STRUCTURAL AND FUNCTIONAL ASPECTS OF SPERM AFTER TRANSIT THROUGH THE FALLOPIAN TUBE OF THE SHEEP

by

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Submitted in partial fulfilment for the degree of

Magister Scientiae

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February 1996

To Taariq

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ABSTRACT

Ejaculated mammalian spermatozoa have the potential for fertilization. The acquisition of the ability to fertilize a fully invested oocyte occurs during the passage through the female reproductive tract. These post-ejaculatory maturational changes include the processes of capacitation and the acrosome reaction. The Fallopian tube has been accepted as the site of completion of these events while their initiation may occur either in the uterus or in the Fallopian tube. The role and/or interaction of the Fallopian tube with sperm to undergo these molecular events, are poorly understood. This study attempts to demonstrate the influence on sperm of the environment of the Fallopian tube which could initiate certain structural and functional alterations with respect to capacitation and the acrosome reaction; this study does not attempt to define the exact nature of the interaction between spermatozoa and the Fallopian tube.

In this study the *in vivo* situation is mimicked, exposing sperm to the oviduct under laboratory conditions. Pre- and postmigratory sperm were compared. The specific structural and functional aspects examined in this study include motility, morphology, localization of N-acetyl-D-glucosamine receptors and the acrosomal status.

Motility is important to ensure successful migration to the site of fertilization. Special attention was focussed on the incidence of hyperactivation after transit through the Fallopian tube. The term hyperactivation has been used to describe

the frantic movement of sperm before they undergo the acrosome reaction and often serves as a biological marker for capacitation. Motility analyses were performed using the CASMA system, the Sperm Motility Quantifier (SMQ). The biological state of hyperactivation occurred in a small population of spermatozoa after transit through the Fallopian tube.

A morphologically normal sperm is a direct product of spermatogenesis and epididymal maturation. No gross morphological alterations have been reported to occur to sperm within the female tract. Instead, elimination of sperm with gross morphological aberrations (whether as a direct function of the female tract or intrinsic sperm factors) has been shown. Measurement of the sperm head dimensions were performed in this study using the Flexible Image Processing System (FIPS). No obvious morphological disparities were present in the samples of ram sperm used. No obvious morphological alterations/selections occurred during transit through the Fallopian tube. Ram sperm head dimensions are defined.

Fusion between spermatozoa and the egg vestments is a crucial step in fertilization. Exposure of fusiogenic structures on sperm is a component of capacitation. The presence of these receptors is important in species-specific interaction and its absence play a significant role in infertility. FITC-conjugated wheatgerm agglutinin was used to identify and localize N-acetyl-D-glucosamine-like receptors on the sperm membrane surface. This surface component is believed to play an important role in sperm-egg interaction. Membrane alterations associated with receptor activity (allowing for sperm-zona binding)

appear to have occurred after transit through the Fallopian tube.

The acrosome reaction has, to date, been recognised as the most reliable indicator of the completion of capacitation. It is generally accepted that the acrosome reaction of the fertilizing sperm occurs at the zona pellucida surface, that previously acrosome-reacted sperm may also bind to the ZP. FITC-conjugated peanut agglutinin was selected as a probe for acrosomal status determination. An increase in the incidence of acrosome-reacted sperm was observed after transit through the Fallopian tube.

A small population of spermatozoa, therefore, appear to have acquired (wholly or partially) fertilizing potential after transit through the Fallopian tube.

ABSTRAK

Uitgestorte soogdier spermatosoa besit die potensiaal om te bevrug. Die verwerwing van die vermoë om 'n ten volle omklede oösiet te bevrug geskied ten tye van die deurvaart van spermatosoa deur die vroulike geslagsisteem. Hierdie post-ejakulatoriese rypwordings veranderinge sluit in die prosesse van kapasitasie en die akrosoomreaksie. Die fallopiese buis word aanvaar as dié plek vir die voltooïng van hierdie gebeurtenisse, terwyl die inisiasie geskied in óf die uterus óf die fallopiese buis. Die rol en/of interaksie van die fallopiese buis met spermatosoa om die molekulêre gebeure te ondergaan, word swak begryp. Hierdie studie poog om die invloed van die fallopiese buisomgewing op spermatosoa te demonstreer in so verre dat die spermatosoa sekere strukturele en funksionele veranderinge ondergaan met betrekking tot kapasitasie en die akrosoomreaksie; die studie poog nie om die presiese karakter van die interaksie tussen spermatosoa en die fallopiese buis te definïeer nie.

Dus probeer hierdie studie die *in vivo* situasie naboots; om spermatosoa aan die ovidukt onder labratoriumkondisies bloot te stel. Pre- en postmigrasie spermatosoa word vergelyk. Die spesifieke strukturele en funksionele aspekte wat in hierdie studie onder die loep kom, sluit in motiliteit, morfologie, lokalisasie van N-asetiel-D-glukosamien reseptore en akrosomale status.

Motiliteit is belangrik om die suksesvolle migrasie na die plek van bevrugting te verseker. Spesiale aandag was gevestig op die voorkoms van hiperaktivering na

migrasie deur die fallopiese buis. Die term hiperaktivering is gebruik om die hoë aktiwiteit beweging van spermatosoa te beskryf voor hulle die akrosoomreaksie ondergaan en dien dikwels as 'n biologiese indikator vir kapasitasie. Motiliteitsanalises was verkry deur die gebruik van die "CASMA" sisteem, die "Sperm Motility Quantifier (SMQ)". Die biologiese toestand van hiperaktivering het slegs in 'n klein populasie van spermatosoa na migrasie deur die fallopiese buis voorgekom.

'n Morfologies normale sperm is 'n direkte produk van spermatogenese en epididimale rypwording. Geen aanduiding van morfologiese veranderinge van spermatosoa binne die vroulike geslagsisteem, kon vasgestel word nie. Die eliminasie van spermatosoa met groot morfologiese afwykings (óf vanweë 'n direkte funksie van die vroulike geslagsisteem óf intrinsieke spermatosoön faktore) word egter aangetoon. Opmetings van die spermkop se dimensies was verkry deur gebruik te maak van die "Flexible Image Processing System (FIPS)". Geen ooglopende morfologiese verskille van die betrokke ram spermatosoa is waargeneem nie. Geen ooglopende morfologiese veranderinge/seleksies het geskied tydens die deurtog deur die fallopiese buis nie. Ram spermkop dimensies is gedefinïeer.

Binding tussen spermatosoa en die eier bedekkings is 'n belangrike stap in bevrugting. Blootstelling aan bindingsstrukture op spermatosoa is 'n komponent van kapasitasie. Die voorkoms van hierdie reseptore is belangrik in spesie-spesifieke interaksie en hul afwesigheid speel 'n onmisbare rol in infertiliteit. FITC-gekonjugeerde koringkiem agglutinien was gebruik om N-asetiel-D-

glukosamien-tipe reseptore op die spermmembraan oppervlakte te identifiseer en te lokaliseer. Dié oppervlakte komponent speel na bewering 'n belangrike rol in die sperm-eier interaksie. Membraan veranderinge wat geassosieer is met reseptor aktiwiteite (wat toelaat vir sperm-zona binding) blyk voor te kom na migrasie deur die fallopiese buis.

Die akrosoomreaksie is, tot op datum, erken as die mees betroubare indikator vir die voltooïng van kapasitasie. Dit word algemeen aanvaar dat die akrosoomreaksie van die bevrugtende sperm geskied op die zona pellucida oppervlakte, maar dat die akrosoom-gereageerde sperm ook mag bind aan die ZP. FITC-gekonjugeerde grondboontjie agglutinien was geselekteer as om akrosomale status aan te dui. 'n Verhoging in die voorkoms van akrosoom-gereageerde spermatosoa was waargeneem na migrasie deur die fallopiese buis.

'n Klein populasie van spermatosoa het blykbaar (geheel of gedeeltelik) bevrugtings potensiaal verwerf na migrasie deur die fallopiese buis.

CHAPTER ONE

General Introduction

1.1 Introduction

Fertilization may be described as the sequence of events that starts with the interaction of the male and female gametes and culminates with the formation of the zygote. The crucial event of fertilization is the fusion of one spermatozoon with one oocyte [Monroy & Rosati, 1983]. The mechanisms leading to fertilization point to the existence of a well-defined sequence of events which includes maturation of both the oocyte and the spermatozoon. Sperm maturation starts within the male reproductive tract and is completed within the female reproductive tract. This maturational process includes the development of the fertilizing capacity of the spermatozoa and involves the processes of capacitation and the acrosome reaction. Subsequent events include sperm passage through the zona pellucida and fusion of the fertilizing spermatozoon with the oocyte [Hafez, 1980].

The sperm cell is unique in that it has evolved to survive many different physiological microenvironments from the epididymis, the vas deferens and

seminal plasma of the male post-testicular reproductive tract, to the vagina, cervix, uterus, Fallopian tube and peritoneal cavity of the female reproductive tract. Although the autonomous migration of sperm possibly plays a large role in the transport of sperm through the different microenvironments of the female reproductive tract, the fusion of sperm and oocyte also depends on the actions of the female tract in which the sperm cell may be a passive participant. These actions are biophysical phenomena and include the muscular contractions of the viscera and the activity of the cilia lining the lumina within the tract. The epithelial surfaces and secretions of the female reproductive tract, as well as the egg vestments, appear to modulate sperm motility and may coordinate the transport and fusion of the gametes [Katz *et al.*, 1989]. The female hormones, secreted during the different phases of the oestrus cycle, may also have an indirect effect on the transport of spermatozoa through the female tract by altering the properties of the secretions found within the female reproductive tract.

The prerequisites for successful fertilization *in vivo* on the part of the sperm cell appear to include:

1) a morphologically normal sperm which is a product of spermatogenesis and/or epididymal maturational processes,
2) vigorous sperm motility to ensure successful migration to the site of fertilization and transit through the egg envelopes (the forces due to flagellar motion assist the sperm in penetrating the egg vestments to reach

the oolemma) [Katz *et al.*, 1989] and

3) exposure of fusiogenic structures (receptors) coupled with species-specific interaction between the spermatozoa and the egg envelopes [Epel, 1980].

Many studies have been undertaken to elucidate the complexity of sperm transport and the prefertilization changes that occur within the female reproductive tract. Some of these studies have focused on microenvironment-induced capacitational changes by studying the effect of various possible contributing factors. However, these factors were studied in isolation and, therefore, it is difficult to appreciate the influence of the female reproductive tract, in its entirety, on these capacitational changes. Possible contributing factors, from the oviduct to the transport and prefertilization changes of sperm, include mechanical activity of the oviducts, oviductal fluid, follicular fluid, oviductal mucosa and ovarian hormones. For obvious reasons, it is very difficult to study these events as they occur *in vivo*. In this study, where the *in vivo* situation was mimicked, sperm were exposed to the oviduct environment under laboratory conditions. Although every possible precaution was taken to accommodate and maintain a constant physiological environment, an obvious drawback to the study is the possible failure of the maintenance of tissue survival outside the body.

Fertilizing spermatozoa are sequestered in the lower part of the oviductal isthmus until ovulation begins so that sperm ascent to the ampulla occurs

synchronously [Yanagimachi, 1994]. The termination of the arrest of spermatozoa in the caudal isthmus results in the release of capacitated sperm [Hunter *et al.*, 1983; Smith & Yanagimachi, 1991]. Although the site of capacitation is uncertain and may vary from species to species, increasing evidence suggests that, in some species at least, capacitation must be completed in the oviduct [Barros, 1968; Bedford, 1972]. Also, Fallopian tube fluid [Barros & Austin, 1967; Barros, 1968] and follicular fluid [Yanagimachi, 1969] have been found to promote capacitation. These results have led me to target the oviduct for my study of structural and functional aspects of sperm after transit through the Fallopian tube.

This study, therefore, examines structural and functional aspects of sperm transit through the oviduct. Although many studies have focused on a single fertilizing requirement of sperm, such as motility or morphology, after exposure to the oviduct, no study thus far has examined structural and functional aspects relating to motility, morphology, receptors and acrosomal status within the same sperm sample.

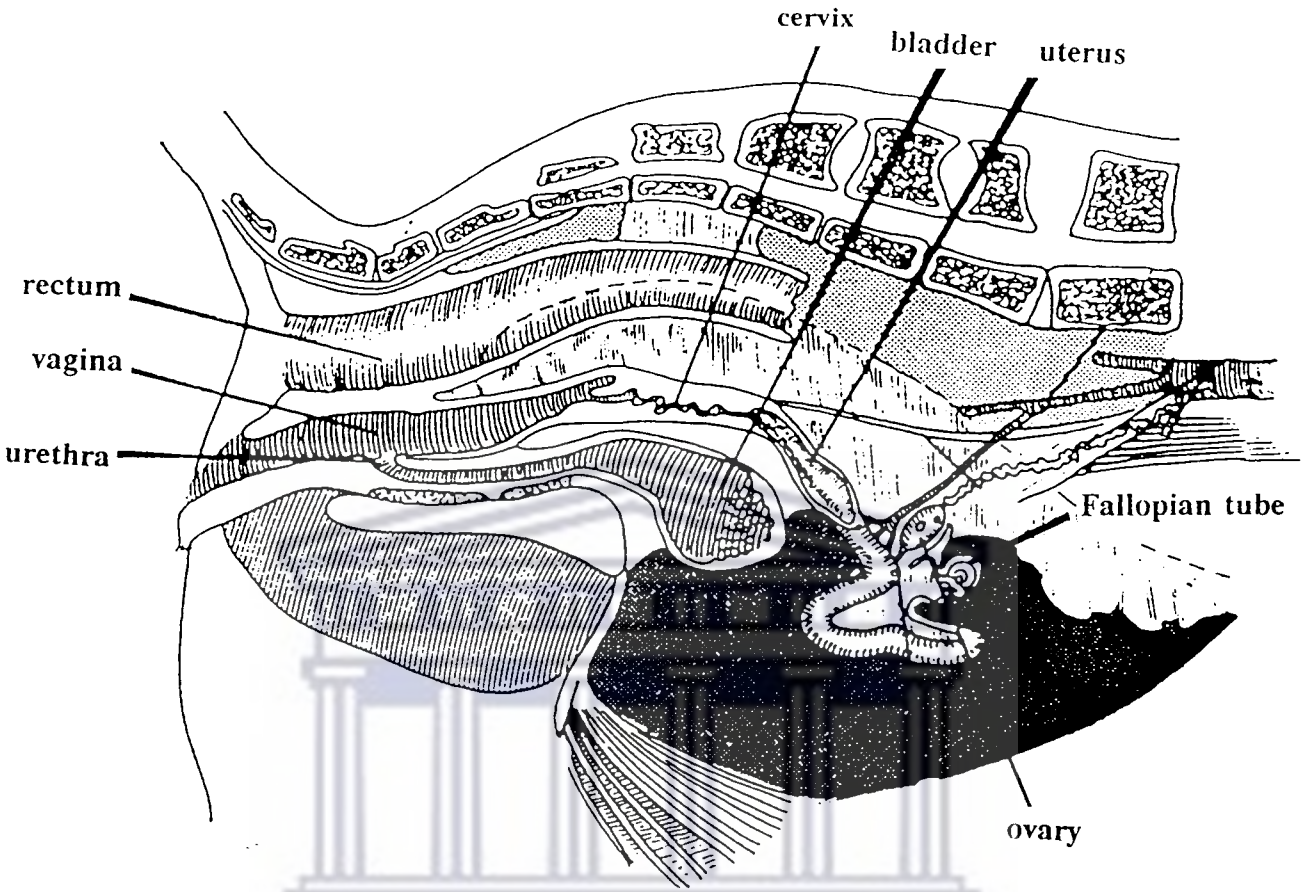


Fig.1.1 Saggital section through pelvic region (view of left side) showing urogenital tract of the ewe [Hafez, 1980]

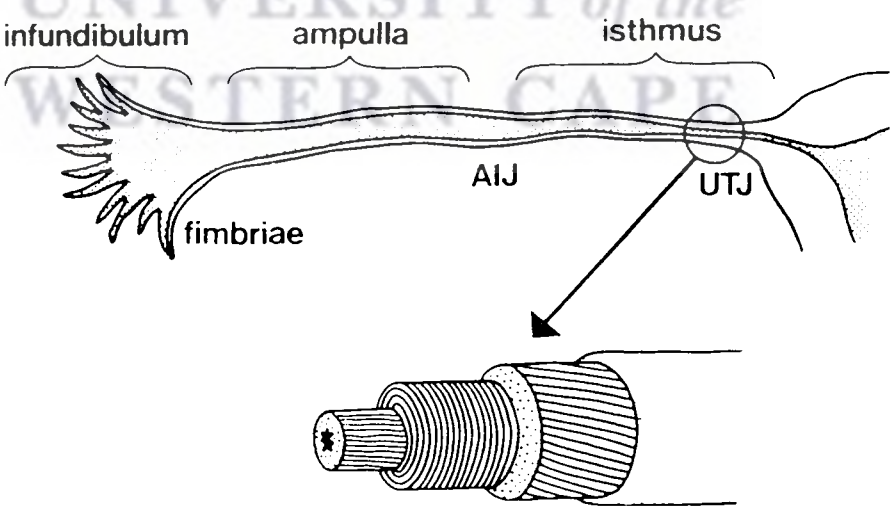


Fig.1.2 Major segment of oviduct [Hafez, 1980]

AIJ - ampullary-isthmic junction; UTJ - uterotubal junction

1.2 Migration through the female reproductive tract

Sperm forward progression is characterized as an interactive process between the cell and its environment. Flagellar movement is a fundamental expression of the vitality of the sperm cell where flagellar bending results in a distribution of local forces against the surrounding fluid. The viscosity of the suspending fluid can influence sperm flagellar motion. If viscosity increases, the flagellum must push harder to achieve the same local velocity as in a simple fluid. The cervical mucus is commonly seen as the first polymeric secretion with complex rheological behaviour which the sperm must traverse [Katz *et al.*, 1989].

Prior to exposure to cervical mucus, spermatozoa are exposed to seminal plasma. Although the properties conferred to sperm by seminal plasma are not fully known, Peitz and Olds-Clarke [1986] have shown that seminal vesicle removal results in decreased fertility in the house mouse. Stegmayr and Ronquist [1982] concluded that particulate elements in human seminal plasma were involved in stimulation of forward motility in spermatozoa, whereas Davis and Hungund [1976] observed that the particles in rabbit seminal fluid originating from the epididymis caused sperm decapacitation and inhibited fertility. The functional importance of the particles in seminal fluid remain contradictory. One of the important physical properties of cervical mucus is the exclusion of the seminal plasma from semen [Katz *et al.*,1989].

Sperm migration through the cervix involves distinct but interrelated factors. The

ability of spermatozoa to penetrate the mucus, the properties of mucus that enable it to participate actively in the process of sperm transport and, also, the morphologic configuration of cervical crypts and clefts that contribute to the storage and preservation of spermatozoa in the cervical canal and their suspended and prolonged release to the upper tract [Blandau & Moghissi, 1973]. Other factors which contribute to sperm penetration at the semen-mucus interface are seminal enzymes and external forces due to visceral contractility. Mechanical properties of the microstructure, the viscosity of the mucus plasma, and the size and shape of the sperm head and flagellum afford mechanical resistance to sperm penetration through the cervical mucus. There is variation among species in these biological factors [Katz *et al.*, 1989].

Subsequent movement of sperm into and through the uterus is probably the result of myometrial contractions rather than significant independent sperm motility [Mortimer, 1978]. The increased uterine activity found during orgasm in women may assist in sperm transport into the cervix and uterus [Edwards, 1980].

The spaces through which sperm migrate *in vivo* may have transverse dimensions of the same magnitude, or they may be smaller than the length of the body of the sperm. In some locations, such as the uterotubal junction and the lower oviductal isthmus, the spaces are small enough to restrict sperm passage physically. The presence of a nearby surface will have a profound effect on

sperm movement. When a sperm is swimming near a solid surface at a distance less than its own body length, the fluid motions induced by the flagellum are constrained by the surface; this is known as the wall effect. This phenomenon causes an increase in the dissipation of hydrodynamic energy and, consequently, the flagellum must push harder to achieve a local velocity equal to that attained in the absence of a nearby boundary. As in the case of increased viscosity, it is reasonable to expect that the flagellar bend propagation mechanism will respond to this wall effect [Katz *et al.*, 1989]. Experimental evidence demonstrating such a response was reported by Suarez *et al.* [1983] that, when sperm become constrained to beat two-dimensionally, their flagellar waves become less symmetrical resulting in circular trajectories. It is likely that these wall effects, so conspicuous *in vitro,* play a role in sperm migration *in vivo*. During much of their journey along the female reproductive tract, spermatozoa move in close proximity to epithelial surfaces. The two-dimensional flagellar wave propagation, with planar orientation of the sperm body, is well suited to migration along such surfaces. These sperm would progress along surfaces but could be trapped and eliminated from transport if led into cul-de-sacs of the epithelium. The presence of fluid currents due to ciliary activity would act to reorientate such sperm; this mechanism, along with visceral contractility, may contribute to sperm transport through the oviduct [Katz *et al.*, 1989; Mortimer & Swan, 1995].

The term hyperactivation has been used to describe the frantic movement of sperm before they undergo the acrosome reaction [Yanagimachi, 1994]. Most

spermatozoa in the ampulla are hyperactivated [Suarez *et al.*, 1983]. Hyperactivation is a useful biological marker as related to capacitation and it may mechanically promote a number of sperm functions, including transport through the oviduct and penetration of the cumulus and zona pellucida. However, despite the increasing attention to this visually striking phenomenon, it still lacks objective kinematic definition: the site, mechanisms and kinetics of its onset are unclear and its role in sperm function is not fully understood. Subjective visual assessments have led to a remarkable set of metaphors to characterize swimming trajectories of hyperactivated sperm. Such motion has been referred to as "bobbing" [Gwatkin & Anderson, 1969], "serpentine", "high amplitude" [Yanagimachi, 1970], "whiplash" [Cooper *et al.*, 1979], "figure-of eight" [Fraser, 1977] and "darting" [Corselli & Talbot, 1986]. Clearly these terms do not permit standardisation in the identification of hyperactivated sperm. Definition and interpretation of hyperactivation should derive from analysis of the sperm flagellar beat, which is the biophysical cause of the motion [Katz *et al.*, 1989].

1.3 Selection of a morphologically normal sperm for fertilization

A normal sperm has a head, neck, midpiece and tail. Two-thirds of the anterior surface of the head is covered by the acrosome. Both the midpiece and the tail are capable of independent motility, even in the absence of the head. The size and shape of the sperm head is species-specific [Van der Horst *et al.*, 1991].

Various morphological abnormalities can occur in semen [Salisbury & VanDemark, 1961].

In humans and certain other mammals, including sheep, sperm is deposited in the upper part of the vagina. The cervical mucus acts as a barrier to spermatozoa during most of the menstrual and oestrus cycle. Only in the periovulatory period does the structure of the cervical mucus alter to permit penetration of the spermatozoa [Blandau & Moghissi, 1973]. The ability of the cervix and its mucus to exclude many of the morphologically abnormal spermatozoa has long been recognised [Bergman, 1955; Botella-Lluisa, 1956]. Information on the morphology of the spermatozoa at the site of fertilization is limited but available literature indicates that there is indeed a morphological selection along the female reproductive tract.

Algren *et al.* [1974] reported that between 79 and 98% of human spermatozoa recovered from the ampullae were morphologically normal, while Asch [1976] reported that no abnormal forms were encountered. However, Mortimer and co-workers' [1982] results show that this may be an over-simplification, and that a few abnormal forms may reach the site of fertilization. Mortimer *et al.* [1982] conclude that the apparent selection of morphologically normal spermatozoa is not a direct function of the female tract, but that spermatozoa can effect their own selection because of differential motility. Detailed evaluation of spermatozoa in their study shows that the selection of sperm is largely achieved

by reductions in sperm with midpiece, tail and other defects which might be expected to impair their motility. A study undertaken by Ragni *et al.* [1985] supports Mortimer's work that the mucus acts as a "passive filter" with selection depending on spermatozoa themselves in relation to motility.

Katz *et al.* [1989] agree that morphologically abnormal sperm, as a group, have inferior motility compared with normal sperm in the same ejaculate, but that this differential swimming ability is not large enough to account for the exclusion of such a large population of abnormal sperm from mucus penetration. The properties of the human sperm surface possibly influence penetration. When antisperm antibodies are present on the sperm surface, sperm with vigorous motility may be unable to swim for more than a few sperm body-lengths into cervical mucus [Fjallbrandt, 1968 & 1969;Jager *et al.*, 1981; Bronson & Cooper, 1987]. Either by physical entanglement or by chemical linkage, antibodies interact with sperm in such a way as to resist forward progression. Similarly, antibodies secreted into the mucus can link to penetrating sperm, causing analogous impediment to motion. Basic hydrodynamic reasoning dictates that the variations in the dimensions of the sperm heads alone will not generate sufficient drag to prevent mucus penetration by these sperm. The origin of the increased resistance to these abnormal sperm may lie in their surface interactions with the mucus macromolecules [Katz *et al.*, 1989]. Studies with capacitated sperm have indicated that a more generalized cellular dysfunction may be associated with morphological abnormalities [Morales *et al.*, 1988]. Surface

changes in the sperm cell are closely related to the functional alterations of capacitation. It is possible, therefore, that the dysfunctional state of the sperm cell is revealed in its earliest interaction with the cervical mucus [Katz & Phillips, 1986].

Fredericcson and Björk [1977] additionally reported that, a barrier which is particularly active against spermatozoa with abnormal heads, exists at the level of the external os. Their study indicates the presence of a female factor in the selection of spermatozoa but it does not attempt to explain a mechanism for this selection. Foldesy *et al.* [1984] reported that there was no selection along the female tract in the rabbit of a special subpopulation of spermatozoa from the ejaculate for fertilization. They indicate that the progressive reduction in numbers during the ascension of spermatozoa through the female reproductive tract does not appear to be an important factor for fertilization but may be important in reducing the incidence of polyspermy. No morphological assessments were done; their study was based on the survival of rabbit embryos after fertilization.

1.4 Molecular events leading to fertilization

1.4.1 Capacitation

Capacitation is primarily an endogenous physiological change of the spermatozoon necessary for mammalian fertilization [Bedford, 1973; Austin,

1975]. It is a membrane event at the molecular level, achieved after spermatozoa have resided in the female genital tract. Sperm capacitation is believed to be a continuation of the maturational process initiated in the epididymis [Hinrichson-Kohane *et al.*, 1984]. Initial alterations in capacitation include modification, redistribution or loss of the epididymal and seminal plasma proteins coating the sperm surface [Brackett & Oliphant,1975; Oliphant, 1976; Kinsey & Koehler, 1978; Koehler,1976 & 1981]. Not only is the sperm plasma membrane surface altered during capacitation but changes intrinsic to the membrane have also been described and these are evidenced by the restriction in the ability of intramembraneous proteins to move laterally within the plasma membrane [O'Rand, 1977]. These intramembraneous modifications, which occur during capacitation, lead to the formation of glycoprotein-rich and glycoprotein-poor areas [Friend *et al.*, 1997]. These areas co-exist in a patchwork-like topography and are consistent with the pattern of membrane fusion seen during the acrosome reaction [Franklin, 1970]. Capacitation is now generally accepted to include all steps required to permit sperm to undergo the acrosome reaction without actually doing so [Bedford, 1970a]. For this reason, in the past, the acrosome reaction had been recognised as the principal event that signalled that capacitation had been completed [Burkman, 1991].

As stated earlier, hyperactivation is a useful biological marker for capacitation. Membrane changes during capacitation may be directly related to changes in flagellar motion, for example, in altering membrane ion conductance [Katz *et al.*,

1989]. Mohri and Yanagimachi [1980] showed that, upon exposure to ATP, demembranated cauda epididymal and ejaculated sperm displayed hyperactivated motility. They suggest that the characteristics of the sperm motor apparatus does not change during capacitation but that the motor apparatus, in fresh epididymal and ejaculated sperm, must merely be prevented by some (intrasperm?) mechanism from effecting the hyperactivated motility.

The site of physiological capacitation within the female reproductive tract is uncertain. In species in which spermatozoa are deposited in the vagina at coitus, sperm capacitation may begin while spermatozoa pass through cervical mucus. "Rubbing off" sperm-surface-adsorbed materials (including seminal plasma proteins) against the mucus network may facilitate capacitation [Gould *et al.*, 1985; Katz *et al.*, 1989]. Barros *et al.* [1988] reports that cervical mucus modifies human spermatozoa, as measured by their interaction with zona-free hamster eggs. They suggest that spermatozoa might achieve a state of partial capacitation while in the cervical mucus. Chang [1951, 1955] established that capacitation of rabbit spermatozoa can take place within the uterus. However, there is increasing evidence that, in some species at least, capacitation must be completed in the oviduct [Barros, 1971; Bedford, 1972]. This idea is further supported by the fact that the cumulus oophorus has been found to be a potent inducer of hamster capacitation *in vitro* [Gwatkin *et al.*, 1972]. In the presence of cumulus oophorus, spermatozoa, attach to cumulus cells , remain associated with them for two to three hours and are then released in a capacitated state. According to

Gwatkin [1977], cellular microfilaments and microtubules appear to be involved in the capacitation process. During this association, the spermatozoa are enveloped by the cumulus cell microvilli and become deeply embedded. Glycosidases released by the cumulus cells, at this stage, appear to alter the sperm plasma membrane [Carter, 1974; Gwatkin & Carter, 1974].

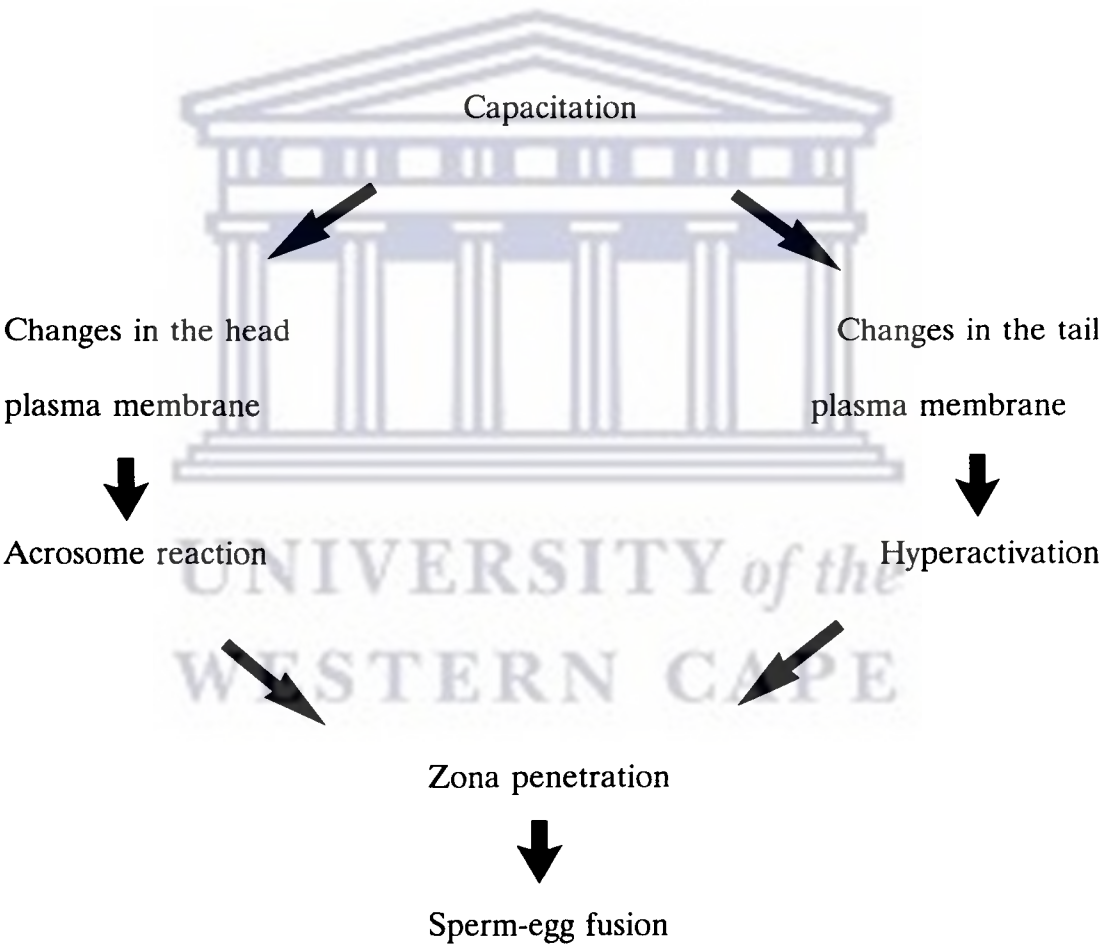


Fig.1.3 Possible relationships among sperm capacitation, acrosome reaction and hyperactivation [Yanagimachi, 1994].

1.4.2 Acrosome reaction

The acrosome of a mammalian spermatozoon is an organelle that covers the anterior portion of the nucleus and contains hydrolytic enzymes including at least one zymogen [Harrison, 1983]. Although a continuous structural element, the region of acrosomal membrane closest to the nucleus is termed the inner acrosomal membrane while that immediately below the sperm plasma membrane (outer limiting membrane) is termed the outer acrosomal membrane. The acrosome reaction results in a release, or at least an exposure, of the enzymes in the acrosome by disrupting acrosomal integrity.

During this reaction the outer acrosomal membrane starts invaginating to form membrane-bound vesicles within the acrosome and, finally, either the plasma membrane is lost [Jones, 1973; Roomans & Afzelius, 1975] or multiple fusions develop between the plasma and the outer acrosomal membranes [Barros *et at.*1967, Franklin *et at.*, 1970; Yanagimachi & Noda, 1972]. These vesiculation processes mark the start of the acrosome reaction and probably allow the release of acrosomal enzymes which presumably facilitate the passage of spermatozoa through the cumulus and corona radiata, dissolving the intercellular matrix [Katz *et at.*, 1989]. The site where the fusion points between the plasma membrane and the outer acrosomal membrane first takes place may vary according to species. It may vary from the frontal margin of the acrosomal cap region to the border of the acrosomal cap region and the equatorial segment of the acrosome. The equatorial segment is not involved in vesication of the membranes during

the acrosome reaction. While the acrosomal cap is loaded with enzymes, the equatorial segment could be enzymatically "empty" [Yanagimachi, 1994].

Two enzymes which appear to occupy an intra-acrosomal location are acrosin and hyaluronidase and, to date, they have the most defined potential roles in the fertilization process. The specific localization of these enzymes is not known, but evidence suggests that the active enzyme is probably membrane-bound [Fraser, 1984]. Acrosin, within the intact sperm, exists as its zymogen, proacrosin, and is activated following initiation of the acrosome reaction [Green, 1978]. Acrosin is believed to enable sperm to penetrate the zona pellucida and/or by causing or aiding the sperm acrosome reaction.

Hyaluronidase, like acrosin, is released concomitantly with the membrane fusion events of the acrosome reaction [Fraser, 1984]. A study by Lewen *et al.* [1982] suggested that at least some hyaluronidase has an extracellular location. The cumulus cells surrounding freshly ovulated eggs are held together by an intra-cellular matrix containing hyaluronic acid residues which could provide a substrate for hyaluronidase. Exposure of cumulus-intact eggs, either to sperm suspensions or solutions of commercially-available hyaluronidase preparations, results in rapid loss of the cellular layers. The bulk of hyaluronidase, however, is located within the acrosome and its release, therefore, requires the acrosome reaction [Fraser, 1984]. There is some dissent as to whether the acrosome reaction of the fertilizing sperm occurs before cumulus penetration or at the

surface of the zona pellucida. Cummins and Yanagimachi [1986] suggest that some spermatozoa may initiate the early stage of the acrosome reaction within the cumulus oophorus, but that fertilizing spermatozoa do not initiate the true acrosome reaction *in vivo* until they come into contact with the zona pellucida.

Storey [1991] has proposed a possible sequence of events of the acrosome reaction in mouse sperm:

In the first stage, a fully intact plasma membrane overlies the outer acrosomal membrane. The acrosomal matrix is completely contained within the outer and inner acrosomal membrane. During the second stage, the outer acrosomal membrane and the plasma membrane make point appositions, but both retain their membrane integrity. The third stage is the punitate stage in which the outer acrosomal membrane and plasma membrane have fused, at the point appositions, to form pores. The acrosomal matrix remains intact. In the fourth stage , the fenestrate stage, the pores widen and the acrosomal matrix disperses. The last stage is the reacted stage, where there is loss of the fused outer acrosomal membrane and the sperm plasma membrane. The inner acrosomal membrane is now the outer limiting membrane.

The consequence of the acrosome reaction is a spermatozoon capable of penetrating the zona pellucida and fusing with the egg plasma membrane [Yanagimachi, 1994].

1.5 Objectives

The present study aims to examine:

1) changes in motility, in terms of progressive activity as well as alterations in swimming patterns, due to transit through the Fallopian tube.

2) whether any morphological selection occurs during Fallopian tube transit, be it intrinsic to the sperm or selection by the female tract.

3) localization of receptors, specifically N-acetyl-D-glucosamine-like sites since these receptor sites have been implicated in capacitational events [Nicolson & Yanagimachi, 1972];

   In addition, Kumar *et al.* [1989] have shown an increase in the amount of N-acetyl-D-glucosamine residues over the sperm acrosomal domains during epididymal maturation in rats, golden hamsters, albino rabbits and goats. Weil and Finkler, as quoted by Nicolson and Yanagimachi [1972], in turn showed that seminal plasma proteins adsorb to epididymal spermatozoa, thereby possibly masking N-acetyl-D-glucosamine sites on the sperm membrane surface. Hence, capacitation appears to involve the unmasking of these receptor sites.

4) the occurrence of the acrosome reaction, which signals the completion of capacitation, due to Fallopian tube transit.

Although the acrosome reaction is recognised as the most reliable indicator of the completion of capacitation, no clear recognizable marker for the initiation or the process of capacitation has been established to date. The structural and functional aspects examined in the present study could infer whether the process

of capacitation (with or without the acrosome reaction) has occurred during transit through the Fallopian tube, thereby indicating the influence of the Fallopian tube on sperm to complete these maturational processes in the merino sheep.

CHAPTER TWO

Materials and Methods

2.1 Introduction

The experimental design is based on a combination of *in vitro* and *in vivo* conditions. A physiological environment (Fallopian tube) is provided to facilitate completion of the events under examination, but under laboratory conditions to ensure more control (to a certain degree). Sperm are placed inside the Fallopian tube thereby fulfilling the *in vivo* aspect. The entire system is then sustained under laboratory "physiological" conditions. As stated earlier [see chpt.1, pg. 3], full cognizance is taken of any defects in this artificial physiological system. The resultant methodology described has proven to be the most efficient and successful in obtaining reproducible results for this study.

2.1.1 Fallopian tube preparation

Fallopian tubes were obtained from 16 ewes. To eliminate any variations in results due to different phases of the cycle, only ewes in oestrus were used. The same semen sample was often used to inseminate Fallopian tubes from more than one ewe.

The oestrus cycles of fertile adult merino ewes were synchronized by administering vaginal sponges impregnated with synthetic progesterone [Repromap sponges, 60 mg medroxy progesterone acetate [Upjohn, South Africa]], for 14 days. The sponges were inserted into the vagina and removed 2 days prior to the excision of the Fallopian tubes. The entire reproductive tract was removed immediately after the slaughter of the ewe. The Fallopian tubes were ligated at both ends to retain tubal fluid, before being separated from the rest of the reproductive tract. A portion of the uterus, adjacent to the Fallopian tube, was removed together with the Fallopian tube. The sperm sample was placed in this portion of the uterus, since the uterotubal junction is an important factor in reducing the number of spermatozoa entering the oviduct [Bedford, 1970b; Overstreet, 1977].

The Fallopian tubes were washed immediately after removal from the ewe with preheated (35°C) Phosphate Buffered Saline (PBS) [Sigma, St Louis, MO], pH 7.4, to remove excess blood. The Fallopian tubes were transported on ice to the laboratory within approximately 1 hour after removal, for experimentation. In the laboratory, the temperature of the tissue was slowly raised to that of room temperature by using a heating pad. The temperature was thereafter maintained at 37°C until completion of the experiment. In initial experiments, the tissue was transported at 37°C in a culture medium (pH 7.4) saturated with O_2. These experiments yielded unsatisfactory results; possibly due to postmortem changes in the tissue after removal from the body.

2.1.2 Sperm Preparation

Semen samples were collected by electroejaculation from proven fertile rams. Electroejaculation was done by intermittent rectal electrostimulation of periods between 4-5 seconds using 6-10 volts. The semen samples were transported to the laboratory at 31°C. In the laboratory, the samples were washed and centrifuged (500 xg, 10 min) twice in Ham's F-10 culture medium (pH 7.4), supplemented with 10% human serum albumin (HSA) [Sigma, St. Louis, MO]. Decapacitation factors present in seminal plasma are removed *in vivo* through semen-cervical mucus interaction [see chapt.1, pg.9]. The double wash-centrifugation method [WHO, 1987] ensures the removal of most of these decapacitating factors and, therefore, establishes the physiological correctness of this *in vitro* situation. Since ram sperm generally have a gross motility of above 80% and forward progression of 4+ (subjective assessment using WHO criteria [appendix 6.1]), procedures to isolate the motile sperm fraction were unnecessary.

2.1.3 Fallopian tube insemination and sperm transit

Washed sperm (100 μl) were placed in the portion of the uterus attached to the Fallopian tube. This end was then ligated to retain sperm inside the Fallopian tube. The sperm-inseminated Fallopian tube was incubated in Ham's F-10 + 10% HSA, pH 7.4, saturated with O_2 at 37°C. After an incubation period of 30 min., sperm were retrieved from the ampullary region. Retrieval of adequate numbers of sperm for analysis of all four parameters was only possible by flushing of the Fallopian tube. The uterine portion was removed and the

Fallopian tube was flushed from the isthmic end using Ham's F-10 +10% HSA. The sperm sample was then collected at the ampullary end. An aliquot of the sperm sample before migration, which represents the control, as well as the retrieved sample were analyzed.

Henceforth, sperm samples exposed to the Fallopian tube will be referred to as the postmigration sample. The sperm samples not exposed to the Fallopian tube represent the control and will be referred to as the premigration sample. The parameters evaluated for both pre- and postmigration are as follows:

a) motility assessment

b) morphological evaluations

c) localizations of WGA-receptors and

d) assessment of acrosomal status

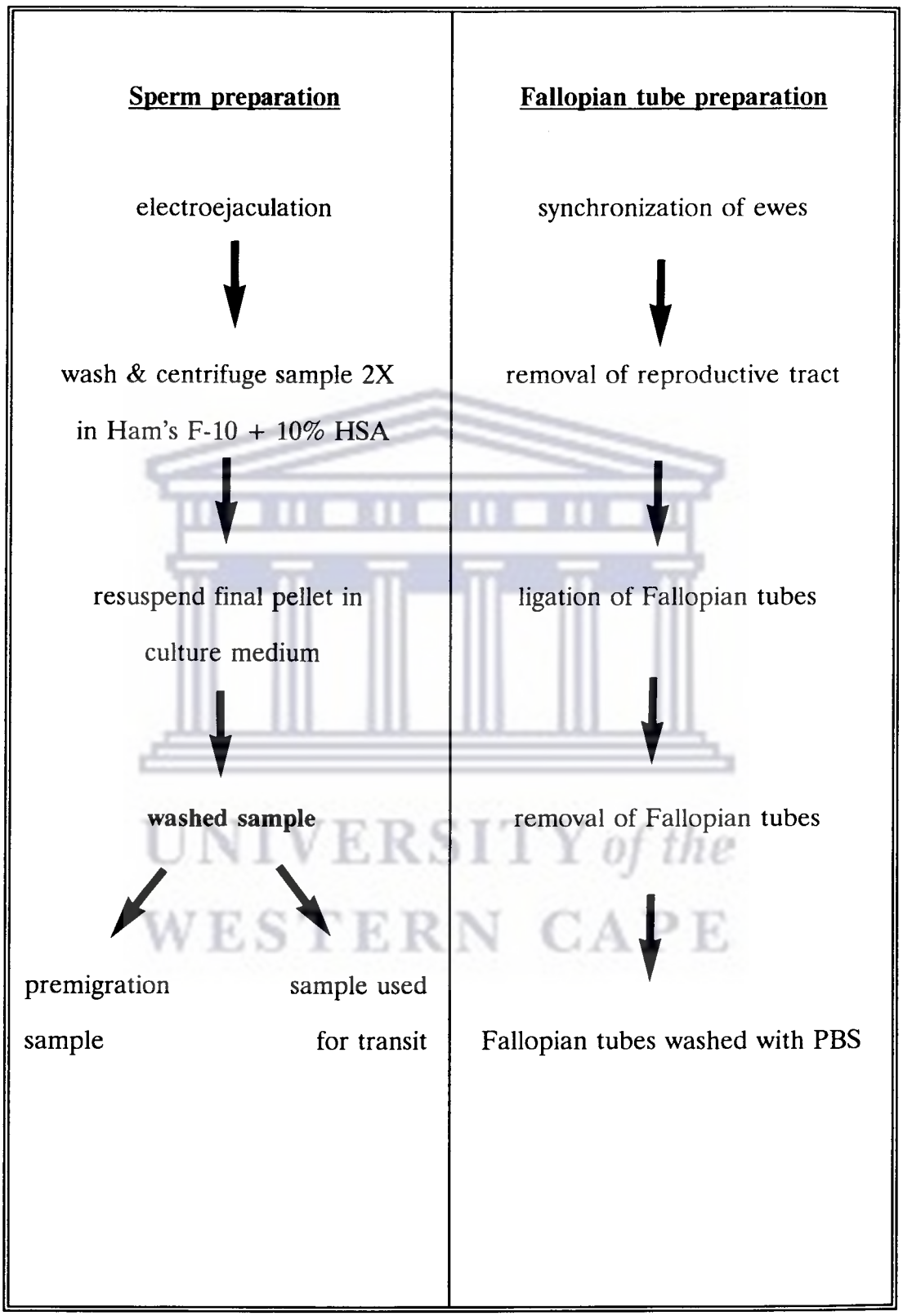


Fig. 2.1 **Flow diagram of experimental procedure for obtaining samples**

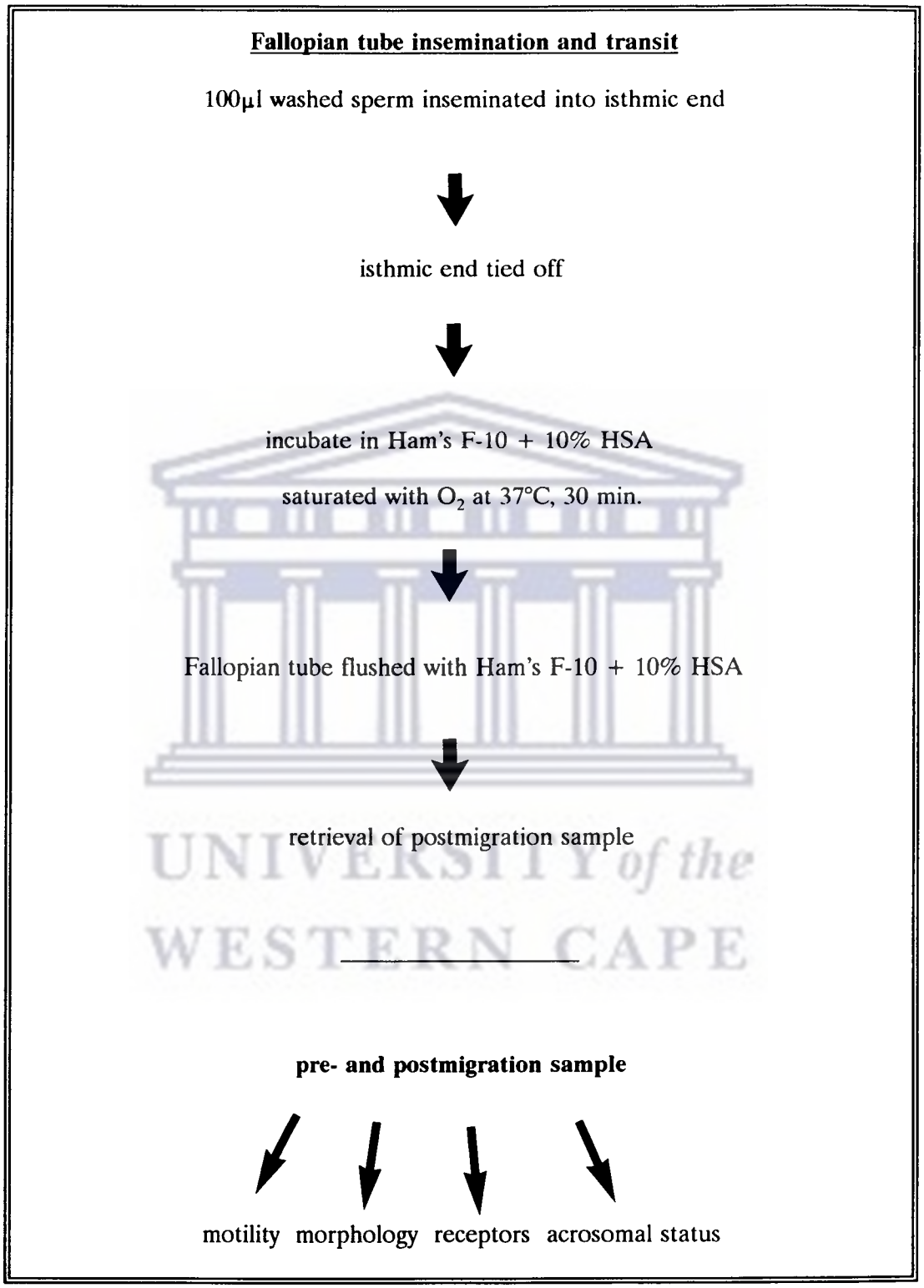


Fig.2.2 Flow diagram of experimental procedure for obtaining samples and inseminating the Fallopian tubes

would not have been sufficient for the needs of the experiment as a sizable amount would be lost during the washing and centrifugation. Although the swim-up step could have been adapted to exclude the wash step, the procedure would have been ineffective as the spermatozoa would still have been exposed to Fallopian tube tissue/cells. The manual function of SMQ proved invaluable in this regard.

Changes in motility patterns e.g. hyperactivation [Gwatkin & Anderson, 1969; Yanagimachi, 1969; Suarez, 1988; Burkman, 1991] which could signify capacitational changes in sperm after transit through the Fallopian tube, were compared in pre- and postmigration samples.

Videomicrography was completed using a Zeiss ICM 405 inverted microscope with 16X objective lens set for pseudo-phase conditions; the images being recorded with a JVC VF-1900E vidoecamera and a U-matic VCR.

2.2 Motility

With the advent of Computer Aided Sperm Motion Analysis (CASMA), a more objective analysis of sperm motility is now obtainable as compared to subjective motility assessment by a technician. The Sperm Motility Quantifier (SMQ) [Wirsam Scientific, South Africa] is one such system and was used for motility analyses in these experiments. SMQ measures 14 motion parameters, some of which are indicative of the speed and forward progression of the sperm. SMQ allows for analysis in the automatic and/or manual mode. All the motility analyses of the postmigration sample was carried out using the manual function of SMQ, whereas the premigration samples were analyzed in the automatic mode. Validation of SMQ as a CASMA system and comparability of results obtained by automatic and manual mode was shown by Van der Horst [1995].

Included in the postmigration sample were epithelial cells of the Fallopian tube lining and other debris. It was extremely difficult to wash this debris from the postmigration sample. A swim-up was attempted to isolate only the motile fraction but was not feasible when the time factor of one hour for a swim-up preparation was taken into account. During the swim-up, the spermatozoa would still be exposed to the epithelial cells and other debris. Possible post-mortem changes of these cells cannot be excluded as factors which could affect the viability of the spermatozoa during this time. Even when negating these post-mortem changes, there is no guarantee that motility changes (due to intrinsic sperm factors) would not occur during this time period. These motility results would then not be a true reflection of the effect of the Fallopian tube environment on sperm. Also, the percentage sperm obtained from the swim-up

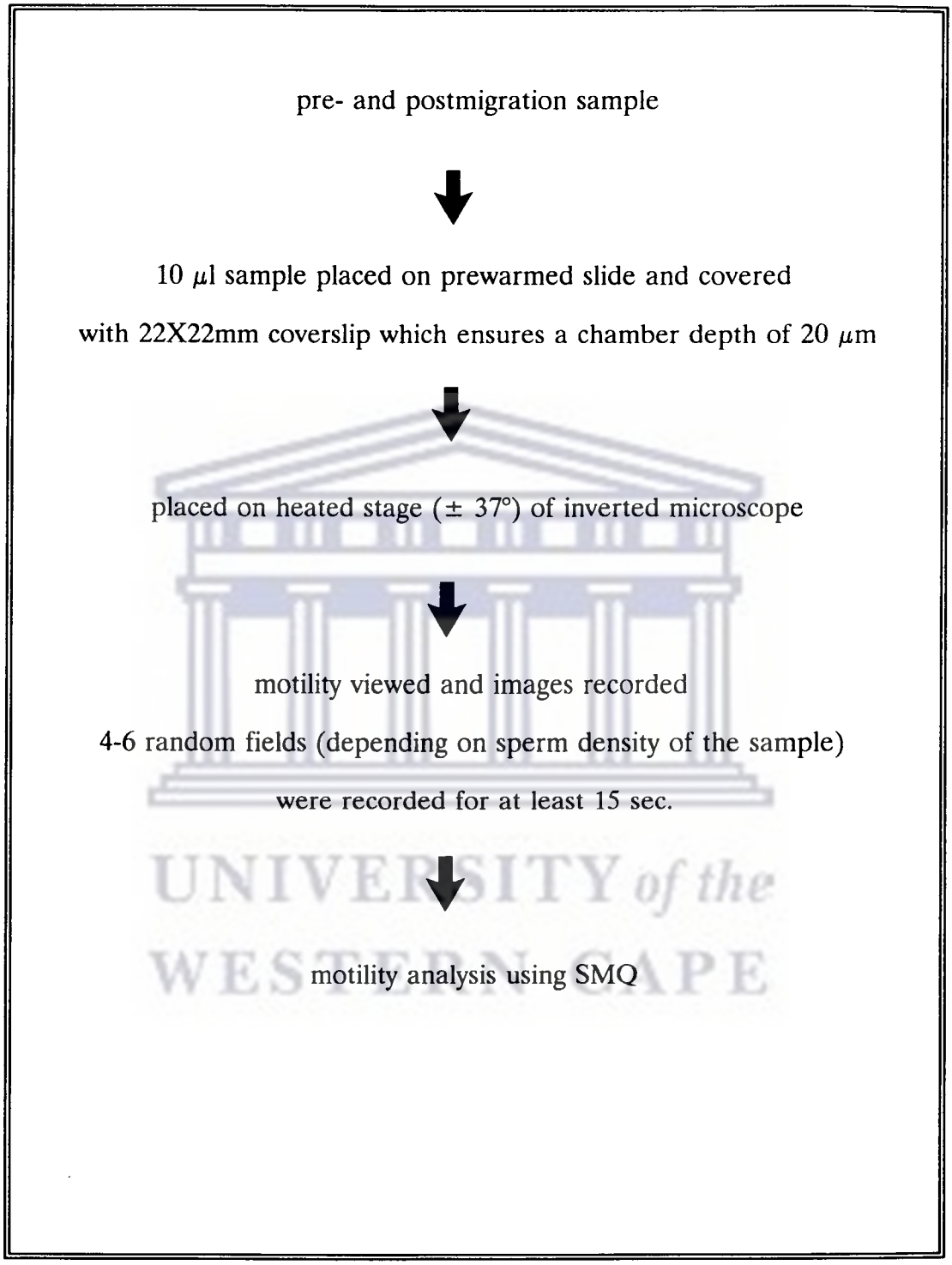


Fig.2.3 Flow diagram of procedure for videomicrography

2.3 Morphology

The objective assessment of abnormalities in morphology present similar difficulties as those experienced in the assessment of motility. For human sperm especially, evaluation has been difficult due to the high percentage and variation of abnormal forms prevalent in semen samples. The World Health Organization [1988] and Tygerberg Group [1990] have set out certain criteria which help to standardize evaluations. However, these criteria are still dependent upon subjective assessment by the technician. The Flexible Image Processing System (FIPS) [CSIR, South Africa] is a new tool which can be used to alleviate the problem of subjective assessment. FIPS is a processing system that allows video images to be captured, characterized and stored. It measures dimensions of required structures. In this instance, the sperm head. All measurements on the screen image are related to actual physical dimensions. These physical dimensions include the surface area, the perimeter, the minimum and maximum ferret, the aspect ratio and the shape factor. The minimum and maximum ferret refer to the minimum and maximum diameter, respectively. The aspect ratio is the ratio of the perimeter to surface area and is given as value between 0 and 1. FIPS assigns a value to the shape of the structure (shape factor) and this among other measurements can be used to set down fixed criteria for head size and shape of a particular species.

Ram sperm do not present the same problem as do human sperm since very few abnormal head forms are present in their semen samples. The use of FIPS in these experiments with regard to morphology are twofold:

1) to determine dimensions of normal ram sperm and

2) to compare pre- and postmigration samples and determine whether any morphological changes have occurred during transit through the Fallopian tube.

The images were displayed by the FIPS program using a Grundig videocamera from a Zeiss transmitted light research microscope fitted with 40X objective lens.

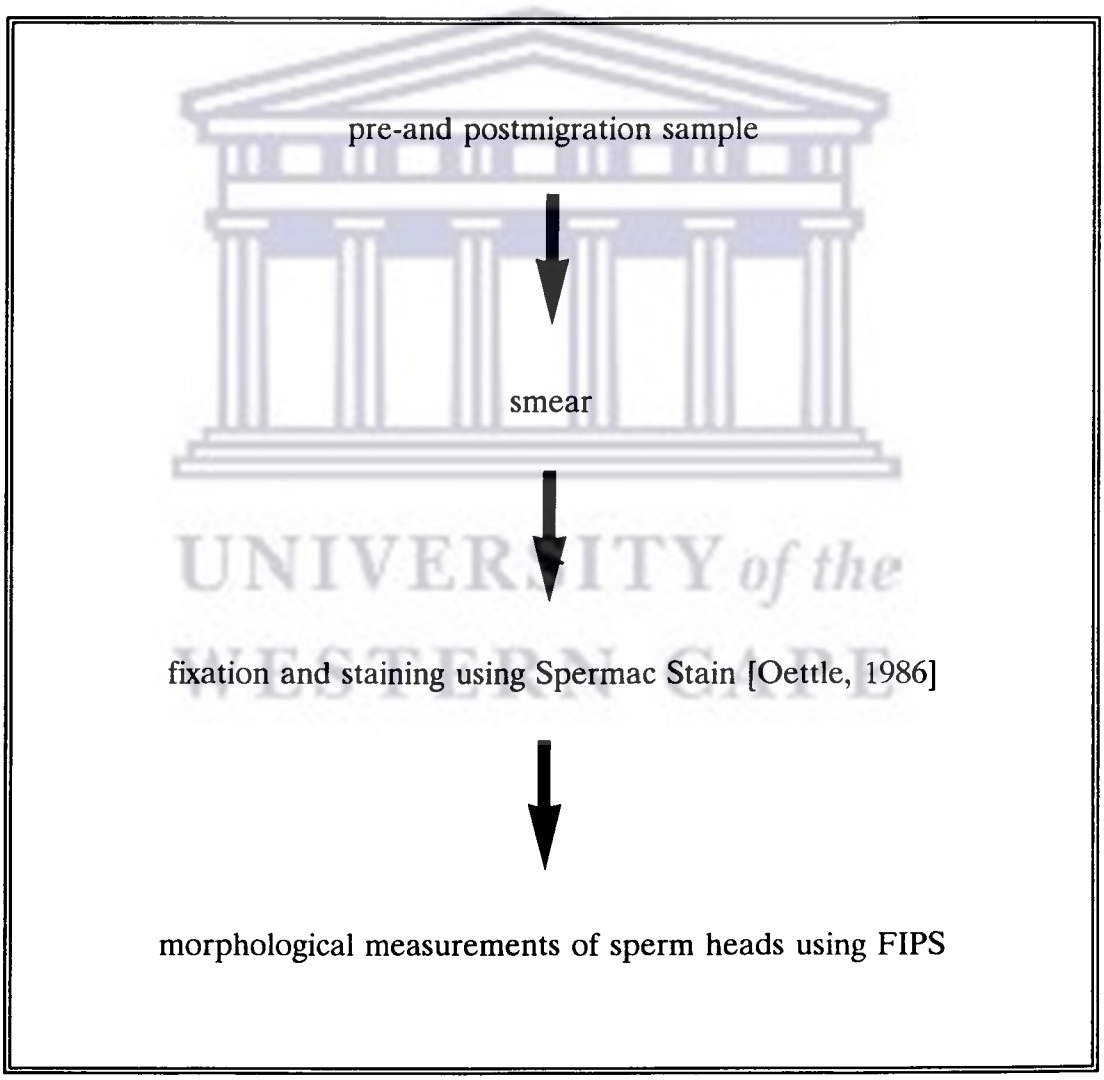


Fig.2.4 Flow diagram of procedure for morphological evaluations

2.4 Localizations of WGA-receptors

Membrane alterations during capacitation appear to be due to molecular alterations involving oligosaccharides. Wheatgerm agglutinin (WGA) is specific for N-acetyl-D-glucosamine and sialic acid [Kallojoki *et al.*, 1984; Kumar *et al.*, 1989]. Both of these sugars are terminal residues in some of these oligosaccharides with sialic acid being more terminal than N-acetyl-D-glucosamine.

Wheatgerm agglutinin was used to localize N-acetyl-D-glucosamine-like sites on the sperm membrane surface indicating the role and/or interaction of the Fallopian tube with sperm during capacitation.

Purified wheatgerm agglutinin extracted from *Triticum vulgaris* labelled with fluoroscein isothiocyanate (FITC) [Sigma, St. Louis] was reconstituted with PBS and diluted to 100μg/ml. A flow diagramme is presented in fig.2.5, illustrating the procedure used for staining. Fluorescence was observed using a Leitz microscope [Leitz, Germany] with epifluorescence attachments and correct filters (excitation filter = BP 390-490, dichroic mirror = RKP 510, barrier filter = LP 515) for FITC excitation.

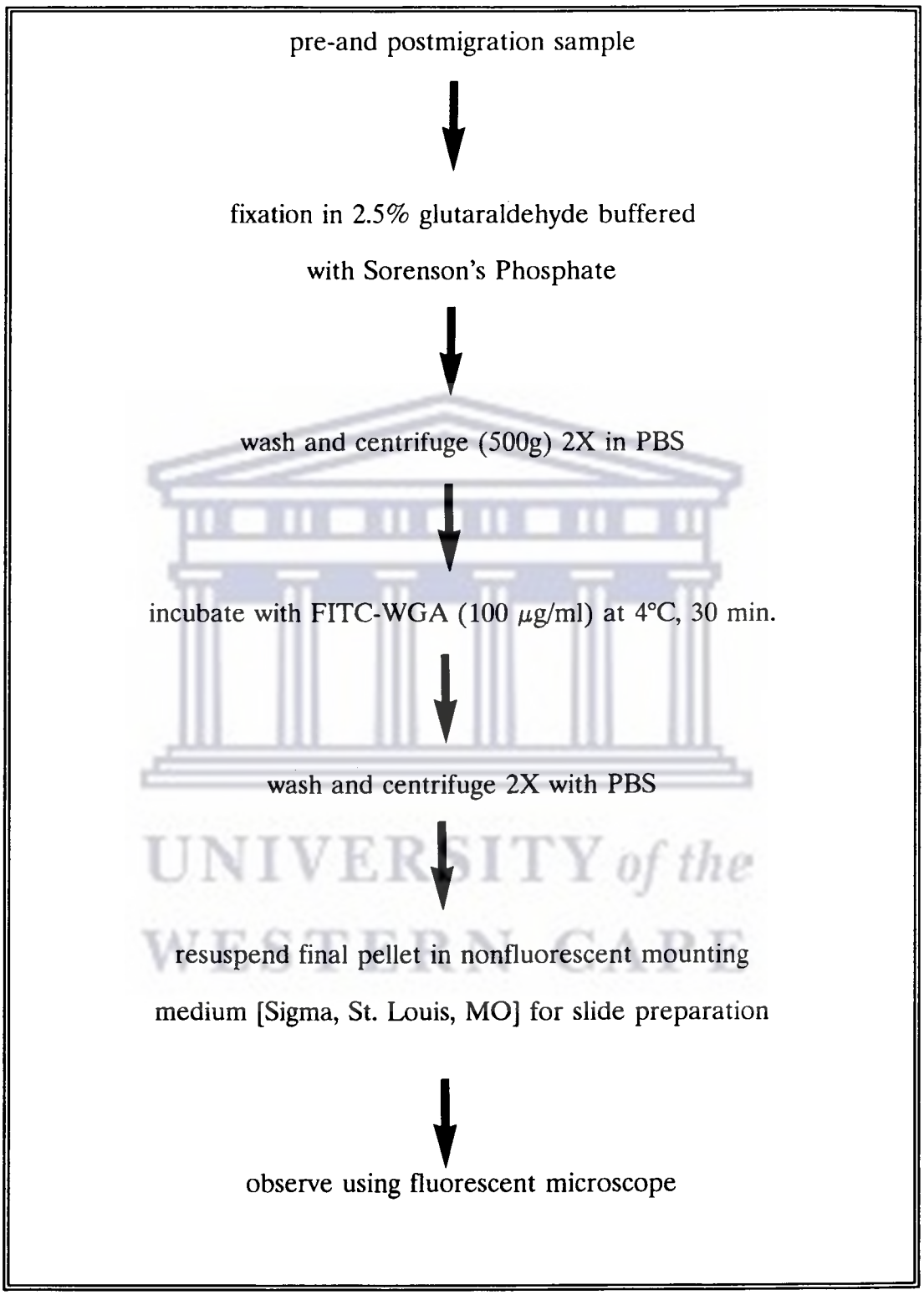


Fig.2.5 Flow diagram of procedure for lectin staining

2.4.1 Evaluation

Evaluation of localization of FITC-WGA receptors was carried out by determining the % positive staining in the different zones of the sperm membrane. No variation in the lectin staining intensity was observed among the different zones.

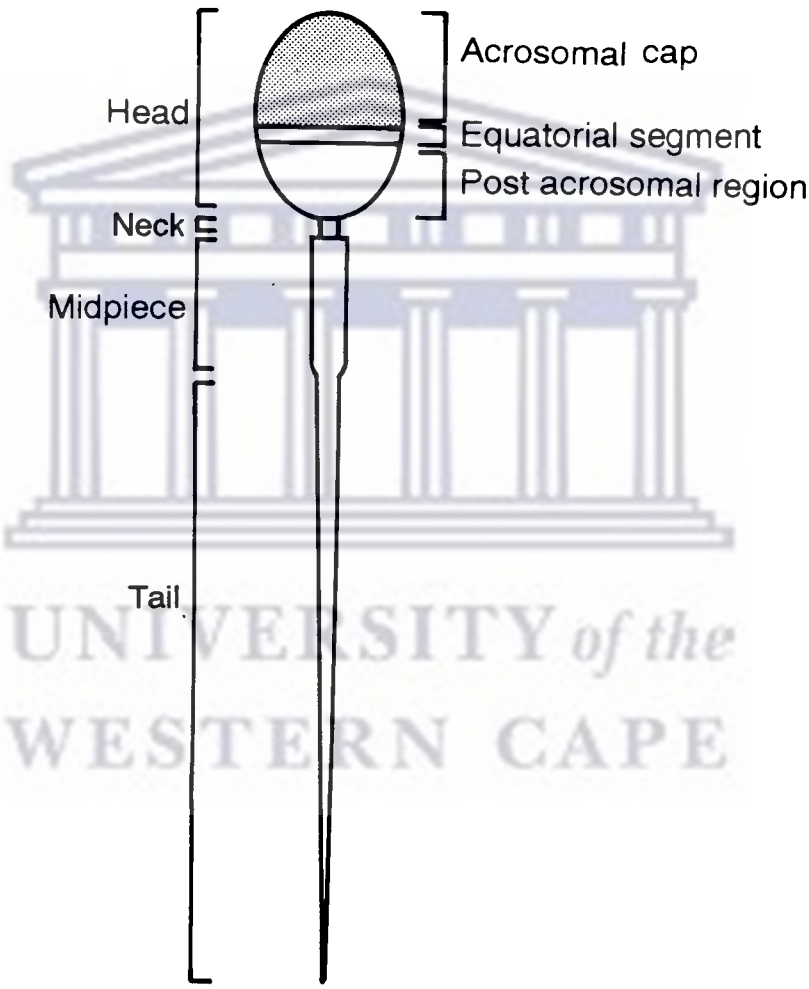


Fig.2.6 **Sperm membrane regions for the evaluation of WGA receptors [Gabriel *et al.*, 1994]**

2.5 Acrosomal Status

The acrosome reaction is recognized as an important indicator of the fertilizing potential of sperm. Despite the research focused on acrosomal status determination, no consensus has been reached on an undisputable method for determining true acrosome reaction. Labelled-lectins are tools which are extensively used [Scacciati De Cerezo *et al.*, 1982; Cross *et al.*, 1986; Mortimer *et al.*, 1987; Mladenovic *et al.*, 1993]. Lectins localize specific glycoproteins on the sperm membrane surface which are often redistributed during maturational processes; the acrosome reaction being one such process. Various studies have selected three lectins with the highest potential to discriminate various stages of sperm capacitation and the acrosome reaction, viz. *Pisum sativum* agglutinin (PSA) which binds to the acrosomal content and inner acrosomal membrane, Concanavalin A (Con A) which also binds to the inner acrosomal membrane and peanut agglutinin (PNA) which binds to the outer acrosomal membrane [Mladenovic *et al.*, 1993]. These lectins, however, have also been shown to bind to other parts of the plasma membrane but with less intensity [Kallojoki *et al.*, 1984]. The binding variability of the lectins is species-specific [Kallojoki *et al.*, 1984], although pretreatment of sperm also influence lectin binding.

PNA was selected for these experiments because it showed a higher precentage staining when compared with PSA. This is probably due to a higher incidence of beta-galactosyl residues, for which PNA is specific, on ram sperm membrane surfaces. Also, Gabriel [1995] suggests that of these three lectins (PNA, PSA and Con A), PNA may be the superior marker for the acrosome reaction. The results of his studies, together with discrepancies in current literature, cast

significant doubt on the reliability of Con A and PSA in the determination of the physiological acrosome reaction.

PNA-FITC reconstitution and staining were performed as for WGA lectin staining [fig.2.4, pg.42].

2.5.1 Evaluation

The percentage positive staining for pre- and postmigration was assessed. Since PNA stains the outer acrosomal membrane, positive staining is indicative of acrosome-intact spermatozoa. An absence of staining of sperm would indicate acrosome-reacted sperm. No variation in the staining intensity was observed among the positively stained spermatozoa.

CHAPTER THREE

Results

3.1 Motility Results

3.1.1 Motion parameters of three motility groups

The motion parameters of the premigration samples and 2 motility groups, isolated from the motile fraction of the postmigration sample, were analyzed. The results for the motion parameters of the premigration sample correlate with baseline data obtained for ram sperm ejaculations by Van der Horst [1995]. A minimum of 100 sperm were analyzed for each premigration sample.

The motile fraction of the total retrieved postmigration sample varied between 25-35% (subjective assessment). Large variation in motility patterns were observed in this motile fraction of the postmigration sample. These patterns included:

a) single, motile spermatozoa, which included straight-swimming and hyperactivated sperm (this sample was referred to as the postmigration sample),

b) aggregations of head-to-head agglutinated sperm with forward, progressive movement (these samples were referred to as the agglutinated samples) and

c) aggregations of head-to-head agglutinated sperm, but with tails facing in the opposite directions. No forward progression resulted from these aggregations of sperm since the progression of each sperm was counteracted by the opposite facing sperm. [See fig. 3.1.1 for sperm associations.]

Motility analyses were performed on the postmigration groups **a** and **b** above as well as the premigration samples.

Table 3.1.1 represents the motion parameters (means + SD) of the three motility groups analyzed. With the exception of mnALH ($p < 0.05$), there was no statistically significant difference between the three groups for the motility parameters.

Fig. 3.1.2 represents trajectories from the three motility groups. The trajectories of the premigration sample are fairly straight. Uniformity of these trajectories were apparent throughout the different premigration samples. The patterns of trajectories in the postmigration sample are as diverse as the swimming patterns of the different sperm present within the sample. The sperm tracks of the agglutinated samples are similar to the premigration sample. During manual analysis of the agglutinated sample, the computer cursor is focused on the

amassed heads of the agglutinated sperm. Fine discrimination of the head movement is not possible, since the region that forms the head area is quite large. Also, when visually assessed, most of the agglutinated samples display straightline swimming patterns. It seems reasonable to assume that the individual sperm cells will not undergo hyperactivation while associated with this agglutinated mass. The swimming patterns of the agglutinated samples would, therefore, be concurrent with that of the premigration sample.

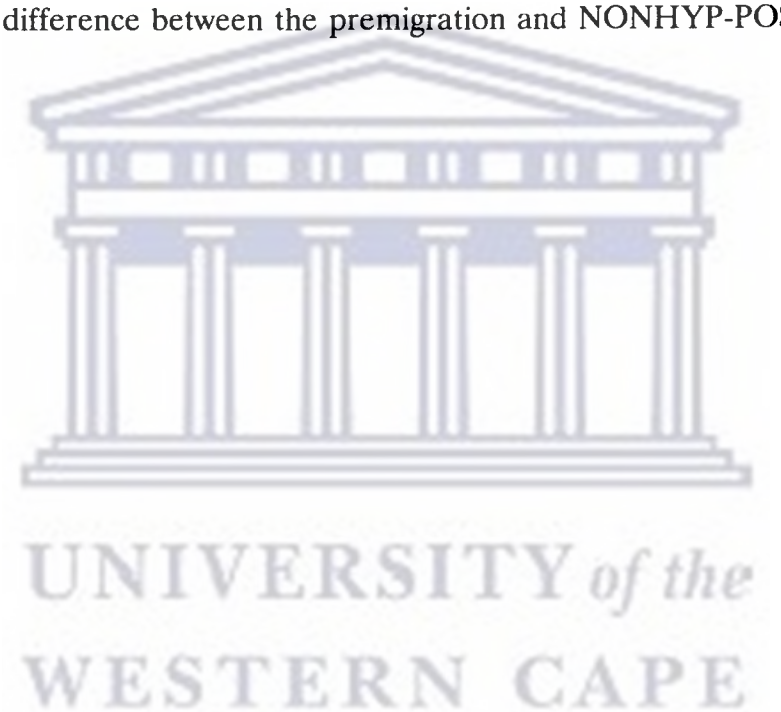
Table 3.1.2 presents correlation analysis for 8 motion parameters of the premigration sample. The motion parameters all show a good correlation with one another, with the exception of CURV which shows no correlation with any of the parameters featured and mnALH which only shows correlation with VCL and BCF. VCL, VSL, LIN, BCF, and VAP all show a positive correlation with one another. VCL, VSL, VAP and BCF are indicative of vigour of motility. Therefore, an increase in the swimming speed of the sperm will result in an increase in these parameters. The correlation analysis, therefore, reiterates this fact. LIN is calculated as the ratio of VCL over VSL and, therefore, any increase or decrease in these two parameters will affect the LIN. MAD shows a negative correlation with all of these parameters. An increase in MAD denotes an increase in the angle that the sperm head moves in each frame; a decrease would, therefore, denote a less angular movement. This is supported by the strong negative correlation between LIN and MAD. An increase in LIN will result in a concomitant decrease in MAD.

3.1.2 Motion parameters of hyperactivated and non-hyperactivated samples within the postmigration sample

The individual samples making up the postmigration sample vary in their motility patterns. The motion parameters analyzed for this sample are, therefore, not a true reflection of the motility patterns of the various groups within the postmigration sample. Separation of the hyperactivated from the non-hyperactivated samples within the postmigration sample was attempted. Receiver Operating Characteristic curve analyses were used to determine cut-off points for hyperactivation within the postmigration sample. The premigration sample was used as the positive group (based on the assumption that the premigration sample is largely non-hyperactivated) with the postmigration sample as the negative group. Five parameters were selected which include: VCL, LIN, mnALH, RAD and CURV. Selection of these parameters were based on the information that they impart with respect to hyperactivated motility patterns [see discussion]. The cut-off values for these parameters obtained from the ROC-curve analyses (presented in table 3.1.4) were then applied to the postmigration sample. Hyperactivated samples were discriminated from non-hyparactivated samples within the postmigration sample using these criteria. A sample was classified as hyperactivated if at least 3 of the 5 criteria for hyperactivation was met.

An example of a ROC-curve analysis is presented in fig. 3.1.3.

Table 3.1.3 presents the revised results for the postmigration sample. For six of the motion parameters (LIN, mnALH, WOB, STR, MAD & CURV) there were significant differences between premigration and hyperactivated-postmigration samples. For the same parameters, with the exception of mnALH, there was statistically significant differences between hyperactivated (HYP-POST) and non-hyperactivated postmigration (NONHYP-POST) samples. There was no significant difference between the premigration and NONHYP-POST.



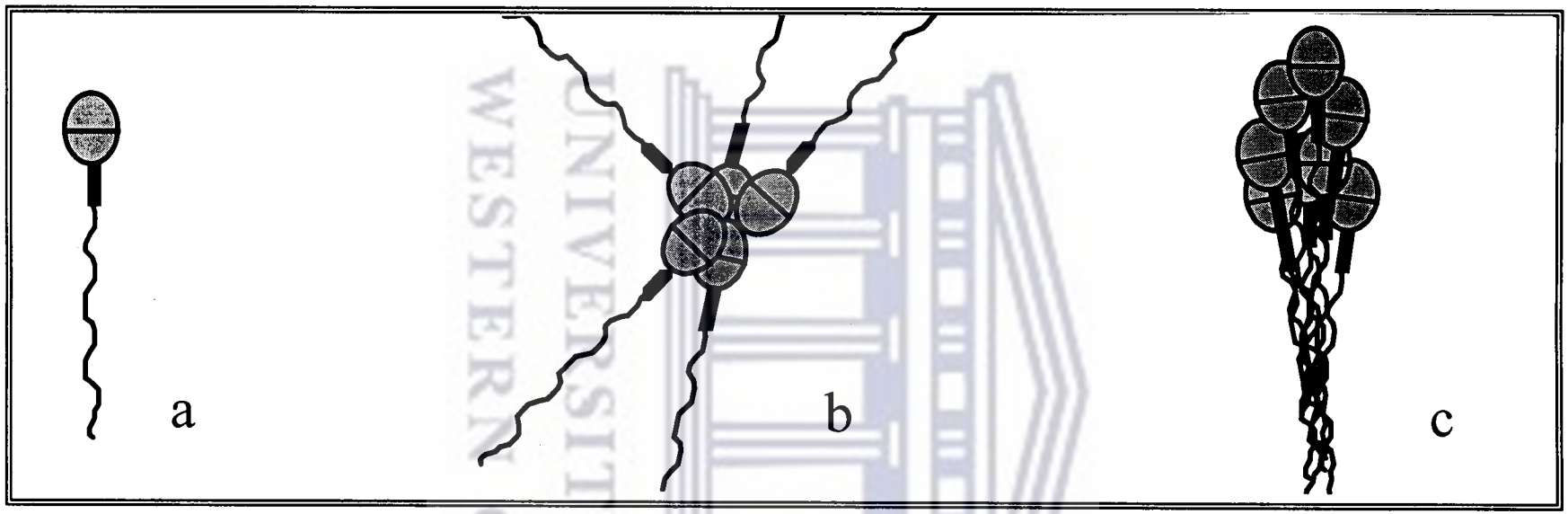


Fig. 3.1.1 **Types of sperm associations found within the postmigration sample.**

a) **Singly, motile spermatozoon.**

b) **Head-to-head agglutinated sperm with tails facing in opposing directions allowing no forward progressive movement.**

c) **Head-to-head agglutinated sperm with tails orientated unidirectionally allowing forward progressive movement.**

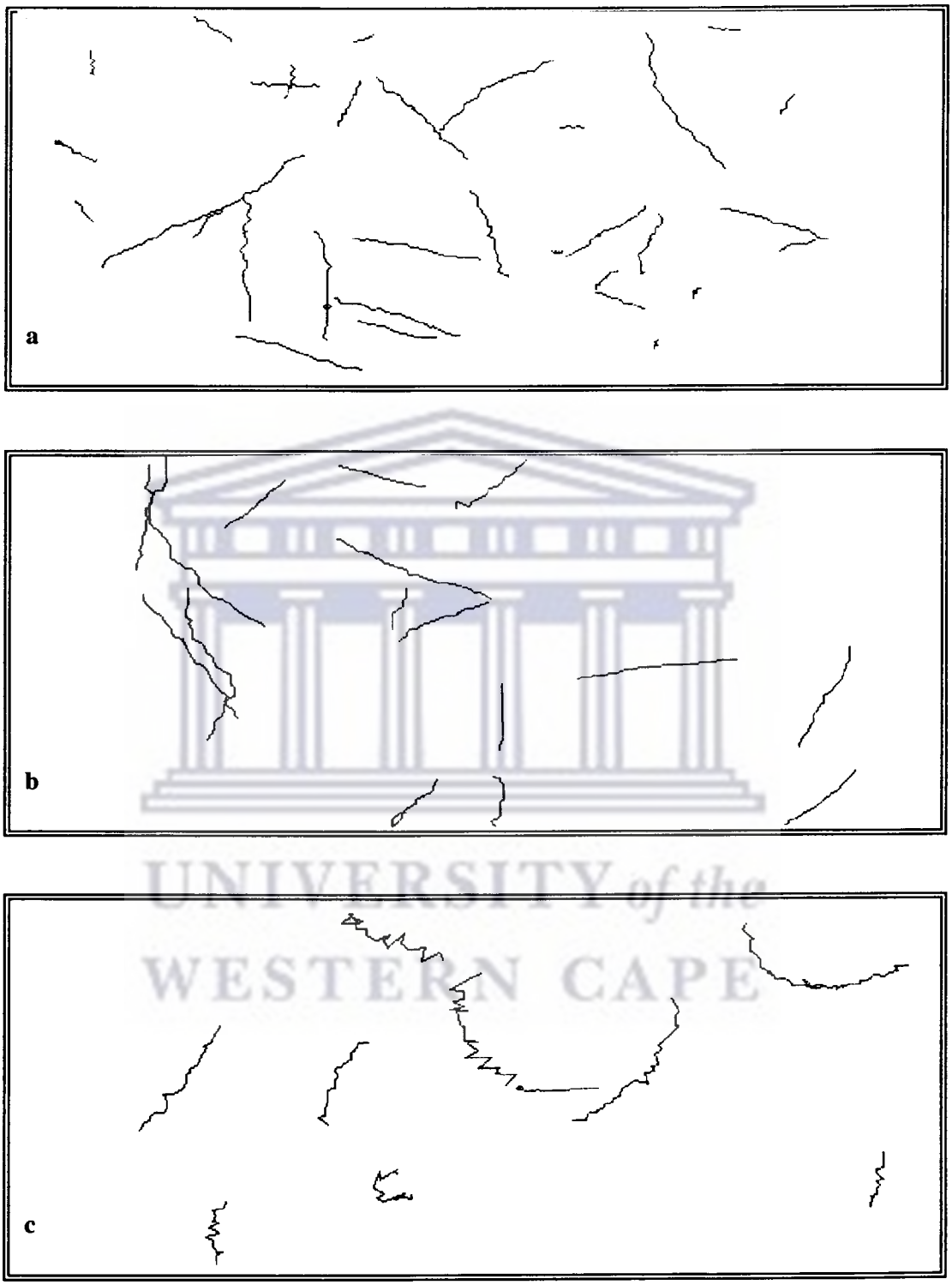


Fig. 3.1.2 **Sperm tracks of premigration, postmigration and agglutinated samples analyzed at 50 Hz.**

a - premigration; b - agglutinated; c - postmigration

Table 3.1.1 Motion parameters of three motility groups (premigration, postmigration and agglutinated) with means ± SD presented.

MOTION PARAMETER	PRE-MIGRATION	POST-MIGRATION	AGGLUTI-NATED
VCL	319.4 ± 35.48	392.19 ± 103.35	362.7 ± 95.81
VSL	262.62 ± 40.06	235.32 ± 102.95	247.51 ± 108.97
LIN	78.87 ± 6.00	66.47 ± 13.68	66.90 ± 18.63
mnALH	4.56 ± 0.05	7.27 ± 2.80	6.47 ± 1.93
mxALH	10.83 ± 0.99	15.49 ± 5.81	13.98 ± 3.40
BCF	40.95 ± 6.50	37.97 ± 12.09	38.95 ± 10.22
DNC	2794.82±1227.94	6543.05±4348.85	5183.83±2667.62
VAP	276.27 ± 38.24	287.37 ± 89.50	274.92 ± 107.71
WOB	85.00 ± 4.00	73.00 ± 13.00	74.00 ± 16.00
STR	93.00 ± 5.00	87.00 ± 8.00	89.00 ± 8.00
MAD	1.48 ± 0.18	1.64 ± 0.39	1.80 ± 0.62
CURV	0.37 ± 0.04	0.44 ± 0.11	0.49 ± 0.17

Table 3.1.2 **Correlation analysis for 8 motion parameters of the premigration sample. (Correlation coefficient plus level of significance (below) is indicated).**

	VCL	VSL	LIN	mnALH	BCF	VAP	MAD	CURV
VCL	1.000	.9438 .0014	.8319 .0014	.8409 .0178	.9906 .0001	.9419 .0015	-.8792 .0091	-.4361 .3280
VSL	.9438 .0014	1.000	.9554 .0008	.6292 .1300	.9640 .0005	.9991 .0001	-.9867 .0002	-.6567 .1091
LIN	.8139 .0203	.9554 .0008	1.000	.4842 .2709	.8552 .0142	.9485 .0011	-.9463 .0012	-.7505 .0519
mnALH	.8409 .0178	.6292 .1300	.4842 .2709	1.000	.7696 .0431	.6177 .1394	-.5172 .2345	-.0367 .9377
BCF	.9906 .0001	.9640 .0005	.7696 .0431	.7676 .0431	1.000	.9666 .0004	-.9022 .0054	-.5353 .2156
VAP	.9479 .0005	.9991 .0001	.6177 .1394	.6177 .1394	.9666 .0004	1.000	-.9744 .0002	-.6702 .0995
MAD	-.8792 .0091	-.9767 .0002	-.9463 .0012	-.5172 .2345	-.9022 .0054	-.9744 .0002	1.000	.6315 .1282
CURV	-.4361 .3280	-.6567 .1091	-7505 .0519	-.0367 .9377	-.5353 .2156	-.6702 .0995	.6315 .1282	1.000

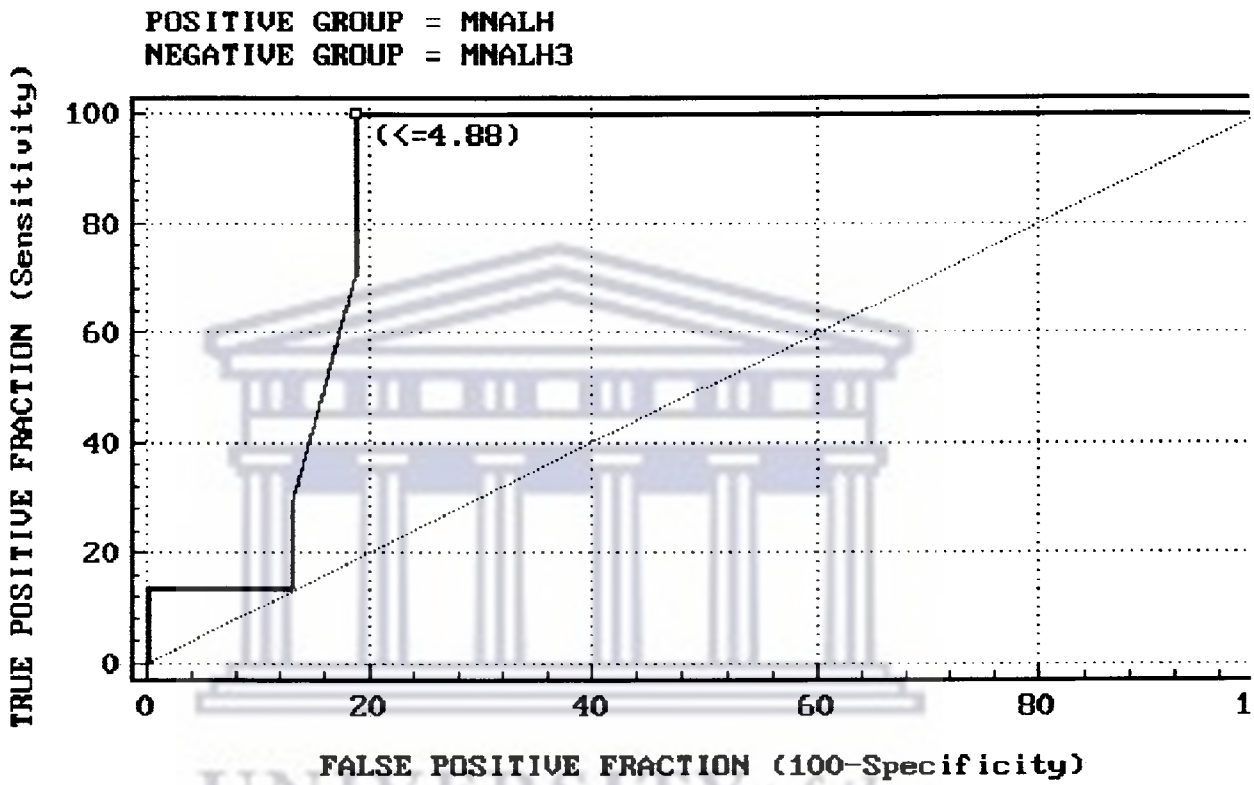


Fig. 3.1.3 **Receiver Operating Characteristic curve for mnALH of premigration (mnALH - positive group) and postmigration (mnALH3 - negative group) showing cut-off point.**

Table 3.1.3 **ROC-curve analysis showing cut-off points between post- and premigration. The cut-off values were used to discriminate between hyperactivated and non-hyperactivated samples within the postmigration sample, e.g. a linearity of below 69.33 would indicate a hyperactivated sample.**

MOTION PARAMETERS	CUT-OFF VALUE	AREA UNDER ROC-CURVE	SENSITIV.	SPECIFIC.
VCL	> 354.23	75	100	69
LIN	< 69.33	73	100	63
mnALH	> 4.88	86	100	81
MAD	> 1.73	67	100	56
CURV	> 0.41	72	100	50

Table 3.1.4 Motion parameters of the hyperactivated postmigration (HYP-POST), nonhyperactivated postmigration (NONHYP-POST) and premigration samples.

MOTION PARAMETERS	PRE-MIGRATION	NONHYP-POST	HYP-POST	P-VALUE
VCL	319.4 ± 35.48	384.28 ± 42.25	395.78 ± 123.53	
VSL	262.62 ± 40.06	303.49 ± 52.68	230.83 ± 87.13	
LIN	78.87 ± 6.00 a	81.41 ± 13.10 b	59.68 ± 11.73 ab	$p < 0.001$
mnALH	4.56 ± 0.05 a	5.29 ± 1.37	8.17 ± 2.86 a	$p < 0.005$
mxALH	10.83 ± 0.99	11.92 ± 3.17	17.11 ± 6.11	
BCF	40.95 ± 6.50	43.98 ± 11.69	35.24 ± 11.76	
DNC	2794.82 ± 1227.94	4209.02 ± 1262.21	7603.97 ± 4874.45	
VAP	276.27 ± 38.24	333.89 ± 63.06	266.22 ± 94.08	
WOB	85.00 ± 4.00 a	85.00 ± 12.00 b	68.00 ± 10.00 ab	$p < 0.005$
STR	93.00 ± 5.00	95.00 ± 3.00 b	84.00 ± 8.00 b	$p < 0.005$
MAD	1.48 ± 0.18 a	1.15 ± 0.24 b	1.86 ± 0.19 ab	$p < 0.001$
CURV	0.37 ± 0.04 a	0.35 ± 0.05 b	0.48 ± 0.1 ab	$p < 0.005$

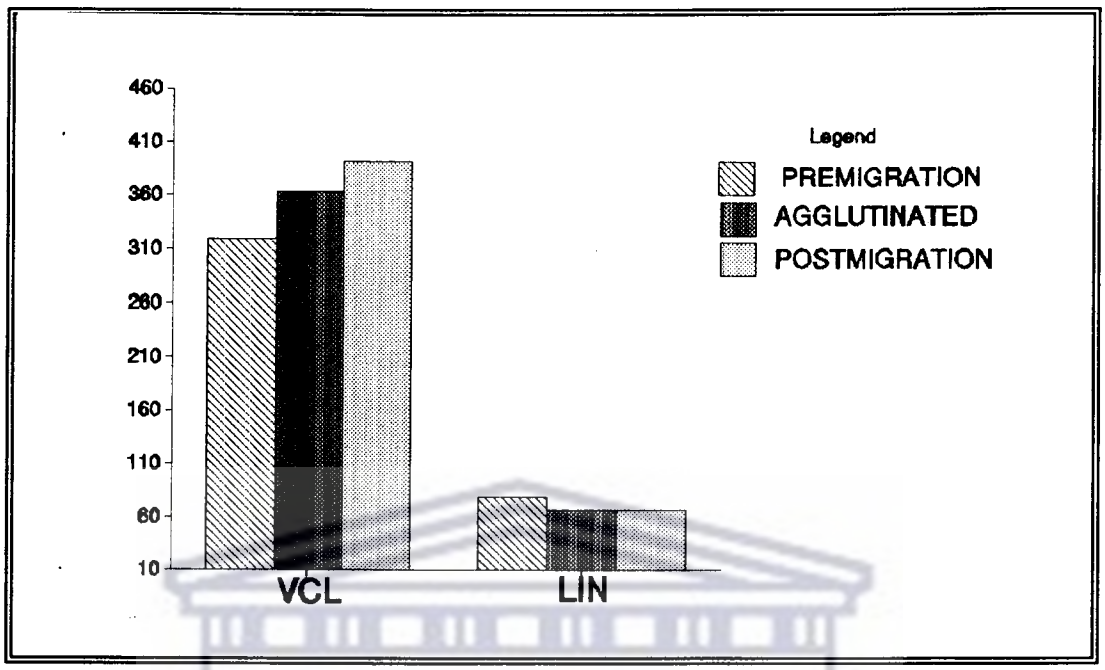


Fig. 3.1.4 **Association of VCL and LIN between the premigration, postmigration and agglutinated samples.**

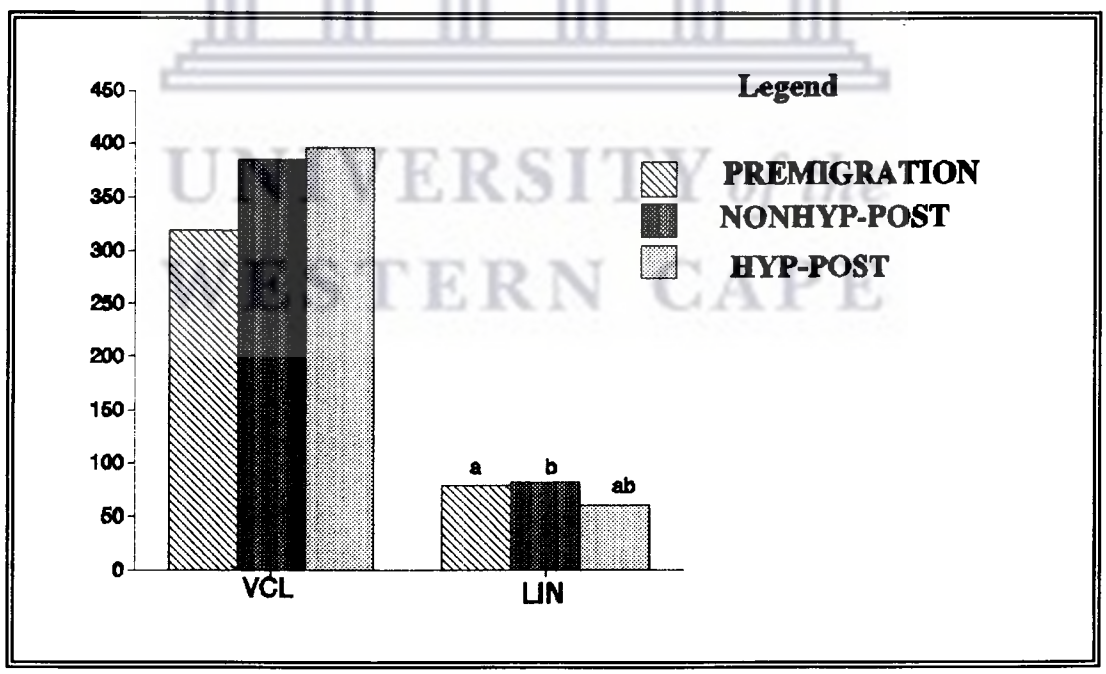


Fig. 3.1.5 **Association of VCL and LIN between the premigration, HYP-POST and NONHYP-POST samples.**

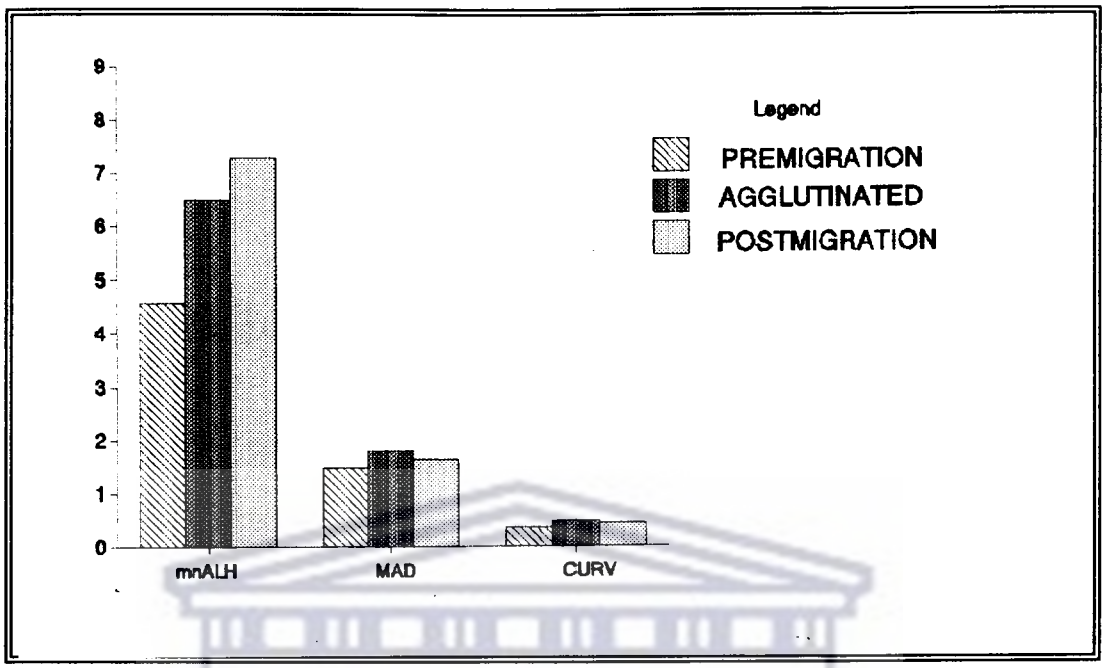


Fig. 3.1.6 **Association of mnALH, MAD and CURV between the premigration, postmigration and agglutinated samples.**

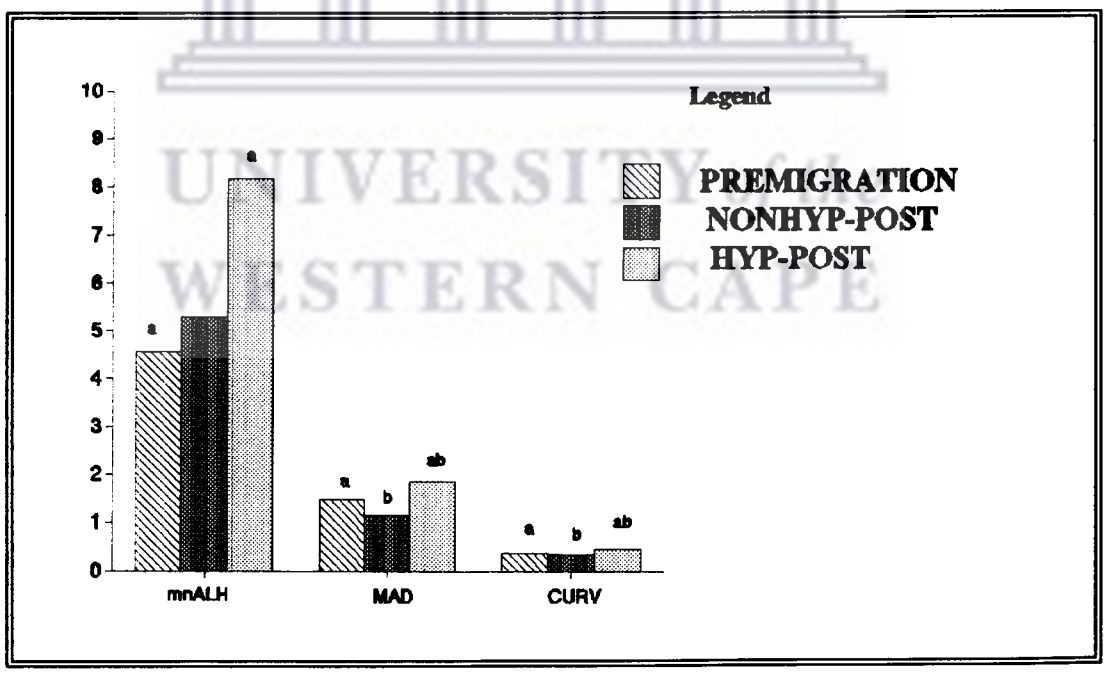


Fig. 3.1.7 **Association of mnALH, MAD and CURV between the premigration, HYP-POST and NONHYP-post samples.**

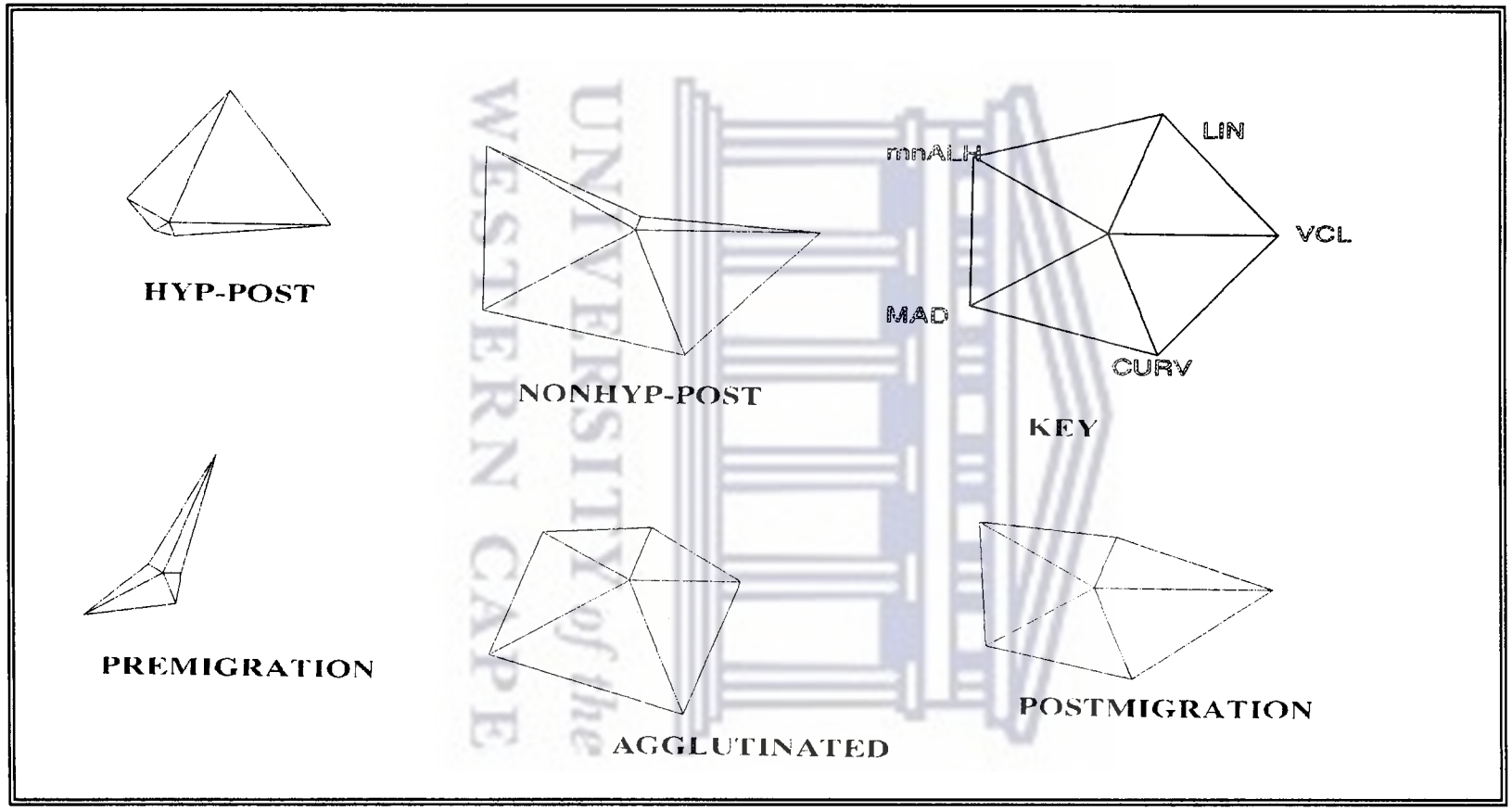


Fig. 3.1.8 Star symbol plot analysis of five motion parameters for premigration, agglutinated, HYP-POST and NONHYP-POST.

3.2 Morphology results

Table 3.2.1 represents the measurement parameters as determined by the Flexible Image Processing System (FIPS) of the pre- and postmigration samples. There was no significant difference between the samples. The perimeter, surface area and diameter (min, max and ave.) of the sperm head are quantified. The shape factor is important in defining the head shape of a particular species and together with the quantified measurements can be used to discriminate the normal from the abnormal forms within a particular sample.

Fig. 3.2.1 are phase contrast micrographs of pre- and postmigration spermatozoa. No obvious, easily discernible differences can be seen between the two samples on a quantitative basis. This visual similarity between the samples is supported by the results in table 3.2.1.

Table 3.2.2 represent correlation analysis within the premigration sample. There is a strong correlation (r^2 between 0.93 and 0.98) between perimeter, area, ave. diameter and shape. This further supports the concept that the shape factor is a good selector for normal forms, since any deviation from the quantified perimeter, area and diameter values, will influence the shape factor.

Table 3.2.1 FIPS measurement parameters of pre- and postmigration spermatozoa.

MEASUREMENT PARAMETER	PREMIGRATION	POSTMIGRATION
PERIM	30.70 ± 3.41	32.50 ± 1.87
AREA	54.37 ± 8.03	59.73 ± 5.67
MIN. DIAMETER	5.90 ± 0.38	6.12 ± 0.25
MAX. DIAMETER	12.68 ± 0.79	12.61 ± 0.90
AVE. DIAMETER	9.27 ± 0.61	9.76 ± 0.38
ASPECT	0.60 ± 0.40	0.47 ± 0.03
SHAPE	0.76 ± 0.06	0.72 ± 0.03

Table 3.2.2 Correlation analysis of the FIPS measurement parameters for the premigration sample.

	PERIM	AREA	AVE_FER	ASPECT	SHAPE
PERIM	-	.9333 < 0.01	.9667 < 0.01	-.1339	-.9373 < 0.01
AREA	.9333 < 0.01	-	.9833 < 0.01	-.0753	-.9500 < 0.01
AVERAGE DIAMETER	.9667 < 0.01	.9833 < 0.01	-	-.1255	-.9333 < 0.01
ASPECT	-.1339	-.0753	-.1255	-	.1925
SHAPE	-.9333 < 0.01	-.9500 < 0.01	-.9333 < 0.01	.1955	-

Fig. 3.2.1 **Micrographs of phase contrast microscopy pre- and postmigration spermatozoa.**

(A) - premigration; (B) - postmigration

3.3 Results of WGA assays

The premigration samples showed uniform lectin binding over most aspects of the cell. These results were significantly different ($p < 0.001$) from the postmigration sample where lectin-binding was restricted to the acrosomal and/or postacrosomal domains. Table 3.3.1 represents the percentage positive lectin staining in the whole-stained sperm and acrosomal and postacrosomal domains for pre- and postmigration. No variation in lectin staining intensity was observed for the different samples.

Fig.3.3.1 shows associations between the three dominant staining patterns of the pre- and postmigration samples. There appears to be redistribution of staining patterns from whole-stained in the premigration sample to the acrosomal and postacrosomal regions in the postmigration sample. Lectin staining occurs predominantly in the acrosomal domain in the postmigration sample. Fluorescent micrographs of the various staining patterns are presented in fig. 3.3.2.

3.4 Results of acrosomal status determination

Table 3.4.1 represents results obtained for acrosome-intact and acrosome-reacted sperm for pre-and postmigration. The results indicate that for acrosomal status, pre- and postmigration spermatozoa are significantly different ($p < 0.05$) from each other. Fluorescent micrographs of acrosome-intact and acrosome-reacted sperm are presented in fig. 3.4.2.

Table 3.3.1 **Percentage positive staining of WGA for pre- and postmigration for the whole-stained sperm and in the acrosomal and postacrosomal domains.**

	PRE-MIGRATION	POST-MIGRATION	P-VALUE
WHOLE-STAINED	81.57 ± 4.47	26.00 ± 11.75	P < 0.001
ACROSOMAL	10.28 ± 2.69	40.14 ± 13.44	P < 0.001
POSTACROSOME	5.42 ± 0.79	28.14 ± 15.06	P < 0.001

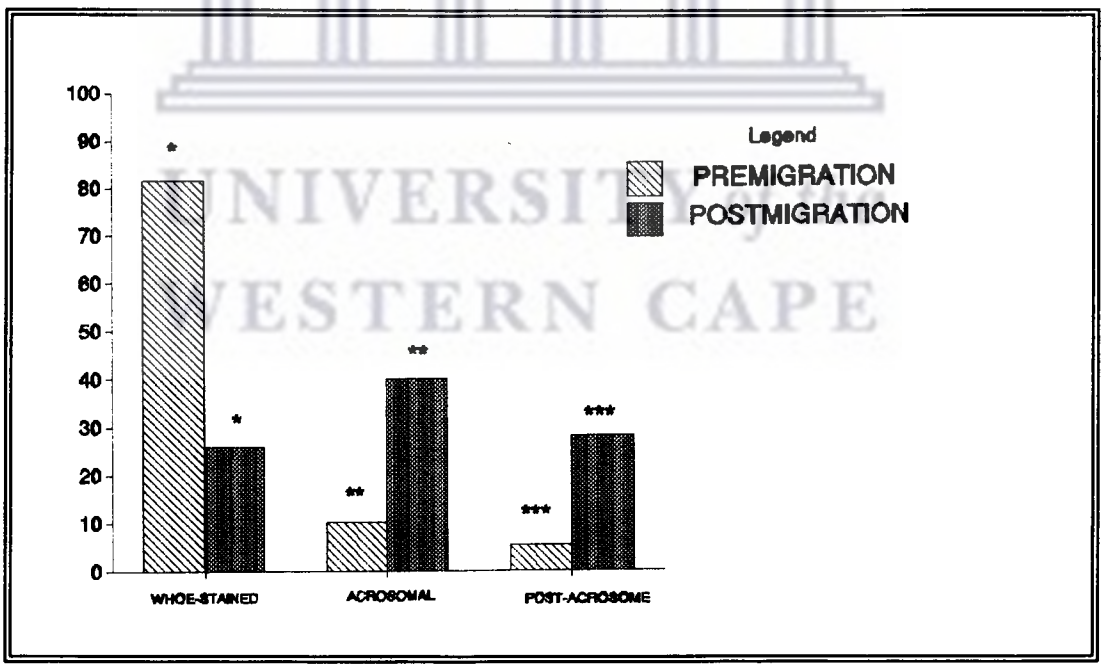


Fig.3.3.1 **Associations between the dominant staining patterns of WGA for pre- and postmigration.**

Fig.3.3.2 **Micrographs of fluorescence microscopy of the various staining patterns for WGA observed in the pre- and postmigration samples.**

US - uniformly stained; AR - acrosomal region; PA - postacrosomal region

Table 3.4.1 **Percentage positive staining for PNA in the pre-and postmigration samples. (A positive reading will indicate an acrosome-intact sperm).**

	PREMIGRATION	POSTMIGRATION	P-VALUE
ACROSOME INTACT	38.33 ± 9.56	27.33 ± 3.72	P < 0.05
ACROSOME REACTED	61.67 ± 9.56	72.67 ± 3.72	P < 0.05

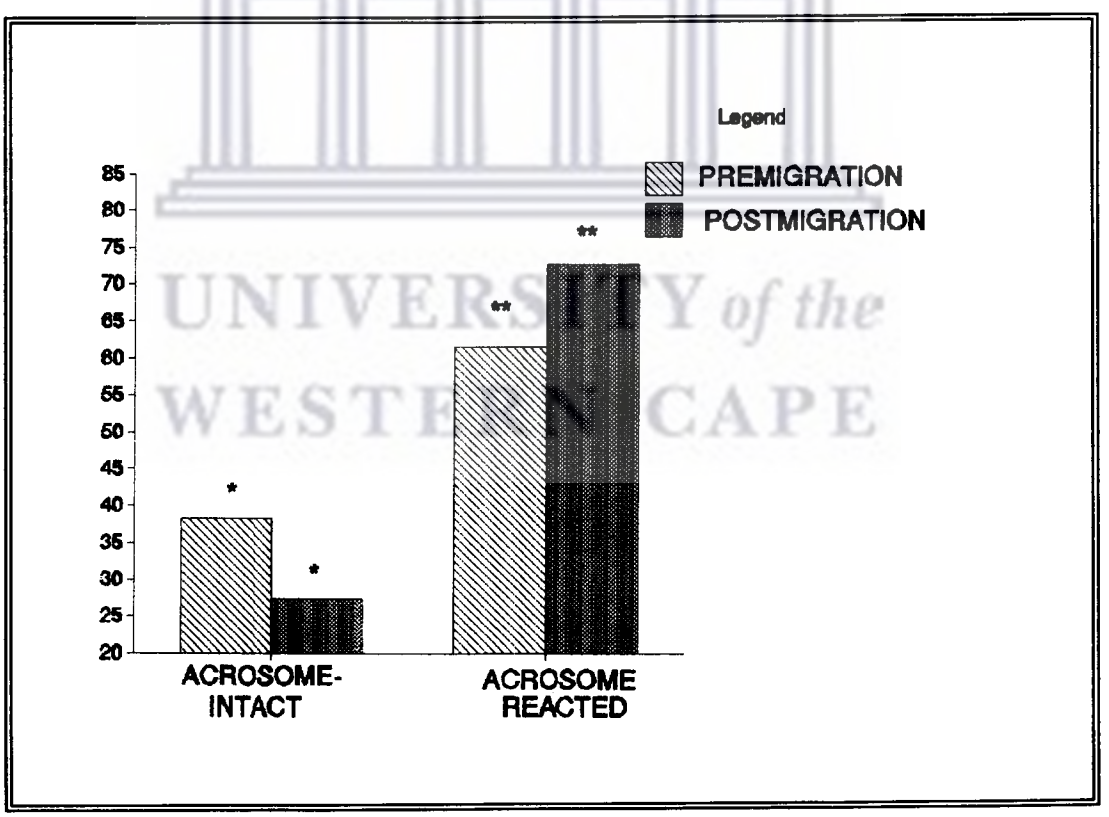


Fig. 3.4.1 **Association between acrosome-intact and acrosome-reacted sperm for pre-and postmigration.**

Fig. 3.4.2 **Micrographs of fluorescent microscopy of acrosome-intact and acrosome-reacted sperm.**

AI - acrosome-intact; AR - acrosome-reacted

CHAPTER FOUR

Discussion

4.1 Motility Assessment

Evidence to date indicates that sperm are sequestered in the caudal isthmus until just before ovulation [Hunter & Nicol, 1983] and that movement of sperm from this region to the ampulla is a highly synchronized event [reviewed by Barrat & Cooke, 1991]. Increasing evidence also suggests that spermatozoa adhere to epithelial surfaces which modulate sperm passage through the female reproductive tract and egg vestments [Katz *et al.*, 1989]. Sperm attachment to the epithelial surfaces may play an important role in maintaining sperm viability in the Fallopian tube [Barrat & Cooke, 1991]. Sequential flushing of sperm before ovulation yielded mainly dead sperm from the first and second flush (containing sperm mainly from the lumen and mucosal surface, respectively), whereas over 50% of sperm from the third flush (sperm mainly in the crypts) were alive [Smith & Yanagimachi, 1991]. Capacitation appears to occur while sperm are sequestered in contact with the epithelial cells of the isthmic portion of the oviduct [Hunter & Nicol, 1983; Suarez, 1987; Yanagimachi, 1994].

Ellington *et al.* [1993] showed that co-culture of oviduct epithelial cells with equine sperm appears to induce capacitation. The sperm in their study maintained flagellar motion over a 4-day period after attachment to the oviduct epithelial cells. During this period there was a slow release of sperm from the oviduct epithelial cells. Investigations by Guerin *et al.* [1991] support these findings; sperm co-cultured with genital tract epithelium monolayers resulted in the release of hyperactivated sperm. Attachment of sperm to the oviduct epithelium after flushing was also noted in the present study. The increase in systemic oestradiol during oestrus could induce the production of sperm binding molecules by the epithelium, while the drop in oestradiol that precedes ovulation could lead to a loss in sperm binding molecules and the gradual release of sperm from the epithelium [Raychoundhury *et al.*, 1993]. This also implies that the Fallopian tube may regulate the speed of sperm capacitation. Smith and Yanagimachi [1989] found that sperm capacitation proceeds at a faster rate when mating occurs after ovulation.

Completion of capacitation in the Fallopian tube, therefore, may result in sperm detachment from the epithelium - a process which may be concordant with the development of sperm hyperactivation [Barrat & Cooke, 1991]. The expression of hyperactivation is an important aspect of sperm movement within the Fallopian tube. Membrane alterations during capacitation may also be directly related to changes in flagellar motion, e.g. in altering membrane ion conductance. The electrochemical properties of the sperm plasma membrane also create a

potential for affinity to epithelial tissue [Katz *et al.*, 1989]. Capacitation-induced changes in surface properties could, therefore, interact with changes in flagellar activity to modulate sperm transport.

The objective identification of hyperactivated motility has been controversial. A need for the establishment of a set of geometric parameters to characterize this form of motion became apparent [see chpt.1, pg.12]. Burkman [1991] defined a new set of criteria for automatically sorting various hyperactivated patterns from non-hyperactivated sperm in the human. Automatic sorting of hyperactivated patterns were achieved with the use of the following criteria:

1. linearity ≤ 65,
2. velocity ≤ 100 and
3. lateral head displacement ≥ 7.5 μm.

Additional criteria were used for identifying each hyperactivated class separately (circling high curvature, thrashing, star and helical patterns).

Although the values for the criteria recommended by Burkman [1991] cannot strictly be applied to ram sperm as their baseline values differ from human, the motion parameters selected by Burkman [1991] can be used to discriminate hyperactivated from non-hyperactivated sperm. In the present study, these selection criteria (VCL,ALH and LIN), in addition to CURV and MAD, were used to discriminate hyperactivated from non-hyperactivated samples within the postmigration samples.

CURV and MAD together with LIN and STR are indicative of progressiveness of sperm movement [see appendix 6.2]. The smaller the curvature (CURV) (expressed as a value between 0-1), the straighter the sperm path. Generally a value > 0.5, will indicate sperm moving in a circular mode. The mean angular displacement (MAD) gives information on the average angle at which sperm deviate from one frame to the next. Curvature, helps to assess the form of the sperm path, whereas, MAD assesses how sperm attain this [SMQ manual]. A decrease in LIN and increase in MAD and CURV, therefore, indicate that the sperm have a more circular trajectory which is one of the hallmarks of hyperactivated sperm.

The amplitude of lateral head displacement (ALH) reflects the extent of oscillation of the sperm head around its average path and inherently determines the linearity, beat cross frequency and several derived parameters such as STR, WOB, DNC, MAD and CURV [Van Der Horst, 1995]. Zouari *et al.* [1993] indicated that ALH amongst all motion parameters showed the highest positive correlation with the outcome of *in vitro* fertilization in humans. ALH was also selected as one of the selection criteria for defining hyperactivated sperm by Burkman [1991].

Four of the five selected motion parameters (LIN, ALH, CURV and MAD) together with WOB and STR showed significant differences between the premigration and hyperactivated-postmigration samples. VCL which is indicative

of vigour of motility, also gives valuable information about hyperactivated sperm, but there is often considerable overlap between hyperactivated and non-hyperactivated sperm for this parameter. Consequently, no significant difference was found between the premigration and hyperactivated-postmigration samples. Burkman [1991] also experienced this when discriminating between hyperactivated and non-hyperactivated samples in her study.

The motion parameters used to analyze the pattern of head motion (LIN, ALH, CURV, MAD, STR) in the present study, all give significant kinematic and mechanical differences between pre- and postmigration. This information can, therefore, be used to establish the biological state of sperm hyperactivation.

An occurrence noted in the postmigration sample, was the presence of large numbers of agglutinated sperm. These sperm exhibited head-to-head agglutination while continuing forward progressive motility. Although these agglutinated samples showed significant differences for certain parameters (STR, MAD and CURV) concurrent with the postmigration sample from the premigration sample, no definite conclusions can be drawn from these results. The number of sperm forming the cluster of agglutinated samples varied from one group to another (as observed microscopically). The contribution of each sperm to the overall value of the motion parameters, cannot be ignored. The number of sperm, therefore, forming the cluster could easily dictate the final result for the motion parameters analyzed. Autoagglutination is a feature that

has been associated with capacitation [Harper, 1990]. Therefore, although the motion parameters of these agglutinated samples cannot be utilized, their autoagglutination may be an expression of a capacitational change which may have occurred due to migration through the Fallopian tube.

The changes in motility patterns, in the present study, lead to the assumption of a capacitational change. Hyperactivation is considered an integral part of the capacitation process [Robertson *et al.*, 1988] and can, therefore, serve as a marker for the progression of capacitation, as capacitation results in no obvious, easily discernible, morphological alterations. The cut-off values obtained by the ROC-curve [table 3.1.3] can tentatively be used for coarse discrimination between hyperactivated and non-hyperactivated sperm in rams. Robertson *et al.* [1988] demonstrated an association between hyperactivation and acrosomal loss. In their study, the percentage of cells which showed hyperactivated motility increased initially to plateau levels, whereas spontaneous acrosomal loss (measured in a second aliquot of the same sample) increased for the entire incubation period after an initial lag period. The development of hyperactivation, therefore, preceded the occurrence of an acrosome reaction in free-swimming sperm.

4.2 Morphological Assessment

Although the potential of sperm morphological characteristics has been

recognised as a prognostic tool for the prediction and assessment of male fertility in humans, no clear standardized criteria exist for 'normal' spermatozoa. The WHO [1987] and Tygerberg strict criteria [Menkveld *et al.*, 1990] have attempted to address this problem. Their criteria, however, can only be applied to human spermatozoa and are, therefore, inadequate when applied to other mammalian species. The flexible image processing system (FIPS), as it has been used in this investigation, can be used to establish baseline values for sperm head measurements.

There was no significant difference occurred in morphology between pre- and postmigration. Very few gross morphological abnormalities were apparent in either of these samples (subjective assessment). This assessment was supported by the results obtained from the FIPS measurements. Both pre- and postmigration data showed uniform distribution with a very narrow range. The values described in table 3.2.1, therefore, quantify ram sperm head dimensions with a shape factor ranging between 0.7 and 0.75. The shape factor describes the regularity of the object. A value of zero would indicate a round sperm. The results obtained in the present study imply that ram sperm have an irregular shape. This irregularity is, however, constant throughout the pre- and postmigration samples. There is a negative correlation between area and shape, implying that the larger the sperm, the less round the sperm will be. This relationship corresponds to the visual image of the sperm.

The absence of gross morphological abnormalities was uniform throughout the samples, between and within samples. According to Salisbury and VanDemark [1961], the occurrence of morphological uniformity or non-uniformity can be ascribed to spermatogenesis, since no recognisable changes in the gross morphology of spermatozoa occurred during their passage through the reproductive tract of the bull. In bull sperm, one abnormality appeared to be limited to a single breed of cattle and was, therefore, presumed to be inherited [Salisbury & VanDemark 1961]. However, Leuchtenberger *et al.* as quoted by Salisbury & VanDemark [1961] found that some morphologically normal sperm cells of infertile bulls were abnormal in that they had a lower-than-normal DNA content. Therefore, a morphologically normal cell may contain genetic aberrations which render it incapable of performing normal function.

4.3 Localization of WGA-receptors

An early step in mammalian fertilization is an adhesion step between the sperm surface and the zona pellucida [Myles & Primakoff, 1991]. Sperm do not exit the testis with an innate ability to interact with, i.e. to bind to the zona pellucida, and to fertilize eggs [Yanagimachi, 1994]. The modifications of the sperm surface may occur both in the male reproductive tract (during epididymal transit) and in the female reproductive tract (during capacitation) [Myles & Primakoff, 1991]. Various independent studies [Magargee *et al.*, 1988 and Kumar *et al.*, 1990] have shown that sperm surface changes do occur during epididymal maturation.

Wheatgerm agglutinin, in particular, showed increased binding as the sperm progressed through the epididymis with the highest percentage binding in the caudal epididymal sperm of ram [Magargee *et al.*, 1988], rat, mouse and hamster [Kumar *et al.*, 1990]. Magargee *et al.* [1988] found that ejaculated sperm retained the intensity and distribution of lectin binding associated with caudal epididymal sperm. Binding was noted over most aspects of the cell. They attributed these changes to exposure or formation of sialyl residues. Sialic acid residues are secreted by the epithelium of the epididymis as terminal sugars of sialoglycoproteins that bind to the sperm surface during epididymal transit [Toshimori *et al.*, 1991].

The results obtained in the present study for the premigration sample correlates with the findings of Kallojoki *et al.* [1985], Cross and Overstreet [1987], Magargee *et al.* [1988], Kumar *et al.* [1990] and Lasalle and Testart [1994]. The results obtained indicate uniform staining of WGA over most aspects of the cell [see fig.3.3.2]. Results of previous investigations [Magargee *et al.*, 1988 and Lasalle & Testart, 1994] indicate that the WGA has bound to sialic acid residues instead of N-acetyl-D-glucosamine (GlcNAc) residues. Lasalle and Testart [1994] demonstrated the expression of sialic acid residues on human sperm plasma membrane. Wheatgerm agglutinin, which specifically recognises both NeuNAc and GlcNAc bound almost all intact motile spermatozoa, whereas succinylated WGA, which recognises only GlcNAc, bound less than 10% intact motile spermatozoa. Treatment of distal cauda sperm with neuraminidase returned the

cellular profile to that of immature cells [Magargee *et al.*, 1988]. Neuraminidase cleaves N-acetyl-neuraminic acid (sialyl moiety). However, the incidence of WGA lectin-binding to GlcNAc in addition to sialic acid residues within the premigration sample cannot be excluded in the present study, since the WGA used recognises both sialic acid and GlcNAc.

The results of the postmigration sample show selective lectin binding predominantly over the acrosomal and postacrosomal domains. The results of the postmigration sample imply that sialic acid residues were cleaved from the sperm plasma membrane during transit through the Fallopian tube with WGA then possibly binding to GlcNAc. Removal of sialic acid residues from the oligosaccharide chain attached to the sperm membrane surface [see fig.4.1], unmasks galactose residues with GlcNAc residues beneath these. Removal of sialic acid residues, as a source of WGA receptors as well as a steric blockage, facilitates binding of WGA to GlcNAc. GlcNAc is believed to be involved in sperm-zona binding; binding was blocked when capacitated sperm were treated with 0.1M GlcNAc *in vitro* [Kumar *et al.*, 1990].

Conclusions drawn from the present study support results of previous investigations [Holt, 1980; Langlais & Roberts, 1985; Lassalle & Testart, 1994]. Initial alterations in capacitation include modification, redistribution or loss of the epididymal and seminal plasma proteins coating the sperm membrane surface [Brackett & Oliphant, 1975; Oliphant, 1976; Kinsey & Koehler, 1978; Koehler,

1976 & 1981]. The removal of sialic acid residues during capacitation would be a logical assumption, since sialic acid residues bind to the sperm surface during epididymal transit [Toshimori *et al.*, 1991]. Also, capacitation is viewed as a reversible process that results in a net decrease in negative surface charge [Langlais & Roberts, 1985]. Holt [1980] suggests that sialic acid was responsible for the net negative surface charge of the spermatozoa so that removal of sialic acid results in the loss of the negative surface charge. Lasalle and Testart [1994] conclude from their studies that the release of sialic acid residues from the sperm plasma membrane could be one of the capacitation events necessary for unmasking certain sperm surface antigens implicated in zona pellucida recognition. The masking of antigens is reported as one of the significant functions of sialic acid [Schauer quoted in Lassalle & Testart, 1994] rendering sperm immune from phagocytosis [Holt, 1980]. Its absence, therefore, leads to the destruction of spermatozoa of abnormal morphology by phagocytosis in the ejaculate and during their transit through the female reproductive tract.

However, no subsequent studies were completed to substantiate the hypothesis that sialic acid, indeed, was cleaved from the sperm membrane surface during Fallopian tube transit.

Distribution of GlcNAc was higher in the acrosomal region (40.14 ± 13.44) than the postacrosomal region (34.14 ± 15.06) for the postmigration sample. These results supports previous findings which tentatively isolated the acrosomal region as the site for sperm-zona binding. Two components, ZP2 and ZP3, have sperm

binding activities. In the mouse, and perhaps many other species, ZP3 is the primary ligand that specifically binds to the plasma membrane over the acrosomal cap of acrosome-intact spermatozoa. ZP2 is the secondary ligand which preferentially binds to the inner acrosomal membrane of acrosome-reacted sperm [reviewed by Yanagimachi, 1994]. Leyton and Saling [1989] hypothesize that receptor aggregation i.e. aggregation of sperm membrane proteins, occurs in response to ligand-receptor (sperm-ZP3) interaction. This receptor aggregation initiates the signal that leads sperm to undergo the exocytotic events of the acrosome reaction.

The lectin staining observed in the postacrosomal region is significantly higher ($p < 0.001$) in the postmigration sample (28.14 ± 15.06) when compared to the premigration sample (5.42 ± 0.79). This higher incidence of lectin staining in the postacrosomal region of the postmigration sample could be attributed to an increase in the number of sperm which have released their plasma membrane i.e. acrosome reacted. Lassalle & Testart [1994] found that treatment of sperm with Triton X100, which releases the sperm plasma membrane, resulted in succinylated WGA binding of equatorial and postacrosomal regions. They conclude that WGA binding sites on the anterior acrosomal region seemed to be released with the plasma membrane. This hypothesis is supported by the results obtained from the acrosomal status determination in the present study. Postmigration sperm show a higher incidence of acrosome reaction when compared to the premigration sample.

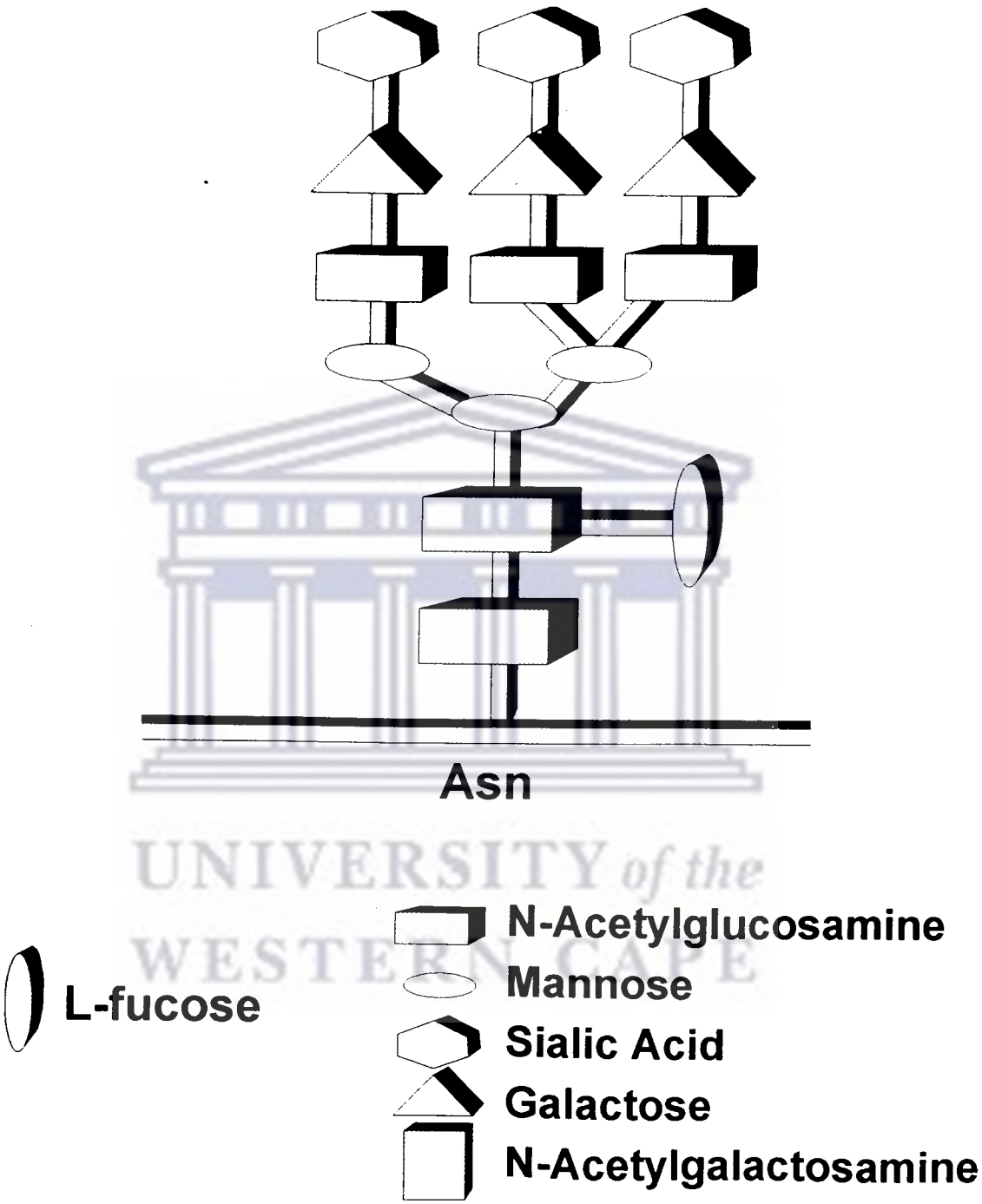


Fig.4.1 Diagrammatic representation of the N-linked complex type oligosaccharide chain attached to the sperm membrane surface

4.4 Acrosomal status

Sperm binding must be transient, allowing the sperm to unbind and pass through the zona pellucida so that the gamete membranes can come into contact with each other. In *in vitro* assays, sperm bind to the zona pellucida both before and after they acrosome react [Myles & Primakoff, 1991]. In some species, including the chinese hamster [Yanagimachi, 1981], rabbit [Kuzan *et al.*, 1987], guinea pig [Myles *et al.*, 1989] and human [Morales et al., 1989], it was shown that both acrosome-intact and acrosome-reacted sperm can initiate binding to the zona pellucida. In mouse sperm, however, it appears that only acrosome-intact sperm can initiate binding to the zona pellucida and that acrosome-reacted sperm can only bind after some sperm have acrosome reacted on the zona pellucida [Myles & Primakoff, 1991]. Therefore, although the ability of the zona pellucida to induce the acrosome reaction has been confirmed in a variety of animals, including the sheep [Crozet & Dumont, 1984], the zona-mediated acrosome reaction is species-specific and most species are able to undergo a true acrosome reaction in the absence of the zona pellucida [Yanagimachi, 1994].

Sperm transit through the Fallopian tube inthe present study resulted in a higher incidence of acrosome-reacted sperm. The incidence of WGA staining in the postacrosomal domain reiterates these findings, since WGA staining in the postacrosomal domain may indicate acrosome-reacted sperm [Lassalle & Testart, 1994]. It is not known whether the high incidence of acrosome reaction, per se, is as a result of spontaneous and/or false acrosome reaction (spontaneous true

as opposed to zona-mediated acrosome reaction).

The false acrosome reaction is defined as the acrosome reaction which occurs as a result of autodigestion of dead or moribund sperm [Yanagimachi, 1994]. The false acrosome reaction, associated with sperm death, occurs all the time in the female reproductive tract as the tract is by no means hospitable to all spermatozoa. Only those in the right place (microenvironment) at the right time can stay alive within the tract. Virtually all viable spermatozoa in the uterus and the oviductal isthmus have intact acrosomes [Yanagimachi, 1994].

The spontaneous acrosome reaction is defined as the acrosome reaction which occurs in actively motile spermatozoa in sperm capacitating media, without any stimulative agent. In the experimental conditions of the present study, though, the sperm were briefly washed in Ham's F-10 and then subjected only to the fluids found in the Fallopian tube. The Fallopian tube environment can, therefore, cause a false (due to sperm death) or spontaneous acrosome reaction (by providing a capacitating environment). According to Yanagimachi [1994], the spontaneous acrosome reaction is an unphysiological event, since most of the evidence indicates that fertilizing spermatozoa do not initiate the true acrosome reaction *in vivo* until they come into contact with the zona pellucida.

The percentage acrosome-reacted sperm in the premigration sample of the present study, however, is also quite high. This could be due to the same reasons

stated above for postmigration i.e. spontaneous and/or false acrosome reaction. Pretreatment of sperm, however, also affect lectin binding. Kallojoki *et al.* [1985] conclude from their studies that paraformaldehyde fixation and airdrying of spermatozoa on slides may disrupt the sperm plasma membrane and expose acrosomal and other intracellular glycoconjugates to lectin binding, thus giving false positive surface results. Gabriel *et al.* [1994] confirmed their results that fixation affects sperm membrane integrity (different procedures affecting it to different degrees). Although the general pattern of localization remains unaltered for specific lectins (for results obtained by different researchers), the actual percentage values of staining are critical when attempting to correlate them with sperm fertilizing potential.

The high percentage of acrosome-reacted sperm present in the premigration sample could, therefore, be due to false positive internal staining, false or spontaneous acrosome reaction or a combination of all three. These factors are also applicable to the postmigration sample. Negation of these factors when comparing the pre- and postmigration sample, however, still indicate a significant difference in results. Sperm transit through the Fallopian tube, therefore, does and/or can cause the acrosome reaction to occur, albeit false or spontaneous.

4.5 Conclusion

The viable subpopulation of sperm retrieved from the Fallopian tube was very

small in the present study presented here. Quantisation of results obtained by Smith and Yanagimachi [1987] revealed the number of sperm that reach the site of fertilization as 1 in 10,000. The low ratio of sperm to eggs (approx. 1:1) in the ampulla at the time of fertilization [rats: Shalgi & Phillips, 1988 ;hamsters: Cummins & Yanagimachi, 1982; sheep: Hunter & Nicol, 1986] suggests strongly that these sperm are likely to be fertile [reviewed by Barrat & Cooke, 1991]. Hunter *et al.* [1991] suggested that the influence of viscous proteinaceous secretions within the uterotubal junction and in the caudal portion of the isthmus act to inhibit sperm flagellar activity and progression, and thereby restrict sperm numbers in the Fallopian tube very significantly. The dilution of sperm along the female tract could be a means of selection to prevent polyspermy. The population of sperm that reaches the ampulla, therefore, probably consists of the most effective sperm to complete fertilization.

The fundamental aim of this study was to demonstrate that capacitation (whether wholly or partially) may have occurred due to the influence of the Fallopian tube. No obvious morphological alterations/selections occurred during transit through the Fallopian tube, but then no obvious morphological disparities occurred prior to exposure to the Fallopian tube. The factors which indicate functional alterations (motility, receptor activity and acrosomal status) appear to have been influenced by migration through the Fallopian tube. The apparent functional alterations which occurred, predisposed the sperm to fulfil the requirements as fertilizing sperm. The biological state of hyperactivation can be assumed to have

occurred in a selected population of the premigration sample after transit through the Fallopian tube. Mohri and Yanagimachi [1980] suggest that hyperactivated motility in fresh epididymal and ejaculated sperm is prevented by an inherent (intrasperm?) mechanism since demembranated cauda epididymal sperm displayed hyperactivation upon exposure to ATP. The characteristics of the motor sperm apparatus, therefore, do not change during capacitation, but removal or alterations of surface components/factors allow exhibition of hyperactivation. The inherent ability of the sperm to display this phenomenon is reiterated.

The membrane surface changes which qualify and equip the sperm for interaction with the egg vestments, appear to have occurred. The occurrence of the acrosome reaction before or after sperm-zona interaction is controversial. Notwithstanding this controversy, the results indicate an increase in the percentage of acrosome-reacted sperm after transit through the Fallopian tube. This could, of course, simply illustrate the natural maturational development after completion of capacitation, irrespective of the presence or absence of an oocyte, within a certain population of sperm.

Collation of these factors point to the occurrence of molecular events that could signify capacitation and/or the acrosome reaction. There are many gaps in our understanding of the molecular events of both capacitation and the acrosome reaction. This lack of knowledge can mean that a molecular event that is called

a part of the acrosome reaction in one study may eventually prove instead to be a step in capacitation [Meizel, 1985]. Although it cannot unequivocally be stated that capacitation and the acrosome reaction (to a lesser degree) have occurred, the results of the present investigation reflect an influence of the Fallopian tube on sperm. *In vitro* incubation of the premigration samples in culture medium concurrent with sperm migration through the Fallopian tube, does not reveal any of the changes observed in the postmigration sample.

Although the Fallopian tube environment may facilitate the transport of sperm to the site of fertilization at certain times coinciding with ovulation (thereby ensuring synchronization of sperm-egg arrival), the sperm require an innate ability to survive. Any genetic defect would manifest itself during the rigorous transit through the female reproductive tract and penetration through the egg vestments. The inherent ability of sperm to undergo the processes of capacitation and acrosome reaction would determine its survival and/or selection by the female tract.

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Appendix

6.1 WHO CRITERIA FOR MOTILITY ASSESSMENT

Grading of forward progression

0	no movement
1	no forward movement
1+	few sperm move now and then
2	undirected slow movement
2+	movement slow but directly forward
3-	movement fast but undirected
3	movement fast and directed forward
3+	very fast forwardly directed motility
4	extremely fast (wave-like) movement directed forward

6.2 SMQ AS CASMA SYSTEM

Seventeen motility characteristics of sperm as well as sperm density (concentration) can be assessed in both the automatic and manual modes by means of SMQ. All 18 of these parameters can be assessed within five minutes in the automated mode when using a 486 (33MH) computer.

A full description of each of these parameters will be discussed.

Refer to text below as well as Figs. 1 and 2 for the explanation of the SMQ parameters. The terminology used is internationally accepted and recently defined by Katz (1991) and now commonly used in recent literature.

I. Velocities

1. curvilinear velocity (VCL)

This represents the time-average velocity of the sperm head along its actual precise path or curvilinear trajectory and is measured in μm/s.

2. Straightline velocity (VSL)

This represents the average velocity of the sperm head as projected along the straight line between its first and final detected positions and is expressed as μm/s.

3. Average path velocity (VAP)

The time average velocity of the sperm head as projected along its spatial average trajectory and is expressed as μm/s.

II. Ratios, amplitudes and frequencies

4. Linearity

A ratio of projected length to total length of the curvilinear trajectory.

5. Amplitude of Lateral Head Displacement (ALH - average)

the average amplitude of lateral distances of the actual sperm head trajectory about its spatial average path.

6. Amplitude of Lateral Head Displacement (ALH - maximum)

The maximum amplitude of lateral distances of the actual sperm head trajectory about its spatial average path. Katz [1991] correctly indicates that there is no unique definition for ALH, even for a fixed definition of average and it should be realized that different CASA systems measure it differently.

7. Beat Cross Frequency (BCF)

The time-average rate at which the curvilinear path crosses its average path. This parameter partly describes sperm vigour.

8. Straightness (formerly known as the linear index) - (STR)

This is the ratio of VAP to VSL and is an expression of the straightness of the average path.

9. Wobble (formerly known as the curvilinear progressive ratio) - (WOB)

This is the ratio VAP to VCL and is an expression of the degree of

oscillation of the curvilinear path about its spatial average path.

10. Dance

This is defined by the product of VCL and mean ALH and expressed as $\mu m^2/s$. This parameter, therefore, describes sperm motion as the space (window) occupied by the sperm head path during 1 second.

11. Dancemean

This is the product of the mean ALH and the ratio of the VCL and the VSL and is expressed in μm.

12. Curvature

This is a new parameter for CASA and has been previously used in semi-automatic analysis (frame lapse photography) By Samuels and Van der Horst [1986]. This parameter also reflects progressiveness of movement and is reflected in the curve 0 - 1 (1 - straightline path μm/curvilinear path μm). The smaller the curve 0-1, the straighter the sperm path and the higher is progressiveness. This parameter also gives information on the mode of movement and a value > 0.5 will indicate sperm swim in a circular mode.

13. Radian - (RAD): New terminology is **Mean Angular Displacement (MAD)**

This is also new parameter for CASA and previously used in semi-automatic analysis by Samuels and Van der Horst [1986]. By using radian (RAD μm), which is the radius of the circle of which the total curvilinear

track (μm) is an arc, circling spermatozoa can be detected. When the average angle of curvature is 180° the RAD (μm) is 3.14 and 6.28 when the average angle of curvature is 360°. The formula for Radians = Radius/3.14 x 180. This parameter gives information on the average angle at which a sperm turn when motile. Curvature, therefore, helps to assess the form of the sperm path whereas RAD assesses how sperm attain this. Virtually no research is available on these parameters and they seem to have great potential.

III. Percentage groupings

14. Percentage motile sperm - (PM)

Percent motility of sperm population in field of view and represents all forms of motility from wiggling sperm to fast and highly progressively motile sperm.

15. Percentage progressively motile sperm - (PPM)

Only sperm that swim in a progressively forward direction with a linearity (LIN) > 30%, VAP > 20μm/s and VCL > 30 μm/s.

16. Percentage hyperactivation

A controversial parameter which can be divided into several subgroups. The most important form of hyperactivation namely "starspin" hyperactivation is defined as: VCL>80μm/s; LIN<19 and DANCEMEAN >17. "Transitional" hyperactivation is defined as: VCL>80μm/s; LIN>19 and <34 and DANCENEAN >17. These parameters have been verified

Robertson, Wolf and Tash [1988] for human sperm and as research progresses may be redefined. Rat sperm hyperactivation is associated with excessive head displacement in relation to the middle piece but LIN may remain high. RAD and/or CURV could be incorporated in the formula for future trials.

17. Sperm density or sperm concentration

sperm density will be determined as 10^6sperm/ml. It should be realized that this can only be accurate determined if the volume of the counting chamber is known and all sperm are in the same focal plane. The dilution factor is also needed when semen is diluted in an extender.

Summary of the SMQ parameters

Section I and II represent swimming characteristics of each individual sperm in a given field whereas III represents an overall view of the motile status of the sperm population in a given field. The above 16 parameters can also be divided into different classes based on functionality:

i) **Vigour of motility:**

VCL, VSL, VAP and BCF

ii) **Pattern of motion:**

LIN, STR, WOB, ALH (mean and max.) DANCE and DANCEMEAN, CURV, MAD (RAD)

iii) **Overall population motility:**

PM, PPM and PHA represents and overall assessment of motility.

iv) **Progressiveness of sperm movement:**

LIN, STR, CURV and MAD (RAD)

v) **Sperm density or concentration:**

Density or sperm count reflects the number of sperm as 10^6/ml.

6.3 LIST OF TABLES

6.4 LIST OF FIGURES

Acknowledgements

I gratefully thank:

Prof. G. Van der Horst for his support, critical review and editorial comment

Dr. F. Van Niekerk for providing the experimental material, his critical review and editorial comment

Leon K. Gabriel for assistance with the experimental procedures, his critical review and motivational support

Shona Grammer for her assistance in the collection of experimental material and support

The staff at Elsenburg Farm, University of Stellenbosch, for assistance with the collection of experimental material

Mr. S. Allie of the Dept. of Physiological Sciences, University of the Western Cape, for providing all necessary equipment and materials

Mrs. L. Van der Merwe of the Experimental Biology Group, Medical Research Council, for her assistance with the fluorescence microscopy and

photomicrography

The Experimental Biology Group, Medical Research Council, for the use of their fluorescent microscope

Thullie Leubane for her assistance with FIPS

Khalied Kasker for his assistance with the motility recordings

Ruby-Ann Levendal for editorial comment

Nazlie Hendricks and Kareemah Allie for their assistance with the typing of this thesis

Melvin Van der Berg for the translation of the abstract

My mother for her devotion and support

Annette Van der Berg for her friendship and motivational support

Mark Van der Berg for his encouragement and unswerving loyalty