTER 3: DESIGN FABRICATION AND VALIDATION OF ASSAY SYSTEM FOR SPERM CHEMOTAXIS

3.1 INTRODUCTION:

It is generally accepted that even in the case of mammals where fertilization is internal, sperm are directed to the egg within the female reproductive tract. Induced organized contractions in the female reproductive tract, thermokinesis and chemotaxis are foremost among several factors that have been proposed to be guiding forces that enable sperm to meet with the egg *in vivo*. Chemotaxis is the oriented movement of the sperm in response to a chemical gradient. Although hormones and follicular fluid have been implicated in sperm chemotaxis, the actual active principles involved have not yet been identified. In order to identify the active principles involved *in vivo* it is imperative to have a mechanism to test the phenomenon *in vitro*. Several authors have described techniques to assess the chemotactic behaviour of cells to test substances *in-vitro*. These procedures have varying degree of sophistication and reliability. In general, most assay systems are designed to cater to the needs of individual laboratories and have their own merits and demerits. However, the common drawback of the assay systems used to monitor sperm chemotaxis is that they are not able to distinguish between the different type of migration of sperm cells (i.e. chemokinesis, chemotaxis, trapping).The most commonly used techniques for studying sperm chemotaxis in mammals is the accumulation assay in which spermatozoa sense an ascending gradient of the attractant and accumulate near or at its source. The basic principle being diffusion of attractant from one chamber to another and migration of cells towards the source of the attractant on sensing increasing gradient of attractant. A number of designs have been reported making use of above stated principle (Zamir *et al.*, 1993; Anderson *et al.*, 1995; Sliwa, 1995; Ralt *et al.*, 1991, 1994).

For our laboratory there was need to design and develop a system that was simple, robust and cost effective and could be used for high throughput operations to screen several chemical/ biomolecules for their chemotactic potential. Therefore, an attempt was made to self-fabricate an apparatus that could suit our purpose. In the present study goat sperm was used to test the chemotaxis apparatus that was designed and fabricated in the laboratory. Using this apparatus preliminary investigations were carried out to monitor sperm migration *in-vitro*. The assay system was designed so that it can distinguish between other mechanisms of accumulation such as chemokinesis and trapping. In this chapter, all the steps taken to systematically design and fabricate a chemotaxis apparatus are described. Data is also presented that validates the assay in comparison with a commercially available apparatus.

3.2 MATERIALS AND METHODS:

3.2.1 Chemicals:

All the chemicals used for conducting the experiments were obtained from SRL, Qualigen, Sigma-Aldrich, Biochemika Fluka and Hi-media. Trans-well plates were procured from Corning Costar (USA).

3.2.2 Tissue Collection:

All procedures and experiments involving animals used in investigations have the approval of the Institutional Animal Ethics Committee. Goat testis along with the epididymis (100 ± 10 g) of mature animals was purchased from the local abattoir from time to time and brought to the laboratory in ice within one hour of slaughter. The testis was perfused through the testicular artery until the epididymis was devoid of blood and became pale white. The epididymis was dissected out and divided into caput (head), corpus (body) and cauda (tail) as shown in Figure

3.1A, B. The cauda segment of the epididymis belonging to 10 animals were pooled at a time and used for the recovery of sperm. Briefly, the tissue was cut (approximately, 1-2 mm thick slices) with a sharp razor blade, suspended in PBS (0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 0.02% KCl, pH 7.4) and agitated in a shaking water bath at 37±1°C to release the sperm. The supernatant containing sperm cells was aspirated, passed through a nylon mesh to get rid of small tissue pieces together with other debris and then centrifuged at 200 x g for 10 min to obtain the sperm as a pellet. Identical methods have been previously used to obtain sperm from the epididymis (Tulsiani *et al.*, 1989, 1992).

3.2.3 Sperm Preparation and Counting:

The loosely pelleted spermatozoa were overlaid with 500-1000 µL of PBS (phosphate buffer saline 0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, and 0.02% KCl, pH 7.4). The overlaid sample was then incubated at 37±1°C in slightly slanted position (45°) for 30 min so that the motile spermatozoa swim up in the media. After incubation the medium portion was carefully aspirated using a positive displacement pipette and pooled together in a sterile plastic tube. The total sperm count was done using hemocytometer and the sperm concentration was adjusted to obtain the requisite number of cells/mL by suspending the pelleted spermatozoa in desired volume of the PBS (pH 7.4).

Principle and procedure of hemocytometer: This involves diluting a fixed volume of liquefied semen/ sperm suspension and counting the preserved sperm suspension in a hemocytometer chamber. Dilutions of 1:9, 1:19 or 1:49 are generally prepared for counting depending of the number of spermatozoa in semen sample/ sperm suspensions.

Dye solution: This reagent consists of 50g $NaHCO_3$, 10 mL of 36-40% formaldehyde solution, and 0.25g of trypan blue (CI 2385) dissolved in water and the final volume was made up to

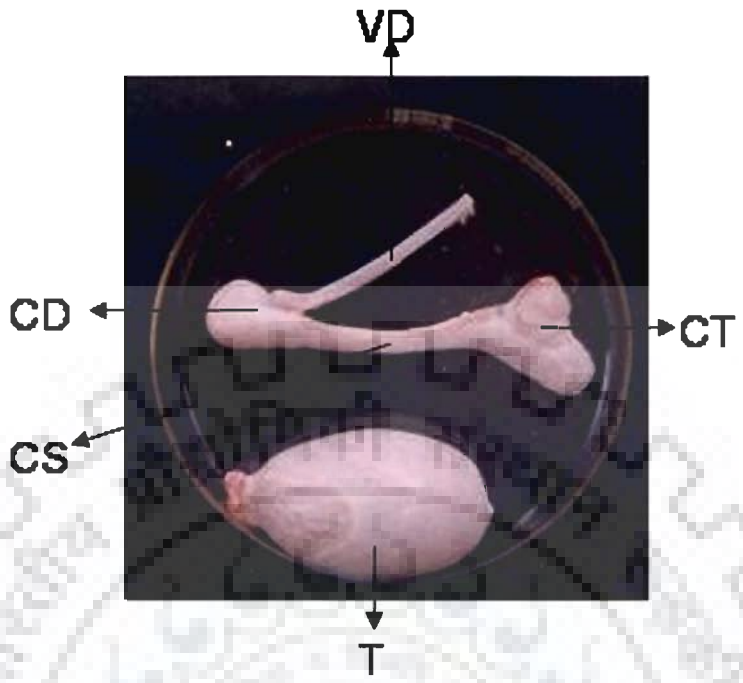


Figure 3.1(A): Dissected region goat testis and epididymis. T-testis, CT-caput, CS-corpus, CD-cauda region of the epididymis and VD-vas deferens.

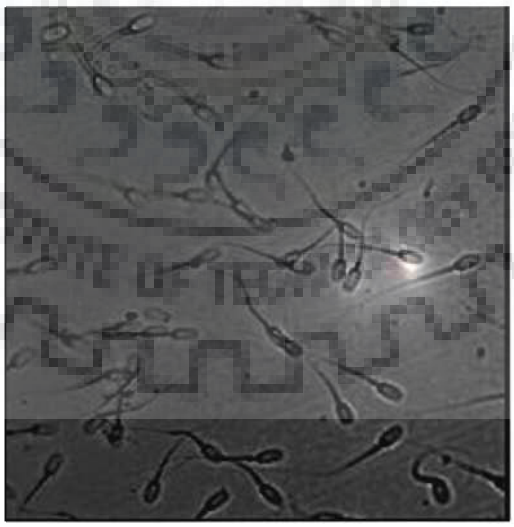


Figure 3.1(B): Light micrograph of spermatozoa obtained from cauda region of epididymis (40X).

1000 mL. This solution was filtered through Whatman No1 paper into a clean bottle and stored at 4°C.

Procedure:

1). A cover slip was fixed over the chamber of a hemocytometer

2). The resuspended sperm sample was gently vortexed for 10 sec. and about 10µL was transferred to the hemocytometer chamber. Samples at each dilution were counted in duplicate

3). The hemocytometer was left alone for 3-4 min in a humidified chamber, during which time the spermatozoa settle down onto the counting grid.

4). The sperm was counted using 20x objective using phase contrast optics. The central square of the grid contains 25 large squares each with 16 smaller squares. The number of squares counted on each grid depends on the number of spermatozoa seen in the first large square counted. For sample containing less than 10 spermatozoa per square, the whole grid (i.e. 25 squares) was counted; for samples containing 10- 40 spermatozoa per square, two horizontal or vertical rows were assessed and for samples containing more than 40 spermatozoa per square, the four corner plus center squares were counted. Spermatozoa lying on the line dividing two adjacent squares were included in the count only when present on the upper or left side of the square being assessed.

Calculation:

1. Compute the mean number of sperm counted in each chamber (i.e. for each of the central counting areas of each chamber).

2. Multiply the mean obtained in (1) by 10,000 to obtain the number of cells per mL of diluted sample.

3. Multiply the count obtained in (2) by the dilution factor. This gives the number of sperm per mL of original sample.

3.2.4 Microscopic Analysis of Sperm Motility:

Spermatozoa that showed feeble motility and forward progression were noted under a light microscope at 100x magnification. An aliquot of the freshly extracted/processed sperm maintained at 37±1°C was assessed for sperm motility in a hemocytometer maintained at 37±1°C. For the microscopic analysis method described by Roy et *al.*, (1985) was followed, all cells which showed some degree of motility and progressive forward motility were counted (expressed as %).

3.2.5 Assessment of Sperm Viability and Acrosome Integrity:

Assessment of live and dead spermatozoa and proportion of acrosome-reacted spermatozoa was according to the triple stain protocol described by Talbot and Chacon, (1981).

1). A 100 µL aliquot of sperm suspension was placed into a 2 mL test tube, and an equal amount of 2% Trypan blue dissolved in PBS was added.

2). The test tubes were then kept in a water bath at 37 °C for 15 min, and then centrifuged (280 × *g* for 3 min).

3). The supernatant was removed and the sperm pellet was washed thrice by re-suspension in 2 mL PBS and centrifuging. The final suspension was clear or pale blue.

4). The samples were centrifuged once again and the pelleted spermatozoa fixed in 1 mL of 3% glutraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min at 4 °C.

5). The spermatozoa was washed free of fixative in distilled water with centrifugation (280 × *g* for 3 min).

6). The pellets were resuspended in PBS; 10–20 µL of this suspension was spread evenly on a glass slide on a surface of approximately 3 cm^2 and air-dried.

7). Slides were then exposed to 8% Bismark brown solution in distilled water (pH 1.8) for 15 min at 37 °C.

8). The glass slides were rinsed with water and stained with 0.8% Rose Bengal solution in 0.1 M Tris–HCl buffer (pH 5.3) for 1 min at room temperature.

9). The glass slides were rinsed with water and examined (100 X) under a light microscope. A total of 100 spermatozoa per slide were evaluated to estimate sperm viability and acrosome integrity. (dead sperm cells- light blue/on colour, live and acrosome intact sperm cells- reddish brown/pink colour, live but acrosome reacted cells- reddish brown/pink colour with no colour or light blue colour at acrosome region)

3.2.6 Fabrication of Apparatus:

To study migration pattern during sperm chemotaxis, self fabrication of a device was made to monitor movement of the sperm along a gradient (chemotaxis). The design was based on the models described previously such as Zigmonds chamber, capillary assay, choice assay and double diffusion assay (Eisenbach *et al.,* 1999).

Principle: The assay chamber was designed based on the principle of diffusion of solute (assays substance) from one compartment (higher concentration) to another (lower concentration).The assay chambers were fabricated locally from a single block of polystyrene material which was tested and found to be non-toxic to the sperm cells. Assay systems were fabricated: consisting of four wells surrounding a central well (as shown in figure 3.2). All the wells were punched at equidistance from the central well and the peripheral wells were connected to the central well through a groove. The wells and the interconnecting groves were ground and polished to

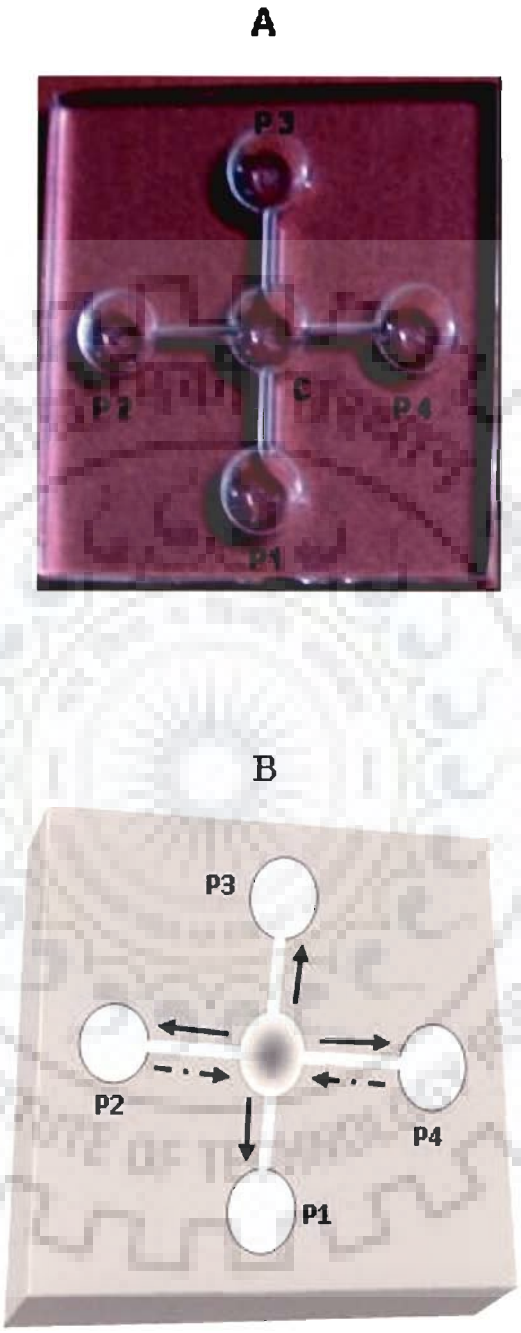


Figure 3.2: (A) Assay plates designed for studying chemotaxis of spermatozoa. (B) Represents the schematic diagram of the assay system (dotted arrow shows the direction of movement of test substance and bold arrow indicates the direction of migration of spermatozoa from central well towards increasing gradient of test chemical.

maintain a scratch free smooth surface to prevent cell trapping. The dimensions of the wells and interconnecting groves between wells of the assay plates were calculated based on the dimensions used by other scientists in their assay systems to monitor sperm migration. However slight modifications in measurements were made to suit our purpose which was standardised and validated in our laboratory. The specifications are provided below:

Assay chamber comprising of four peripheral wells (P) and one central well (C):

Depth of the well P1, P2, P3, P4 and C = 5mm,

Diameter of the wells =10 mm,

Interconnecting grove between the wells =1mm deep,

Width of the interconnecting groove= 3mm

3.2.7 Validation Test of Assay Chamber:

3.2.7.1 Evaluation of Toxicity of material used in the apparatus:

To prove proper functioning of the assay chambers some preliminary tests were performed. The first one was to check whether the material used for designing the assay chambers is non-toxic to the sperm cells. For this the live and motile spermatozoa obtained by swim-up method (described above) was employed. The sperm cells approximately 10^4 cells were pipetted in the wells containing BWW media (pH-7.4). The chambers were then incubated in an atmosphere of 5% CO_2 for 1 h at 37±°C. The sperm cells from the well were analysed for live and dead cells after regular interval of time (15, 30 and 60 min) using the simple trypan blue dye exclusion test.

3.2.7.2 Evaluation of Gradient formation:

The second test was performed to check if the alleged chemotactic factor after being placed in the experimental well created a concentration gradient and did not move out of the well at once.

This was tested by monitoring the diffusion of Bovine Serum Albumin (BSA) from one compartment to another. Initially, all the wells together with the grooves were primed with PBS. This was done by adding 250 µL of PBS in each of the wells which ensures that the wells are half full and the grooves are irrigated with the buffer. Now, 50 µL of protein (2 mg/mL BSA) in PBS was dispensed into the central well. The assay plates were then left alone on a flat surface at room temperature. The protein diffuses slowly from the central well into peripheral wells with time. The change in concentration of protein was estimated (Bradford, 1976) at regular intervals of time (0, 10, 20, 30, 40 and 45 min) by drawing out small volume from the peripheral well.

3.2.7.3 Sperm Chemotaxis by the Choice assay:

A third experiment was performed to demonstrate and validate chemotaxis with this model. From literature it was gathered that a tri-peptide, formyl-met-leu-phe, induces sperm as well as leukocyte chemotaxis (Vijayasarthy *et al.*, 1980; Gnessi *et al.*, 1986). This peptide was used to study chemotactic behavior of the goat spermatozoa. Briefly, suspension of known concentration of tri-peptide (10, 30, 50, 70, 90 and 110µM) in BWW (pH 7.2) was made. Medium containing known number of spermatozoa (10^4 cells) were placed in the central well where as the peripheral wells contained either buffer or solution containing tri-peptide. The assay chamber was then incubated at 37±1°C for 1 h. Following the incubation, the buffer from experimental wells was collected after blocking the channel connecting the central well. The suspension was then centrifuged at 600 x g for 10 min and the sperm pellets were resuspended in BWW (pH 7.4) buffer. The number of cells that migrated from the central well towards the solution containing chemoattractant or buffer was determined by counting the number of cells using haemocytometer (section 3.2.3). All the validation experiments were conducted in triplicate and the results were statistically analysed.

3.2.8 Sperm Chemotaxis by the Trans-Well Method:

For evaluating sperm migration (chemotaxis/chemokinesis), Corning-costar trans-well plates were used as per manufacturer's instructions. . The trans-well plates consisting of two chambers are separated by a polycarbonated membrane having 12 μm diameter pore size. The bottom chamber (trans) was filled with BWW buffer containing 10, 30, 50, 70, 90 and 110μM dilution of tri-peptide, formyl-met-leu-phe and without tripeptide (control). The upper chamber (cis) consisting of an insert with the membrane stretched across its base, was lowered into the bottom chamber. The chamber was filled with a 0.5 mL aliquot of sperm suspension and the system was incubated for 60 minutes at 37±1°C, following incubation the insert was removed and the buffer from the lower chamber was collected and centrifuged. The pellets were re-suspended in buffer, and were counted for sperm using haemocytometer. All the assay data obtained were statistically analyzed and represent mean and standard error of mean for at least three experiments.

3.3 RESULTS:

The sperm collected from the cauda segment of the epididymis of 10 individual samples were tested for basic spermatozoa characteristics which included sperm motility (random and feeble motile cell), progressive forward motility, acrosome reacted cells and dead cells. The relative distribution of sperm of various categories is presented in figure 3.3. Of the total spermatozoa collected only 43.5% showed some kind of motility when observed under microscope. Among this only 18.9% were found to exhibit progressive motility. When cell vitality was tested by staining an aliquot from each sample with triple stain/ trypan blue, 3.7% of the total

spermatozoa were found to be dead, and 35.6% of the total spermatozoa were found to have intact acrosome. The differential staining patterns obtained based upon which the above data was summarised is shown in figure 3.4.

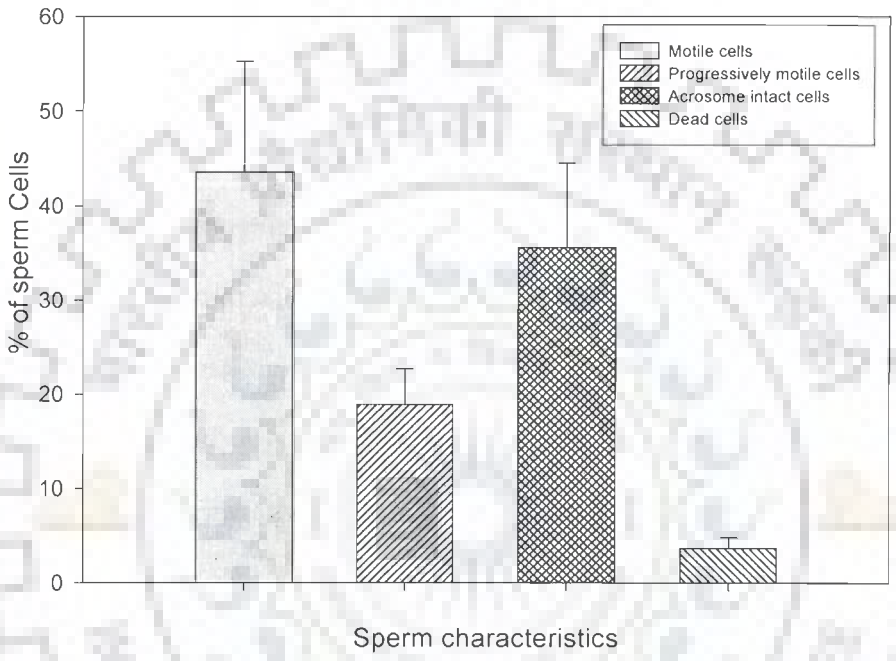


Figure 3.3: The relative distribution of sperm exhibiting different functional and morphological characteristics.

Since the number of live and progressively motile spermatozoa were less for conducting further experiments (As per WHO guidelines at least 50% progressively motile spermatozoa are required) sperm isolated from all 10 animals were pooled together. The pooled sample was then overlaid with modified PBS buffer and the swim up protocol was used to enrich the live and progressively motile spermatozoa that could be used in the validation of the chemotaxis assay. As shown in figure 3.5 most of the spermatozoa obtained after enrichment had much larger population of live and progressively motile cell (82%) whereas 12.5% of the cells were found to

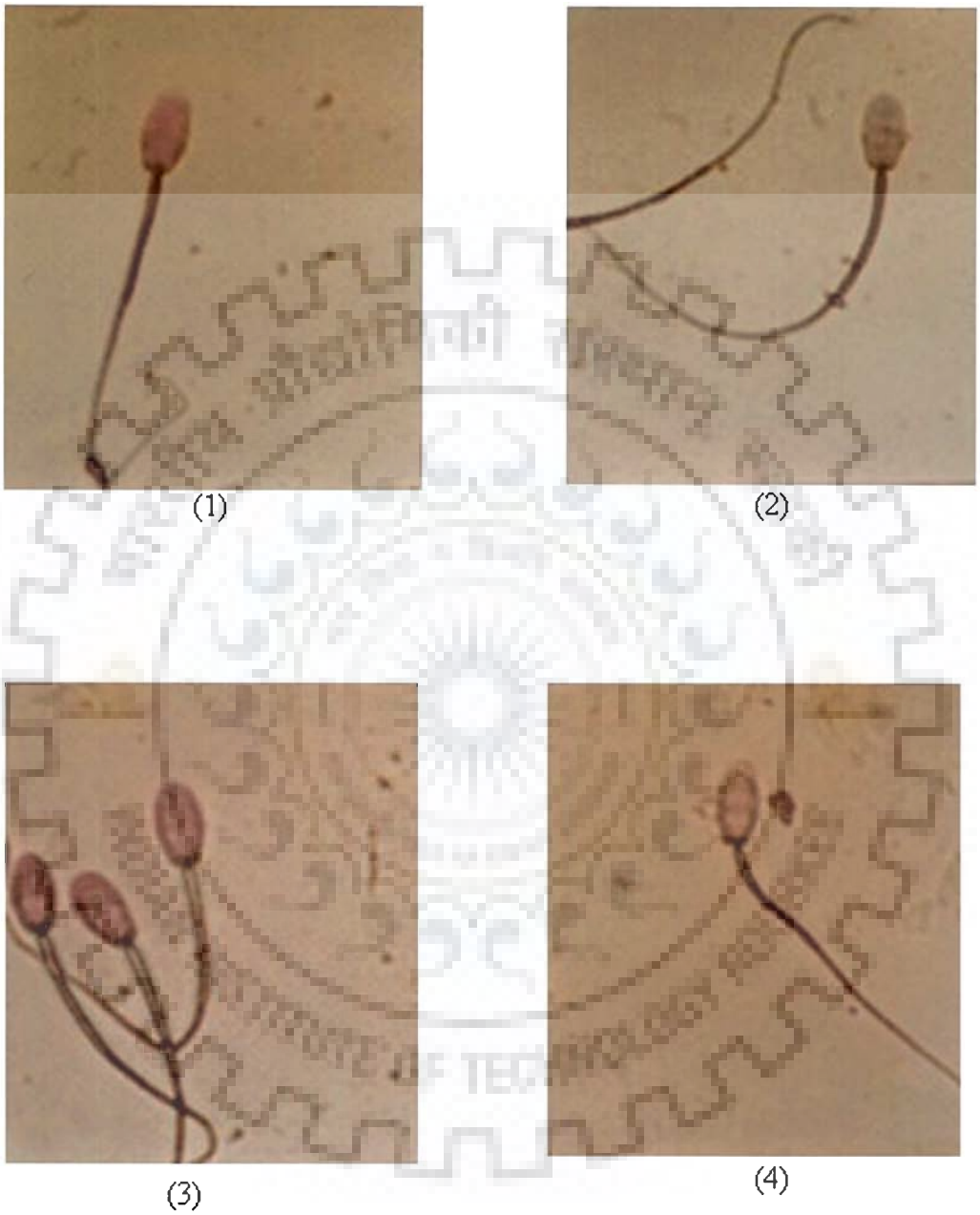


Figure 3.4: Differential staining pattern of goat spermato (100 X). 1). live spermatozoon acrosome reacted, 2). dead spermatozoon with an intact acrosome, 3). live spermatozoa with intact acrosomes. 4). live spermatozoa with acrosome partially intact

be live but showed very less or no motility at all. A small proportion of the spermatozoa (5.5%) were found to be dead.

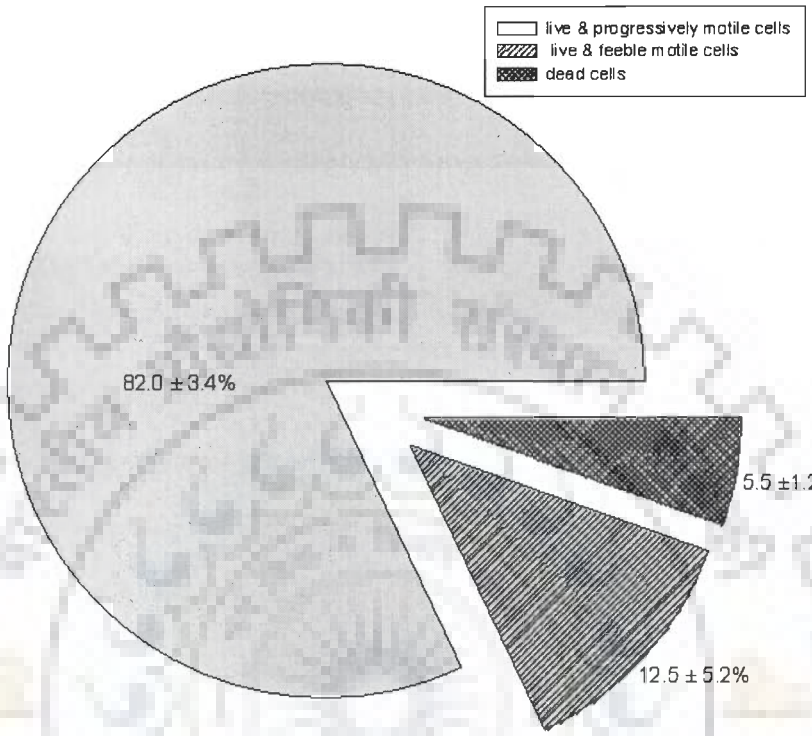


Figure 3.5: Spermatozoa characteristics of goat spermatozoa after enrichment process. The data shown is % mean ± SEM of at least 3 experiments.

The spermatozoa obtained by enrichment method was further used in the evaluation of the chemotaxis chamber that was locally designed (figure 3.2). In order to check if the material used in fabrication of the apparatus interferes with the characteristics of sperm, suspensions of spermatozoa (10^3 cells) were incubated in the wells of the plates at 37±1°C for varying periods of time. The summary of the number of live cells that were placed before and after the incubation is shown in figure 3.6. There was a 5 % drop in the number of live cells after 15 min which increased to 8% after 1 h incubation.

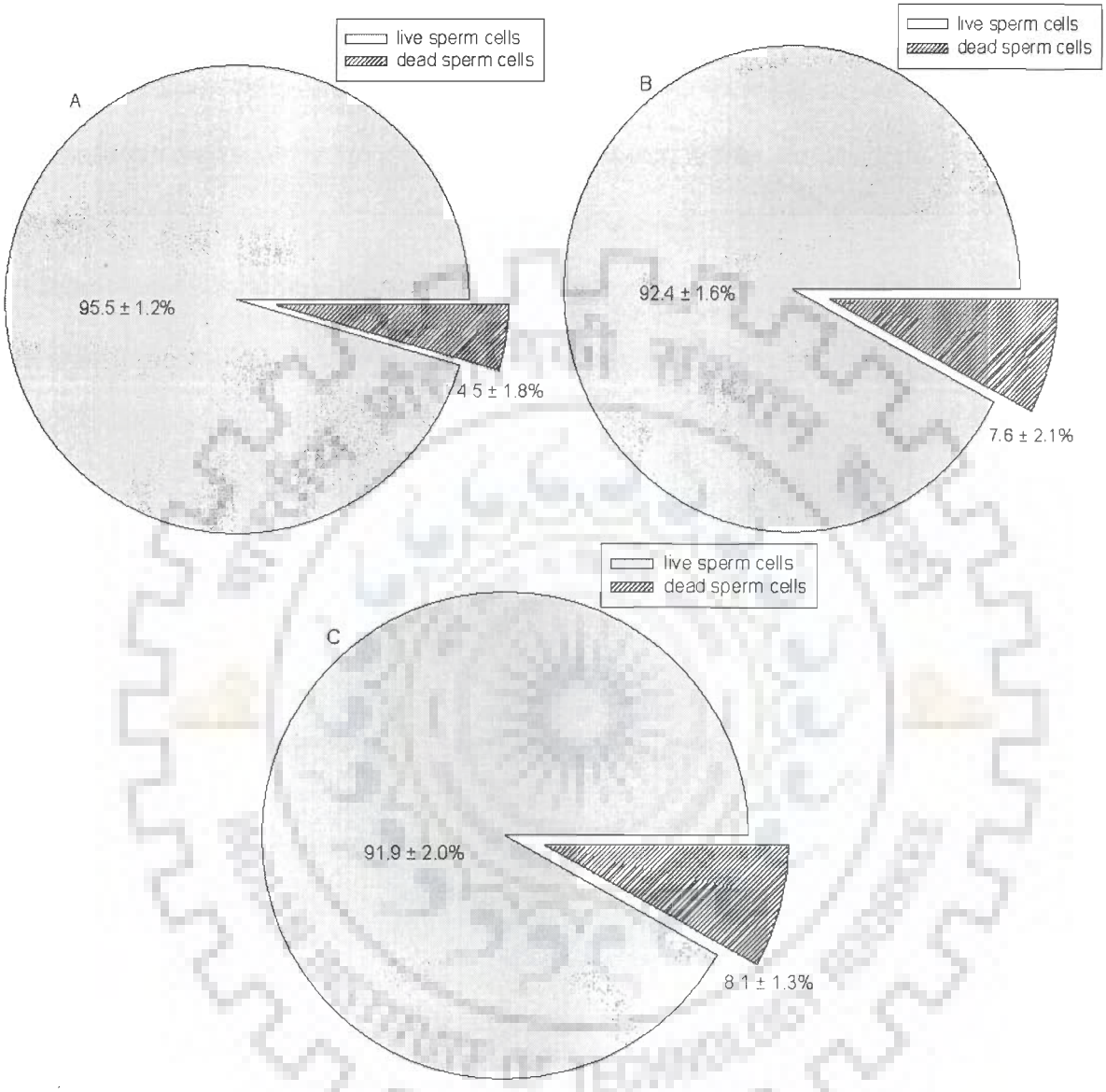


Figure 3.6: Percentage of cells that were scored for live and dead after incubation in assay plate. A).15 min. of incubation, B). 30 min.of incubation C). 60 min.of incubation. All the data shown are % mean ± SEM of at least 3 experiments.

The second validation test of the assay chamber designed to test chemotaxis, was to check the formation of gradient of solute along a defined region surrounding the central well. The rate of

diffusion of BSA from the central well towards the surrounding wells was monitored. As indicated in figure 3.7 soon after mixing the protein solution in the central well of the assay chamber the buffer, no protein was detected in the peripheral well. After 10 minutes of incubation at room temperature trace amount of protein was detected in buffer collected from peripheral well. The concentration of protein in the peripheral well gradually increased with time, after approximately 40 minutes of incubation the protein content of the wells reached maximum level. Incubation beyond 45 minutes at room temperature did not show any significant change in the protein content of the wells.

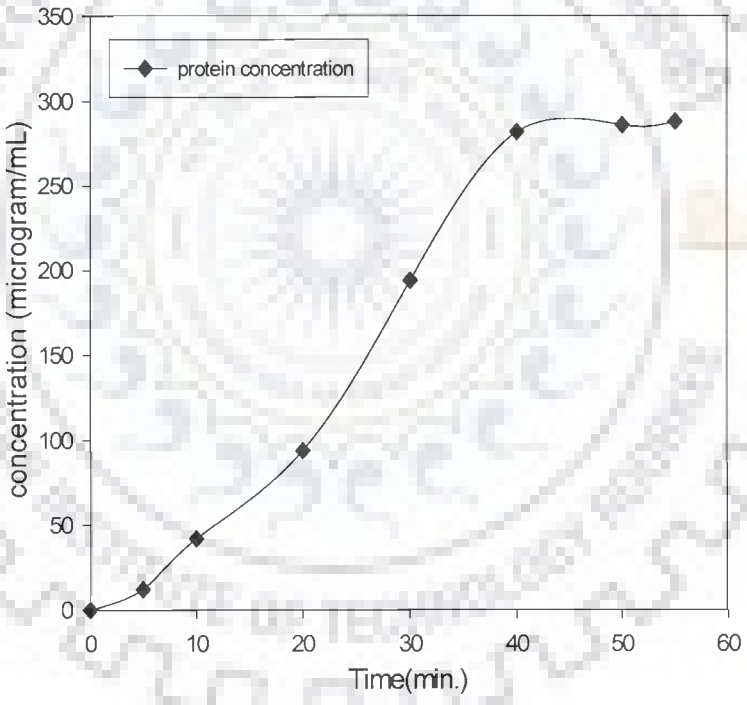


Figure 3.7: Profile of protein gradient generated in the peripheral chambers after regular interval of time.

The third validation assay was conducted to prove that the assay chamber can be used to monitor sperm chemotaxis *in vitro*. Figure 3.8 shows the chemotactic effect of tri-peptide

(Formyl-met-leu-phe) on goat spermatozoa. Dose dependent study of the tri-peptide after 60 minutes of incubation at 37±1°C showed that with increasing concentration of tri-peptide the number of spermatozoa moving towards the well containing test substance increased. A steady increase in the chemotactic response of spermatozoa to tri-peptide was noticed at concentrations starting from 10 to 50μM. A significantly higher number of cells showed migration towards well containing a concentration of 50μM. There after with increasing concentration of tri-peptide (70 to 100μM) a sharp fall in the chemotatic response of the spermatozoa was observed.

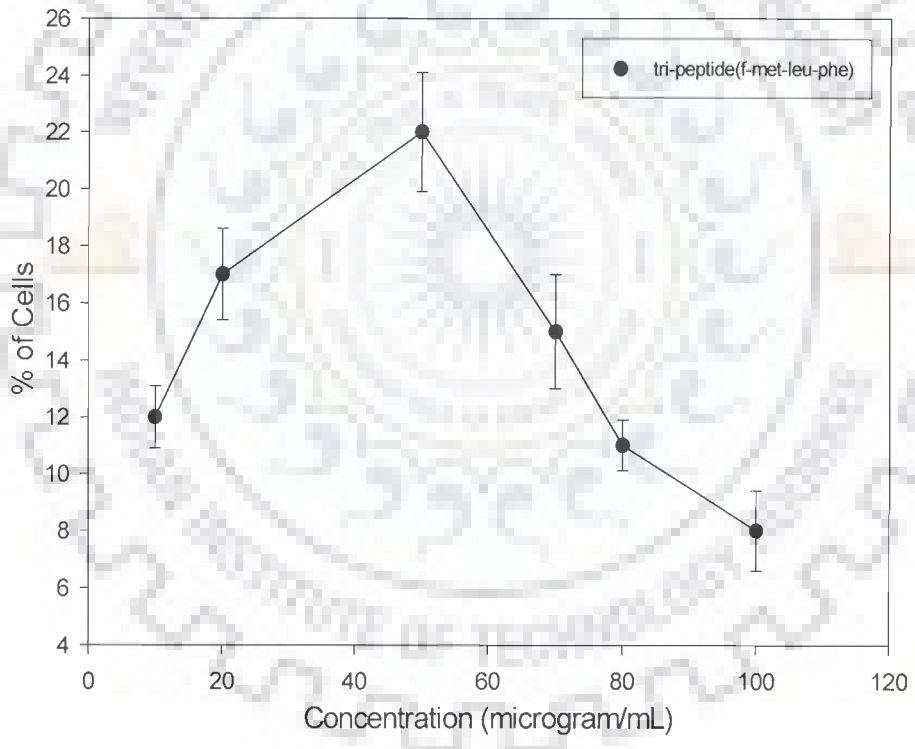


Figure 3.8: Dose dependent chemotaxis assay performed on self fabricated assay chamber. All the data shown are % mean ± SEM of at least 3 experiments. (Student's t test $p < 0.001$)

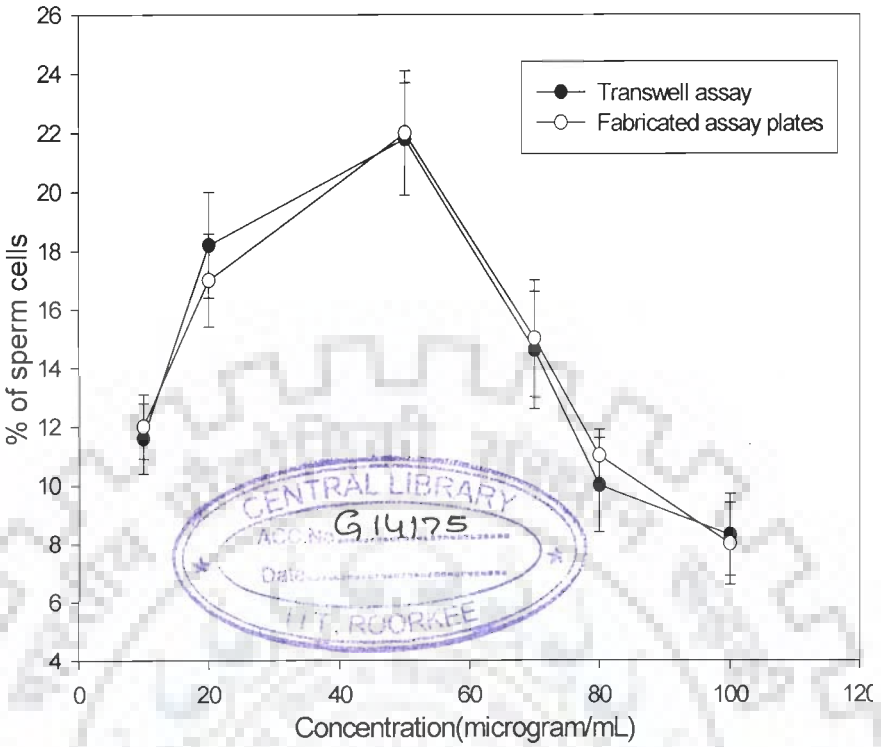


Figure 3.9: Comparison of chemotaxis assay performed on trans-well assay plates and assay plates designed in the lab.

3.4 DISCUSSION:

For the development of an *in vitro* assay to monitor sperm chemotaxis, it is imperative to start with a sperm population which is of the highest quality. In the present study sperm was collected from the cauda segment of the goat epididymis. However, the yield of sperm extracted from a single animal was low and the number of motile sperm was insufficient to be used in successive assays so that meaningful comparisons can be made. This necessitated the pooling of samples from 10 different animals and selecting a population of sperm that exhibited good motility characteristics. Enrichment was done by the swim-up protocol that ensured a good yield of motile sperm. At this stage, it may be mentioned that even in nature when millions of sperm

are ejaculated into the female reproductive tract only a few with high motility characteristics manage to reach the oviduct to effect fertilization (Hunter, 1993). This implies that under *in vivo* conditions only a select population of sperm (the fittest) participate actively in the process of chemotaxis.

While designing the chamber for use in chemotaxis assay it is essential that the material used does not alter the motility characteristics of the sperm sample being tested. From the results it is clear that the polystyrene material did not drastically alter the motility of sperm for periods up to 1 h of interaction. Since the ultimate assay time was within this time frame the polystyrene material could be considered inert to sperm.

The basic design of the chemotaxis apparatus consisted of wells and grooves punched in a block of polystyrene. The dimensions of the grooves and wells were standardized to ensure that multiple assays could be conducted in the five-well chamber which includes a control for comparison purposes. The time frame for the migration of sperm was also considered while making these calculations.

As mentioned before, one problem commonly encountered in devices of this kind is that it is difficult to distinguish between sperm trapping, chemokinesis and chemotaxis. This was circumvented in the present case by machine grinding and polishing of the wells and grooves. Such a treatment made the chamber scratch free and prevented sperm trapping. Since the design permitted chemical gradients to be established prior to the testing of sperm samples, it is possible to conclude that the sperm movement from the central to peripheral wells was largely on account of sperm chemotaxis and not chemokinesis. The creation of gradients between the central and peripheral wells was confirmed by the decolourization of phenol red and the diffusion data of BSA in separate experiments. The results obtained in case of apparatus

designed and fabricated in our laboratory prove that the apparatus system designed can be used to monitor unidirectional migration of cells *in-vitro.* Since the system based on choice assay can distinguish between chemokinesis and chemokines it may be inferred that results obtained are more reliable as compared to other systems as suggested by other investigators (Makler *et al.,* 1992; Sliwa *et al.,* 1993; Eisenbach *et al.,* 1999).

One of the most reliable assays available for evaluation of chemotaxis is the one using the Corning Trans well apparatus. However, this turns out to be very expensive for routine and repeated use. To evaluate the performance of the choice assay using the self-fabricated apparatus, a tripeptide, f-met-leu-phe was employed as a chemoattractant. Formyl-met-leu-phe is a known chemoattractant of neutrophil and bull spermatozoa and has been used in the past to evaluate chemotaxis (Becker *et al.,* 1976; Iqbal *et al.,* 1980). Preliminary experiments done in our laboratory confirmed that this peptide is effective as a chemoattractant for goat sperm at concentrations up to 50 μg/mL. Using this peptide, the results obtained for the self-fabricated choice assay were compared with those of the commercially available Trans well assay. The similarity in the pattern of response obtained by the two procedures only goes to prove that the self fabricated apparatus could be a cost effective substitute for the commercial device.

Concluding remarks:

The self-fabricated chemotaxis choice bioassay described in this chapter is highly sensitive, selective and reproducible. The technique is able to validate biological responses to various concentrations of test solutions simultaneously. Since t*his in vitro* chemotactic chamber uses small volume of samples for assay, it allows testing a large number of samples in replicate.

Chapter-4

CHAPTER 4: EFFECTS OF FOLLICULAR FLUID, HORMONES, FREE AMINO ACIDS AND SUGARS ON GOAT SPERM MIGRATION/ CHEMOTAXIS AND ITS CORRELATION WITH CAPACITATION

4.1 INTRODUCTION:

The occurrence of sperm chemotaxis (that is a response of motile cells to the gradient of a chemical stimulus, resulting in the modulation of the direction of movement so as to move towards the attractant or to move away from the a repellent) is a common phenomenon which is mostly seen in animals where fertilization is external (Miller 1985; Cosson 1990; Eisenbach *et al.,* 1994). But in case of species were fertilization is internal this phenomenon is still under study. Since follicular fluid contains secretions of the egg and its surrounding cells, it is considered as a potential source of soluble factors that are secreted from the egg or surrounding cells that can attract sperm cells (Villanueva-Diaz *et al.,* 1990). Recent studies on follicular fluid from humans and mice (Giojalas *et al.,* 1998; Oliveira *et al.,* 1999) have shown the occurrence of this phenomenon *in-vitro*. In humans most of the cells which are chemotactically responsive are very few (approximately 10%) (Cohen-Dayag *et al.,* 1994) and this chemotactically responsive subpopulation consists of capacitated spermatozoa (Cohen-Dayag *et al.,* 1995). This stage of the spermatozoa remains for a very short time (50-240 min. in humans) (Eisenbach, 1999). It has been proposed that in mammals (at least in humans) a steady state of capacitated spermatozoa is maintained. Moreover, a selective recruitment of capacitated spermatozoa and its continuous replacement may be to prolong the time during which these spermatozoa are available in the female reproductive tract (Eisenbach *et al.,* 1994).The objective of the present study was to determine the effect whether spermatozoa are attracted towards factors released by egg or its surrounding environment. Although a large number of studies on human sperm

chemotaxis concluded that chemotaxis to follicular fluid occurs, but different groups had sometime reported conflicting results (Eisenbach *et al.*, 1999). These conflicting results may be due to the use of non-diluted follicular fluid and perhaps the use of follicular fluid from immature follicles. Therefore follicular fluid was chosen for study. The effect of follicular fluid collected from immature as well as mature ovaries was tested separately at varying concentrations. Efforts were further made to correlate association between capacitation and chemotaxis and to distinguish between chemotaxis and chemokinesis. Along with this effect of hormones both sex steroid and other hormones, free amino acids and sugars were also analyzed.

4.2 MATERIALS AND METHODS:

4.2.1 Chemicals:

All the chemicals used for conducting the experiments were obtained from Sisco Research laboratory (SRL), Qualigen, Sigma-Aldrich, Merck, Biochemika Fluka, Bio-Rad, Amersham and Hi-media.

4.2.2 Extraction and Isolation of Sperm from Goat Epididymis:

All procedures and experiments involving animals used in investigations have the approval of the Institutional Animal Ethics Committee. Sperm was extracted from the cauda segment of the goat epididymis collected from the local abattoir when animals were slaughtered for meat consumption. The sperm collected from several animals was pooled and the live sperm population was enriched by the swim up protocol (for details section 3.2.2; 3.2.3).

4.2.3 Extraction of Follicular Fluid from Goat Ovary:

Healthy adult goats with normal reproductive tract were included for this study. Ovaries were collected immediately after slaughter on three different days of the same week. Ovaries were transported to the laboratory in ice, washed twice in cooled 0.9% NaCl (4°C) and blotted dry.

Small follicles (≤ 2 mm diameter) and large follicles (≥ 4mm diameter) were tapped for drawing follicular fluid. Follicular fluid was collected by aspiration with a 26G needle and a 1 ml syringe and pooled separately according to follicle size. For each animal and follicle class, a different needle and syringe were used. Haemorrhagic and morphologically atretic follicles, identified macroscopically according to the method of Kruip and Dieleman (1982), were not sampled. Follicular fluid (at least 0.3 ml per sample) was centrifuged (10,000 × *g*, 7 min) and the supernatant was saved for analysis. Samples were frozen immediately after centrifugation and stored at -80°C for further assay. Assays were conducted within one week of sample collection.

4.2.4 Induction of Capacitation:

The Biggers, Whitten and Whittingham (BWW) medium (95 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 3.5 % HAS/BSA, pH 7.4) was employed to induce capacitation of spermatozoa. The sperm sample was incubated in BWW medium for 3 hours at 37±1°C under 5% CO_2 in CO_2 incubator (Bigger *et al.*, 1971).

4.2.5 Decapacitation of Spermatozoa:

Acrosome reacted cells were prepared from subpopulation of capacitated spermatozoa as described by Jaiswal *et al.*, (1999) and Fabro *et al.*, (2002). An aliquot of capacitated spermatozoa was incubated with 10 µM calcium ionophore A23187 (Sigma chemicals Co. St. Louis, MO) for 30 min at room temperature. Initially the ionophore was dissolved in minimum volume of DMSO and then adjusted so that the final DMSO concentration is 0.2%. After incubation, acrosome-reacted cells were scored by the triple staining procedure. Dead cells were not included in counting. Total number of live cells that were counted was considered as 100%. Live, dead and proportion of acrosome-reacted spermatozoa was assessed of according with the triple stain protocol described by Talbot and Chacon (1981) (for detail section 3.2.5).

4.2.6 Chemotaxis Assay (Choice Assay):

Sperm chemotaxis was quantified as using assay plates designed and fabricated locally in the laboratory. The assay plates consist of four peripheral well surrounding a central well connected to the central well by an interconnecting grove (details given in section 3.2.6). Before filling the chambers the interconnecting grove were temporarily blocked. A bolus of soft agarose (0.2%) containing different dilutions (10^{-1}-10^{-8}) of follicular fluid was made (Serrano *et al.*, 2001) and placed onto the floor of the peripheral chambers which was then filled with modified BWW (400 µL).The central chamber was filled with an equal volume of BWW buffer containing sperm cell (1-3x10^4 cells). Immediately after adding cell suspension the interconnecting groves were opened, and the system was incubated for 60 min. at 37±1°C. Following incubation, the buffer from peripheral wells were aspirated and centrifuged separately. The sperm pellet thus obtained was then resuspended in BWW media. The resuspended pellet was counted for sperm using a haemocytometer. All assay data in the figures represents the percent mean and standard error of mean for at least three experiments.

4.2.7 Chemotaxis Assay (Corning Trans-well Plate Assay):

Sperm chemotaxis was quantified as described by Al-Anzi *et al.*, (1998). Commercially available corning-costar transwell plates, consisting of two chambers separated by a polycarbonate membrane having 12 µm diameter pore size were used. A bolus of soft agarose (0.2%) containing appropriately diluted follicular fluid (10^{-1}-10^{-8}) was made (Serrano *et al.*, 2001) and placed onto the floor of the bottom chamber (trans) which was then filled with modified BWW (1.2 mL). The upper chamber (cis), consisting of an insert with the membrane stretched across its base, was lowered into the bottom chamber. The chamber was filled with a 0.5 ml aliquot of capacitated sperm suspension, and the system was incubated for 60 min. at

37±1°C. Following incubation, the insert was removed, the lower chamber buffer was collected separately and centrifuged (300 x g) and the pellets obtained were then resuspended in BWW buffer. The resuspended pellet was counted for sperm using a haemocytometer. All assay data in the figures represents the mean and standard error of mean for at least three experiments.

4.2.8 Chemokinesis Assay:

Sperm chemokinesis assay was performed on corning costar transwell plates with slight modification (Devreotes *et al.,* 1988) of the chemotaxis assay protocol mention above. The bottom chamber was filled with 1.5mL of appropriately diluted follicular fluid (10^{-1}-10^{-6}) in BWW medium (pH-7.4). The upper chamber was lowered into the bottom chamber and filled with the same concentration of follicular fluid in BWW media (pH-7.4) as used in the lower chamber. An aliquot (0.5mL) of capacitated sperm suspension was then added to the upper chamber and the system was incubated for 60 min at 37±1°C. Following incubation, the insert (upper chamber) was removed, the lower chamber buffer was collected separately and centrifuged (300 x g), and the pellets obtained were then resuspended in BWW buffer. The resuspended pellet was counted for sperm using a haemocytometer.

4.2.9 Biochemical Assay of Follicular Fluid:

4.2.9.1 Enzyme Immunoassay for the Quantitative Determination of Sex Steroids

Major sex steroid hormones (Progesterone, Testosterone Estrone and Estradiol) present in follicular fluid was measured using commercially available kits (Pathozyme kits, Omega diagnostics Ltd. UK).

Principle of the Test: The kit is based on the principle of competitive binding between the hormone in the test specimen and hormone-HRP Conjugate for a constant amount of rabbit antibody raised against the hormone. In the incubation, goat anti-rabbit IgG-coated wells are

incubated with hormone standards/ samples (follicular fluid), hormone-HRP Conjugate reagent and rabbit anti-hormone reagent. During the incubation, a fixed amount of HRP-labelled hormone competes with the endogenous hormone (progesterone, testosterone, estrone and estradiol) in the standard/ sample/ quality control serum for a fixed number of binding sites of the specific hormone-antibody. Thus, the amount of hormone-peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of specific hormone in the specimen increases. Unbound hormone-peroxidase conjugate was then removed and the wells washed. The substrate (TMB) was added, resulting in the development of blue colour. The colour development was stopped with the addition of stop solution, and the absorbance measured spectrophotometrically at 450 nm. The intensity of the colour formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled hormone (progesterone, testosterone, estrone and estradiol) in the sample. A standard curve was obtained by plotting the concentration of the standard versus the absorbance. The hormone concentration of the specimen can be calculated from the standard curve.

Assay Procedure

1. All the kit components and the test sample were brought to room temperature (20°C to 25°C) prior to the start of the assay.

2. One set of standards was run with each batch of test sample. The desired number of coated wells was secured in the holder.

3. Unused strips were resealed in the foil bag containing the desiccant, using the resealing zip-lock before being replaced at 2°C to 8°C.

4. 25 µl of standards, test samples and controls were dispensed into the appropriate wells.

5. 100 µl working solution of hormone-HRP Conjugate reagent was dispensed to each well.

6. 50 µl of rabbit anti-hormone reagent was dispensed to each well and thoroughly mixed for 30 sec.

7. The plates were incubated at room temperature (20°C to 25 °C) for 90 min.

8. At the end of the incubation period, the contents of the wells were discarded by flicking plate contents into a Biohazard container. The wells were then sharply stroked against absorbent paper.

9. Washing: The wells were first filled with a minimum of 300 µl of distilled water per well and the contents of the plate were flicked into a Biohazard container. The wells were sharply stroked against absorbent paper. The empty wells were washed 5 times.

10. The wells were sharply stroked onto absorbent paper or paper towel to remove all residual water droplets.

11. 100µl of Substrate solution was now dispensed into each well and gently mixed for 5 sec.

13. The plates were then incubated in the dark at room temperature (20°C to 25°C) for 20 min.

14. The reaction was stopped by adding 100µl of Stop solution to each well.

15. Upon gentle mixing for 30 sec. all the blue colour changes to yellow colour immediately.

16. The absorbance was read at 450 nm with a microtitre well reader within 10 min.

4.2.10 Identification of Free Amino Acids in Follicular Fluid:

A. Amino acid standard.

A 21-compound calibration mixture (standard AA) used for the experiment was prepared by adding glutamine, asparagine, and taurine (25 nmol each) to a commercially available 18 amino acid calibration mixture (kit). A sample (10 µl) of the 21-compound mixture of amino acids in 0.1 N HCl containing ammonium sulfate containing 25 nmol each of the 21 amino was dried in a small test tube and dissolved in 100µl of coupling buffer (acetonitrile: pyridine: triethylamine:

H_2O, 10:5:2:3). This solution was dried by rotary evaporation and the residual amino acids were dissolved once more in 100 µl of coupling buffer. To this solution was added 5 µl of PITC (phenylisothiocyanate). After a 5 min reaction at room temperature, the solution was evaporated to dryness by rotary evaporation under high vacuum (vacuum pump; 50-100 mtorr). The resulting PTC amino acids were dissolved in 250 µl of 0.05 M ammonium acetate, water, or water: acetonitrile (7:2). Amino acid analysis of the sample was performed on the reverse-phase HPLC as described below.

B. Extraction of free amino acid from follicular fluid.

Follicular fluid was collected from mature follicle as described earlier (section 4.3). The pooled sample (2 mL) was deproteinized by filtering through a 3 kDa molecular weight cut-off ultrafiltration membrane filter (Ultrafree-MC, Millipore, Bedford, MA 01730) by centrifuging at 1500 ×g for 15 min. The deproteinized sample was then used for detection of free amino acid.

Coupling of free Amino Acids from follicular fluid with PITC (phenylisothiocyanate)

The precolumn derivatization of free amino acids present in deproteinized follicular fluid sample was accomplished with phenyl isothiocyanate to produce phenylthiocarbamyl amino acids. 100µl of deprotenized sample was evaporated to dryness by rotary evaporation under high vacuum pressure (vacuum pump; 50-100 mtorr). The dried sample was dissolved in 1mL of coupling buffer (acetonitrile: pyridine: triethylamine: H_2O, 10:5:2:3). The solution was again dried by rotary evaporation and the residual amino acids were dissolved once more in 1000 µl of coupling buffer. To this solution was added 50 µl of PITC (phenylisothiocyanate). After a 5-min reaction at room temperature, the solution was evaporated to dryness by rotary evaporation under high vacuum. The resulting PTC-amino acids were dissolved in 200 µl of 0.05 M

ammonium acetate: water, or water: acetonitrile (7:2). Amino acid analysis of the sample was performed on the reverse-phase HPLC as described below.

4.10.1. Chromatography Conditions:

Reverse phase HPLC separation of the of the PTC amino acid was performed with HPLC system (Waters Model 484) with three-headed pump, ternary solvent system with low pressure mixing, and a variable wavelength detector at 254 nm. The column (Octadecyl (C18) columns, 25 cm in length) and injection valve was maintained at room temperature. The solvent used for separation was (A) water (B) 0.1 M ammonium acetate, pH 6.8, in 50% (v/v) acetonitrile: water. The step-gradient used for separation was:

Time (min.)	%A	%B
0	100	0
15	85	15
30	50	50
40	20	80
50	0	100

4.2.11 Analysis of Sperm Migration in Response to Hormones, Amino Acids and Sugars:

Sperm chemotaxis was quantified using the self-fabricated assay plates (described in section 3.2.6). Before filling the wells, all the interconnecting groves were temporarily blocked. The central well was filled with 400 µl of BWW medium containing capacitated sperm sample (1-3x10^4). The peripheral wells were filled with an equal volume of the following fluids: BWW only (control well) and BWW containing the test substance (hormones, free amino acids and sugars in three concentrations). After filling the wells the grooves were opened and the plates

were incubated at 37±1°C for 1h. After incubation, the buffer from each well was aspirated separately and the number of cells counted in a haemocytometer. All the experiments were conducted in triplicate and the results were statistically analyzed using Student's t-test.

4.2.11.1 Hormones Used In Sperm Migration Studies:

Stock solutions (0.5 mg/mL) of all steroid based hormones (progesterone, testosterone, estrone and estradiol) were prepared in BWW medium (pH-7). Each hormone was first dissolved in minimum volume of DMSO and then made-up to 1mL (the final DMSO concentration was less then 0.2%). The required concentration (0.1 µg/mL, 0.5 µg/mL and 1.0 µg/mL) of the individual hormones for assay was prepared by diluting appropriate volume from stock solution in BWW medium (pH-7.4).

Similarly, stock solutions (0.5 mg/mL) of all other hormones that were investigated- adrenalin (biochemika fluka), insulin (biochemika fluka), oxytocin (Sigma), thyroxine (Sigma), glucagon (biochemika fluka), calcitonin (Sigma), acetylcholine (Sigma) and melatonin (Sigma) were prepared separately in BWW medium. For chemotaxis assay appropriate volume was taken and diluted in BWW medium so that the final concentration of each hormone was (0.1 µg/mL, 0.5 µg/mL and 1.0 µg/mL). A vial of oxytocin (50 IU/mL) was diluted in BWW medium to give (0.1 IU/mL, 0.5 IU/mL, and 1.0 IU/mL).

4.2.11.2 Free Amino Acids Used In Sperm Migration Assay:

Stock solution (0.5 mg/mL) of individual free amino acids (glutamic acid, aginine, Serine, lysine, aspartic acid, alanine, glycine, leucine, cystine, methionine, valine, isoleucine, tyrosine, tryptophan and histidine) was prepared in BWW medium (pH-7.4), separately. For performing chemotaxis assay three different concentrations (0.01 µg/mL, 0.1 µg/mL and 1 µg/mL) of each amino acid was used. All the amino acids were purchased from SRL India.

4.2.11.3 Sugars Used In Sperm Migration Assay:

A stock solution of sugars (0.5 mg/mL) mostly hexose (glucose, fructose, mannose, sorbitol and ascorbic acid) was prepared in BWW medium (pH 7.4), separately. For conducting sperm chemotaxis assay three different concentrations (0.01, 0.1 and 1 µg/mL) of each sugar was used. Before conducting the chemotaxis assay appropriate dilution of each sugar was made from stock solution in BWW medium. (All the sugars used were purchased from Himedia, India).

4.2.12 2-D Gel Electrophoresis:

Proteomic analysis of the crude follicular fluid extracted from two different stages of development of follicle small (≤ 2mm) and large (≥ 4mm) was done according to the method described by O'Farrell (1975) and Bio-rad instruction manual with slight modification.

(A) Iso-Electric Focussing (IEF)

1. Crude follicular fluid (300-500 µg) sample was purified using PlusOne 2-D clean-Up Kit Amersham), as per the manufacturer's protocol.

2. The protein pellet was Re-suspended in 185 µl of re-hydration/Sample Buffer.

3. Re-hydration of an 11-cm long IPG strip (pH 4-7) with the protein sample on a disposable re-hydration/equilibration tray was carried out for overnight at the room temperature. Each strip was over laid with 2-3 ml of mineral oil to prevent evaporation during the rehydration process.

4. Electrode Wicks (Bio-Rad) was placed on anode and cathode ends of electrode wires on an IEF tray. The wicks were wet by adding 9 µl of nano-pure water.

5. The IPG strip was removed from rehydration tray and oil drained before laying it on wet electrode wicks. Each strip was overlaid with 2-3 ml of mineral oil to prevent evaporation during IEF process.

6. IEF on Protean IEF cell (Bio-Rad) at 20°C was carried out using the following step

(a) Step 1 250 volts, 20 min, and linear ramp

(b) Step 2 8000 volts, 2.5 hr, and linear ramp

(c) Step 3 8000 volts, 20,000 V-hr, rapid ramp

Total: ~30,000 V-hr for a period of 5.3 h

(B) 2nd Dimension

7. After IEF the IPG strips was thawed at room temperature for 10-15 minutes and then equilibrated with 3-4 ml/strip of Equilibration Buffer-I, followed by Equilibration Buffer-II for 10 minutes each. (as per manufacturers protocol)

8. The IPG strip was dip in Electrode Buffer before placing it on a 12% SDS-PAGE. Electrophoresis was carried out at 200 volts using Criterion Dodeca Cell (Bio-Rad) attached to a cooling water bath circulator.

Staining of 2nd dimension gel

The gel was removed and washed with dd H2O twice and then stained with 20 ml of Pierce silver stain. Stain the gel till spots become visible.

4.3 RESULTS:

To identify the population of goat spermatozoa that are chemotactically responsive to follicular fluid the dynamic response of washed and capacitated spermatozoa was investigated. The data obtained with different dilutions of follicular fluid (mature) is presented in figure 4.1(a). The chemotaxis assay was performed in assay plates designed locally in the laboratory as well as by the corning co-star transwell plates and the data are provided side by side for comparison purposes (figure 4.1 a & b). By both the procedures the results obtained are analogous. From the

data, it is abundantly clear that both the washed and capacitated populations of sperm showed a very similar pattern of response to follicular fluid. The response of the sperm at high concentrations of follicular fluid had no significant influence on sperm chemotaxis. At lower dilutions (10^{-5}, 10^{-6}), both washed and capacitated sperm showed chemotactic responses. However, the number of chemotactically-responsive sperm was substantially higher in the capacitated batch. Further dilutions resulted in a decline in the sperm migration towards the follicular fluid.

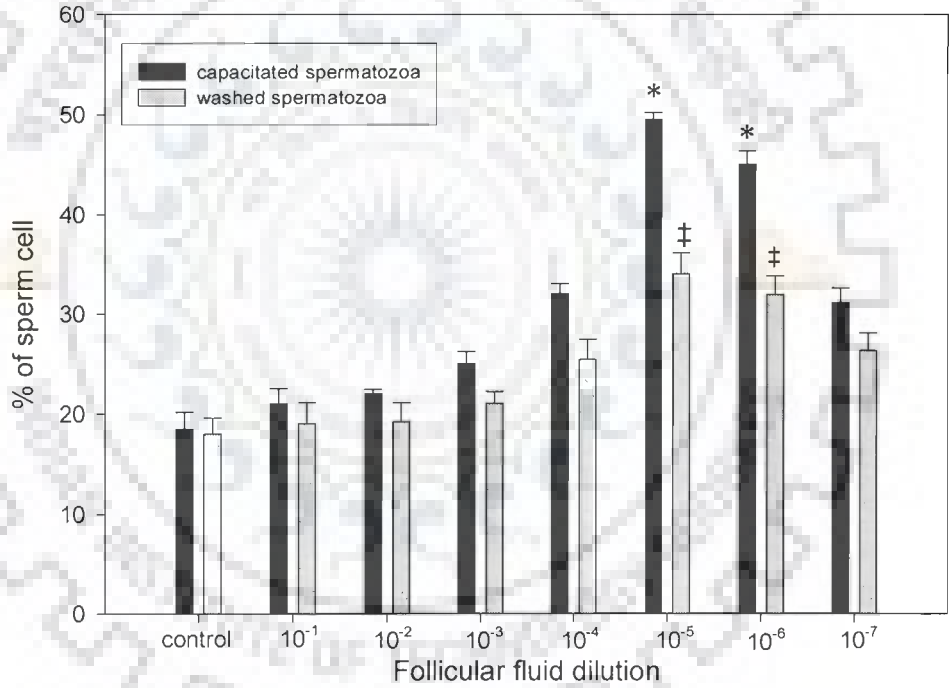


Figure 4.1(a): Chemotactic response of washed and capacitated sperm to follicular fluid collected from mature follicles. The total number of spermatozoa taken was considered as 100%. All data are presented as mean ± SEM. All comparisons are made with control (*$p<0.01$, Student's t-test).

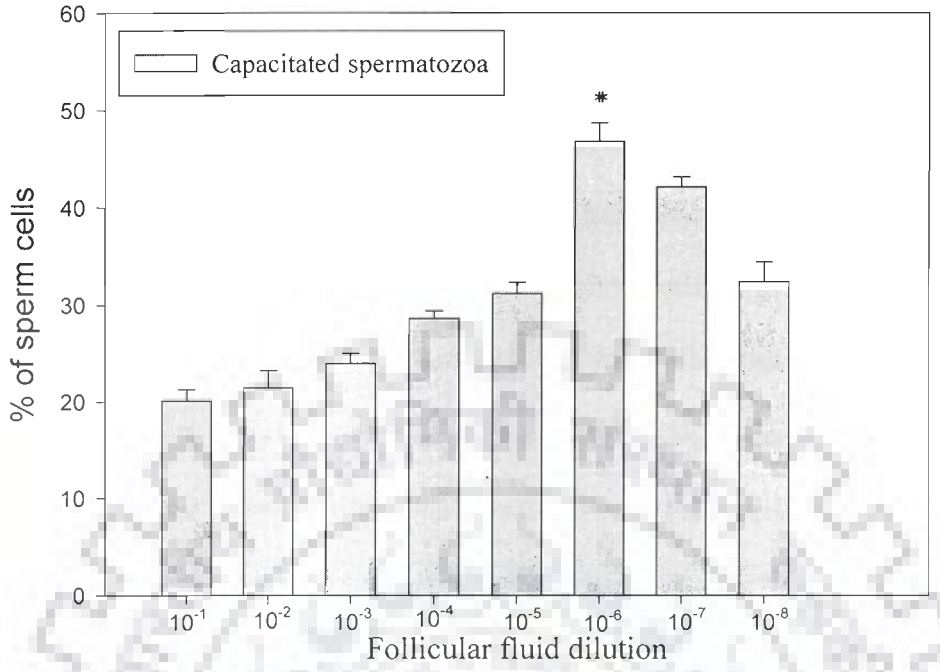


Figure 4.1(b): Chemotactic response of capacitated spermatozoa measured using transwell plates. The total number of spermatozoa taken was considered as 100%. All data are presented as mean ± SEM. All comparisons are made with control (*$p<0.01$, Student's t-test).

To further investigate the effect of follicular fluid on de-capacitated (acrosome–reacted) spermatozoa, a population of spermatozoa was first capacitated in BWW medium and then decapacitated with calcium ionophore A23187. The chemotactic response of these three classes of sperm was tested and the results are presented in table 4.1. The results suggest that chemotactic activity is manifested by the population of spermatozoa that are capacitated ($p<0.001$) where as de-capacitated sperm lose their ability to respond to external stimuli once they are exposed to A23187. Since it became clear that the capacitated sperm showed a much higher chemotactic response than any other population of sperm only capacitated sperm were used in further experiments.

S.No.	Experiment	Capacitated cells (%)	Cells showing response to follicular fluid (%)
1	Washed sperm collected by swim- up protocol (Control)	2.4 ± 1.1	16.2 ± 2.4
2	Sperm capacitated in BWW medium	64.5 ± 4.2	52.1 ± 3.1*
3	Acrosome-reacted decapacitated sperm after treatment with Calcium ionophore A23187	-	12.5 ± 1.2

Table 4.1: Chemotactic response of normal, capacitated and decapacitated sperm to follicular fluid collected from mature follicles. All data are presented as mean ± SEM (n=3). All comparisons are made with the control group (*$p<0.001$, Student's t-test)

Follicular fluid from both mature and immature follicles was tested for their chemotactic potential and the results are summarized in figure 4.2. It is obvious from the results that the follicular fluid collected from immature follicles did not influence sperm chemotaxis at any of the several dilutions tested. Once again the results reiterate that goat sperm responds chemotactically only to follicular fluid derived from mature follicles at high dilutions.

To further investigate the possible relation between chemotaxis and chemokinesis an experiment was performed by slightly modifying the procedure used for evaluating the chemotactic effect of follicular fluid on spermatozoa using corning co-star transwell plates (pore size 12 μm). In the absence of a gradient, it was found that the percentage of spermatozoa cells that migrated into lower well was statistically significant ($p<0.01$) only at the highest concentration of follicular fluid (figure 4.3). At lower concentration of follicular fluid, the number of cells migrating into the lower wells was not substantially different from that of control.

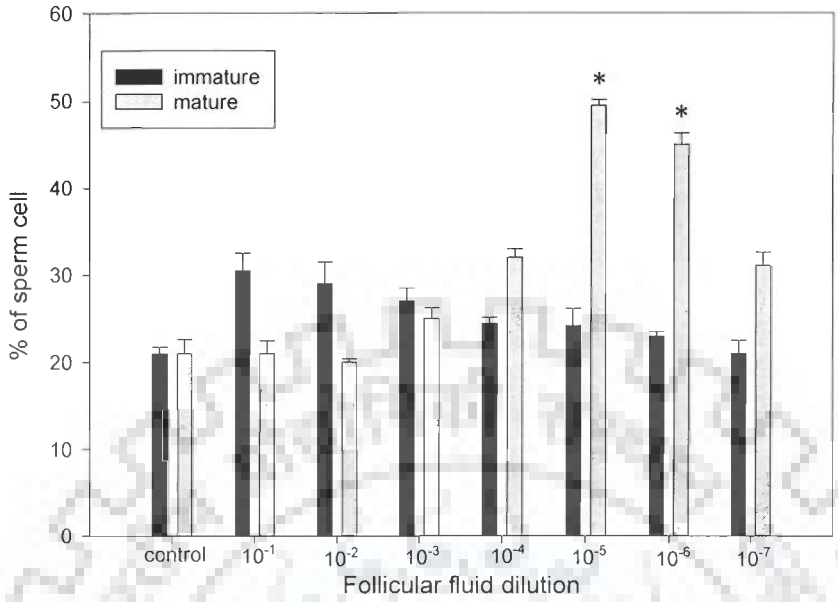


Figure 4.2: Chemotactic response of capacitated goat sperm to follicular fluid collected from mature and immature follicles. The original number of spermatozoa taken was considered as 100%. All data are presented as mean ± SEM (n=3) All comparisons are made with control (*p<0.01, Students t-test)

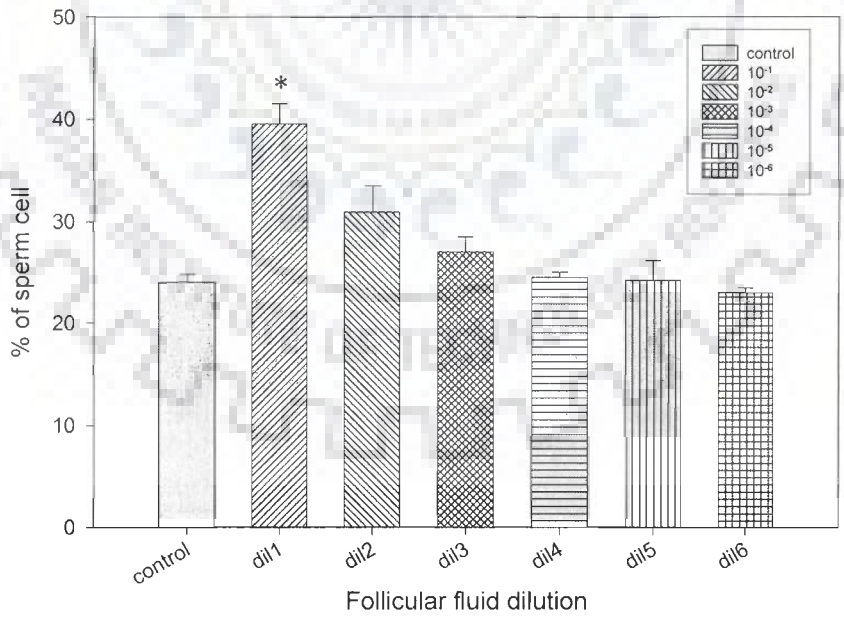


Figure 4.3: Chemokinetic response of goat sperm to follicular fluid. Total number of spermatozoa taken was considered as 100%. All data are presented as mean ± SEM and comparisons have been made with control. (*p<0.01, Student's t-test)

Identification and quantification of sex steroid hormones:

Follicular fluid was aspirated from goat ovaries of two different follicular sizes: large (mature: ≥4 mm in diameter) follicles and small (immature: ≤ 2 mm in diameter) follicles. Follicular fluid was assayed for major sex hormones progesterone, testosterone, estradiol and estrone (table 4.2). When the data of the follicular fluid collected from the two types of follicles were compared, the concentration of progesterone was significantly higher ($p<0.001$) in the follicular fluid collected from the mature follicles. At the same time the concentration of estradiol was substantially reduced ($p<0.001$). There was no change detected in the concentrations of testosterone and estrone.

Diameter of follicles	Number of follicles	FF concentration of sex steroid hormone (ng/mL)			
		Progesterone	Testosterone	Estrone	Estradiol
Immature (< 2mm)	10	67.8±10.4	17.7±3.9	12.7±2.9	127±18.4
Mature (> 4mm)	10	289±60.4*	21.8±8.4	13.3±1.2	14.2±1.6*

Table 4.2: Hormonal changes in follicular fluid collected from two different stage of follicular development. All values are Mean ± SEM (n=5) Comparisons have been made between the immature and mature groups (*$p< 0.001$, Student's t-test)

Migratory response of sperm to sex steroids:

The self fabricated apparatus with one central and four peripheral wells was used for this experiment. Sperm was loaded in the central well and the peripheral wells had a control and hormone at three different concentrations loaded in them. The relative migrations of sperm into

the peripheral wells are consolidated in figure 4.4. Of all the sex steroid hormones that were investigated, significant number of sperm migrated into wells containing progesterone particularly at the lower concentrations 0.1 μg/mL and 0.5 μg/mL ($p<0.01$). With regard to testosterone the results were just the opposite. The relative movement of sperm was towards the buffer rather than towards the hormone suggesting if any, some sort of a negative chemotaxis ($p<0.01$). Sperm migration was not influenced by estradiol and estrone.

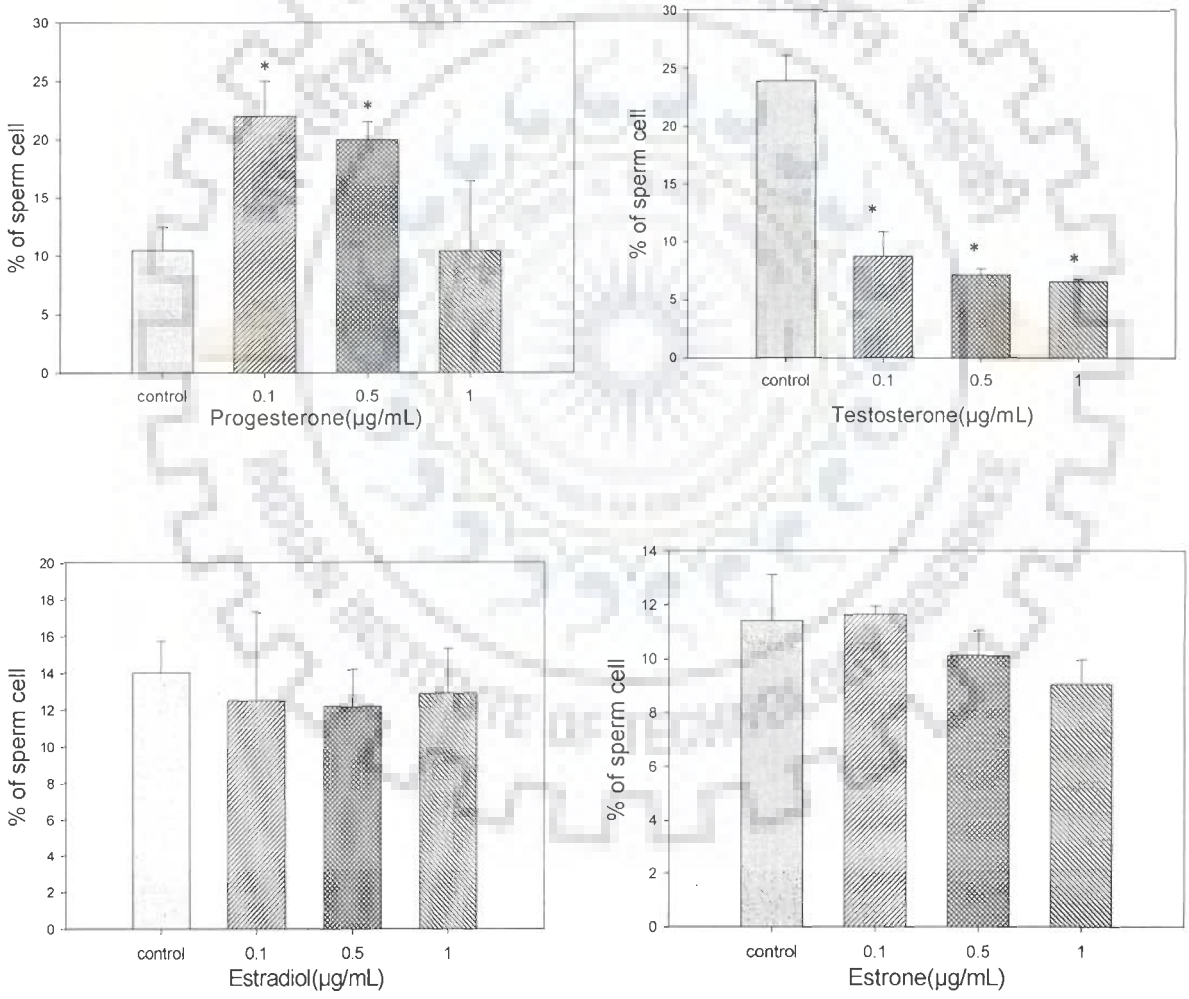


Figure 4.4: Effect of sex steroid hormones on the relative migration of goat sperm. All data are presented as mean ± SEM. All comparison are made with the respective controls (* $p<0.01$, Student's t-test).

Migratory response of Goat sperm to other hormones:

Investigations with hormones other than sex steroid were also carried to see their effect on the migratory behavior of sperm cells. The results are grouped in three categories those that produced a stimulatory response (figure 4.5), those that induced a negative response (figure 4.6) and those that did not influence the response (figure 4.7). Sperm was attracted to adrenalin at a concentration of 0.1μg/mL ($p<0.01$) with approximately 25% of spermatozoa migrated into the well. At higher concentration of adrenalin no significant migration took place. The other hormone that showed significant ($p<0.01$) increase in sperm cell migration as compared to control was oxytocin at 0.1 and 0.5 μg/mL. Once again, the effect was not significant at higher concentration. Calcitonin, on the other hand, showed a significantly ($p<0.01$) higher concentration of hormone (0.5 and 1 μg/mL) where as at lowest concentration (0.01 μg/mL) the result was not significantly different from that of control.

A statistically significant ($p<0.01$) decrease in migration spermatozoa was also recorded in wells containing hormone glucagon at 0.5 and 1 μg/mL concentrations. At the lower concentration of glucagon (0.1 μg/mL) the decrease in sperm migration was not statistically significant. A reduction in the migration of spermatozoa was observed with Insulin at all the three concentration 0.1, 0.5 and 1 μg/mL tested ($p<0.01$).

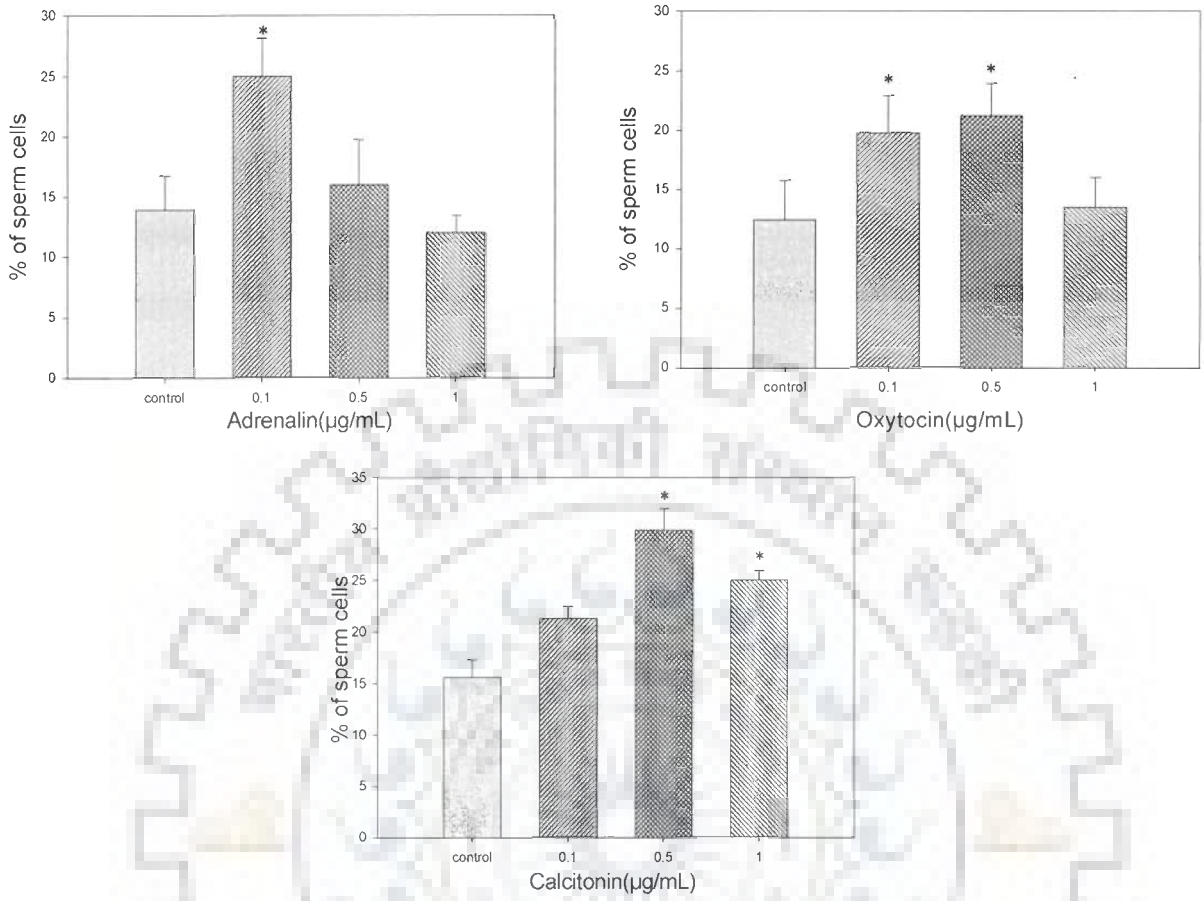


Figure 4.5: Effect of hormones that had a positive impact on goat sperm migration *in vitro*. All data are presented as mean ± SEM (n=3). All comparisons are made with the respective controls (*p<0.01, Student's t-test).

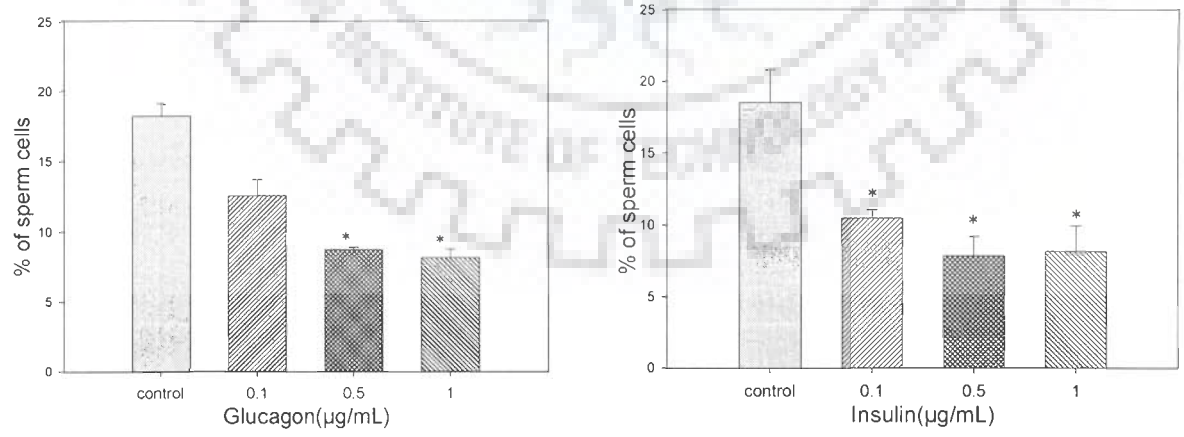


Figure 4.6: Effect of hormones having negative impact on goat sperm migration *in vitro*. All data are presented as mean ± SEM (n=3). All comparisons are with the respective controls. (*p<0.01 Student's t-test).

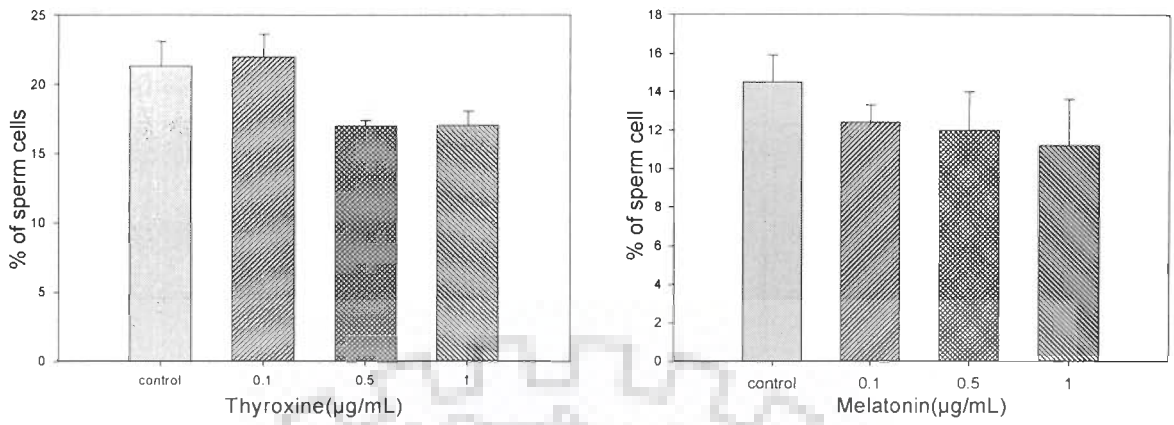


Figure 4.7: Effect of hormones that did not influence goat sperm migration *in-vitro*. All data are presented as mean ± SEM (n=3).

Thyroxine and melatonin did not have any significant effect on the migration pattern of goat spermatozoa as compared to control.

Identification of free amino acid in follicular fluid:

Qualitative analysis of follicular fluid obtained from mature follicles was performed to determine whether follicular fluid contains free amino acids. For this the deproteinized sample was derivatized with phenylisothiocyanate and then analyzed by HPLC. In all eighteen major peaks were obtained from derivatized follicular fluid sample. As shown in figure 4.8 these peaks when matched with that of chromatogram obtained from standard (sigma) (PITC derivatized form) showed the presence of aspartate, glutamate, asparagine, glutamine, serine, threonine, glycine, alanine, arginine, valine, methionine, isoleucine, leucine, tryptophan, cystine, lysine, histidine and tyrosine. In addition to these, some minor peaks were also seen which did not match with standard. All major amino acid were identified except proline, phenylalanine and taurine.

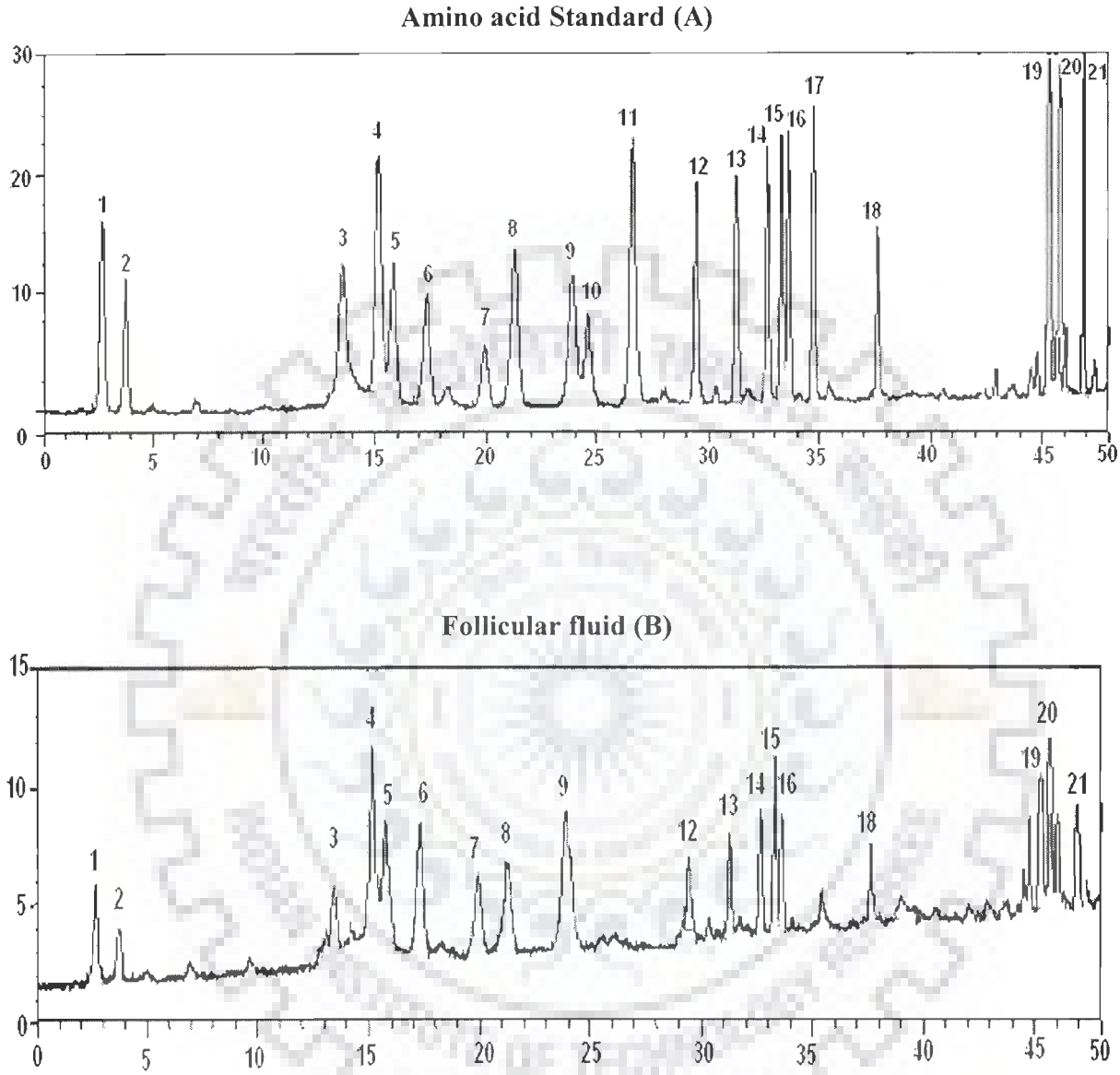


Figure 4.8: HPLC separation of PITC coupled free amino acids. Chromatograms of PITC derivatives of the amino acid standard mixture (A) and follicular fluid sample from mature goat ovary (B) are shown. The amino acids corresponding to the peaks are: 1, aspartate; 2, glutamate; 3, asparagine; 4, glutamine; 5, serine; 6, threonine; 7, glycine; 8, alanine; 9, arginine; 10, taurine; 11, proline; 12, valine; 13, methionine; 14, isoleucine; 15, leucine; 16, tryptophan; 17, phenylalanine; 18, cystine; 19, lysine; 20, histidine; 21, tyrosine.

Migratory response of sperm to free amino acids:

Beside hormones, free amino acids were also tested for its effect on *in-vitro* sperm migration. In general, no significant change in sperm migration pattern was observed with most of the free amino acids that were investigated. However, glutamic acid at 0.01 μg/mL concentration showed significant ($p<0.01$) increase in the migration of spermatozoa as compared to control. Wells containing higher concentration (0.1 and 1μg/mL) of glutamic acid did not cause any significant change in sperm migration (figure 4.9). Cystine at a concentration of 1μg/mL also caused increase in migration of sperm migration ($p<0.01$) as compared to control. The lower two concentrations (0.01 and 0.1 μg/mL) did not have any significant effect.

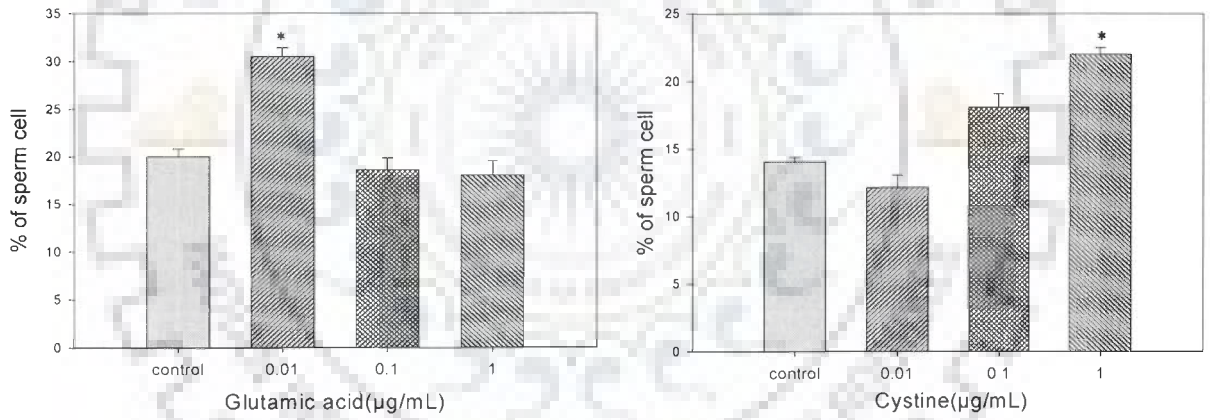


Figure 4.9: Effect of free amino acids having positive impact on goat sperm migration *in-vitro*. All data are presented as mean ± SEM (n=3) Comparisons are made with control.(* $p<0.01$, Student's t-test).

A similar pattern in the sperm migratory response was seen with methionine, glycine and alanine (figure 4.10). When these three amino acids were tested, sperm migration was retarded at the 1μg/mL concentration as compared to control ($p<0.01$). The other concentrations of amino acids did not influence sperm migration.

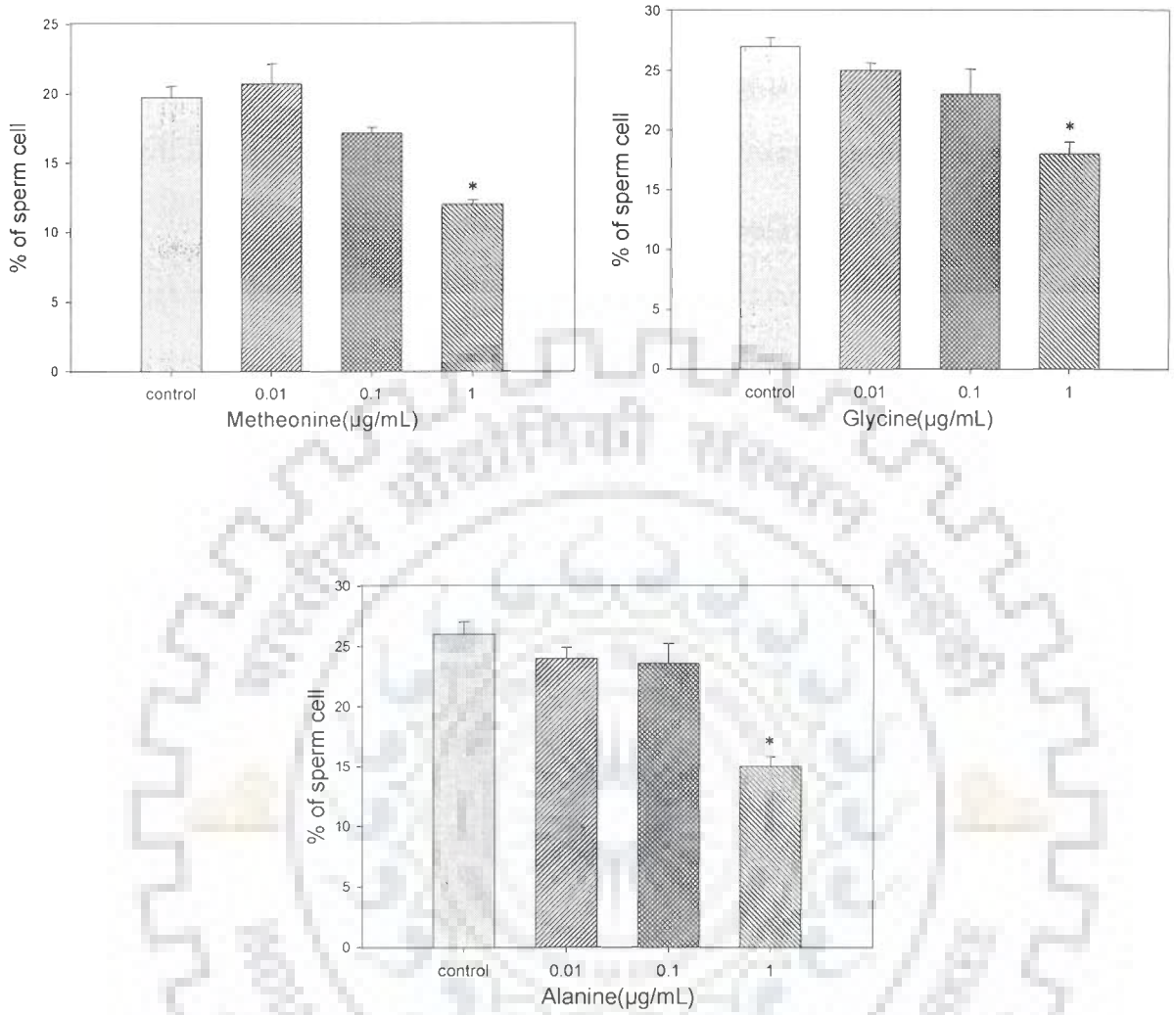
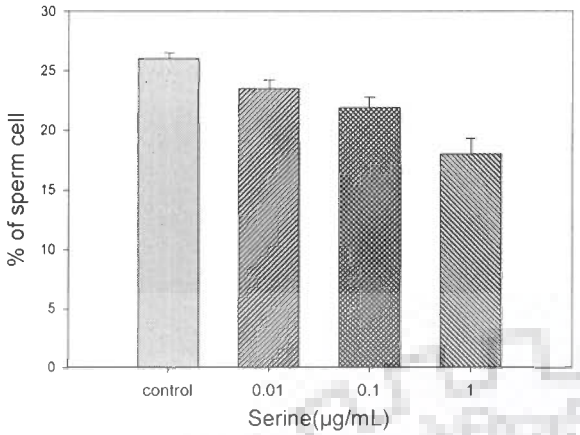
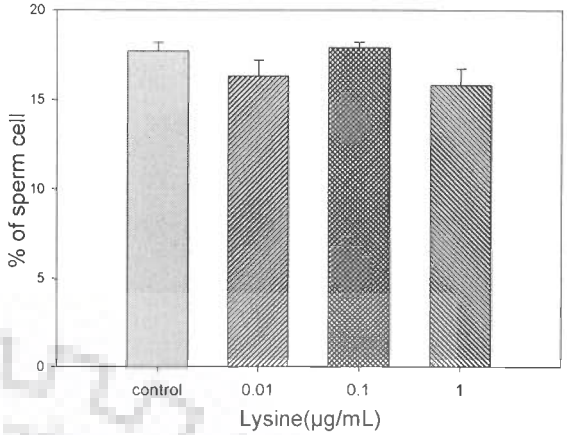
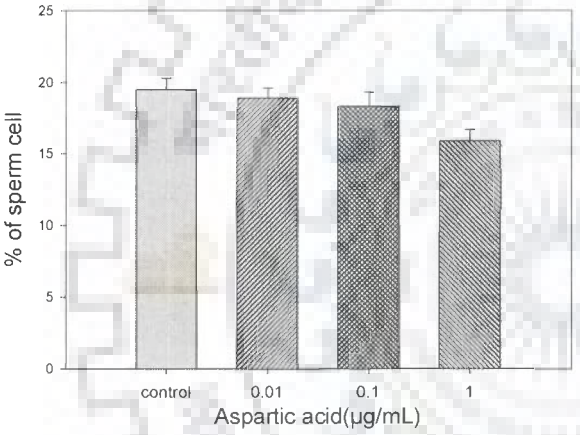


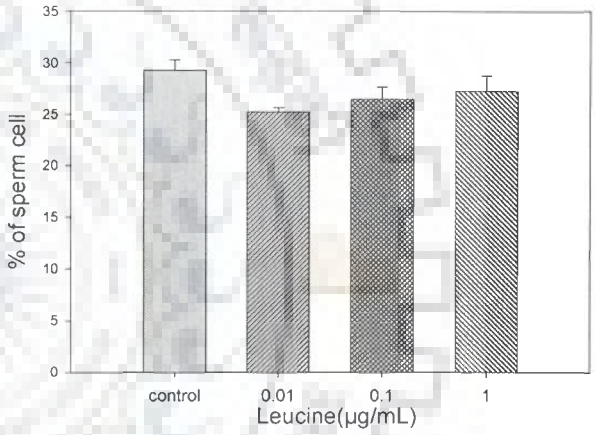
Figure 4.10: Effect of free amino acids having negative impact on goat sperm migration *in-vitro*. All data are presented as mean ± SEM (n=3). All comparisons are made with control (* $p<0.01$, Student's t-test).

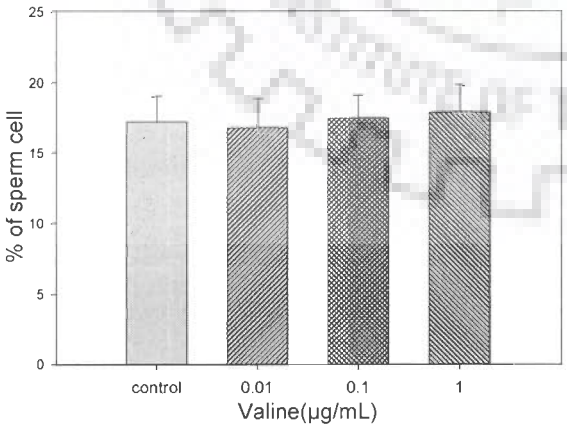
All other amino acids that were tested (serine, lysine, aspartic acid, leucine, valine, isoleucine, tryptophan histidine, tyrosine and arginine) did not show any significant influence on the migration of spermatozoa irrespective of the concentration used (figure 4.11).

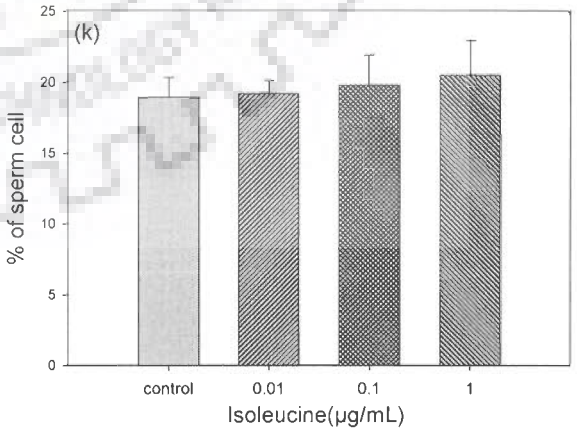

% of sperm cell
30
25
20
15
10
5
0
control
0.01
0.1
1
Serine(μg/mL)


% of sperm cell
20
15
10
5
0
control
0.01
0.1
1
Lysine(μg/mL)


% of sperm cell
25
20
15
10
5
0
control
0.01
0.1
1
Aspartic acid(μg/mL)


% of sperm cell
35
30
25
20
15
10
5
0
control
0.01
0.1
1
Leucine(μg/mL)


% of sperm cell
25
20
15
10
5
0
control
0.01
0.1
1
Valine(μg/mL)


(k)
% of sperm cell
25
20
15
10
5
0
control
0.01
0.1
1
Isoleucine(μg/mL)

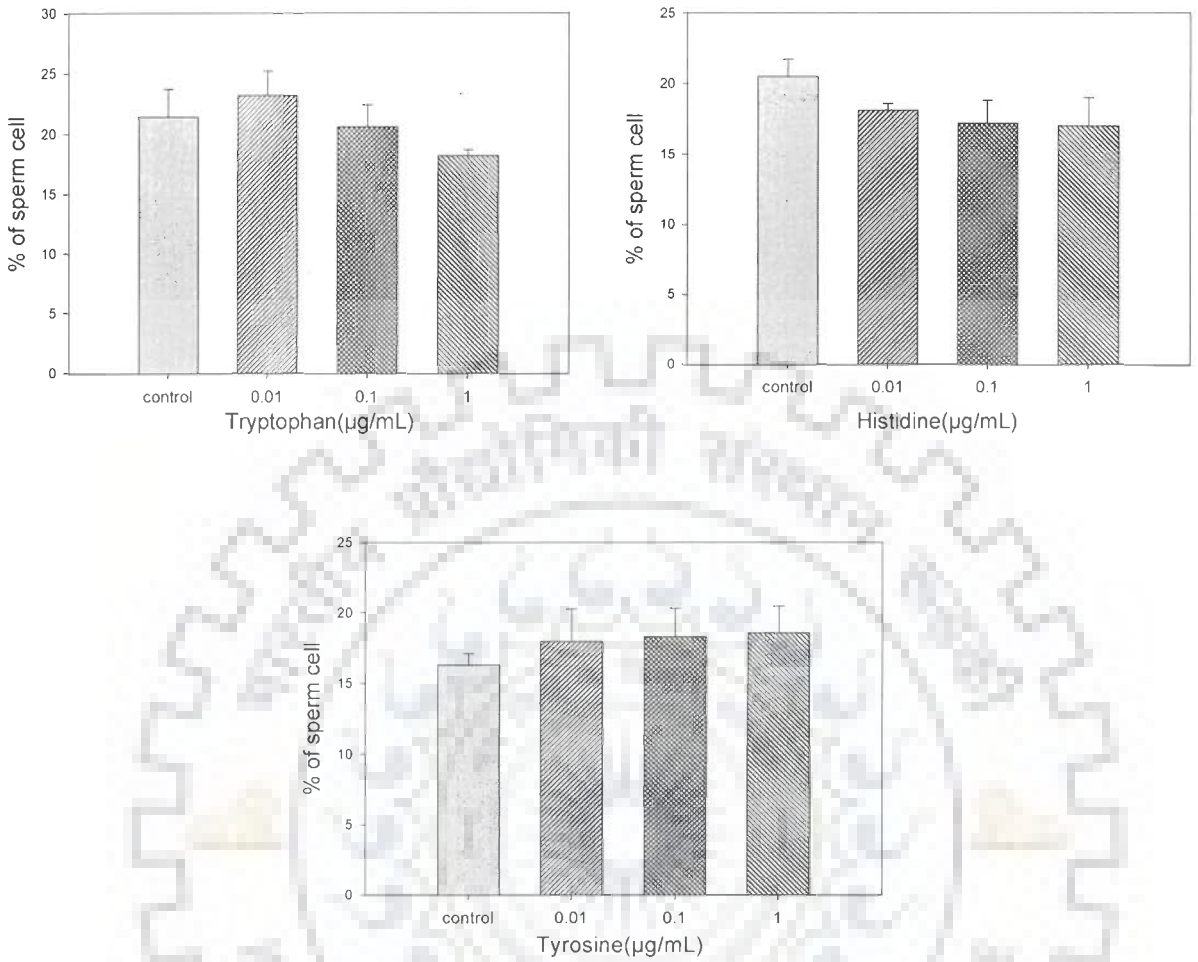


Figure 4.11: Effect of free amino acids showing no effect on goat sperm migration *in-vitro*. All data are presented as mean ± SEM (n=3). All comparisons are made with the respective controls.

Migratory response of goat sperm to simple sugars:

Sugars mainly monosaccharide were also investigated for their effect on migration of goat spermatozoa, the results of which are consolidated in figure 4.12. Increase in the migration of spermatozoa was observed in case of glucose and fructose. Even with these two sugars the results were statistically significant only at specific concentrations: 1 µg/mL of glucose 0.1 µg/mL of fructose ($p<0.05$). None of the other sugars (mannose, sorbitol and ascorbic acid) showed any significant influence on the migration of spermatozoa as compared to control.

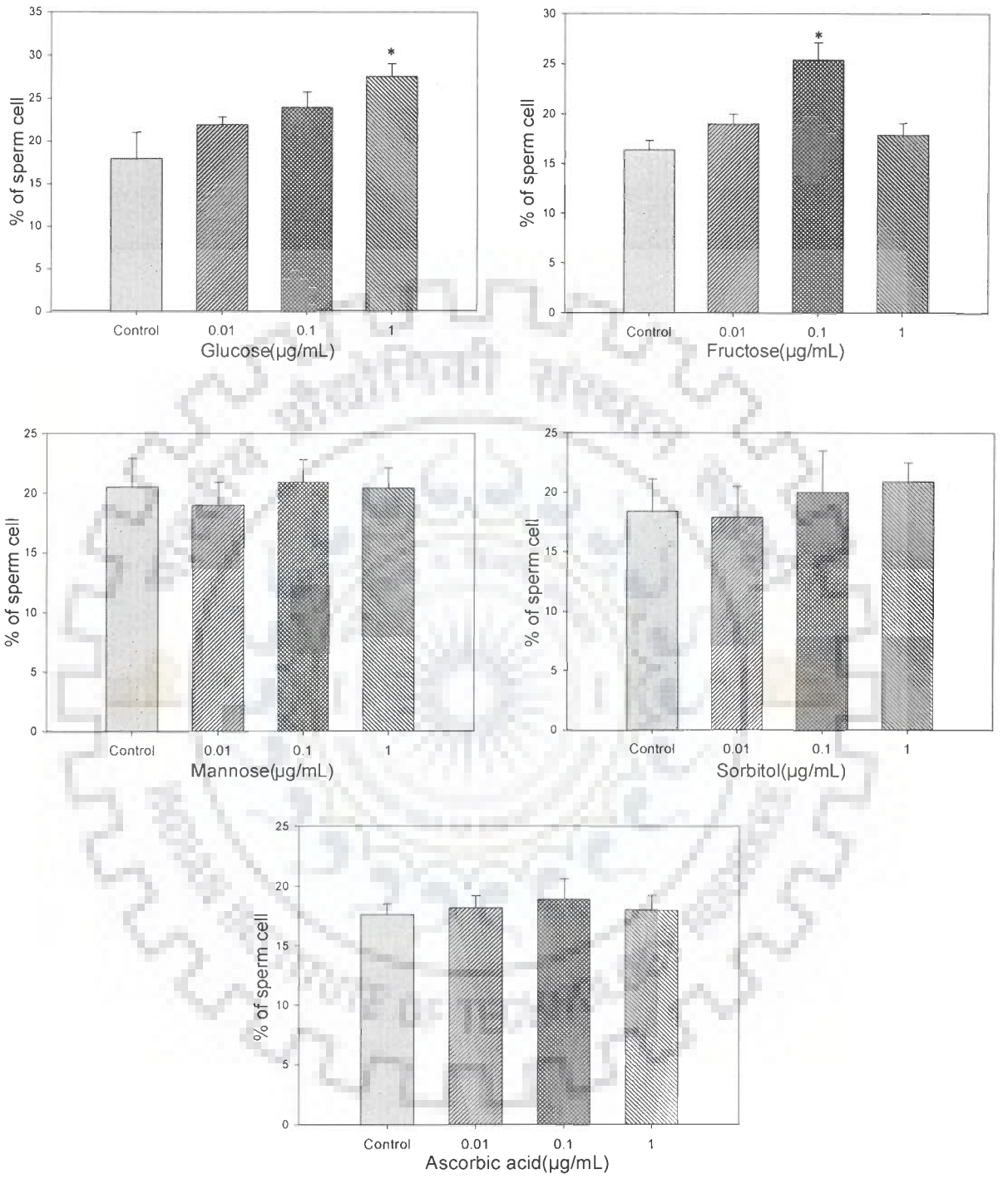


Figure 4.12: Effect of simple sugars on goat sperm migration *in-vitro*. All data are presented as mean ± SEM (n=3). All comparisons are made with respective controls. (*p<0.05, Student's t-test).

Proteomic analysis of follicular fluid:

Follicular fluid extracted from two different stage of development (immature ≤ 2mm and mature follicles ≥ 4mm) was analyzed for its total protein using 2D electrophoresis (figure 4.13 and 4.14). Eighty seven spots were detected on 2D gel of mature follicular fluid by using pH 4–7 IPG strips and analysis by PD Quest software. Two patterns of protein expression were observed which could be directly correlated with oocyte development. From these two different expression patterns, at least 7 varied protein spots (marked in small circles) were found in mature follicular fluid that were not detected follicular fluid of immature follicles. Three spots (marked by oval) with a mass of around 14 kDa were differentially expressed.

4.4 DISCUSSION:

While developing and testing a method to monitor the migratory behavior of sperm *in vitro*, it is essential to identify and employ an enriched population of chemotactically responsive spermatozoa. Mammalian spermatozoa cannot penetrate the egg immediately after ejaculation until they undergo a period of incubation either in the female genital tract or *in vitro* acquire the ability to do so, a process called capacitation (Yanagimachi 1994). During this incubation period, the sperm cells undergo several sequential molecular processes which are essential for fertilization. In the present study the results indicate that in goat, as in other mammals, only capacitated spermatozoa are chemotactically active. When assayed for chemotaxis a significant percentage of capacitated spermatozoa migrated into the well containing diluted follicular fluid (10^{-5}). This was 39.6% higher, as compared to sperm cells that were completely decapacitated using the ionophore A23187. These results indicate a possible role of capacitation in mediating

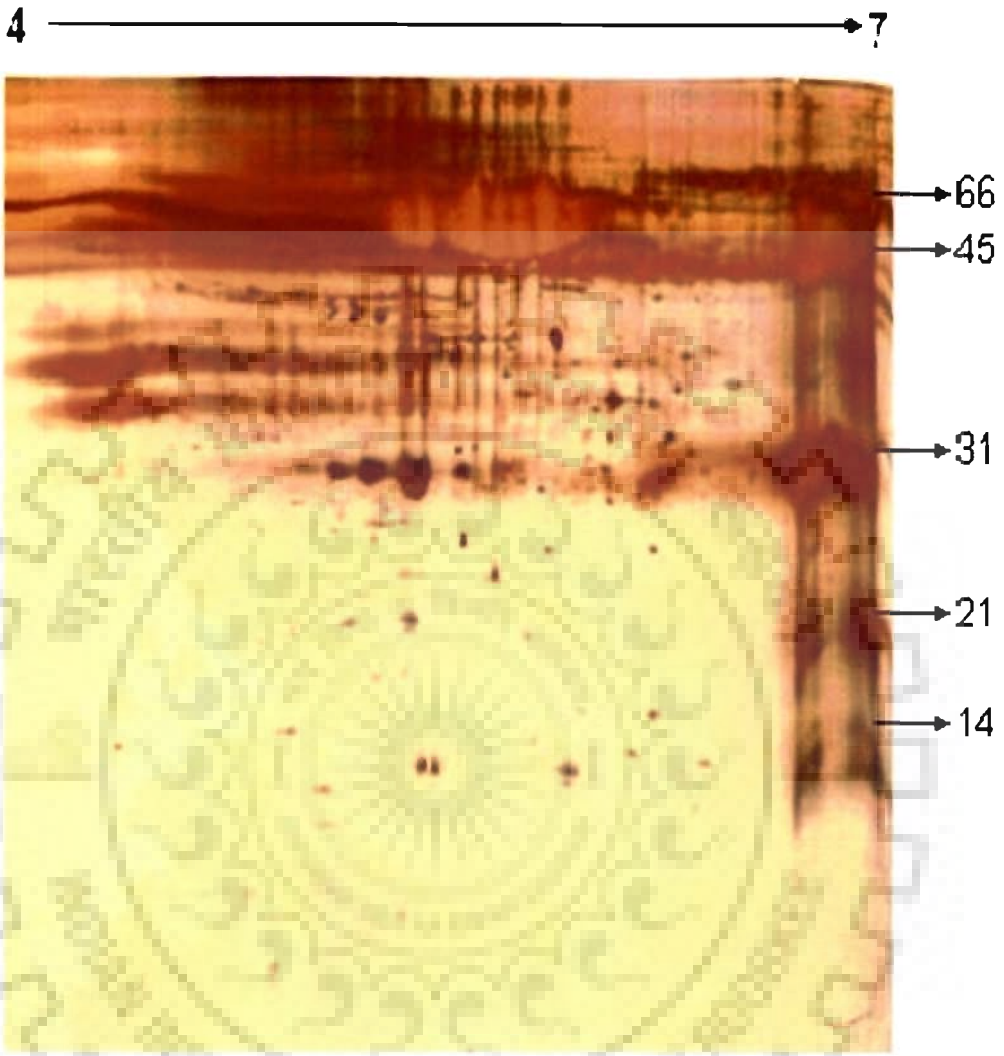


Figure 4.13: 2D Electrophoretic profile of follicular fluid extracted from immature follicles. (Molecular weight marker kDa)

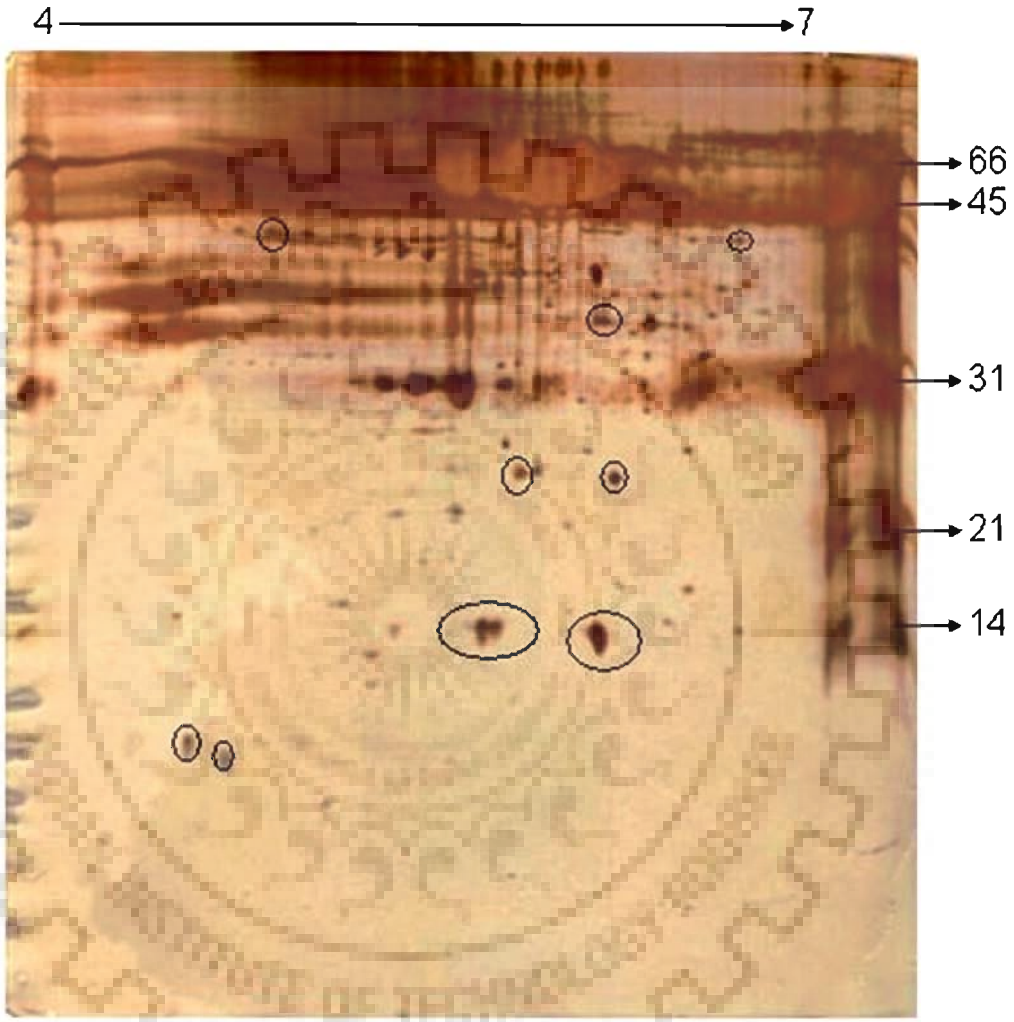


Figure 4.14: **2D Electrophoretic profile of follicular fluid extracted from mature follicles.** Spots marked show differential expression as compared to immature follicular fluid. (Molecular weight marker kDa)

chemotatic response and support the notion that goat spermatozoa acquire their chemotactic responsive as a part of the capacitation process and loses it when the capacitated stage ends.

Experiments with human spermatozoa have convincingly shown that chemotactic responsiveness to FF is acquired in parallel to or as part of the capacitation process (Tacconis *et al.,* 2001). Both the chemotactic responsiveness and the capacitated state are transient and only a small proportion of sperm are chemotactically active at any one point of time. Perhaps the continuous replacement of the population of capacitated sperm ensures that sperm are available in the female reproductive tract for prolonged periods of time for fertilizing the egg (Eisenbach, 1999). Rabbit spermatozoa, like human spermatozoa are chemotactically responsive to FF factor(s) and acquire this responsiveness as part of the capacitation process (Fabro *et al.,* 2002). These authors also analyzed acrosome-reacted sperm by a directionality-based chemotactic assay and provided evidence for the total loss of chemotactic response (Fabro *et al.,* 2002).

This is of physiological significance since in goat where fertilization is internal, considerable fraction of the spermatozoa ejaculated into the female reproductive tract is retained with reduced motility at storage sites. When ovulation occurs, some of the spermatozoa resume high motility and travel the distance between the storage site and the fertilization site within minutes (Harper 1973; Ralt *et al.,* 1991). A very similar view has been expressed in humans, where it has been proposed that chemotactic activity is a dynamic process and at a given time, a different population of sperm undergoes capacitation (Eisenbach and Ralt 1992; Eisenbach *et al.,* 1994; Cohen-Dayag *et al.,* 1995). The role of sperm chemotaxis combined with enhanced motility may be to select capacitated sperm and bring them to the egg (Cohen-Dayag *et al.,* 1994). Therefore only capacitated sperm were used in subsequent *in vitro* assays to study sperm migration.

Using a self fabricated apparatus and a commercially available corning transwell assay system it has been demonstrated in this study that follicular fluid plays a vital role in the activation of goat spermatozoa *in vitro*. The effect of isolated follicular and oviductal fluids on the dynamic parameters and chemotaxis of spermatozoa has been examined before in mice. In these investigations it has been convincingly shown that the motility and directionality of sperm are influenced by these fluids (Oliveira *et al.,* 1999). Under *in-vivo* conditions the role of follicular fluid may be to increase the population of active fractions of spermatozoa and to increase the motility of spermatozoa (both chemotactic and chemokinetic). A general criterion which distinguishes chemotaxis from chemokinesis is the presence of an attractant gradient, a prerequisite for the occurrence of chemotaxis but not chemokinesis. Both chemotactic and chemokinetic activities were observed with goat spermatozoa and could be attributed to factor(s) contained in the follicular fluid of mature follicles. The complex nature of the follicular fluid makes one believe that the migratory activity of goat sperm is not governed by the existence of any one chemical substance and that many factors could contribute to the same.

It was anticipated that the composition of the follicular fluid is not constant during the development of the follicle. A marked change in the pattern of migration was observed when the data of immature and mature follicular fluids were compared. The difference between the two groups was distinctly high at 10^{-5} and 10^{-6} dilution of follicular fluid indicating a chemotactic response of sperm to mature follicular fluid. These results are in accordance with the results obtained by other workers in studies conducted on human sperm (Ralt *et al.,* 1994), mice (Oliveria *et al.,* 1999) and rabbit (Fabro *et al.,* 2002). The migration of spermatozoa is perhaps related to the fertilizability of the egg as seen from the differential accumulation of spermatozoa in wells containing follicular fluid from mature and immature follicles. This difference in the

sperm attracting ability of follicular fluid from two different stages of follicular development may be due to factors released at the time of ovulation. It is generally accepted that the preovulatory follicles contain substances that could act as chemoattractants (Ralt *et al.,* 1991). In fact, it has been suggested that the origin of these chemical substances could be the mature oocyte and the surrounding cumulus cells (Sun *et al.,* 2005). Receptors expressed on the sperm surface could be intricately involved and facilitate sperm chemotaxis (Adler 1973; Zigmond 1978; Devreotes *et al.,* 1988).

Fluctuating level of hormones during follicular development may also have a vital role to play. Of the four sex hormones assayed, the level of progesterone alone was found to be increased during follicular development. *In vitro* study with goat sperm confirmed a response to progesterone suggestive of both chemokinesis and chemotaxis. On a previous occasion the response of human spermatozoa to follicular fluid, progesterone, estradiol and mifepristone was investigated by the Transwell system (Wang *et al.,* 2001). It was found that follicular fluid and progesterone induced the accumulation of spermatozoa while estradiol had no effect. Mifepristone abolished the progesterone induced accumulation of spermatozoa. Whether progesterone serves as a chemoattractant or induces hyperactivation of sperm has been a subject of much debate (Jaiswal *et al.,* 1999). Nevertheless, in the past when several steroids were tested in dose response curves, progesterone was shown to have the strongest ability to influence sperm motility (Vadillo *et el.,* 1994; Villanueva-Diaz *et al.,* 1995). Although recent findings have provided evidence for the presence of progesterone receptors on the sperm membranes, the action could only be non-genomic because of the transcriptional inactivity of sperm (Correia *et al.,* 2007).

Immunohistochemical investigations have shown that progesterone is localized inside the cumulus cells and in this light it has been suggested that the cumulus oophorus could be a potential place for sperm chemotaxis mediated by progesterone *in vivo* (Teves *et al.*, 2006). It has been proposed that thermotaxis is yet another essential mechanism in mammalian species guiding spermatozoa released from the cooler reservoir sites towards the warmer fertilization sites (Bahat and Eisenbach, 2006). The thermogenic nature of progesterone (Kobayashi *et al.*, 2000) makes one to believe that a thermo gradient is generated by progesterone within the female reproductive tract. This view adds an interesting angle to the role of progesterone in leading sperm to the site of fertilization.

Testosterone on the other hand had a negative impact on direction-based sperm motility. When three concentrations of testosterone and BWW medium (control) were tested for chemotaxis in a single assembly, the sperm preferentially moved into the BWW medium. Also the percentage of sperm migration was inversely related to the concentration of testosterone in the wells. Such a dose-dependent inhibition of motility has been described in experiments with ram spermatozoa and has been attributed to the uncoupling of fructolytic energy production in the sperm tail by testosterone (Ailenberg *et al.*, 1984)

Regarding the other hormones investigated, adrenaline, oxytocin and calcitonin had a positive impact while insulin and glucogon had a negative impact on sperm migration. The sensitivity of the goat sperm to these hormones was however not the same since the response to these hormones occurred at very different concentrations. Until now no receptors/ binding sites have been reported for these hormones on the sperm surface. In addition, pathways involved in signal transduction are unknown. Therefore, at this point of time it is difficult to comment on the mechanism by which these hormones control the motility characteristics of sperm.

Of the several monosaccharides tested in the present study, only fructose and glucose were able to influence the migration of goat sperm. These results are not surprising since the two sugars are the major components of semen and serve as the source of energy for sperm during their journey to meet with the egg. In the dog increased sensitivity of freshly ejaculated sperm to fructose and glucose is also documented (Rigau *et al.,* 2001). The effect of artificial medium containing various concentrations of sugars on washed human sperm motility also produced similar results with glucose and fructose alone stimulating sperm motility (Nagai *et al.,* 1982). In another study the existence of a strong correlation between the percentage of motile spermatozoa and fructose levels in normozoospermia has been demonstrated (Patel *et al.,* 1988). Taken together, it is most likely that fructose and glucose serve as chemoattactants. The sperm are known to carry enzymes of the glycolytic pathway (Harrison 1971; Gandhi and Anand 1982; Miki, 2007) and therefore it is possible that sperm are attracted to these two sugars in order to utilize them as substrates for producing energy. This is supported by the fact that addition of fructose or glucose to sugar free medium increased the ATP concentration and motility of human sperm (Williams and Ford, 2001).

The HPLC analysis suggests that goat follicular fluid is a rich source of amino acids. The presence of as many as 19 amino acids was report in the mature group of goat follicular fluid. This is not unexpected since amino acids are building blocks of proteins and during maturation of the follicles protein synthesis occurs in abundance. Amino acid composition of follicular fluid at different developmental stages has been studied in pigs (Chang *et al.,* 1976), cows (Orsi *et al.,* 2005) mares (Gerard *et al.,* 2002; Harris *et al.,* 2005) and humans (Velazquez *et al.,* 1977). In each case both qualitative and quantitative variations were seen. Taurine, glycine, alanine, glutamine and glutamate were the major amino acids detected in murine follicular fluid.

Irrespective of follicle size, GLY, GLU, ALA, GLN, and PRO were the most abundant amino acids in pig follicular fluid (pFF). However, the data obtained on goat follicular fluid can only suggest that there is a large pool of free amino acids available from which a variety of proteins can be synthesized during follicular development.

The follicular fluid is a dynamically changing milieu that possibly supports the growth of the follicle during maturation. The 2-D electrophoretic protein profile obtained in the immature and mature follicular fluids supports this contention. A careful analysis reveals that during follicular maturation a large number of proteins are added or differentially expressed. The origin of these proteins could be ovarian granulose cells and oocytes (Kim *et al.,* 1992; 1993). Alternatively, they could be synthesized elsewhere and transported to the follicular fluid through the blood stream. Several investigators have reported that serum and follicular fluid share a large number of proteins (McGaughey, 1975). Specific sperm chemoattractants in the follicular fluid have not been identified. However, cytokines and growth factors have been described as chemoattractants. In an *in vitro* model it has been demonstrated that transforming growth factors (TGF) contained in follicular fluid influences the chemotactic behavior of mouse sperm (Sliwa, 2003). One of the chemokines present in follicular fluid, RANTES (Regulated on Activation Normal T Expressed and Secreted Chemokine) was found to have profound chemotactic activity on human sperm. Anti-RANTES rabbit IgG partially neutralized the chemotactic effect of follicular fluid. (Isobe *et al.,* 2002). A sperm motion stimulator was isolated and characterized from the human follicular fluid (Fetterolf *et al.,* 1994). It is possible many more biologically active proteins exist whose functional significance is yet to be established. More detailed investigations are needed to improve our understanding of direction-based sperm migration.

Chapter-5

CHAPTER 5: ISOLATION, PURIFICATION, CHARACTERIZATION AND PHYSIOLOGICAL SIGNIFICANCE OF MOTILITY STIMULATOR PROTEIN FROM GOAT FOLLICULAR FLUID.

5.1 INTRODUCTION:

Follicular fluid is a complex fluid which contains the secretions of the egg and its surrounding cells as well as substances which diffuse from blood plasma. It is believed to contain a large number of bioactive substances (Manor, 1994; Ralt *et al.,* 1994). A variety of substances such as proteins, metal ions, enzymes, steroids, hormones and proteoglycans are believed to be present in follicular fluid (Yen, 1986). Follicular fluid has been shown to affect sperm motility (Chao *et al.,* 1991), capacitation reaction (Gwatkin and Anderson, 1969) and acrosome reaction (Suarez *et al.,* 1986). Since follicular fluid is released into the fallopian tube following ovulation therefore it can be hypothesize that follicular fluid might play a role in the fertilization process in vivo.

Mammalian spermatozoa just after its formation in testis are immotile and incapable of fertilizing ova. The male gametes undergo a maturation process during transit through epididymis when they acquire the forward motility and the fertility potential. The molecular basis of the initiation of flagellar motility in epididymis is not well-understood (Majumder *et al.,* 1999). Hoskins *et al.,* (1978) have suggested that elevated level of intra-sperm c-AMP and exogenous forward motility protein derived from the epididymal plasma are essential for the initiation of sperm flagellar motility during its transit from the epididymis. Other important factors such as extracellular bicarbonate and pH have been shown to affect the flagellar motility (Kann and Serres, 1980; Pinto *et al.,* 1984; Cornwall *et al.,* 1986; Jaiswal and Majumder, 1996a, b). Ralt *et al.,* (1991) have shown that one of the low molecular weight protein components is

responsible for the chemoattracting activity manifested by mouse follicular fluid. Similarly Gaur *et al.*, 1975 partially purified a small molecular weight factor (dialyzable) from human seminal plasma that promotes fertility in mouse sperm. Although sperm attracting peptides from invertebrate eggs have been isolated and well characterized (Suzuki, 1995; Miller, 1985) for example Resact isolated from the sea urchins egg jelly. This peptide is highly potent and binds to an unidentified receptor on the sperm flagellum and mediated sperm movement towards the egg (Shimomura *et al.*, 1986; Singh *et al.*, 1988).

Much progress has been made in understanding sperm motility initiating factors or chemoattractant and its components over the past few years. Several studies have reported that follicular fluid exhibits sperm attracting activity which can be co-related to fertilization success (Ralt *et al.*, 1991; Eisenbach *et al.*, 1999), other than follicular fluid blood serum has also been reported to be a rich source of factors that can stimulate sperm motility (Yanagimachi, 1970).In the present investigation we report purification and characterization of sperm motility stimulator from goat follicular fluid to apparent homogeneity. The motility stimulator was further checked for its cross species motility stimulating activity using CASA.

5.2 MATERIALS AND METHODS:

5.2.1 Chemicals:

All the chemicals used were procured from Hi-media, SRL India, Qualigen and Merck. Column for protein purification was procured from GE India, chemicals for isoelectric focusing were obtained form Pharmacia fine chemicals, SDS markers, protein estimation kit were purchased from Bangalore Genie, PVDF membrane was obtained from Millipore and Trypsin sequencing grade from promega.

5.2.2 Tissue Collection:

All procedures and experiments involving animals used in investigations have the approval of the Institutional Animal Ethics Committee. Goat testis along with the epididymis (100 ± 10 g) of mature animals was purchased from the local abattoir from time to time and brought to the laboratory in ice within one hour of slaughter. Spermatozoa from testis were collected as mentioned in section 3.2.2 using method described by (Tulsiani *et al.,* 1989, 1992).

5.2.3 Spermatozoa Preparation and Counting:

The sperm collected from several animals was pooled and the live sperm population was enriched by the swim up protocol (details section 3.2.3). The total sperm count was done using hemocytometer (details section 3.2.3) and the sperm concentration was adjusted to obtain the requisite number of cells/ml by suspending the pelleted spermatozoa in desired volume of BWW media (excluding $CaCl_2$) for further assay.

5.2.4 Preparation of Human Spermatozoa for CASA:

Human semen sample were obtained from 3 healthy male volunteers. All subject abstained from sexual activity for 48 hours before producing sample. The samples were obtained by masturbation into a dry wide-mouthed sterile plastic container. The samples were allowed to liquefy at room temperature for 20-25 min before routine semen analysis was performed according to the world health organization (WHO, 1992) criteria. The spermatozoa were prepared for the study by a swim-up technique from liquefied semen. Each sample was divided into 1 mL aliquots and gently over-layered by an equal volume of Ham's-10 media (pH-7.2) in a 15 mL centrifuge tube. The tubes were kept inclined at 45° and incubated at 37±1°C for 30 min. After incubation the supernatant was removed taking care not to disturb the seminal fluid zone underneath it. The supernatant was collected in a 15 mL sterile centrifuge tube. This was then

centrifuged at 200 x g for 10 min. Following this, the supernatant was discarded and the pellet was resuspended in Ham's-10 media (pH-7.4) to give a final concentration of 10-40 $x10^6$ cells/mL.

5.2.5 Ovary Collection and Processing of Follicular Fluid:

Adult goats in good health with normal reproductive tract after slaughter were used for this study. Follicular fluid was collected from ovaries as mentioned in section 4.3. Follicular fluid (at least 1 mL per aliquot) was centrifuged (10,000 × *g*, 7 min) and the supernatant was collected for analysis. Samples were frozen immediately after centrifugation and stored at -80°C for further assay. Assays were conducted within one week of sample collection.

5.2.6 Purification of Motility Stimulator Protein:

Follicular fluid prepared as above was fractionated to 0-80% saturation, using ammonium sulfate. Each fraction obtained was centrifuged for 15 min at 15000 x g and the pellet was dissolved in tris-buffer (50 mM, pH-7.4) while the supernatant was subjected to further saturation by addition of solid salt. The fraction from 60-75% was pooled and dialyzed extensively (over night at 4°C) against tris-buffer (50 mM, pH-7.4). The dialyzed fraction was further separated on the heparin-sepharose (GE) affinity column (5ml) which was pre-equilibrated with tris-buffer (50 mM, pH-7.4) containing 0.1M NaCl. The column was washed with the equilibrating buffer (50 mM, 30 mL, pH-7.4) and the bound proteins were eluted at a flow rate of 1ml per min with a step-gradient of 0.1M-2M NaCl in tris-buffer (50 mM, pH-7.4) using Akta prime (GE). Each fraction was collected and dialyzed overnight (4°C) against tris-buffer (50 mM, pH-7.4) and then tested for sperm motility-stimulating activity.

5.2.7 RP-HPLC:

Fraction obtained from affinity chromatography was further purified by reverse phase HPLC. RP-HPLC was performed on a C_{18} semi-preparative waters column. Protein was eluted with a linear gradient obtained by mixing two solvents both containing 0.1% trifluoroacetic acid (TFA): A, water; B, 95% acetonitrile, 5% water at a flow rate of 1ml/min and the detection was at 254 nm. The protein from eluted peak was recovered by vacuum drying and tested for its activity.

5.2.8 Protein Estimation:

The protein content of the samples and all the fractions collected were estimated according to method described by Bradford (1976), using bovine serum albumin (BSA) as standard.

5.2.9 Electrophoretic Analysis of Purified Protein:

Electrophorectic analysis of the crude sample as well as purified peptide was checked by native and SDS polyacrylamide gel electrophoresis as per Lamelli, (1970) method with slight modification, SDS-PAGE electrophoresis was performed on 12% polyacrylamide gel.

Reagents

Acrylamide/bisacrylamide monomer stock solution (30%T, 2.6%C).

4 X Running gel buffer (1.5 M Tris-HCL, pH 8.8).

4 X stacking gel buffer (500 mM Tris-HCL, pH 6.8).

SDS Stock Solution 10% (w/v).

Ammonium persulfate initiator solution 10% (w/v).

5 X Electrode (running) buffer (125mM Tris-HCL, 960mM glycine, 0.5% w/v SDS, pH 8.3).

5 X Non-reducing buffer (62.5 mM Tris-HCL, pH 6.8, 20 % (v/v) glycerol, 2% w/v SDS).

5 X Reducing buffer (62.5mM Tris-HCL, pH 6.8, 20% (v/v) glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol).

Staining of gel Coomassiee-Colloidal stain: After removing the gel was washed with distilled water twice and then stained with coomassiee blue for 4-5 hours. The gel was destained with methanol:acetic acid:water (40:20:60) for 1-2 hr.

Procedure

1. SDS-PAGE electrophoresis unit (Biorad protean) was assembled according to manufacturer's instructions.

2. Glass plates, aluminium backings, plastic combs and 1.5 mm plastic spacers were cleaned with detergent and then with 70% (v/v) ethanol. The glass plates, spacers and aluminium backings were assembled in a gel caster, the bottoms sealed and tested for leakage.

3. Acrylamide monomer, dd.H_2O, gel buffer and SDS was mixed with fresh ammonium persulfate and TEMED as indicated for the running gel, the gel mixture was then loaded into sealed gel caster and allowed to polymerize, dd.H_2O can be used to overlay the gel mixture.

4. After polymerization, the dd.H_2O/isopropanol was decanted and the stacking gel, made as described in above, and layered on top of the polymerized running (resolving) gel. Plastic 10 well combs was inserted into the stacking gel and polymerization allowed to occur (~30 min).

5. The combs was then removed, gels assembled into electrophoresis units and pre-electrophoresed (for 30 min, ~30 mA) before sample loading, to remove excess free radicals. Alternatively, wells can be thoroughly washed with water to remove acrylamide and excess radicals instead of pre-electrophoresis.

6. During electrophoresis the gel was cooled using a circulating water bath (4°C) and protein

samples were separated at 15 mA initially, then 30 mA constant current. The gel was further stained for protein detection.

5.2.10 Iso-Electric Focusing (IEF)

The approximate pI of the protein was determined according to the manufacturer's procedure given by (Amersham Biosciences). 5% acrylamide IEF slab gel was prepared containing pharmalyte (pH 3-10). Broad range calibration pI calibration kit (Amersham Bioscience, GE) was used to measure the isoelectric point of the protein. The iso-electric focusing was performed for 1.5-2 hrs at 60 W, 3000 V, current 150 mA, with coolant temperature 5° C for 45 min and then at 12 °C for remaining duration.

Determination of pH gradient and pI of the protein: Using a piece of graph paper with 1 cm markings and 0.1 cm subdivisions, position the gel over the grid so the origin of the grid is lined up with the cathode end of the gel. Using the grid, the distance of each protein pI marker from the cathode was determined. The pH curve was prepared by plotting the pI value of marker versus its distance from the cathode.

Staining of gel: The gel after iso-electric focusing was removed and washed with distilled water for 10 min. after washing the gel was stained with 20 mL of Pierce silver stain till spots were visible.

5.2.11 Protein Electroblotting on Immobilon P (PVDF):

Purified protein sample was run on a 12% SDS-PAGE and then electroblotted onto a PVDF membrane according to the standard protocol of (Towbin and Gordon, 1979). Briefly the gel was removed from the electrophoresis cell and soaked in 100 mL of electroblotting Buffer (25mM Tris base, 192 mM glycine, 10% (v/v) methanol (pH 8.3) for 5 min. The transblot cassette was assembled in the following order starting from anode side: pre wet sponge, filter

paper, sheets of PVDF, gel, filter paper, and sponge. The transblot cassette sandwich was inserted in transblot cell containing 1L of cooled transfer buffer (Tris-Gly) and electroblotted for 2 hrs at 300 mA in cold room. After transfer PVDF was removed from transblotting sandwich and rinsed with distilled water before staining.

5.2.12 Periodic Acid–Schiff Staining:

Periodic acid–Schiff (PAS) staining was done to detect glycosylation of proteins. Two identical membranes were blotted simultaneously, one stained with Coomassie R-250 and the other used for PAS staining for glycoproteins. For PAS staining the membrane was fixed in 12% trichloroacetic acid for 1 hr, and then treated with a 7% trichloroacetic acid solution containing 2% potassium meta-periodate and kept at 4°C for 1 hr. The membrane was then immersed in Schiff's reagent and kept in the dark at 4°C overnight. The background was destained in methanol; glycoproteins appeared as pink/purple bands.

5.2.13 Assay of Peptide Activity:

The forward stimulating activity of protein from each step of purification was measured by the standard procedure (Mandal *et al.*, 1989) with some modifications. Spermatozoa (10^3-10^4 cells) were incubated with purified peptide (0.2 mg/mL protein) at 37±1°C for 1 min in a total volume of 0.5 ml of BWW medium (free of $CaCl_2$). A portion of the cell suspension was then applied into the hemocytometer maintained at 37±1°C. Immediately spermatozoa that showed moderate motility, feeble and defined forward motility (excluding cells that moved in small or large circles) (FM cells) and total cell numbers were counted under a phase contrast microscope at 250x magnification. The percentage of cells showing moderate motility and FM cells were then calculated. Systems lacking peptide served as the blanks in all assays. A unit of the activity was defined as the amount of the factor that induced FM in 10% of the cells under the standard assay

conditions.Concentration dependent and time dependent study of sperm motility with purified protein was carried out by incubating sperm sample with different concentration 10 μg/mL, 15 μg/mL, 30 μg/mL, 75 μg/mL and 90 μg/mL protein in BWW buffer (excluding $CaCl_2$) as mentioned above.

5.2.14 Effect of Temperature:

Thermostability of the purified motility stimulating protein was checked by boiling the purified protein solution at 100°C for 5 min. After boiling the protein was tested for its motility stimulatory activity as mentioned above. Non heated protein served as the positive control.

5.2.15 Evaluation of Sperm Chemotaxis by Trans-Well Method:

Sperm chemotaxis was quantified as described by (Al- Anzi *et al.,* 1998) corning-costar transwell plates, consisting of two chambers separated by a polycarbonate membrane having 12 μm diameter pore size. A bolus of soft agarose (0.2%) containing different concentrations (10, 50, 90, 120 μg/mL) of purified peptide was made (Serrano *et al.,* 2001) and placed onto the floor of the bottom chamber (trans) which was then filled with modified BWW (1.2 mL). The upper chamber (cis), consisting of an insert with the membrane stretched across its base, was lowered into the bottom chamber, the chamber was filled with a 0.5 mL aliquot of a sperm suspension as obtained by swim-up method, and the system was incubated for 60 min. at room temperature. Following incubation, the insert was removed, the lower chamber buffer was collected separately and centrifuged (300x g) for 10 min. and resuspended in BWW buffer. The resuspended pellet was counted for sperm using a haemocytometer. All assay data in the figures represents the mean and standard error of mean for at least three experiments.

5.2.16 Sperm Motion Analysis (CASA):

Computer assisted sperm analysis (CASA) was carried out using Hamilton-thorn research (US)

HIM Ivos Motility analyzer (version 8.1c). Motility parameters were assessed by incubating the sperm suspension with 50 ng/mL and 100 ng/ml concentration of purified protein mixed in Ham's-10 media for 10 min. For each measurement samples each 7 µl were pipetted onto a pre-warmed micro-slide chamber- Makler (10 µm). In accordance with WHO (WHO, 1992) guidelines three to six fields were examined for each experiment. Following parameters were set for the analysis; image type-bright field; frames acquired-30; frame rate-30/sec; minimum contrast-8; minimum size- 6; LO/HI size gate- 0.6- 1.6; non motile head size- 10; non-motile intensity- 20.

5.2.17 In-Gel Digestion:

For in-gel digestion of protein standard protocol of Shevchenko *et al.*, (2006) was followed with some modifications. Coommassiee blue stained gel was washed with MilliQ water for 1h.the desired band was excised and cut into 0.5–1.0 mm^3 cubes in a small, (0.5 ml) placed in a silanized, eppendorf tube and macerated. Cut a piece of the blank gel region for background substraction. The gel was destained by agitating in 100–200 µl 25 mM Ammonium bicarbonate, 50% Acetonitrile for 10–15 min at 37°C supernatant was removed by pipette (use preferably gel-loading tips) and 100-200 µl 100% Acetonitrile was added. Gel pieces shrink and turn opaque/ white as acrylamide is precipitated by Acetonitrile. The precipitated gel piece was dried in a Speedvac for ≈ 10–30 min. Longer time maximises rehydration volume, but may cause some proteins to precipitate.

Reduction and Alkylation: The SH-groups was reduced by heating the gel piece with 100 µl 20 mM (3.08 mg/ml) DTT in 0.1 M Ammonium bicarbonate, 30 min at 56 °C. Excess liquid was removed and the gel was precipitated as mentioned above using acetonitrile. The alkylation of cystein was performed by adding 100µl of 55 mM (10.2 mg/ml) iodoacetamide in 0.1 M

Ammonium bicarbonate, and incubated for 15 – 45 min with occasional vortexing in the dark. Excess liquid was removed, and the gel piece was washed with 100 µl 0.1 M Ammonium bicarbonate and shrunk with Acetonitrile as above.

Trypsin Digestion: Trypsin digestion was performed by mixing 10µl Promega porcine modified trypsin (Sequencing grade, 5000 U/mg), 0.1 µg/µl solution (or 0.05–1.0 µg/µl, depending on protein quantity in the sample) in 50 mM Ammonium bicarbonate and allow to absorb for 10 min at room temperature. A small volume (10 µl) of 50 mM Ammonium bicarbonate 10% Acetonitrile was added to just completely cover gel pieces and the sample was incubated overnight (12–16 h) at 37 °C.

5.2.18 Peptide Mass Fingerprinting:

Mass spectra of the trypsin digested fragments of the protein was recorded by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on Autoflex II Mass Spectrometer (Bruker Daltonics, Germany), using a-cyano hydroxyl cinnamic acid as matrix. Peak detection was performed after calibration of the mass spectrum with internal standards. (The mass finger printing analysis was performed by TCGA New Delhi)

5.2.19 Data Base Analysis:

The list of peptide masses generated as mentioned above was transferred into the peptide mass fingerprinting (PMF) search program Mascot as data file or by copy/paste of the mass list into query linking to http://www.matrixscience.com. Following parameters were set for the search. Enzyme: Trypsin, Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M) Mass values: Monoisotopic, Protein Mass: Unrestricted, Peptide Mass Tolerance: ±100 ppm, Fragment Mass Tolerance: ±1 Da, Max Missed Cleavages: 1.

5.3 RESULTS:

The summary of the purification steps followed is shown in table 5.1 after ammonium sulfate fractionation (60-75% saturation) recovery of motility stimulating activity was approximately 92%. Following this step the motility stimulating peptide was purified around 10 fold. The active fraction was further purified using heparin-agarose affinity column chromatography. The motility stimulating peptide bound to the resin was eluted with a 0.1-1.5 M NaCl step gradient. A typical elution profile of the affinity column is shown in figure 5.1. Two peaks as shown in figure 5.1 was found to elute at a salt gradient of about 48%; peak A (major fraction) and about 67% peak B. Both the peaks were collected and tested for their motility stimulating activity. Only fraction from peak A was found to have sperm motility stimulating activity (table 5.1). The motility stimulating peptide was purified further to homogeneity by semi-preparative RP-HPLC. The motility stimulating peptide resolved into two distinct peaks I (minor) and II (major) (figure 5.2). The minor peak I eluted at 9.5 minutes. The activity test from this peak did not result any significant motility stimulation of spermatozoa. Retention time of peak II was 18 minutes and it represents the major portion and when tested for its activity maximum sperm stimulating activity was observed (table 5.1).

Step	Fraction	Total activity (units)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Fold of purification
I	Follicular fluid	27000	11250	2.4	100	1
II	Ammonium sulphate precipitation (60%-75% saturation)	24800	1035	23.9	91.8	9.95
III	Heparin Affinity chromatography	12280	62	198	49.5	82.5
IV	RP-HPLC	5125	9.8	522.9	18.98	217.8

Table 5.1: Purification of motility stimulating factor from goat follicular fluid

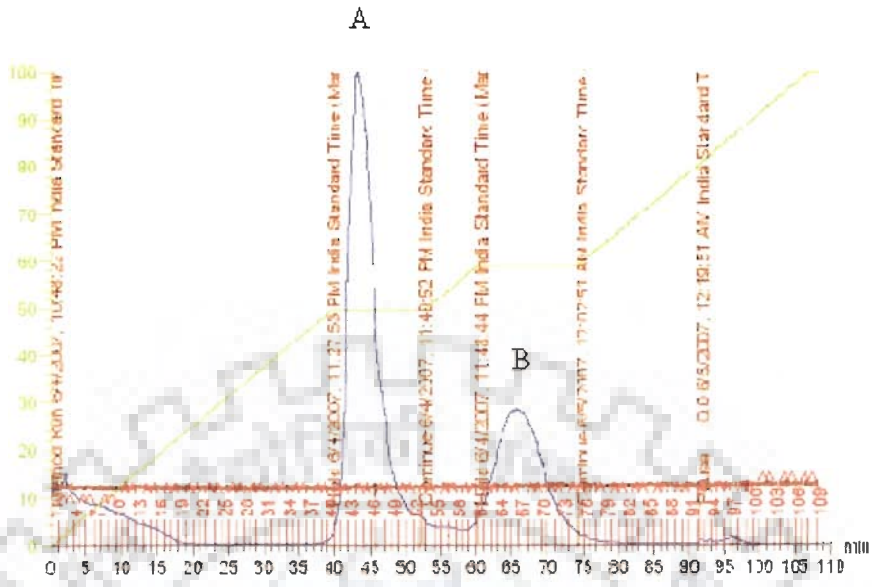


Figure 5.1: Elution profile of the sperm motility stimulator by heparin-agarose column (5mL column). The column was equilibrated with 50mM Tris-HCl buffer (pH- 7.4) containing 0.1M NaCl before running sample. Protein was eluted with a step gradient from 0.1 to 1.5M NaCl in the equilibrating buffer. Fraction A and fraction B were dialysed before assaying for sperm motility stimulating activity.

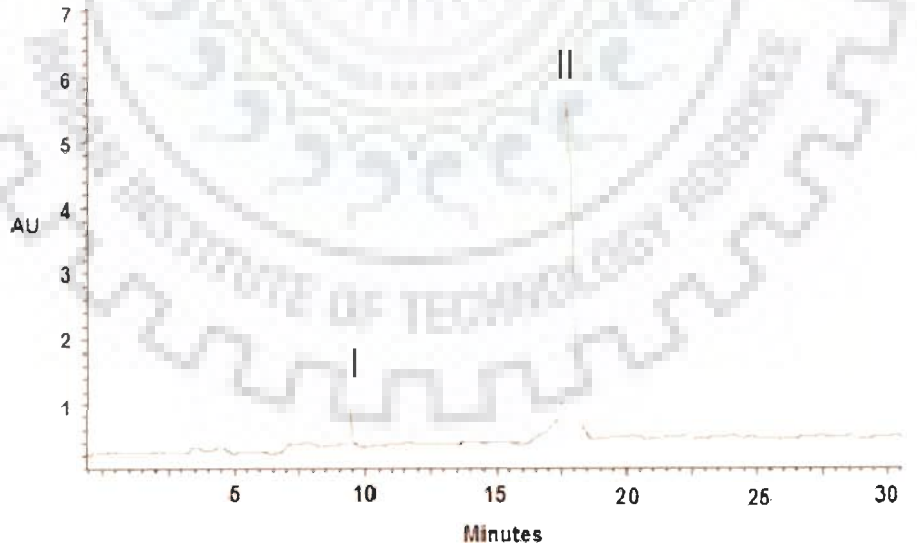


Figure 5.2: RP-HPLC purification of motility stimulating peptide (fraction A of affinity chromatography) from goat follicular fluid. Protein eluted with a linear gradient obtained by solvent A (water) and solvent B (95% acetonitrile, 5% water) both containing 0.1%TFA. Detection was at 254 nm.

The fraction showing maximum activity obtained from HPLC step of purification was analyzed by SDS-PAGE. The fraction showed the presence of single band with molecular weight of 52 kDa (figure 5.4). Purity of the active fraction was further checked by native PAGE and the electrophoretogram showed a single band of about around 52 kDa (figure 5.5). These results indicate that this protein is composed of a single polypeptide. To ascertain the nature of the polypeptide periodic acid-schiff's reagent was used to stain the protein electroblotted onto a PVDF membrane. The peptide exhibited a clear pink band (figure 5.6) indicating that the sperm motility stimulator is a glycoprotein. The iso-electric point of the motility stimulator protein was determined by analytical iso-electric focusing using broad pI calibration kit on a 5% gel containing pharmalyte pH 3-10. As shown in figure 5.7 the protein was found to stabilize at around 6.5. This further proved the purity of the peptide.

The forward motility activity of the peptide was analyzed by monitoring the motility pattern of the goat spermatozoa under phase contrast microscope (250 x) in the presence and absence (control) of purified peptide. The freshly extracted spermatozoa (10^4) were incubated in BWW ($CaCl_2$ free) buffer (pH-7.4) with different concentration of purified protein (10 µg/mL, 15 µg/mL, 30 µg/mL, 75 µg/mL and 90 µg/mL). As shown in figure 5.8 overall motility (cells showing moderate + feeble motility) of the spermatozoa showed gradual increase with increase in the concentration of peptide, approximately 28% increase in motility was found as compared to control with highest concentration of peptide (90 µg/mL). Addition of peptide also significantly enhanced the spermatozoa forward motility (figure 5.8) with increase in the concentration of the peptide. At a concentration of 50 µg/mL more than 50% of the total spermatozoa showed forward progressive motility, beyond this with increase in concentration only slight increase in forward motility was recorded. Further when tested for the time

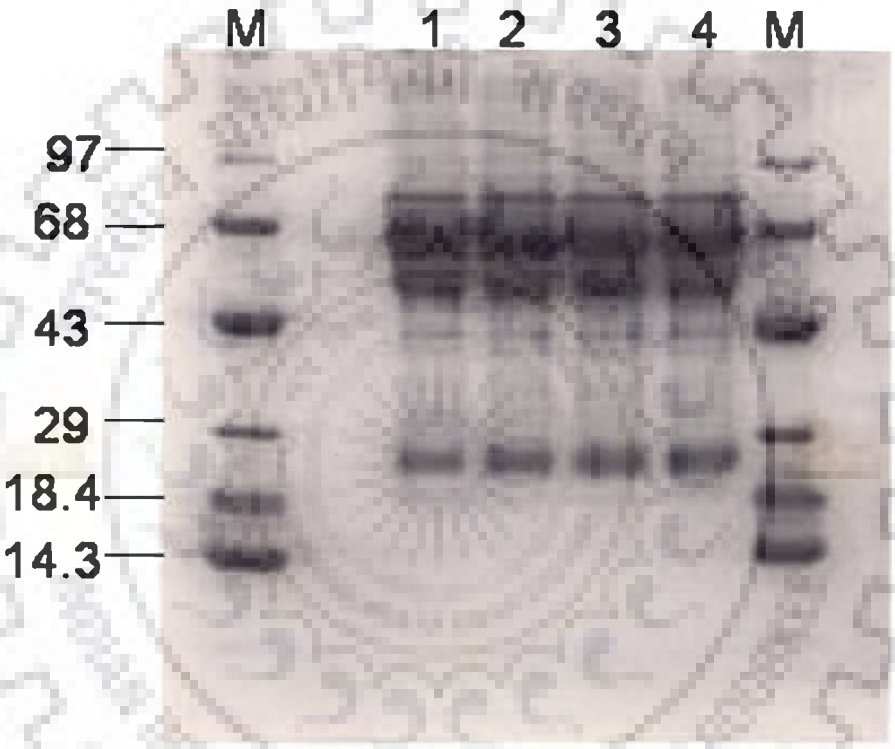


Figure 5.3: **Electrophoretogram of crude follicular fluid extracted from mature follicles of goat ovary**. Lane M-molecular weight marker (kDa); lane 1- 4 follicular fluid.

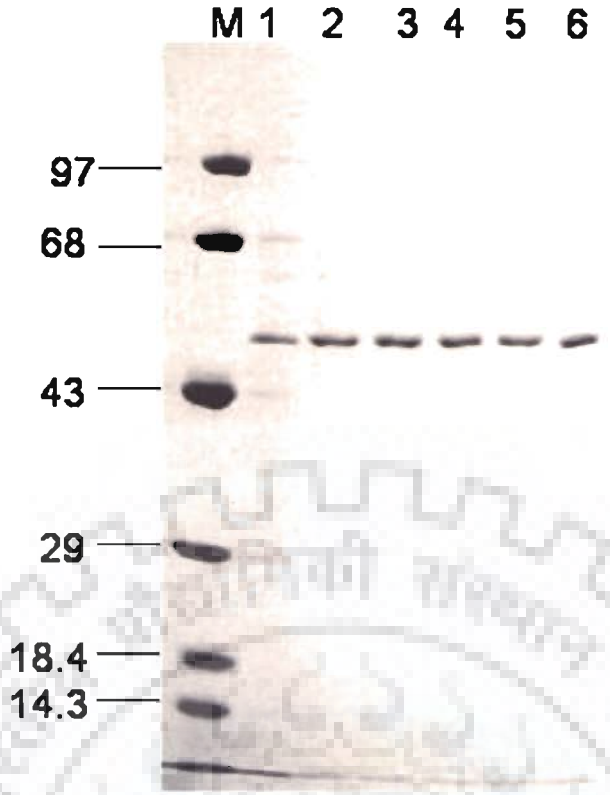


Figure 5.4: SDS-polyacrylamide gel (12%) electrophoretogram of purified sperm motility stimulator. Lane 1, Protein MW marker phosphorylase-b (97 kDa), BSA (68 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and Lysozyme (14.3 kDa). **Lane 1-6 purified protein (52 kDa)**

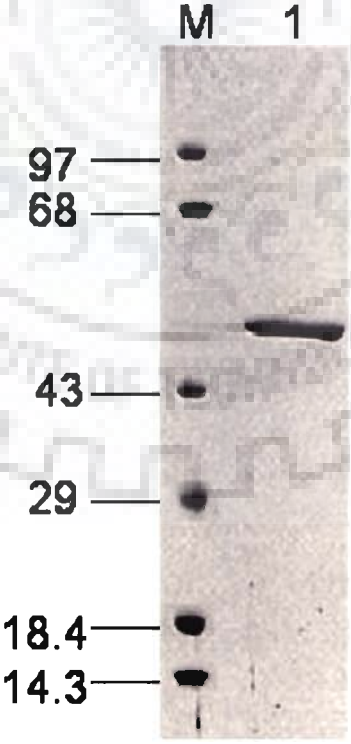


Figure 5.5: Native PAGE (10%) electrophoretogram of purified sperm motility stimulator. Lane 1, Protein MW marker phosphorylase-b (97 kDa), BSA (68 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and Lysozyme (14.3 kDa). **Lane 1 purified protein (≈ 52 kDa)**

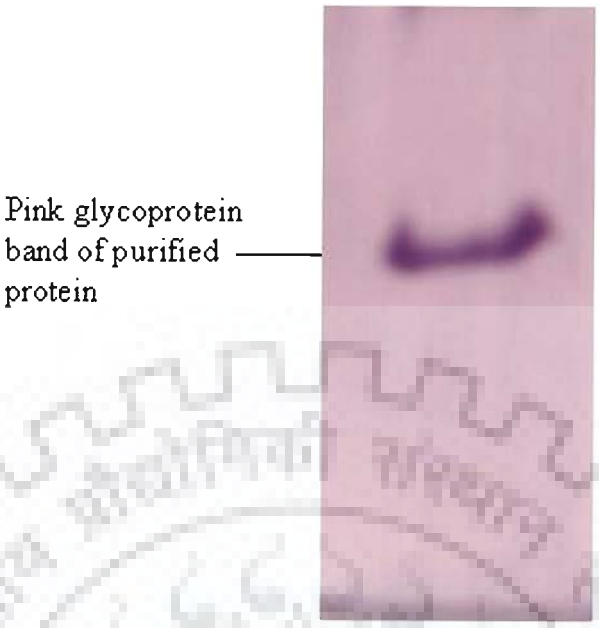


Figure 5.6: PAS staining of sperm motility stimulator protein subjected to native PAGE and glycoprotein band detected on a PVDF membrane.

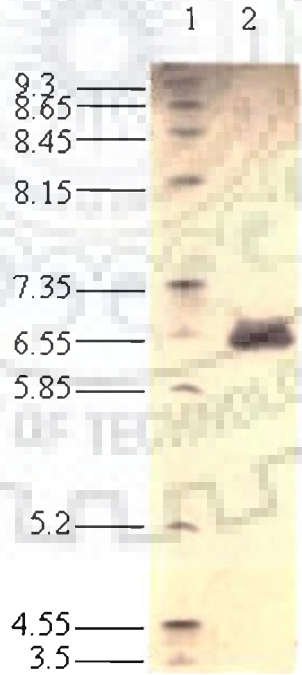


Figure 5.7: Iso-electric focusing of purified protein on 5% acrylamide gel containing pharmalyte pH 3-10. Lane 1- Trypsinogen (pI 9.3), lentil lectin basic band (pI 8.65), Lentil lectin middle band (pI 8.45), Lentil lectin acidic band (pI 8.15), Horse myogloblin basic band (pI 7.35), Human carbonic anhydrase B (pI 6.55), Bovine carbonic anhydrase B (pI 5.85), β-lactoglobulin-A (pI 5.20), Soya bean trypsin inhibitor (pI 4.55), Amylo-glucosidase (pI 3.50). **Lane 2- purified protein (pI 6.5).**

dependent activity of the spermatozoa with peptide (50 μg/mL) rise in forward motility was observed till 10 minutes after which no significant increase was observed (figure 5.9). As shown in figure 5.9 there was rapid rise in forward motility of the spermatozoa till 4 min after which gradual increase was observed till 10 minutes. On treating the protein at 100°C for 10 min. a significant decrease in the activity of protein by 40-45% (figure 5.10) was observed, suggesting partial thermostable nature of the protein.

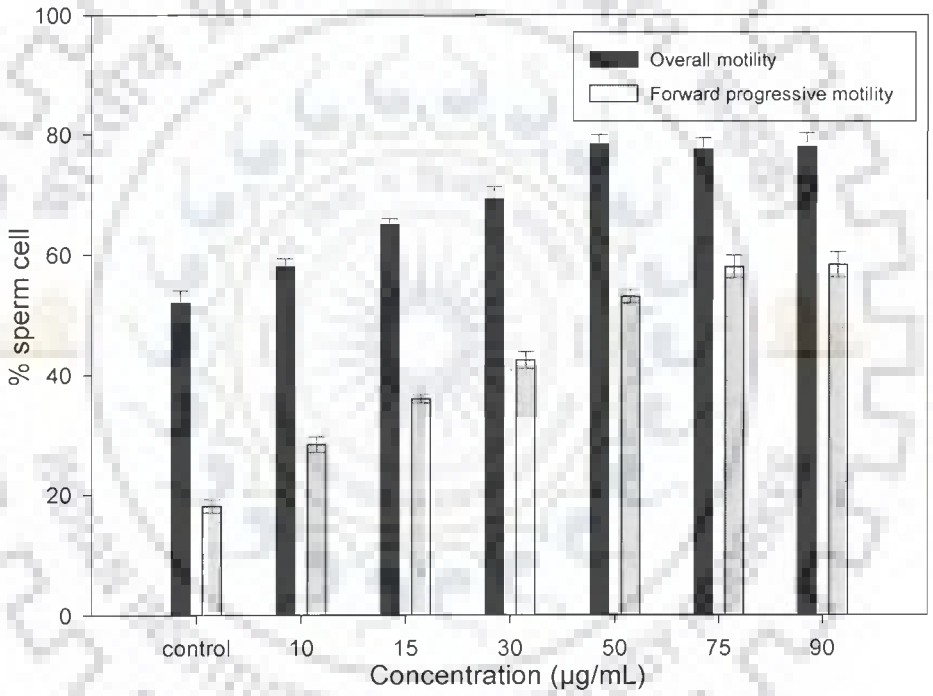


Figure 5.8: Effect of purified protein on goat spermatozoa at different concentrations on sperm motility (overall and forward motility) under standard assay condition (paired t-test $p<0.01$).

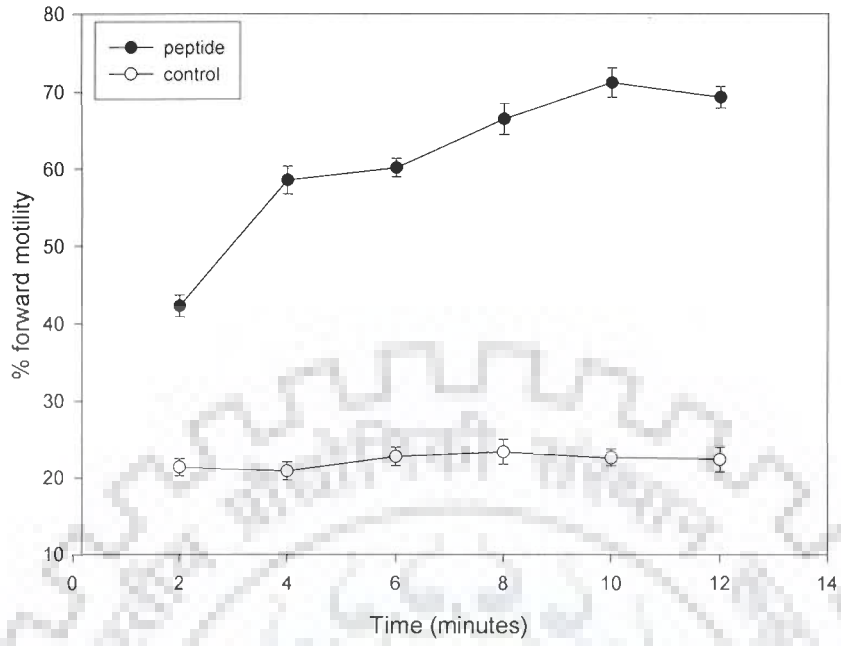


Figure 5.9: Effect of purified protein (50 µg/mL) on goat spermatozoa at different time interval (upto 10 minutes) under standard assay conditions.

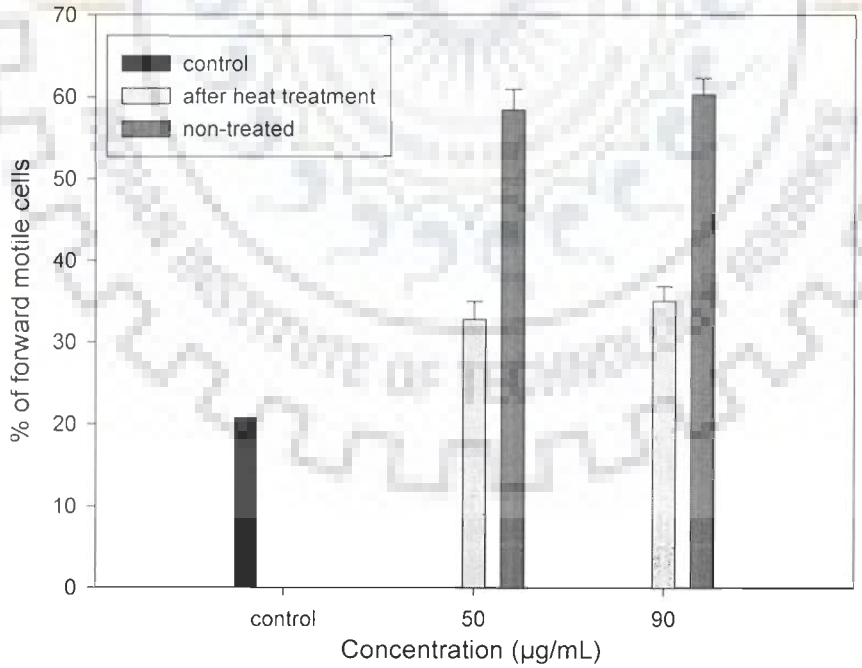


Figure 5.10: Effect of heat treatment (at 100°C for 5 min) on the forward motility activity of purified protein. (paired t-test $p<0.01$).

In order to ascertain the chemotactic effect of the protein on goat spermatozoa, chemotaxis assay using corning transwell plates was performed. The bolus of soft agarose impregnated with different concentration of purified peptide (10, 50, 80 and 100μg/mL) was kept at the bottom of the transwell chamber. The slow release of protein from bolus formed a gradient between two chambers (lower and upper). After 60 min of incubation at 37±1°C the number of cell that migrated in to the lower well containing peptide was calculated. As shown in figure 5.11 there was increase in the number of spermatozoa migrating into wells containing peptide with increasing concentration of peptide as compared to control.

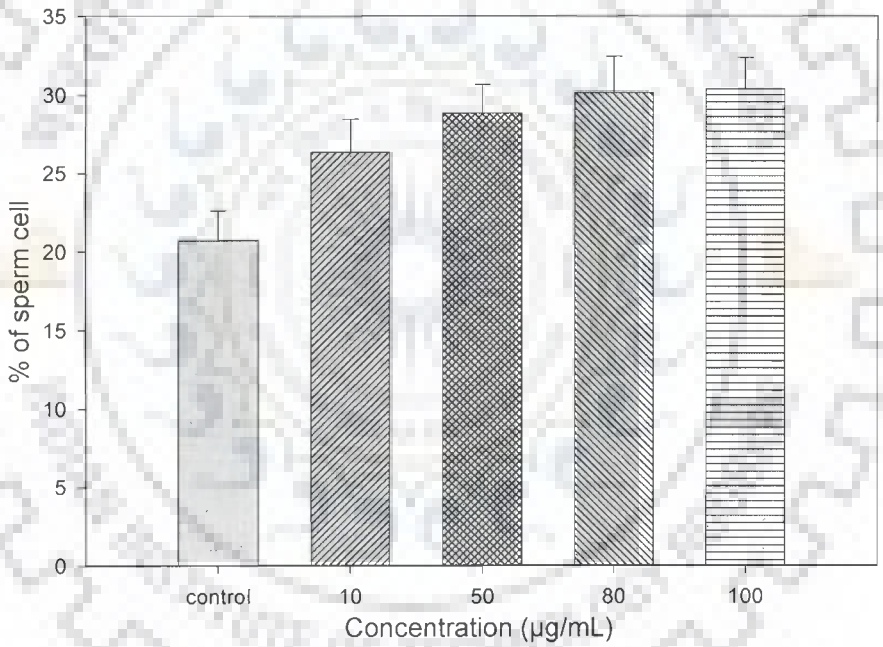


Figure 5.11: Chemotactic effect of purified protein on goat spermatozoa at different concentration under standard assay conditions for 60 minutes.

The cross species motility stimulating effect of the purified protein on human spermatozoa (with normal semen) was also studied. The sperm motion was assessed by computer assisted semen analysis (CASA). The spermatozoa were incubated for 10 min with two different concentrations (50 μg/mL and 100 μg/mL) of purified protein in Ham'10 media

(pH-7.4). The CASA results showed an over all increase in mean VSL, progressive motility, Mean ALH, linearity (LIN), mean straightness (STR) and BCF with increasing concentration of purified protein as compared to control (Table 5.2, 5.3 and 5.4**).** The concentration dependent enhancement in mean VCL, BCF and over all percentage of progressively motile sperm cells showed significant increase ($p<0.05$) as compared to control.

DATA SUMMARY			
	Conc.(M/ml)	Sample (M)	Count
Total	204.7	798.3	108.6
Motile	155.9	609.4	108.6
Prog.	91.1	355.2	63.3
Motility	75.6%		
Progressive	45.3%		
Velocity (VAP)	32.6 mic/sec		
Velocity			
Rapid	51.3%		
Medium	16.3%		
Slow	9%		
Static	23%		
SUPPLEMENTARY DATA			
Mean track speed (VCL)	39.3 mic/sec		
Mean progressive velocity (VSL)	29.6 mic/sec		
Mean linearity (LIN)	72.3%		
Mean straightness (STR)	86.7%		
Mean ALH	2.4 microns		
Mean beat frequency (BCF)	12.7 hz		
Sort fraction	0%		

Table 5.2: Kinematic response of human spermatozoa (control)

DATA SUMMARY			
	Conc.(M/ml)	Sample (M)	Count
Total	166.3	648.7	105
Motile	120.8	471.1	77.3
Prog.	74.2	289.3	48
Motility	79.3%		
Progressive	52%		
Velocity (VAP)	33 mic/sec		
Velocity			
Rapid	54.6%		
Medium	20%		
Slow	11%		
Static	14.6%		
SUPPLEMENTARY DATA			
Mean track speed (VCL)	41.2 mic/sec		
Mean progressive velocity (VSL)	30.3mic/sec		
Mean linearity (LIN)	78.6%		
Mean straightness (STR)	90.6%		
Mean ALH	2.5 microns		
Mean beat frequency (BCF)	13.4 hz		
Sort fraction	0%		

Table 5.3: Kinematic response of human spermatozoa to purified protein (50 µg/mL)

DATA SUMMARY			
	Conc.(M/ml)	Sample (M)	Count
Total	319.3	1247.4	213
Motile	275.9	1075.9	184.3
Prog.	162.3	632.9	108.6
Motility	87.6%		
Progressive	54.3%		
Velocity (VAP)	35 mic/sec		
Velocity			
Rapid	59%		
Medium	19.3%		
Slow	7.6%		
Static	14%		
SUPPLEMENTARY DATA			
Mean track speed (VCL)	43.6 mic/sec		
Mean progressive velocity (VSL)	31 mic/sec		
Mean linearity (LIN)	78.6%		
Mean straightness (STR)	89.3%		
Mean ALH	2.53 microns		
Mean beat frequency (BCF)	15.36 hz		
Sort fraction	0%		

Table 5.4: Kinematic response of human spermatozoa to purified protein (100 µg/mL)

To know the probable sequence of the purified protein, the HPLC purified protein was subjected to trypsin digestion and the experimental array of peptide resulting from digestion was detected by MALDI TOF/TOF (figure 5.12). The experimental mass obtained was further subjected to a MASCOT (database-MSDB, taxonomy-other-mammalia) search in order to identify the probable similar sequences. The summary of the search result is represented in table 5.5. The spectrum best matched with the theoretical spectra in the database to antithrombin III precursor – sheep (Score: 200 Expect: 4.9e-16, Figure 5.13). The overall sequence similarity with sheep antithrombin III was found to be around 68%.The other matches were also to antithrombin III of Bos tourous, Pig (table 5.5).

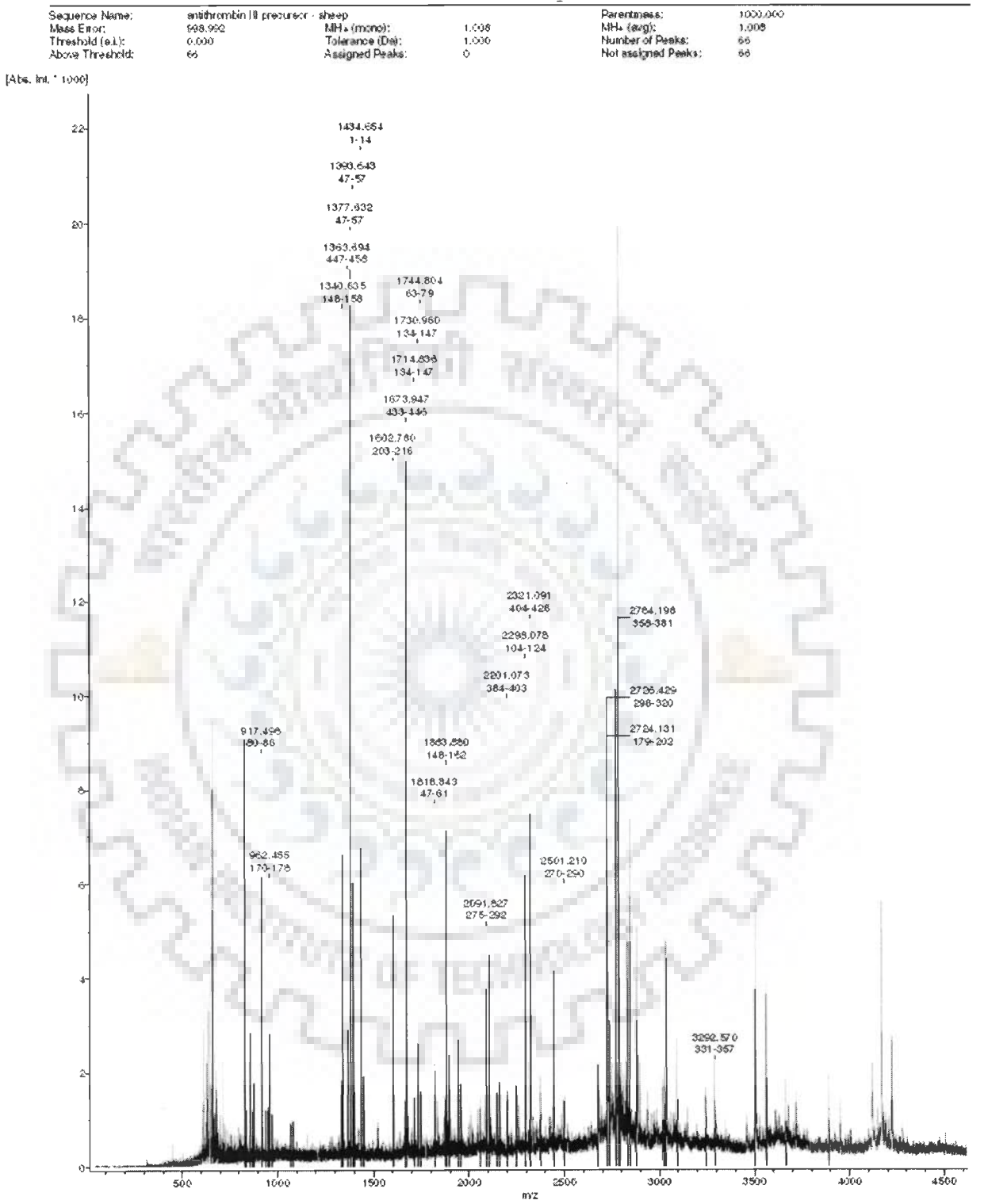


Figure 5.12: Trypsin digested MALDI-ToF spectra of sperm motility stimulator protein

1 MISNGIGTVT TGKRSMCLFP LLLIGLWGCV TCHRSPVEDI CTAKPRDIPV

51 NPMCIYRSPE KKATEGEGSE QKIPGATNRR VWELSKANSH FATAFYQHLA

101 DSKNNNDNIF LSPLSISTAF AMTKLGACNN TLKQLMEVFK FDTISEKTSD

151 QIHFFFAKLN CRLYRKANKS SELVSANRLF GDKSITFNET YQDISEVVYG

201 AKLQPLDFKG NAEQSRLTIN QWISNKTEGR ITDVIPPQAI DEFTVLVLVN

251 TIYFKGLWKS KFSPENTKKE LFYKADGESC SVPMMYQEGK FRYRRVAEGT

301 QVLELPFKGD DITMVLILPK LEKPLAKVER ELTPDMLQEW LDELTETLLV

351 VHMPHFRIED SFSVKEQLQD MGLEDLFSPE KSRLPGIVAE GRNDLYVSDA

401 FHKAFLEVNE EGSEAAASTV ISIAGRSLNL NRVTFQANRP FLVLIREVAL

451 NTIIFMGRVA NPCVN

Figure 5.13: Sequence matching of MALDI generated trypsin digested purified protein with Antithrombin III precursor- sheep (MSDB database MASCOT). Matched segments of peptide shown in bold.

S.No.	Accession	Mass	Score	Description	% Homology
1	S28219	52979	200	antithrombin III precursor - sheep	68
2	A61435	49437	86	antithrombin III - bovine	40
3	AAI02748	52828	70	BC102747 NID: - Bos taurus	32
4	ANT3_BOVIN	52827	70	Antithrombin-III precursor (ATIII).- Bos taurus (Bovine).	32
5	1ATTB2	40787	63	antithrombin III (synchrotron radiation), chain B, fragment 2 - bovine	38
6	1ATTA2	41546	62	antithrombin III (synchrotron radiation), chain A, fragment 2 - bovine	37
7	Q19AZ5_PIG	52866	62	Antithrombin protein.- Sus scrofa (Pig).	28
8	JX0364	49248	55	antithrombin III - pig	27

Table 5.5: The percent similarity of the trypsin digested MALDI spectra with MASCOT (MSDB database). The best 8 hits are recorded.

5.4 DISCUSSION:

It is well documented that forward movement of sperm is essential for the mammalian fertilization. In mammals, spermatozoa are produced in the testis, transit through the epididymis and are stored in the cauda region. Finally, during ejaculation these cells are largely diluted with reproductive fluid derived from the various accessory sex organs before being deposited into the female reproductive tract. Earlier workers have reported the presence of multiple unidentified sperm motility regulating factors in the male reproductive fluid and blood serum (Yanagimachi, 1970; Morita and Chang, 1971; Morton and Chang, 1973; Bavister, 1975; Morton *et al.,* 1979; Mandal *et al.,* 1989).

The stimulatory effect of follicular fluid on sperm motility has been previously shown (Chao *et al.,* 1991; Isobe *et al.,* 2002). In follicular fluids from mammals, proteins with both small (Fetterolf *et al.,* 1994) and large (Lee *et al.,* 1992; Aleporou-Marino *et al.,* 2001) molecular mass have been identified as sperm motion stimulators. In the present study the purification of a sperm motility stimulator protein from ovarian follicular fluid of goat to apparent homogeneity is shown for the first time. The physical property of the purified protein was studied to ascertain the molecular weight and nature of protein. Both the native and SDS-PAGE analysis of purified protein showed a single band corresponding to 52 kDa which clearly indicates that the forward motility stimulating protein obtained in the present study is a monomer.

PAS staining of the purified 52 kDa protein isolated from the goat follicular fluid indicates that the protein is glyco-conjugated. Likewise, a 52 kDa sperm motility protein isolated from porcine ovarian follicular fluid was also reported to be a glycoprotein (Lee *et al.,* 1992). In another study, a forward motility stimulating protein (FMSP-I) isolated from the

buffalo blood serum was also characterized to be a glycoprotein by PAS staining (Mandal *et al.*, 2006). The protein has an affinity for activating forward motility of goat cauda epididymal spermatozoa. Further investigations revealed that this protein lost its activity when the carbohydrate part was enzymatically cleaved showing that the sugar part of the protein was essential for biological activity. In an earlier study a heat stable protein from human follicular fluid was partially purified (Kao, 1991). The thermostability of the forward motility stimulating protein further does not clarify if the protein residue of the FMSP is involved in the motility stimulating activity. In the present study, the described 52 kDa protein decreases forward motility stimulatory activity by around 40-45% as compared to control. It is possible therefore that the sperm motion characteristics influenced by this glycoprotein resides in the protein segment. However, detailed investigations would be needed to conclude whether the sperm stimulatory effects observed in the present study with the 52 kDa protein isolated from goat follicular fluid are due to the carbohydrate moiety, protein component or both.

Our results of cross species motility assay with human sperm using CASA suggests that the 52 kDa goat glycoprotein is capable of inducing forward motility in human spermatozoa as well as goat sperm. A significant increase in forward motility of the goat spermatozoa was also recorded in both concentration dependent and time dependent studies. The results indicate that the spermatozoa can be induced to increase certain motion characteristics in presence of the purified protein. There was significant increase in the forward motility of the goat spermatozoa as compared to control (approximately 28% increase with 90 µg/mL of protein). Further, the time dependent study suggests that the motility promoting activity increased the forward motility rapidly up to 4 min following incubation with 50 µg/mL of protein). Beyond this time, a slow and moderate increase was observed. The pattern of goat

sperm migration in a concentration gradient of the protein also suggests that the molecule is a mild chemoattractant. A very similar protein, also a 52 kDa glycoprotein isolated from porcine ovarian follicular fluid showed a stimulatory effect on porcine sperm motility (Lee *et al.,* 1992). This protein stimulated motility and acted as a chemoattractant of washed boar sperm as well (Lee *et al.,* 1994). Thus, it may be concluded that species specificity of most molecules involved in egg-sperm interaction is of little significance in mammals where fertilization is internal.

A glycoprotein of 50 kDa was isolated from the human follicular fluid (Aleporou-Marino *et al.,* 2001). However, it is unlikely that it has resemblance to the protein isolated in the present study since the N-terminal sequencing data indicates that it is akin to apolipoprotein H. Sperm motion analysis by CASA showed that it significantly increased straight line velocity (VSL) and the amplitude of lateral head displacement (ALH) but does not increase the number of progressively motile sperm. Therefore, the functional activity of this protein is also not exactly the same as our protein.

The primary analysis of the stimulator protein by protein mass finger printing and homology analysis using MSDB database (MASCOT matrix inter-science) reveals that the 52 kDa protein shares maximum identity with antithrombin III precursor of sheep (68%). Moreover when searched for other matches, similarity with antithrombin III - bovine (40%) and Antithrombin-III precursor (AT-III). - Bos taurus (Bovine) (32%) was seen suggesting that the protein shares some common sequence domains with antithrombin III molecule. Moreover, the pI of motility stimulating protein was found to be around 6.5. Antithrombin III molecule from other sources (antithrombin III precursor-sheep- 6.44, bovine- 6.02, bos Taurus-7.01) also showed comparable pI value with that of the protein under this study, further confirming that they are closely related to the one reported here. Thus, it may be inferred that the purified goat

protein is an antithrombin III-like molecule. This suggestion assumes significance since porcine antithrombin III (AT-III) has been reported to be a sperm motility stimulator protein and a chemoattractant of washed boar spermatozoa (Lee *et al.*, 1994). It is proposed that antithrombin III and related molecules could play an important role in influencing sperm motion characteristics and thus the mammalian fertilization process in vivo.

Based on the similarity with antithrombin III, it may be deduced that the protein is either derived from the blood or it is secreted by the surrounding cells of the egg during maturation. Kinetic immunonephelometric measurements conducted in human follicular fluid has revealed that the concentration of antithrombin III was significantly increased in mature follicles compared to immature follicles (Gulamali-majid *et al.*, 1987). The same authors have shown, the concentration of antithrombin III in fluid from follicles with mature oocytes to be higher than in plasma suggesting that the 'blood-follicle' barrier actively transports antithrombin III into the follicles. It has also been shown that IgG, α_1-antitrypsin and antithrombin III reaches their highest levels in human endometrial fluid during ovulatory phase of the menstrual cycle (Zavos *et al.*, 1982). Glycosaminoglycans are present in the uterine fluid (Lee and Ax, 1984) and in follicular fluid (Bushmeyer *et al.*, 1985) and may be released from the ovulatory follicles into the ipsilatory oviduct at the time of ovulation, thus providing a final thrust to the sperm cells in their journey towards the egg. Antithrombin III may have a similar role to play in guiding the sperm to the egg.

To sum up, a glycoprotein of 52 kDa with sperm motility stimulatory activity was purified from goat follicular fluid. The purified protein showed close resemblance with antithrombin III as indicated by peptide mass finger printing results. It is proposed that the protein is either secreted by the matrix in the cumulus cells surrounding the zona-pellucida of the oocyte or it

may be transported from blood serum and the presence of the motility stimulator protein in the follicular fluid may aid in the movement of the spermatozoa towards the egg. It is still not clear as to how the forward motility stimulating protein interacts with the sperm triggering the flagellar motility. Therefore future work should be directed towards understanding the basic mechanism of action of the sperm motility stimulator so that such natural proteins could be used to select high quality sperm in semen samples.

Until now several artificial substances like pentoxifylline, theophylline and bicarbonate have been used as sperm motility stimulators in assisted reproduction especially in patients with azoospermia since they trigger movement of testicular sperm, permit easier identification of vital sperm, shorten the procedure, improve fertilization rates and increase the number of embryos. However, the long term effects of these substances on the developing embryos have not been studied. The physiological role of sperm chemotaxis in mammals is thought to be the recruitment of selected population of capacitated sperm to fertilize the oocyte. The use of sperm motility proteins from natural sources as a substitute would certainly be safer since they provide a mechanism for selection of sperm in vivo. Although the isolated protein is of goat origin, the CASA results suggest that there is no species barrier as far as this protein is concerned since it works just as well on human sperm and could be used in assisted reproduction programs.

Chapter-6

SUMMARY

CHAPTER 6: SUMMARY

Chemically guided movement of spermatozoa is well established in case of marine animals where fertilization is external (Miller, 1985). But, of late *in-vitro* studies conducted on guided movement of spermatozoa of higher animals have also shown the presence of this phenomenon in them (Fabro *et al.*, 2002; Jaiswal *et al.*, 1999). It is now generally accepted that for fertilization to occur in mammals, ejaculated spermatozoa must reach the egg, following ovulation and the prospect of male spermatozoa reaching the female egg are very slim in the absence of some guidance mechanism usually chemical in nature (Krikman-Brown *et al.*, 2003). Thus, it appears that sperm chemotaxis is a general guidance mechanism, irrespective of whether the fertilization is external, like in most marine species, or whether it is internal, as in mammals. Inspite of the generality, there are some basic difference between the sperm chemotaxis of mammals and that of marine invertebrates. Until not too long ago, it was believed that in mammals, following ejaculation into female reproductive tract, large numbers of spermatozoa race towards the egg and compete to fertilize it (Eisenbach, 2004). But recent studies have shown that few of the ejaculated spermatozoa (in humans approximately 250, range 80-1400 spermatozoa) succeed in entering the fallopian tube (Williams *et al.*, 1993; Barratt and Cooke, 1991). The numbers of spermatozoa that can fertilize the egg are even smaller. In mammals, spermatozoa ejaculated in the female reproductive tract must undergo capacitation here to get functionally activated and therefore the fraction of spermatozoa that ultimately reaches the egg is small (Cohen-Dayag *et al.*, 1994; 1995). These physiological factors along with non-consistency in the results obtained in *in-vitro* studies due to non availability of standard assay method used to measure sperm chemotaxis has hampered research work on direction based migration of sperm. The work presented in this thesis is an effort to address

issues such as design of reliable assay methods for measuring sperm chemotaxis, differences between capacitated and non capacitated spermatozoa in sperm chemotaxis, impact of various free biomolecules (amino acids, sugars and hormones) on sperm chemotaxis and physiological role of follicular fluid in sperm migration. Besides this, the isolation, purification and characterization of a novel protein molecule from goat follicular fluid that affects sperm motion characteristics are described. The major contribution of the reported work is outlined below.

First part of the thesis addresses the design, fabrication and validation of assay system to measure sperm chemotaxis. The basic design of the chemotaxis apparatus consisted of wells and grooves punched in a block of polystyrene. The dimensions of the grooves and wells were standardized to ensure that multiple assays could be conducted in the five-well chamber which includes a control for comparison purposes. One problem commonly encountered in devices of this kind is that it is difficult to distinguish between sperm trapping, chemokinesis and chemotaxis. This was circumvented in the present case by machine grinding and polishing of the wells and grooves. Such a treatment made the chamber scratch free and prevented sperm trapping. Since the design permitted chemical gradients to be established prior to the testing of sperm samples, it is possible to conclude that the sperm movement from the central to peripheral wells was largely on account of sperm chemotaxis and not chemokinesis. The creation of gradients between the central and peripheral wells was confirmed by the decolourization of phenol red and the diffusion data of BSA in separate experiments. The results obtained with the apparatus designed and fabricated in our laboratory prove that the apparatus can be used to monitor unidirectional migration of cells *in-vitro*. Since the system based on choice assay can distinguish between chemokinesis and chemotaxis it may be inferred that results obtained are more reliable as compared to models suggested by other investigators (Makler *et al.,* 1992;

Sliwa *et al.,* 1993; Eisenbach *et al.,* 1999). One of the most reliable assays available for evaluation of chemotaxis is the one using the Corning Trans well apparatus. However, this turns out to be very expensive for routine and repeated use. To evaluate the performance of the choice assay using the self-fabricated apparatus, a tripeptide, f-met-leu-phe was employed as a chemoattractant. Formyl-met-leu-phe is a known chemoattractant of neutrophil and bull spermatozoa and has been used in the past to evaluate chemotaxis (Becker *et al.,* 1976; Vijayasarthy *et al.,* 1980). Preliminary experiments done in our laboratory confirmed that this peptide is effective as a chemoattractant for goat sperm at concentrations up to 50 µg/mL. Using this peptide, the results obtained for the self-fabricated choice assay were compared with those of the commercially available Trans well assay. The similarity in the pattern of response obtained by the two procedures only goes to prove that the self fabricated apparatus could be a cost effective substitute for the commercial device.

Second part of the thesis explores the migratory behaviour of sperm *in vitro*, with relation to different physiological states of sperm (capacitated, non-capacitated), and response to follicular fluid (mature, immature). Along with this influence of free amino acids, sugars, hormones (steroid, non-steroid) was also investigated. Finally, the whole proteome characterization was done using 2-D Electrophoresis. The results indicate that in goat, as in other mammals, only capacitated spermatozoa are chemotactically active. When assayed for chemotaxis a significant percentage of capacitated spermatozoa migrated into the well containing diluted follicular fluid (10^{-5}) where as with decapacitation of sperm cell, number of cell migrating towards follicular fluid decreased indicating a possible role of capacitation in mediating chemotactic response in goat spermatozoa. The complex nature of the follicular fluid makes one believe that the migratory activity of goat sperm is not governed by the existence of

any one chemical substance and that many factors could contribute to the same. It was anticipated that the composition of the follicular fluid is not constant during the development of the follicle. A marked change in the pattern of migration was observed when the data of immature and mature follicular fluids were compared. The difference between the two groups was distinctly high at 10^{-5} and 10^{-6} dilution of follicular fluid indicating a chemotactic response of sperm to mature follicular fluid. This difference in the sperm attracting ability of follicular fluid from two different stages of follicular development may be due to factors released from the follicles at the time of ovulation *in vivo*. Fluctuating level of hormones during follicular development may also have a vital role to play in mediating sperm chemotaxis. Of the four sex hormones (progesterone, testosterone, estradiol and estrone) assayed, the level of progesterone alone was found to be increased during follicular development. *In vitro* study with goat sperm confirmed a response to progesterone suggestive of both chemokinesis and chemotaxis. Testosterone, on the other hand, had a negative impact on direction-based sperm motility. Regarding the other hormones investigated, adrenaline, oxytocin and calcitonin had a positive impact while insulin and glucagon had a negative impact on sperm migration. The sensitivity of the goat sperm to these hormones was however not the same since the response to these hormones occurred at very different concentrations. Until now no specific receptors/ binding sites have been reported for these hormones on the sperm surface. In addition, pathways involved in signal transduction are unknown.

HPLC analysis of follicular fluid suggests that goat ovarian follicular fluid is a rich source of amino acids from which a variety of proteins can be synthesized during follicular development. Of the several free amino acid tested for its effect on migatory behaviour of sperm cell only glutamic acid and cystine showed positive effect where as alanine, glycine and

methionine had a negative effect on the migratory behaviour of goat spermatozoa. Of the several monosaccharides tested in the present study, only fructose and glucose were able to influence the migration of goat sperm. The follicular fluid is a dynamically changing milieu that possibly supports the growth of the follicle during maturation. The 2-D electrophoretic protein profile obtained in the immature and mature follicular fluids supports this contention.

In the last part of the thesis the isolation and characterization of an active factor (protein) from ovarian follicular fluid capable of stimulating sperm forward motility is described. It is for the first time that a protein having sperm motility stimulator activity has been purified to apparent homogeneity from ovarian follicular fluid of goat. The PAGE (native and denaturing) analysis of the protein suggests that it is monomeric with molecular mass of around 52 kDa. The glyco-conjugate nature was revealed by the PAS staining of the native protein. The biological activity of the protein on goat spermatozoa showed significant increase in forward motility of goat spermatozoa in both concentration and time dependent manner. However, heat treatment of this protein substantially decreased its potency. Although the isolated protein is of goat origin, the CASA results with human spermatozoa suggest that there is no species barrier as far as sperm motion stimulating activity of this protein is concerned. The MALDI-ToF and mass finger printing of the protein showed a close resemblance of the protein to antithrombin III. Based on this, is it is concluded that the protein is an antithrombin III like molecule. It has been suggested that this sperm motion stimulator protein could be used for selection of high quality sperm in assisted reproduction programs.

REFERENCES

REFERENCES

1. Adler JA. Methods for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by Escherichia coli. J Gen Microbiol 1973; 74: 77-91.

2. Ailenberg M, Bartoov B, Mayevsky A. The effect of testosterone on ram spermatozoa collective motility treated by antimycin A. Acta Endocrinol (Copenh). 1984; 106(1):133-140.

3. Al-Anzi B, Chandler DE. A sperm chemoattractant is released from Xenopus egg jelly during spawning. Dev Biol. 1998; 198(2): 366-375.

4. Aleporou-Marinou V, Pappa H, Yalouris P, Patargias T. Purification of apolipoprotein H (beta 2-glycoprotein I)-like protein from human follicular fluid. Comp Biochem Physiol B 2001; 128(3): 537-542.

5. Anderson RA, Feathergill KA, Rawlins RG, Mack SR, Zaneveld LJD. Atrial natriuretic peptide: a chemoattractant of human spermatozoa by a guanylate cyclase-dependent pathway. Mol Reprod Dev. 1995; 40: 371–378.

6. Arnoult C, Cardullo RA, Lemos JR, Florman HM. Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci USA 1996a; 93: 13004–13009.

7. Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, Florman HM. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proc Natl Acad Sci USA 1999; 96: 6757–6762.

8. Asai H, Kasai H, Matsuda Y, Yamazaki N, Nagawa F, Sakano H, Tsuboi A. Genomic structure and transcription of a murine odorant receptor gene: differential initiation of transcription in the olfactory and testicular cells. Biochem Biophys Res Commun 1996; 221(2): 240-247.

9. Bahat A, Eisenbach M. Sperm thermotaxis. Mol Cell Endocrinol 2006; 252(1-2): 115-119.

10. Bahat A, Tur-Kaspa I, Gakamsky A, Giojalas LC, Breitbart H, Eisenbach M. Thermotaxis of mammalian sperm cells: a potential navigation mechanism in the female genital tract. Nat Med. 2003; 9(2): 149-150.

11. Baldi E, Luconi M, Bonaccorsi L, Muratori M, Forti G. Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. Front Biosci 2000; 5: E110–E123.

12. Ballesteros LM, Delgado NM, Rosado A, Correa C, Hernandez-Perez O. Binding of chemotactic peptide to the outer surface and to whole human spermatozoa with different affinity states. Gamete Res. 1988; 20(2): 233-239.

13. Barratt CLR, Cooke ID. Sperm transport in the human female reproductive tract-a dynamic interaction. Int J Androl. 1991; 14: 394-411.

14. Bavister BD. Properties of the sperm motility stimulating component derived from human serum. J Reprod Fertil. 1975; 43(2): 363-366.

15. Becker EL. Some interrelations of neutrophil chemotaxis, lysosomal enzyme secretion, and phagocytosis as revealed by synthetic peptides. Am J Pathol. 1976; 85(2): 385-394.

16. Bedford JM, Kim HH. Cumulus oophorus as a sperm sequestering device, in vivo. J of Exp Zoo. 1993; 265: 321-328.

17. Bentley JK, Tubb DJ, Garbers DL. Receptor-mediated activation of spermatozoan guanylate cyclase. J Biol Chem. 1986 Nov 15; 261(32): 14859-62.

18. Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JD (ed.), Methods in mammalian embryology. San Francisco: Freeman; 1971: 86-116.

19. Blackmore PF, Lattanzio FA. Cell surface localization of a novel non-genomic progesterone receptor on the head of human sperm. Biochem Biophys Res Commun. 1991; 181: 331–336.

20. Bohmer M, Van Q, Weyand I, Hagen V, Beyermann M, Matsumoto M, Hoshi M, Hildebrand E, Kaupp UB. Ca^{2+} spikes in the flagellum control chemotactic behaviour of sperm. EMBO. 2005; 24(15): 2741-2752.

21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248–254.

22. Brenner BM, Ballerman BJ, Gunning ME, Zeidel ML. Diverse biological actions of atrial natriuretic peptide. Physiol Rev. 1990; 70: 665–699.

23. Brokaw CJ. Chemotaxis of bracken spermatozoids. The role of bimalate ions J Exp Zoo.1958; 35: 192-196.

24. Bronson R, Hamada Y. Gamete interactions in vitro. Fertil and Sterility 1977; 28: 570-576.

25. Burkman LJ. Hyperactivated motility of human spermatozoa during in vitro capacitation and implication for fertility. In: Gangnon C (eds), control of sperm motility: biological and clinical aspect. Boca Raton: CRC Press; 1990: 303-329.

26. Bushmeyer SM, Bellin ME, Brantmeier SA, Boehm SK, Kubajak CL, Ax RL. Relationships between bovine follicular fluid glycosaminoglycans and steroids. Endocrinology. 1985; 117(3): 879-885.

27. Chang SC, Jones JD, Ellefson RD, Ryan RJ. The porcine ovarian follicle: I. Selected chemical analysis of follicular fluid at different developmental stages. Biol Reprod. 1976 Oct; 15(3): 321-328.

28. Chao HT, Ng HT, Kao SH, Wei YH, Hong CY. Human follicular fluid stimulates the motility of washed human sperm. Arch Androl. 1991; 26: 61-65.

29. Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science. 2000; 289: 625– 628.

30. Christen R, Schackmann RW, Shapiro BM. Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin (*Strongylocentrotus purpuratus*). J Biol Chem. 1982; 257: 14881–14890.

31. Cohen-Dayag A, Ralt D, Tur-Kaspa I, Manor M, Makler A, Dor J, Mashiach S. Eisenbach M. Sequential acquisition of chemotactic responsiveness by human spermatozoa. Biol Reprod. 1994; 50(4): 786-790.

32. Cohen-Dayag A, Tur-Kaspa I, Dor J, Mashiach S, Eisenbach M. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. Proc Natl Acad Sci U S A. 1995; 92 (24): 11039-11043.

33. Coll JC, Miller RL. The nature of sperm chemoattractants in coral and star fish. In: Baccetti B (ed), comparative spermatology; 20 years after. New York: Raven press, 1992:129-134.

34. Cook SP, Brokaw CJ, Muller CH and Babcock DF. Sperm chemotaxis: egg peptides control cytosolic calcium to regulate flagellar responses Dev Biol. 1994; 165: 10-19.

35. Cooper GW, Overstreet JW, Katz DF. The motility of rabbit spermatozoa recovered from the female reproductive tract. Gamete Res. 1979; 2: 35–42.

36. Cornwall GA, Smyth TS, Vindivich D, Harter C, Robinson J, Chang TS. Induction and enhancement of progressive motility in hamster caput epididymal spermatozoa. Biol Reprod. 1986; 35: 1065–1074.

37. Correia JN, Conner SJ, Kirkman-Brown JC. Non-genomic steroid actions in human spermatozoa. "Persistent tickling from a laden environment". Semin Reprod Med. 2007; 25(3): 208-219.

38. Cosson MP. Controls of sperm motility: Biological and clinical aspects. In: Gagnon C (ed), Boca Raton Florida: CRC Press; 1990: 103-135.

39. Darszon A, Beltrán C, Felix R, Nishigaki T, Treviño CL. Ion transport in sperm signaling. Dev Biol. 2001; 240(1): 1-14.

40. Devreotes PN, Zigmond SH. Chemotaxis in eukaryotic cells: a focus on leukocytes and Dictyostelium. Annu Rev Cell Biol. 1988; 4: 649-686.

41. Drobnis EZ, Overstreet JW. Natural history of mammalian spermatozoa in the female reproductive tract. In: Mulligan SR (ed), Oxford Reviews of Reproductive Biology. Oxford: Oxford Univ. Press; 1992: 1–45.

42. Eisenbach M, Ralt D. Precontact mammalian sperm-egg communication and role in fertilization. Am J Physiol. 1992; 262(5): 1095-1101.

43. Eisenbach M, Tur-Kaspa I. Do human eggs attract spermatozoa? Bioessays. 1999; 21(3): 203-210.

44. Eisenbach M, Tur-Kaspa I. Human sperm chemotaxis is not enigmatic anymore. Fertil and Steril. 1994; 62(2): 233-235.

45. Eisenbach M. Chemotaxis London: Imperial College Press; 2004.

46. Eisenbach M. Sperm changes enabling fertilization in mammals. Current Opinion Endocrinology and Diabetes 1995; 2: 468-475.

47. Eisenbach M. Sperm chemotaxis. Rev Reprod 1999; 4: 56-66.

48. Eisenbach M. Towards understanding the molecular mechanism of sperm chemotaxis. J Gen Physiol 2004; 124: 105–108.

49. Fabro G, Rovasio RA, Civalero S, Frenkel A, Caplan SR, Eisenbach M, Giojalas LC. Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. Biol Reprod. 2002; 267(5): 1565-1571.

50. Fetterolf PM, Sutherland CS, Josephy PD, Casper RF, Tyson JE. Preliminary characterization of a factor in human follicular fluid that stimulates human spermatozoa motion. Hum Reprod. 1994; 9(8): 1505-1511.

51. Flesch FM, Gadella B M. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta. 2000; 1469: 197–235.

52. Fraser LR. Cellular biology of capacitation and the acrosome reaction. Hum Reprod. 1995; 10(1): 22–30.

53. Fukuda N, Yomogida K, Okabe M, Touhara K. Functional characterization of a mouse testicular olfactory receptor and its role in chemosensing and in regulation of sperm motility. J Cell Sci. 2004; 117: 5835–5845.

54. Gaddum-Rosse P. Some observations on sperm transport through the uterotubal junction of the rat. Am J Anat. 1981; 160: 333–341.

55. Gandhi KK, Anand SR. Fructolysis and respiration of buffalo spermatozoa. J Reprod Fertil. 1975; 45(1): 185-188.

56. Gandhi KK, Anand SR. Regulation of glycolysis/fructolysis in buffalo spermatozoa. J Reprod Fertil. 1982; 64(1): 145-150.

57. Garbers DL. Molecular basis of fertilization. Ann Rev Biochem. 1989; 58: 719–742.

58. Gaur RD, Talwar GP. Further studies on the fertility-promoting factor from human seminal plasma. Int J Fertil. 1975; 20: 133–136.

59. Gerard N, Loiseau S, Duchamp G, Seguin F. Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (1H NMR). Reproduction. 2002; 124(2): 241-248.

60. Giojalas LC, Rovasio RA. Mouse spermatozoa modify their dynamic parameters and chemotactic response to factors from oocyte microenvironment. Int J Androl. 1998; 21: 201-206.

61. Gnessi L, Fabbri A, Silvestroni L, Moretti C, Fraioli F, Pert CB, Isidori A. Evidence for the presence of specific receptors for N-formyl chemotactic peptides on human spermatozoa. J Clin Endocrinol Metab. 1986; 63(4): 841-846.

62. Gnessi L, Ruff MR, Fraioli F, Pert CB. Demonstration of receptor-mediated chemotaxis by human spermatozoa. A novel quantitative bioassay. Exp Cell Res. 1985; 161(1): 219-230.

63. Gomendio M, Harcourt AH, Roldan ERS. Sperm competition and sexual selection. In: Brikhead TR, Moller AP (eds), London: Academic Press; 1998: 667-751.

64. Gulamali-Majid F, Ackerman S, Veeck L, Acosta A, Pleban P. Kinetic immunonephelometric determination of protein concentrations in follicular fluid. Clin Chem. 1987; 33(7): 1185-1189.

65. Gwatkin RB, Andersen OF. Capacitation of hamster spermatozoa by bovine follicular fluid. Nature. 1969; 224(5224): 1111-1112.

66. Hansen C, Srikandakumar A, Downey BR. Presence of follicular fluid in the porcine oviduct and its contribution to the acrosome reaction Mol Reprod Dev. 1991; 30: 148-153.

67. Harper CV, Barratt CL, Publicover SJ. Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of $[Ca^{2+}]i$ oscillations and cyclical transitions in flagellar beating. J Biol Chem. 2004; 279: 46315–46325.

68. Harper MJ. Relationship between sperm transport and penetration of eggs in the rabbit oviduct. Biol Reprod. 1973; 8(4): 441-450.

69. Harper MJK. Sperm and egg transport. In: Austin CR, Short RV (eds), Germ cells and fertilization. Cambridge, England: Cambridge University Press; 1982:102-127.

70. Harper MJK. Gamete and zygote transport. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, 2nd ed. New York: Raven Press; 1994:123–187.

71. Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogenology. 2005; 64(4): 992-1006.

72. Harrison RA. Glycolytic enzymes in mammalian spermatozoa. Activities and stabilities of hexokinase and phosphofructokinase in various fractions from sperm homogenates Biochem J. 1971; 124(4): 741-750.

73. Ho HC, Suarez SS. An inositol 1, 4, 5-trisphosphate receptor-gated intracellular Ca^{2+} store is involved in regulating sperm hyperactivated motility. Biol Reprod. 2001; 65(5): 1606-1615.

74. Ho HC, Suarez SS. Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction. 2001; 122(4): 519-526.

75. Hoskins DD, Brandt H, Acott TS. Initiation of sperm motility in the mammalian epididymis. Fed Proc. 1978; 37: 2534–2542.

76. Hunter RHF. Sperm: egg ratios and putative molecular signals to modulate gamete interactions in polytocous mammals. Mol Reprod and Dev. 1993; 35: 324-327.

77. Hunter, RHF. In: The Fallopian Tubes: Their Role in Fertility and Infertility. New York: Springer-Verlag; 1988: 53–80.

78. Iqbal M, Shivaji S, Vijayasarthy S, Balaram P. Synthetic peptides as chemoattractants for bull spermatozoa: structure activity correlations. Biochem Biophys Res Commun. 1980; 96: 235-242.

79. Isobe T, Minoura H, Tanaka K, Shibahara T, Hayashi N, Toyoda N. The effect of RANTES on human sperm chemotaxis. Hum Reprod. 2002; 17(6): 1441-1446.

80. Jaiswal BS, Eisenbach M, Tur-Kaspa I. Detection of partial and complete acrosome reaction in human spermatozoa: which inducers and probes to use? Mol Hum Reprod. 1999; 5 (3): 214-219.

81. Jaiswal BS, Eisenbach M. Capacitation. In: Hardy DM, Garbers DL (eds), fertilization. San Diego: Academic press; 2002: 57-117.

82. Jaiswal BS, Majumder GC. Cyclic AMP phosphodiesterase: A regulator of forward motility initiation during epididymal sperm maturation. Biochem Cell Biol. 1996a; 74: 669–674.

83. Jaiswal BS, Majumder GC. In vitro initiation of forward motility in testicular spermatozoa. Int J Androl. 1996b; 19(2): 97–102.

84. Jaiswal BS, Tur-Kaspa I, Dor J, Mashiach S, Eisenbach M. Human sperm chemotaxis: is progesterone a chemoattractant. Biol Reprod. 1999; 60(6): 1314-1319.

85. Johnson CH, Clapper DL, Winkler MM, Lee HC, Epel D. A volatile inhibitor immobilizes sea urchin sperm in semen by depressing the intracellular pH. Dev Biol. 1983; 98: 493–501.

86. Kann ML, Serres C. Development and initiation of sperm motility in the hamster epididymis. Reprod Nutr Dev. 1980; 20: 1739–1749.

87. Kao SH. Purification, characterization and mechanism of action of a sperm-motility stimulator from human follicular fluid. Taipei: Institute of Biochemistry, National Yang-Ming Medical College; 1991. Thesis.

88. Katz DF, Drobnis EZ, Overstreet JW. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. Gamete Res. 1989; 22: 443–469.

89. Kaupp UB, Solzin J, Hildebrand E, Brown JE, Helbig A, Hagen V, Beyermann M, Pampaloni F, Weyand I. The signal flow and motor response controling chemotaxis of sea urchin sperm. Nat Cell Biol. 2003; 5(2): 109-117.

90. Kirkman-Brown JC, Sutton KA, Florman HM. How to attract a sperm. Nat Cell Biol. 2003; 5(2): 93-96.

91. Kim SH, Cho KW, Lim SH, Hwang YH, Ryu H, Oh SH, Seul KH, Jeong GB, Yoon S Presence and release of immunoreactive atrial natriuretic peptide in granulosa cells of the pig ovarian follicle. Regulatory Peptide 1992; 42: 153–162.

92. Kim SH, Cho KW, Oh SH, Hwang YH, Lim SH, Ryu H, Seul KH, Jeong GB, Yoon S Immunoreactive atrial natriuretic peptides in the oocyte. Comparative Biochemistry and Physiology: Comparative Physiology 1993; 104:219–223.

93. Koyama S, Amarie D, Soini HA, Novotny MV, Jacobson SC. Chemotaxis assays of mouse sperm on microfluidic devices. Anal Chem. 2006; 78(10): 3354-3359.

94. Kobayashi T, Tamura M, Hayashi M, Itano Y, Tanabe H, Katsuura Y, Ohta T, Komoriya K. Effects of Progesterone on the Elevation of Tail Skin Temperature in Ovariectomized Rats. Japanese Journal of Physiology. 2000; 50: 651-656.

95. Kruip TA, Dieleman SJ. Macroscopic classification of bovine follicles and its validation by micromorphological and steroid biochemical procedures. Reprod Nutr Dev. 1982; 22(3): 465-473.

96. Laemmli UK.. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature.1970; 227: 680–685.

97. Lauffenburger DA, Tranquillo RT, Zigmond SH. Concentration gradients of chemotactic factors in chemotaxis assays. Methods Enzymol. 1988; 162: 85-101.

98. Lee CN, Ax RL. Concentrations and composition of glycosaminoglycans in the female bovine reproductive tract. J Dairy Sci. 1984; 67(9): 2006-2009.

99. Lee HC, Johnson C, Epel D. Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. Dev Biol. 1983; 95: 31–45.

100. Lee SL, Kao CC, Wei YH. Antithrombin III enhances the motility and chemotaxis of boar sperm. Comp Biochem Physiol Comp Physiol. 1994; 107(2): 277-282.

101. Lee SL, Kuo YM, Kao CC, Hong CY, Wei YH. Purification of a sperm motility stimulator from porcine follicular fluid. Comp Biochem Physiol B. 1992; 101(4): 591-594.

102. Lefebvre R, Suarez SS. Effect of capacitation on bull sperm binding to homologous oviductal epithelium. Biol of Reprod. 1996; 54: 575-582.

103. Luconi M, Bonaccorsi L, Maggi M, Pecchioli P, Krausz C, Forti G, Baldi E. Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. J Clin Endocrinol Metab. 1998; 83(3): 877-885.

104. McGaughey RW. A comparison of the fluids from small and large ovarian follicles of the pig. Biol Reprod. 1975; 13(2): 147-153.

105. Maier I, Muller DG. Sexual pheromones in algae Biol Bulletin. 1986; 170: 145-175.

106. Majumder GC, Jaiswal BS, Nath D, Banerjee S, Barua M, Sarkar M, Rana APS, Mitra S, Dutta P, Chatterjee T, Mishra S, Ghosh A. Biochemistry of sperm motility initiation during epididymal maturation. In: Joy KP, Krishna A, Haldar C, (eds), Comparative endocrinology and reproduction. New Delhi, India: Narosa Publishing House. 1999: 242–259.

107. Makler A, Reichler A, Stoller J, Feigin PD. A new model for investigating in real time the existence of chemotaxis in human spermatozoa Fertil and Steril. 1992; 57: 1066–1074.

108. Makler A, Stoller J, Reichler A, Bluemenfeld Z, Yoffe N. Inability of human sperm to change their orientation in response to external chemical stimuli Fertil and Stril.1995; 63: 1077-1082.

109. Makler A, Stoller J, Reichler A, Blumenfeld Z, Yoffe N. Inability of human sperm to change their orientation in response to external chemical stimuli. Fertil and Steril. 1995; 63(5): 1077-1082.

110. Mandal M, Banerjee S, Majumder GC. Stimulation of forward motility of goat cauda epididymal spermatozoa by a serum glycoprotein factor. Biol Reprod 1989; 41: 983–989.

111. Mandal M, Saha S, Ghosh AK, Majumder GC. Identification and characterization of a sperm motility promoting glycoprotein from buffalo blood serum. Journal of Cellular Physiology 2006; 209:353–362.

112. Manor M. Identification and Purification of Female-Originated Chemotactic Factors. Israel: The Weizmann Institute of Science; 1994. Thesis.

113. Meizel S, Turner KO. Progesterone acts at the plasma membrane of human sperm. Mol Cell Endocrinol. 1991; 77:1–5.

114. Miki K. Energy metabolism and sperm function. Soc Reprod Fertil Suppl. 2007; 65: 309-325.

115. Miller RL. Sperm chemo-orientation in the metazoan. In: Metz CB, Monroy A (eds), Biology of fertilization. New York: Academic Press; 1985: 275-337.

116. Miller RD, Vogt R. An N-terminal partial sequence of the 13 kDa Pycnopodia helianthoides sperm chemoattractants 'Startrak' possesses sperm attracting activity J Exp Biol. 1996; 199: 311-318.

117. Miller RL. Synthetic peptides are not chemoattractants for bull sperm Gamete Res. 1982; 5: 395-401.

118. Morisawa M. Cell signaling mechanisms for sperm motility. Zool Sci. 1994; 11: 647–662.

119. Morita Z, Chang MC. Maintenance of the motility of rat epididymal spermatozoa in the presence of male accessory secretions. J Reprod Fertil. 1971; 24(2): 247-254.

120. Morton B, Chang TS. The effect of fluid from the cauda epididymidis, serum components and caffeine upon the survival of diluted epididymal hamster spermatozoa. J Reprod Fertil. 1973; 35(2): 255-263.

121. Morton BE, Fraser CF, Sagadraca R. Initiation of hamster sperm motility from quiescence: effect of conditions upon flagellation and respiration. Fertil and Steril. 1979; 32(2): 822-827.

122. Munoz-Garay C, de la Vega-Beltran JL, Delgado R, Labarca P, Felix R, Darszon A. Inwardly rectifying K(1) channels in spermatogenic cells: Functional expression and implication in sperm capacitation. Dev Biol. 2001; 234: 261–274.

123. Nagai T, Yamaguchi K, Moriwaki C. Studies on the effects of sugars on washed human sperm motility. J Pharmacobiodyn. 1982; 5(8): 564-7.

124. Nagdas SK, Skudlarek MD, Orgebin-Crist MC, Tulsiani DR. Biochemical alterations in the proacrosin-acrosin system during epididymal maturation of the rat spermatozoa. J Androl. 1992; 13(1): 36-43.

125. Oehninger S, Lanzendorf S, Burkman LJ, Sueldo C, Mohony M, Alexander NJ, Hodgen GD. A sequential analysis of the effect of progesterone on specific sperm functions crucial to fertilization in vitro in infertile patients Hum Reprod. 1994; 9: 1322-1327.

126. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem. 1975; 250(10): 4007-4021.

127. Okamura N, Tajima Y, Soejima A, Masuda H, Sugita Y. Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. J Biol Chem. 1985; 260: 9699–9705.

128. Olds-Clarke P, Wivell W. Impaired transport and fertilization in vivo of calcium-treated spermatozoa from +/+ or congenic tw32/+ mice. Biol Reprod. 1992; 47: 621–628.

129. Oliveira RG, Tomasi L, Rovasio RA, Giojalas LC. Increased velocity and induction of chemotactic response in mouse spermatozoa by follicular and oviductal fluids. J Reprod Fertil. 1999; 115(1): 23-27.

130. Orsi NM, Gopichandran N, Leese HJ, Picton HM, Harris SE. Fluctuations in bovine ovarian follicular fluid composition throughout the oestrous cycle. Reproduction. 2005; 129(2): 219-228.

131. Overstreet JW, Drobnis EZ. Sperm transport in female tract. In: Barratt CLR, Cooke (eds), Advances in donor insemination. Cambridge, England: Cambridge university press; 1991: 33-49.

132. Overstreet JW, Cooper GW. Sperm transport in the reproductive tract of the female rabbit: I. The rapid transit phase of transport. Biol. Reprod. 1978a; 19: 101–114.

133. Overstreet JW, Cooper GW. Sperm transport in the reproductive tract of the female rabbit: II. The sustained phase of transport. Biol Reprod. 1978b; 19: 115–132.

134. Overstreet JW, Katz DF. Interaction between the female reproductive tract and spermatozoa. In: Gagnon C (ed), Controls of Sperm Motility: Biological and Clinical Aspects. Boca Raton, Florioda: CRC Press; 1990: 63–75.

135. Parmentier M, Libert F, Schurmans S, Schiffmann S, Lefort A, Eggerickx D, Ledent C, Mollereau C, Gerard C, Perret J. Expression of members of the putative olfactory receptor gene family in mammalian germ cells. Nature. 1992; 355(6359): 453-455.

136. Patel SM, Skandhan KP, Mehta YB. Seminal plasma fructose and glucose in normal and pathological conditions. Acta Eur Fertil. 1988; 19(6): 329-332.

137. Pfeffer W. Lokomotorische Richtungsbewegungen durch chemische Reize. Untersuchungen aus d. Botan. Inst. Tübingen. 1884; 1: 363–482.

138. Pinto O, Amir D, Schindler H, Hurwitz S. Effect of pH on the metabolism and fertility of turkey spermatozoa. J Reprod Fertil. 1984; 70: 437–442.

139. Ralt D, Goldenberg M, Fetterolf P, Thompson D, Dor J, Mashiach S, Garbers DL, Eisenbach M. Sperm attraction to a follicular factor(s) correlates with human egg fertilizability. Proc Natl Acad Sci USA. 1991; 88(7): 2840-2844.

140. Ralt D, Manor M, Cohen-Dayag A, Tur-Kaspa I, Ben-Shlomo I, Makler A, Yuli I, Dor J, Blumberg S, Mashiach S. Chemotaxis and chemokinesis of human spermatozoa to follicular factors. Biol Reprod. 1994; 50(4): 774-785.

141. Rigau T, Farré M, Ballester J, Mogas T, Peña A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology. 2001; 56(5): 801-815.

142. Robertson L, Wolf DP, Tash JS. Temporal changes in motility parameters relates to acrosomal status; identification and charaterization of populations of hyperactivated human sperm Biol Reprod.1988; 39: 797-805.

143. Roy N, Majumder GC, Chakrabarti CK. Occurrence of specific glycoprotein factor(s) in goat epididymal plasma that prevent adhesion of spermatozoa to glass. Andrologia. 1985; 17: 200–206.

144. Ruskoaho H. Atrial natriuretic peptide: synthesis, release, and metabolism. Pharmacol Rev. 1992; 44: 481–602.

145. Santi CM, Darszon A,Hernandez-Cruz A. A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+}current carrier in mouse primary spermatocytes. Am J Physiol. 1996; 271: 1583–1593.

146. Suarez SS. Hyperactivated motility in sperm. Journal of Andrology. 1998; 17: 331-335.

147. Schiffmann E, Corcoran BA, Wahl SM. N.formylmethiony peptides as chemoattracatnts for leucocytes Proc Natl Acad Sci USA. 1975; 72: 1059-1062.

148. Serrano H, Canchola E, García-Suárez MD. Sperm-attracting activity in follicular fluid associated to an 8.6-kDa protein. Biochem Biophys Res Commun. 2001; 283: 782–784.

149. Shalgi R, Smith TT, Yanagimachi R. A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through the uterotubal junction. Biol Reprod. 1992; 46: 419–424.

150. Shao B, Nascimento JM, Shi LZ, Botvinick EL. Automated motile cell capture and analysis with optical traps. Methods Cell Biol. 2007; 82: 601-627.

151. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2006; 1(6): 2856-2860.

152. Shimomura H, Dangott LJ, Garbers DL. Covalent coupling of a resact analogue to guanylate cyclase. J Biol Chem. 1986; 261(33): 15778-15782.

153. Silvestroni L, Palleschi S, Guglielmi R, Croce CT. Identification and localization of atrial natriuretic factor receptors in human spermatozoa. Arch Androl. 1992; 28: 75–82.

154. Sinclair ML, Wang XY, Mattia M, Conti M, Buck J, Wolgemuth DJ, Levin LR. Specific expression of soluble adenylyl cyclase in male germ cells. Mol Reprod Dev. 2000; 56:6–11.

155. Singh S, Lowe DG, Thorpe DS, Rodriguez H, Kuang WJ, Dangott LJ, Chinkers M, Goeddel DV, Garbers DL. Membrane guanylate cyclase is a cell-surface receptor with homology to protein kinases. Nature. 1988; 334(6184): 708-712.

156. Sliwa L. Chemotactic effect of hormones in mouse spermatozoa. Arch Androl. 1994; 32: 83–88.

157. Sliwa L. Chemotaction of mouse spermatozoa induced by certain hormones. Arch Androl. 1995; 35(2): 105-110.

158. Sliwa L. Effect of transforming growth factors (TGFalpha and TGFbeta) on human sperm chemotactic migration in vitro. Arch Androl. 2003; 49(2): 155-159.

159. Sliwa L. Heparin as a chemoattractant for mouse spermatozoa. Arch Androl. 1993b; 31: 149–152.

160. Sliwa L. Hyaluronic acid and chemoattractant substance from follicular fluid: in vitro effect of human sperm migration. Arch Androl. 1999; 43: 73–76.

161. Sliwa L. Substance P and beta-endorphin act as possible chemoattractants of mouse sperm. Arch Androl. 2001; 46: 135–140.

162. Sliwa L.. Effect of heparin on human spermatozoa migration in vitro. Arch Androl. 1993a; 30: 177–181.

163. Smith TT, Yanagimachi R. Attachment and release of spermatozoa from the caudal isthmus of the hamster oviduct J of Reprod and Fertil. 1991; 91: 567-573.

164. Spehr M, Gisselmann G, Poplawski A, Riffell JA, Wetzel CH, Zimmer RK, Hatt H. Identification of a testicular odorant receptor mediating human sperm chemotaxis. Science. 2003; 299(5615): 2054-2058.

165. Spehr M, Schwane K, Riffell JA, Barbour J, Zimmer RK, Neuhaus EM, Hatt H. Particulate adenylate cyclase plays a key role in human sperm olfactory receptor-mediated chemotaxis. J Biol Chem. 2004; 279(38): 40194-40203.

166. Suarez SS, Wolf DP, Meizel S. Induction of acrosome reaction in human spermatozoa by a fraction of human follicular fluid. Gamete Res. 1986; 14: 107-109.

167. Suarez SS. Hyperactivated motility in sperm. J Androl. 1996; 17: 331–335.

168. Suarez SS. The oviductal sperm reservoir in mammals: mechanisms of formation. Biol Reprod. 1998; 58(5):1105-1107.

169. Sun F, Bahat A, Gakamsky A, Girsh E, Katz N, Giojalas LC, Tur-Kaspa I, Eisenbach M. Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants. Hum Reprod. 2005; 20(3): 761-767.

170. Sun F, Giojalas LC, Rovasio RA, Tur-Kaspa I, Sanchez R, Eisenbach M. Lack of species-specificity in mammalian sperm chemotaxis. Dev Biol. 2003; 255(2): 423-427.

171. Sundfjord JA, Forsdahl F, Thibault G. Physiological levels of immuno-reactive ANH-like peptides in human follicular fluid. Acta Endocrinol. 1989; 121: 578–580.

172. Suzuki N. Structure, function and biosynthesis of sperm-activating peptides and fucose sulphate glycoconjugate in the extracellular coat of sea urchin eggs. Zoological Science. 1995; 12: 13-27.

173. Tacconis P, Revelli A, Massobrio M, Battista La Sala G, Tesarik J. Chemotactic responsiveness of human spermatozoa to follicular fluid is enhanced by capacitation but is impared in dyspermic semen. J Assist Reprod Genet. 2001; 18(1): 36-44.

174. Talbot P and Chacon RS. A triple-stain technique for evaluating normal acrosome reactions of human sperm. J Exp Zool. 1981; 215: 201.

175. Teves ME, Barbano F, Guidobaldi HA, Sanchez R, Miska W, Giojalas LC. Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. Fertil and Steril. 2006; 86(3): 745-749.

176. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979; 76(9): 4350-4354.

177. Tranquillo RT, Lauffenburger DA, Zigmond SH. A stochastic model for leukocyte random motility and chemotaxis based on receptor binding fluctuations. J Cell Biol. 1988; 106(2): 303-309.

178. Tso WW, Lee WM, Wong MY. Spermatozoa repellent as a contraceptive. Contraception. 1979; 19(3): 207-212.

179. Tulsiani DR, Skudlarek MD, Orgebin-Crist MC. Novel alpha-D-mannosidase of rat sperm plasma membranes: characterization and potential role in sperm-egg interactions. J Cell Biol. 1989; 109(3): 1257-1267.

180. Vadillo-Ortega F, Villanueva-Diaz C, Arias Martinez QB, Bermejo L, Bustos Lopez H, Chemotactic factor for spermatozoa : a new biological function of progesterone. Ginecol Obstet Mex. 1994; 62: 127-130.

181. Vanderhaeghen P, Schurmans S, Vassart G, Parmentier M. Specific repertoire of olfactory receptor genes in the male germ cells of several mammalian species. Genomics 1997; 39: 239–246.

182. Velazquez A, Reyes A, Chargoy J, Rosado A. Amino acid and protein concentrations of human follicular fluid. Fertil Steril. 1977; 28(1): 96-100.

183. Vijayasarthy S, Shivaji S, Iqbal M, Balaram P. Formyl-met-leu-phe induces chemotaxis and acrosomal enzyme release in bull sperm. FEBS Lett 1980; 115: 178-180.

184. Villanueva-Díaz C, Arias-Martínez J, Bermejo-Martínez L, Vadillo-Ortega F. Progesterone induces human sperm chemotaxis. Fertility and Sterility 1995; 64: 1183–1188.

185. Villanueva-Díaz C, Arias-Martínez J, Bustos-López H, Vadillo-Ortega F. Novel model for study of human sperm chemotaxis Fertility and Sterility 1992; 58: 392–395.

186. Villanueva-Diaz C, Vadillo-Ortega F, Kably-Ambe A, Diaz-Perez MA, Krivitzky SK. Evidence that human follicular fluid contains a chemoattractant for spermatozoa. Fertil and Steril. 1990; 54:1180-1182.

187. Visconti P E, Tezon JG. Phorbol esters stimulate cyclic adenosine 3', 5'-monophosphate accumulation in hamster spermatozoa during in vitro capacitation. Biol Reprod. 1989; 40: 223–231.

188. Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, Kopf GS. Cholesterol efflux-mediated signal transduction in mammalian sperm. beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. J Biol Chem. 1999; 274: 3235–3242.

189. Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development. 1995; 121: 1139–1150.

190. Vredenburgh-Wilberg WL, Parrish JJ. Intracellular pH of bovine sperm increases during capacitation. Mol Reprod Dev. 1995; 40: 490–502.

191. Walensky LD, Roskams AJ, Lefkowitz RJ, Snyder SH, Ronnett GV. Odorant receptors and desensitization proteins co-localize in mammalian sperm. Mol Med. 1995; 1: 130–141.

192. Wang Y, Storeng R, Dale P O, Abyholm T, Tanbo T. Effect of follicular fluid and steroid hormones on chemotaxis and motility of human spermatozoa in vitro. Gynecol Endocrinol. 2001; 15(4): 286-92.

193. Ward GE, Browkow CJ, Garbers DL and Vacquvier VD. Chemotaxis of Arbacia punctulata spermatozoa to resact, a peptide from the egg jelly layer J of Cell Biol. 1985; 101: 2324-2329.

194. White DR, Aitken RJ. Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete Res. 1989; 22: 163–177.

195. Williams AC, Ford WC. The role of glucose in supporting motility and capacitation in human spermatozoa. J Androl. 2001; 22(4): 680-695.

196. Williams M, Hill CJ, Scudamore I, Dunphy B, Cooke ID, Barratt CL. Sperm numbers and distribution within the human fallopian tube around ovulation. Hum Reprod. 1993; 8(12): 2019-2026.

197. World health organization. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 3rd ed. Cambridge, Cambridge University press 1992.

198. World Wide Web (URL:http://www.matrixscience.com/).

199. Yanagimachi R, Mammalian Fertilization. In: Knobil E, Neill JD (eds), The Physiology of Reproduction. 2nd ed. New York: Raven Press; 1994: 189–317.

200. Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote. 1994; 2(4): 371-372.

201. Yanagimachi R. In vitro capacitation of golden hamster spermatozoa by homologous and heterologous blood sera. Biol Reprod. 1970; 3(2): 147-153.

202. Yanagimachi R. The movement of golden hamster spermatozoa before and after capacitation J Reprod Fertil. 1970; 23: 193-196.

203. Yen SS. Estrogen withdrawal syndrome. J Am Med Assoc. 1986; 255 (12): 1614-1615.

204. Yoshida M, Inaba K, Ishida K, Morisawa M. Calcium and cyclic AMP mediate sperm activation, but Ca^{2+} along contributes sperm chemotaxis in the ascidian, *Ciona savignyi* Dev Growth and Differentiation. 1994; 36: 589-595.

205. Yoshida M, Inaba K, Morisawa M. Sperm chemotaxis during the process of fertilization in the ascidians Ciona savignyi and Ciona intestinalis Dev Biol.1993; 157: 497-506.

206. Zamir N, Riven-Kreitman R, Manor M, Makler A, Blumberg S, Ralt D, Eisenbach M. Atrial natriuretic peptide attracts human spermatozoa in vitro. Biochem Biophys Res Commun. 1993; 197(1): 116-122.

207. Zavos PM, Tauber PF, Schumacher GFB. In: Biochemistry of mammalian reproduction Zaneveld LJD, Chatterton RT (eds), New York, John Willey, 1982:186-189.

208. Zeng Y, Clark EN, Florman HM. Sperm membrane potential: Hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. Dev Biol.1995; 171: 554–563.

209. Zeng Y, Oberdorf JA, Florman HM. pH regulation in mouse sperm: Identification of Na(+)-, Cl(-)-, and HCO_3(-)-dependent and arylaminobenzoate-dependent regulatory

mechanisms and characterization of their roles in sperm capacitation. Dev Biol. 1996; 173: 510–520.

210. Zigmond SH. Chemotaxis by polymorphonuclear leukocytes. J Cell Biol.1978; 77(2): 269-87.