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Identification and Functionality of Antigens Reacting with Antisperm Antibodies and their role in Male Immunoinfertility

A Dissertation Submitted to the University of Dublin

for the Degree of Doctor of Philosophy

July 2014

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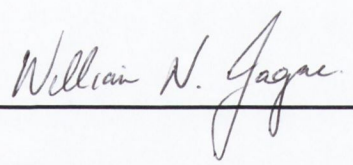

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List of Abbreviations

2-D	Two-dimensional
ADP	Adenosine diphosphate
AKR	Aldo-keto reductase
AKR1B1	Human aldose reductase
AMP	Adenosine monophosphate
ASA	Antisperm antibodies
ATP	Adenosine triphosphate
β_2m	β_2-microglobulin
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CFH	Complement factor H
CM-sepharose	Carboxymethyl-sepharose
CRISP	Cysteine rich secretory protein
DAO	Diamine oxidase
DC	Dendritic cell
DCCD	Dicyclohexylcarbodiimide
DEAE-cellulose	Diethylaminoethanol-cellulose
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC_{50}	Median effective concentration
ELISA	Enzyme-linked immunosorbant assay
FA-1	Fertility Antigen-1
Fas L	Fas Ligand
FCS	Feotal calf serum
FDP-lysine	N$^{\epsilon}$-(3-formyl-3,4-dihydropiperidino)lysine
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
FSH	Follicle stimulating hormone
hCG	human chorionic gonadotrophin
HNE	hydroxynonenal
HRP	horseradish peroxidase
IEF	Isoelectric focusing
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IPTG	Isopropyl -D-1-thiogalactopyranoside

IUI	Intrauterine insemination
IVF	*in vitro* fertilisation
LB	Luria Bertani
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
mABs	Monoclonal antibodies
MAR	Mixed agglutination reaction
MHC	Major histocompatibility complex
mrtCD52	Male reproductive tract specific CD52
MVDP	Mouse vas deferens protein
NK	Natural killer
PAGE	Polyacrylamide gel electrophoresis
PAO	Polyamine oxidase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE	Prostaglandins of the E series
PHA	Phytohaemagglutinin
PNGase	Peptide-N-glycosidase
PSA	Prostate-specific antigen
PVDF	Polyvinylidene fluoride
PVP	Polyvinylpyrollidone
RBCs	Red blood cells
RNase	Ribonuclease
RP-HPLC chromatography	Reversed-phase high performance liquid
RSPH 44	Radial spoke head 44
SDS	Sodium dodecyl sulphate
SI	Sperm Immobilising
SPACA	Sperm acrosome associating proteins
TBS	Tris-Buffered Saline
TCA	Trichloroacetic acid
TGFβ	Transforming growth factor-β
TLC	Thin layer chromatography
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
Treg	Regulatory T cells
UV	Ultra-violet
ZP	Zona pellucida

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ABSTRACT

Antisperm antibodies (ASA) are a leading cause of male associated infertility. The male partner of 1 in 10 couples tests positive for sperm bound antibodies with immunoglobulins of the IgG class being found in all patients and IgA to a lower extent. This study used several approaches to identify novel sperm surface antigens. Firstly, reactivity to mouse reproductive tract antigens were invesigated, indentifying CRISP1 and MVDP as targets for human ASA. Both proteins were purified to homogeneity. CRISP1 was shown to be involved in sperm-egg binding, as polyclonal anti-CRISP1 antibodies blocked mouse ova fertilization *in vitro*. MVDP, a member of the aldo-keto reductase family, was revealed to function in the detoxification of acrolein, a reactive aldehyde produced by the catabolism of the polyamines, spermine and spermidine, which are abundant in seminal plasma. Secondly, human seminal plasma proteins were explored as targets for ASA. Purification of a peptide from seminal plasma followed by mass spectrometric analysis identified semenogelin 1 as an ASA target. Purification of Semenogelin 1 along with its homologue Semenogelin 2 (the two main clot forming proteins of seminal plasma) showed semenogelin 1 to be uniquely reactive to ASA. Thirdly, a novel pull down experiment, revealed a number of sperm surface proteins reactive with ASA. Finally, probing of the oligosaccharide moieties of sperm surface glycolipids reavealed them to be targets of ASA. Enzymatic degradation of the oligosaccharide with galactosidase greatly reduced ASA reactivity, whereas removal of the terminal sialic acids with neuraminadase increased binding. ASA patient seminal plasma was shown to have reduced ability to inhibit mitogen-stimulated proliferation of T cells when compared to normal seminal plasma. The activity was traced to a low molecular weight peptide, that binds divalent metal ions and is cationic in nature, and which can suppresses IL-8 secretion from PBMCs, while inducing IL-10 release. Sequencing of the peptide showed it was an internal fragment of semenogelin 1. Recombinant peptide was used to demonstrate inhibition of CD3 stimulation of T cell proliferation. The activity of the peptide requires both transcription and translation for functionality and appears to affect cell cycle progression. It would appear therefore that ASA directed against semenogelin 1 fragments are the cause of reduced immunosuppression in ASA patient seminal plasma.

CHAPTER 1

Introduction

1.1 Spermatogenesis and Spermatozoa Structure.

Spermatogenesis takes place in the seminiferous tubules of the testes (Eddy, 1988) (Figure 1.1.1). The spermatozoon, the end product of this process, has three main components; the head, midpiece and tail. The head consists of the acrosome, the nucleus and lesser amounts of cytoskeletal structures and cytoplasm. It is subdivided into various domains; the acrosome, equatorial segment and postacrosomal region (Eddy, 1988) (Figure 1.1.2).

The acrosome is a membranous structure containing enzymes necessary for the sperm to penetrate and/or fuse with the plasma membrane of the egg to achieve fertilisation. This structure sits as a cap over the nucleus in the anterior part of the sperm head (Eddy, 1988) (Figure 1.1.2).

The tail contains a centrally placed axoneme, which is a highly ordered complex of microtubules surrounded by dense fibres extending from the head to the near posterior end of the axoneme (Eddy, 1988). The mid-piece lies at the base of the head and consists of helically wrapped mitochondria around the outer dense fibres of that part of the sperm tail. This is the spermatozoon's 'power house' (Eddy, 1988). The whole sperm is tightly wrapped by the plasma membrane.

After release from the seminiferous epithelium in the testes, the spermatozoa pass into a network of spaces constituting the rete testes, and thence, via the efferent ducts, into the epididymis. The epididymis consists of three main parts; the caput (head), corpus (body) and cauda (tail). It is essentially a long tube of varying diameter that is greatly convoluted to form a compact body that is only about 1/100th of its fully

extended length. The epididymal duct is lined internally by columnar epithelium that differs in height and cellular composition along its length (Austin, 1985) (Figure 1.1.1).

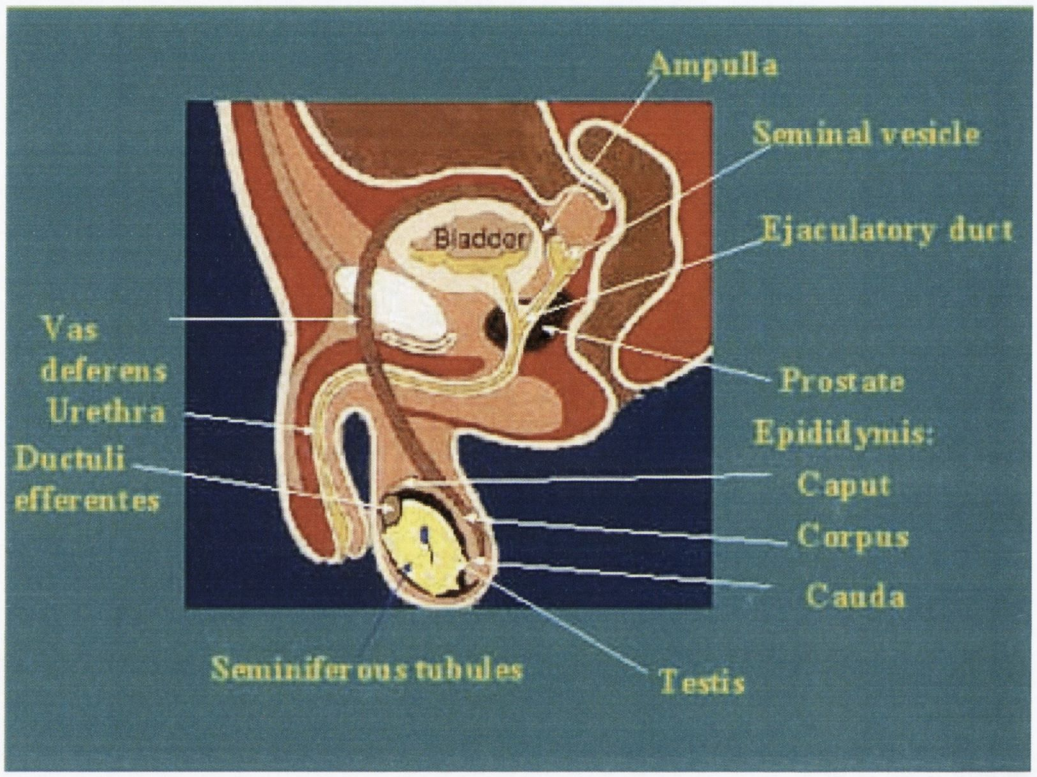


Figure 1.1.1 Diagrammatic Representation of the Male Reproductive Tract. Different segments of the epididymis are also indicated; the caput, corpus and cauda. (Taken from Schlegal, 1987).

From the cauda epididymus, spermatozoa are transported into the vas deferens and, on ejaculation, into and through the urethra.

Throughout this journey the spermatozoa are suspended in the transudations and secretions of each region; the chemical and physical nature of this medium progressively changes. During their transport from the seminiferous tubules to the egg the spermatozoa are also changing structurally, chemically and behaviourally. This process is termed 'maturation' (Austin, 1985).

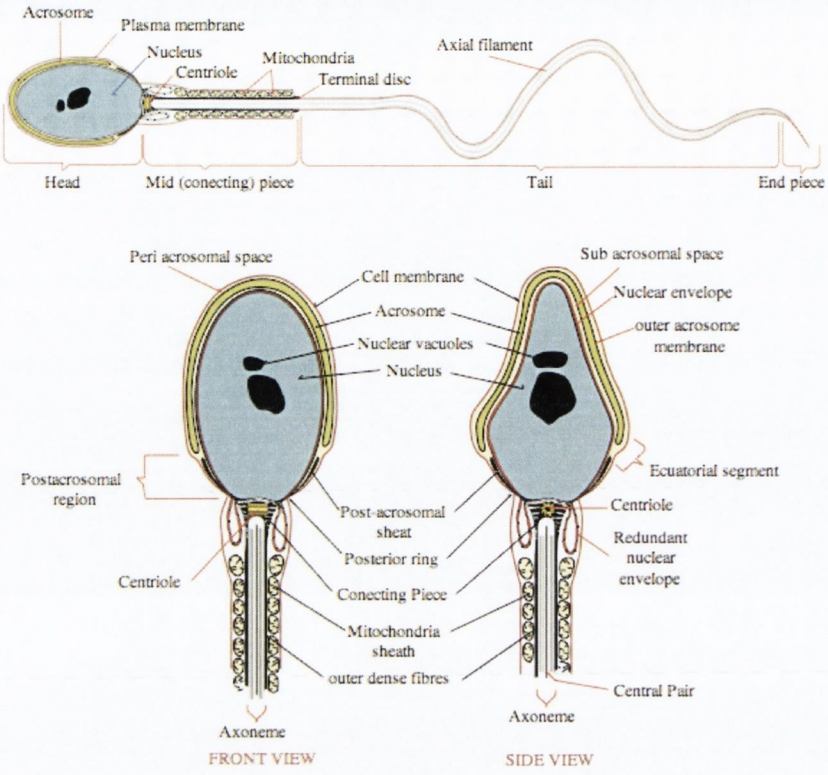


Figure 1.1.2 Drawing of a Human Spermatozoon. Indicated are its various domains; the acrosome, equatorial segment, posterior head (postacrosomal region), midpiece and tail. (Taken from Villarreal, 2014).

1.2 Spermatozoa Maturation.

Spermatozoa leaving the seminiferous tubules have little or no motility of their own. The plasma membrane is coated with many macromolecules that may be lost or modified as they pass from the caput to the cauda epididymis. Also, new macromolecules of epididymal origin are absorbed onto or into the plasma membrane. This process of epididymal maturation produces motile sperm in the cauda epididymis (Austin, 1985).

These motile sperm pass into the vas deferens and on ejaculation enter the vagina; swim through the cervical mucus and onwards to the uterus and oviduct.

During their stay in the uterus and oviduct they undergo 'capacitation'. This involves a destabilisation of the plasma membrane of the sperm head without any visible morphological changes. These changes are necessary to allow the final maturational change, the so called 'acrosome reaction', to take place (Austin, 1985).

This reaction involves fusion of the outer acrosomal membrane and the plasma membrane of the spermatozoon, thereby permitting release, through the pores formed, of the hydrolytic enzymes (Eddy, 1988), (Figure 1.2.1). This allows penetration and fertilisation of the egg (Eddy, 1988) (Figure 1.2.2).

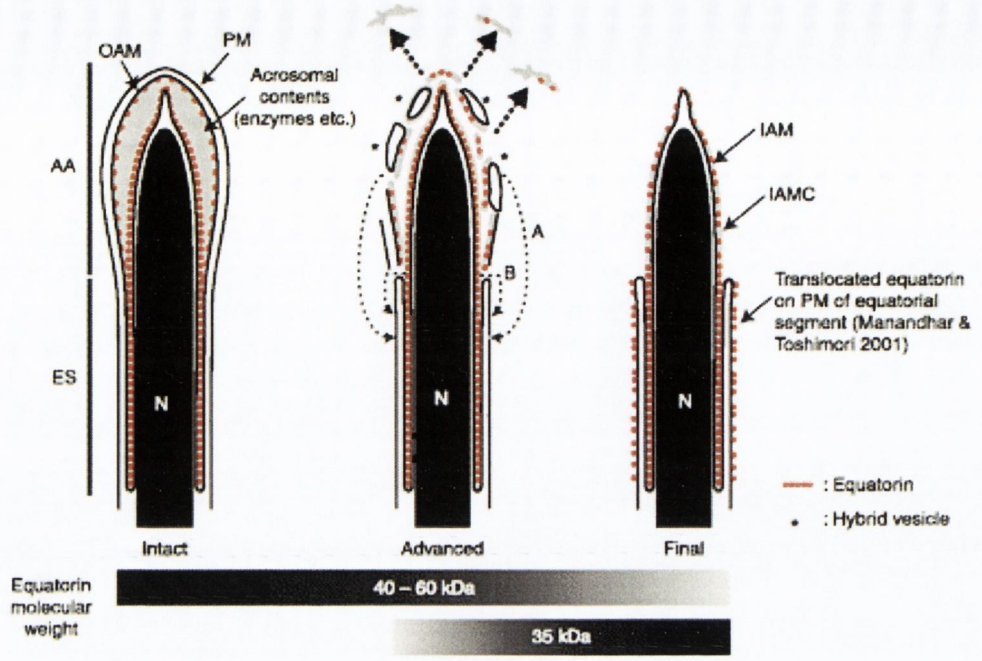


Figure 1.2.1 Diagrams Showing the Progression of a Typical Acrosome Reaction with Equatorin as Marker of Progression. IAM, inner acrosomal membrane; N, nucleus; OAM, outer acrosomal membrane; PM, plasma membrane. As shown in the left-hand panel, before the acrosome reaction has started (intact), equatorin (red) is rich on the IAM and rather sparse on the OAM. There are several possible routes for the appearance of equatorin on the sperm surface. Firstly as indicated in the middle panel by A (reassociation model), as the acrosome reaction proceeds, equatorin on the OAM at the anterior acrosome (AA) detaches and reassociates with the sperm surface, presumably undergoing molecular processing; some amounts of equatorin are associated with hybrid vesicles (asterisk) and surrounded by amorphous substance (acrosomal contents). Secondly as indicated by B (lateral diffusion model), equatorin in the acrosomal lumen diffuses to the sperm surface along the acrosomal and plasma membranes. When the acrosome reaction is complete, equatorin is found localized on the surface of the PM over the equatorial segment (ES; also shown in (Manandhar and Toshimori, 2001); this mechanism is unclear. Some amounts of equatorin remain on the IAM on the AA. During the acrosome reaction progression, low molecular mass equatorin (35 kDa) appears in addition to the 40–60 kDa range equatorin and increased in the signal intensity. This process may be attributed to biochemical modifications such as deglycosylation and/or cleavage. (Taken from Yoshida, 2010).

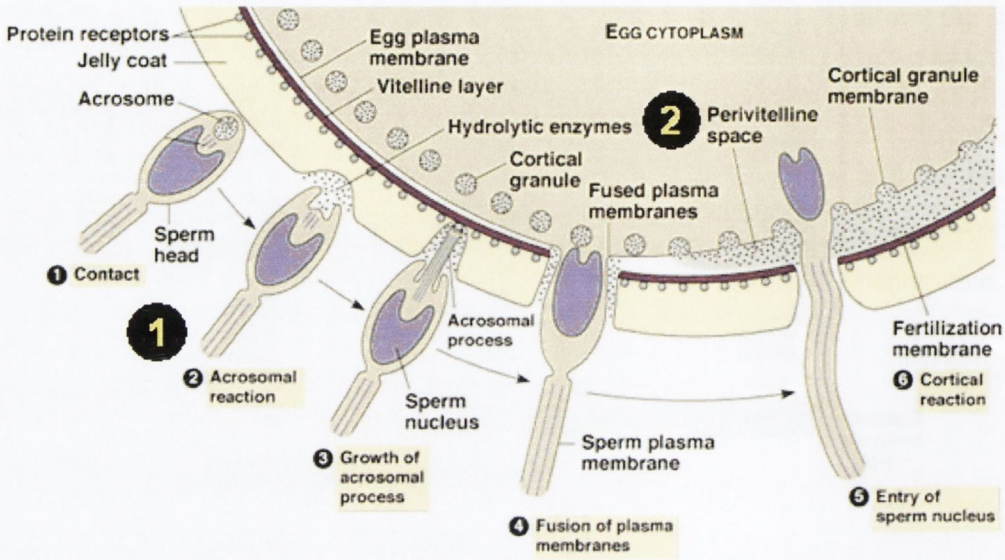


Figure 1.2.2 Schematic Representation of Sperm Penetration of the Egg. As sperm bind to the egg through a number of receptors, the acrosome reaction is induced to allow access through the zona pellucida (ZP) by the release of hydrolytic enzymes. This is followed by fusion of the plasma membranes and entry of the sperm nucleus. (Taken from Inoue et al., 2005)

1.3 Antisperm Antibodies.

1.3.1 Historical Perspective.

At the beginning of the last century, (Landsteiner, 1900) and (Metchnikoff, 1899) first demonstrated that antibodies to spermatozoa could be induced experimentally. However, it was not until 1954 that the possible role of autoimmunity to sperm as a cause of infertility was first described, when (Rumke, 1954), successfully demonstrated the presence of sperm agglutinating and sperm immobilising antibodies in the sera of a small number of men with infertility. These patients also exhibited spontaneous agglutination of their spermatozoa in their semen. In 1964, sperm agglutinins were revealed in the sera of women who had unexplained infertility (Franklin and Dukes, 1964). The presence of antisperm antibodies (ASA) has been associated with reduced fertility in a large number of individuals over the last few decades (Haas, 1987b). The human immune system is complex and therefore, the understanding and thorough investigation of the role of ASA in fertility has progressed rather slowly. The diagnostics of reproductive immunology are often complex or practically difficult and therefore can produce discordant results. In reality, immuno(in)fertility is a clinical science yet to be properly investigated.

In retrospect, male fertility has been ignored and neglected over the past decades, with most work in the field of infertility being carried out by gynaecologists into the field of female infertility, which therefore has been disproportionately investigated.

The quest to investigate and understand both autoimmunity and isoimmunity (alternatively referred to as alloimmunity wherein the body gains immunity to another individual of the same species) to human spermatozoa has been an emerging interest parallel to progress in the treatment and diagnostics of general human fertility. The understanding of the causes and roles of ASA is at an early stage, yet the scientific world continues to base its treatment on the older, unreliable, inaccurate and inconclusive diagnostic probes into reproductive disease processes (Haas, 1987a, Hjort et al., 1985).

1.3.2 Review of Attempts to Remove Antisperm Antibodies.

Previously, once men were diagnosed as being antisperm antibody positive all hope of a successful pregnancy was forsaken and only perseverance or adoption were advocated. Physical as well as chemical methods were adopted in an effort to reduce or completely remove the effects of the ASA *in vitro.* The method of processing semen by washing and centrifugation was concluded to be ineffective because of the intricate, complex binding mechanisms and the high affinity of these immunoglobulins for sperm surface antigens. The futility of repeated washings was exhibited when 18 consecutive washings had no apparent positive effect (Haas et al., 1988). More recently, an updated technique of removing sperm bound antibodies from the sperm surface *in vitro* was devised (Grundy et al., 1992). Other methods of decreasing the antibody levels or reducing their deleterious effects on sperm included exposure to lower pH or high ionic solutions, to induce the antibodies to dissociate. However, these techniques were unwise since they decreased sperm motility and thus their viability. In the long run these efforts were unlikely to be adopted into everyday laboratory practice, as the techniques yielded no overall advantages.

High levels of serum in media preparations used for washing sperm appeared to induce a reduction in antisperm antibody titres. However, this result emerged to be false as the antibodies become effective again in serum free media (Grundy et al., 1991, Hinting et al., 1989). Ejaculation directly into buffer was also seen as a possible method of controlling the deleterious effects of ASA. The ejaculate containing the antibody bound sperm would be diluted and it was believed that in theory the number of sperm that would then become antibody bound would also be reduced. However, this hypothesis was proved incorrect in practice.

The simultaneous use of thawed frozen sperm from antibody-negative donors as an immunosorbent and a subsequent swim up into overlying buffer was also suggested (Bronson et al., 1984). Because of the negative effects ASA have on motility and progression and therefore the sperm's ability to swim, this method also had little practical use (Kiser et al., 1987).

Intrauterine insemination (IUI) with antibody coated sperm in order to bypass the cervical canal and mucus has also been attempted with some success (Allen et al., 1985, Margalloth et al., 1988). Sperm, which are incapable of swimming due to antibodies bound to their surface, would find navigating the cervical canal a very difficult task. Thus IUI would help to overcome some of the effects of reduced sperm transport. However, the sperm, which are then delivered directly to the uterus, are still antibody bound and their motility and ability to fertilise the awaiting ovum would still be below par. In cases of severe autoimmunity to sperm (all sperm anti-body coated), 110 rounds of IUI in 19 couples gave no pregnancies (Francavilla et al., 1992), while a control group without ASA achieved a pregnancy rate of 25.6%. This suggests that severe levels of antibody coating reduce sperm viability or sperm-egg interaction within the female reproductive tract.

Sperm washing (Shulman et al., 1978), the use of condoms to reduce the exposure of the uterine cavity to sperm cells, IUI, *in vitro* fertilisation (IVF), gamete intrafallopian tube transfer (GIFT), and zygote intrafallopian tube transfer (ZIFT) were never completely successful and results were poor (Hinting et al., 1989, Horvath et al., 1989).

It has been shown by numerous clinical investigations that c. 8-10% of men attending assisted fertility clinics have reduced fertility or are sterile due to immunological factors (Clarke et al., 1985, Francavilla et al., 1997b, Mandelbaum et al., 1987). ASA can be detected in the serum of 8-14% of subfertile men (Halim and Antoniou, 1973, Hendry et al., 1977). In recent years the realisation that ASA are of extreme importance in male infertility has increased and so the quest for a complete understanding of the etiology and pathology of ASA has begun (Haas, 1987a). Through these investigations a suitable and effective treatment will be discovered.

1.3.3 Etiology of Antisperm Antibody Formation.

Antibodies to human spermatozoa can exist both in men and women. The factors that induce the presence of these antibodies in serum are varied. Antisperm immune responses occur as a result of a breakdown or imbalance in normal protective mechanisms, which include the blood/testis barrier system in men, and the seminal

plasma immunosuppressive factors and active immuno-regulatory mechanisms operative in both men and women. Frequently ASA are detected in the blood and semen of men who have endured an injury or insult to the reproductive tract; either chemical or physical (i.e. vasectomy) or infection (i.e. mumps).

In women, induction of ASA can occur due to a trauma to the endocervical canal or the introduction of excessive numbers of spermatozoa into the uterine cavity during artificial insemination. Other than in cases of vas deferens occlusion (Phadke and Padukone, 1964) however, the cause of sperm antibody formation and its appearance in the ejaculate remain unclear. The inoculation of male guinea pigs with testis extracts also appeared to lead to a lowered sperm output, associated with autoimmune orchitis. A number of other studies have suggested that there is a definite association between sperm autoantibodies and epididymitis and prostatovesticulitis (Fjallbrant, 1968). Sera obtained from normal, fertile animals of many species, including rabbits, mice and humans have been found to contain antibodies that react with sperm of their own species and, to a lesser degree, with sperm of other species (Menge, 1971). The mechanism by which binding to spermatozoa occurs appears to be through Fc receptors, that have been demonstrated on alcohol-fixed sperm of both rabbits and pigs but were not present on the surface of living sperm of those species (Jager et al., 1981). While binding of the intact immunoglobulins of the immunoglobulin G (IgG) class was noted, Fab fragments obtained from digests of purified rabbit IgG failed to bind to sperm. It has been demonstrated that a preparation if IgG from serum of unimmunized rabbits mediates a complement dependent sperm immobilisation process. This finding suggests that a naturally occurring antibody directed against a specific sperm associated antigen is also present within the sera of some normal female bucks.

Naturally occurring ASA have been identified by immunofluorescence in 90% of sera from children of both sexes before the onset of puberty. The incidence declines thereafter to about 60% and remains throughout life. These naturally occurring antibodies were readily absorbed by sperm and testicular extracts but not by any other human tissues. The constant staining pattern with several hundred samples of serum also suggested that they were unlikely to be alloantibodies (Fjallbrant, 1969). They were directed against neither blood group antigens nor sperm-coating antigens, since the antibodies were not absorbed by seminal plasma. Of special importance, these sperm-

reactive naturally occurring antibodies did not stain the surface of viable sperm but rather were directed against intracellular antigens. Against this background of naturally occurring sperm-reactive antibodies, there has been much confusion over the role of ASA in impaired human reproduction.

An injury to the testes can occur quite commonly during sport or routine work activities and results in exposing the sperm to the serum via the ruptured blood/testes barrier (Johnson, 1973). However, routine surgery to correct a varicocoele or hernia can also induce an immune response to spermatozoa in men. Autoimmune diseases of the testis may be prevented by sequestration of autoantigens on germ cells by the presence of a blood/testis barrier. However, not all such antigens appear to be sequestered, since it is possible for them to be detected in the seminiferous tubules, where they can come into contact with circulating antibodies and T cells. Active systemic or local immunoregulatory mechanisms must then be operative. These mechanisms may include (a) activity of suppresser cells, (b) non-specific suppression in the testis, (c) failure of antigen presentation in the testis, or (d) lymphocyte trafficking bypassing the testis.

The existence of antigens on sperm is vital to the consideration of their immunogenicity in the reproductive tract, much the same as is the presence of antigens on the placental trophoblast. Sperm antigens could theoretically induce either auto- or alloimmunity, and either type could result in partial or total infertility. Alternatively, these antigens could be non-immunogenic in the normal environment of the reproductive tract.

In, women, IUI has been shown to increase the number of spermatozoa in the uterine cavity from the normal range of values after natural intercourse of 0-22,000 to several million (Mortimer and Templeton, 1982). In theory it can be agreed that the continued and prolonged exposure of greater than natural numbers of washed spermatozoa into the uterine cavity may indeed induce ASA in the female recipient.

Spermatozoa are not produced normally until puberty; therefore, they are highly antigenic to the already mature immune system of the man producing them. Experimentally, auto- or alloimmunization to spermatozoa can in fact result in relative

infertility in either the female or the male. Naturally occurring autoimmune reactions to sperm are uncommon but have become increasingly recognised as a consequence of vasectomy and in infertile couples. Recent findings suggest that potentially harmful immune responses associated with spermatozoa as antigens are inhibited by naturally occurring local and systemic immuno-regulatory mechanisms.

ASA may be caused by a local phenomenon such as genital inflammation. This primary source of antisperm antibody initiation may occur in certain people who are genetically prone to such infections (Law et al., 1979). However, later work indicated that ASA do not appear to be linked with chronic inflammation or infectious diseases of the male reproductive tract (Marconi et al., 2008, Marconi et al., 2009), but have been associated with other autoimmune diseases (Shiraishi et al., 2009), though no individual autoimmune disease appears to be a risk factor.

A possible secondary source of antisperm antibody production may be an injury to the testes, which causes obstruction to the testicular tracts i.e. post vasectomy. Spermatozoa continue to be produced daily after vasectomy, degenerate in the male reproductive tract, and leak soluble products through distended rete testis, epididymis, and efferent ductules into the interstitial spaces which are accessible to immunological mediators in the lymphatic system leaving the testes (Ball and Setchell, 1983). These stimulate antibody production in the abdominal lymph nodes, which result in high titres of ASA. However, only 40-60% of vasectomised men develop detectable levels of ASA; non-responder individuals could be genetically predisposed to low immunological response to sperm antigens. An association has been reported between ASA in vasectomised men and the human leukocyte antigen (HLA) phenotypes A-28 and B-22 (Law et al., 1979).

It is of great importance to know whether it is a primary or secondary stimulant, which is resulting in the production of ASA. Firstly, there is a significant difference in which type of immunoglobulin is produced by each of the two causes. It has been shown that primary stimulated antibodies are of the immunoglobulin A (IgA) class and secondary immunoglobulins belong to the classes IgG and immunoglobulin M (IgM). IgA has been deemed to be more harmful (Hendry, 1992).

Therefore the production of ASA can be induced by a variety of causes ranging in nature from sports injuries to varicocele or vasectomy surgeries. The final result is the same and will most likely initiate an immune response.

1.3.4. Role of Antisperm Antibodies in Infertility.

Antibodies directed against sperm antigens will result in reduced fertility in men. Results from several large studies on the presence of sperm agglutinins in fertile men place the cut off point for a significant titre of sperm-agglutinating antibodies at approximately 1:32 (Francavilla et al., 1984). Sperm agglutination is almost always associated with ASA (Cerasaro et al., 1985, De Almeida et al., 1986) but as agglutination post semen liquefaction rarely includes a high proportion of motile sperm, it does not represent an important mechanism for reduction of fertility in most patients.

Evidence exists that sperm-reactive autoantibodies can impair sperm penetration into cervical mucus from seminal plasma. This can be shown both clinically at postcoital testing and *in vitro* (Menge and Beitner, 1989). Thus, there is a decrease in the number of viable spermatozoa, which may reach the uterine cavity for egg penetration and fertilisation (Jager et al., 1981). Some studies suggest a role for IgA-ASA in poor cervical mucus penetration (Jager et al., 1980, Sinton et al., 1991). However, it appears that the Fc portion of both IgA and IgG-ASA lead to an abnormal interaction between the sperm surface ASA and cervical mucus giving the characteristic shaking movement of sperm and their inability to penetrate the mucus (Jager et al., 1981). A cervical mucus protein of approximately 15kDa with an ability to bind all immunoglobulins has been demonstrated (Hirano et al., 1999) and may be responsible for the entrapment of sperm within the mucus.

The severe impairment of sperm motility by antibodies has been exhibited (Devine et al., 1993) and also the deleterious effect of antibodies on sperm capacitation and egg penetration (Rajah et al., 1993, Vazquez-Levin et al., 1997). Spermatozoa bound by immunoglobulins at the acrosomal and postacrosomal regions of the sperm head may be impaired in their ability to fertilise eggs, even if they reach the site of fertilisation within the distal ampulla of the uterine tube. Species-specific receptors for the ZP have been identified on the plasma membrane of sperm and antibodies to sperm

membranes block attachment to the zona in animal experiments. Spontaneously occurring autoantibodies in men from infertile couples directed against surface antigens of the sperm head have also been shown to impair the ability of the human sperm to attach to the ZP of the human ova. In addition, diminished IVF rates of human eggs with intact zona have been observed if the woman's serum used in culture medium had antisperm antibody titres of greater than 1:10 or if more than 70% of sperm were bound to autoantibodies (Gonzales et al., 1992).

It has been suggested that men suffering from asthenozoopermia (reduced sperm motility) who are considering IVF should always be screened for antibody presence. Also azoospermic (lack of measurable sperm) or oligospermic (a low concentration of sperm) men have been observed to show a very high antibody titre, which appears to be caused by a type of cell mediated immune orchitis (Rumke, 1981).

It is known that not all infertility due to antibodies is absolute and not all antibodies to spermatozoa inhibit sperm function and so their ability to fertilise is not always directly affected (Hammitt et al., 1988). Many types of ASA of different classes that are directed against many different types of antigens undoubtedly have varying effects on spermatozoa.

Many ASA may bind to surface antigenic sites on spermatozoa, but they may result in varying degrees of immune response and so not always result in spermatozoan incapacitation, immobilisation or agglutination. However, no available techniques are available to define the relative severity of the immunologically mediated infertility or define the antibodies related to spermatozoan interference or incapacitation.

The immunoglobulin isotype (i.e. IgA, IgM, IgG) represents one key characteristic of ASA that contributes to infertility, since structural differences between the isotypes affects sperm function in many different ways (Grundy et al., 1991). This is exemplified by the fact that all classes of ASA cause sperm transport inhibition yet IgA, which is non complement fixing, seems to be the most inhibitory to cervical mucus penetration (Parslow et al., 1985). Conversely, complement binding IgM and IgG antibodies interfere more with the sperm capacitation and acrosome reaction (Bronson et al., 1982a).

The nature of the different classes of immunoglobulins covering the sperm cells has been evaluated (Bronson et al., 1982b). It was found that IgG and IgA occurred more commonly than IgM (Clarke et al., 1985, Shulman et al., 1985). IgG was shown to be bound predominantly to the head or both head and tail, IgA bound predominantly to the tail and IgM did not bind at all (Carson et al., 1988, Shulman et al., 1985).

Most agglutinating antibodies to sperm in seminal plasma are of the IgA class and may exist only in seminal plasma, whereas immobilising antibodies are predominantly IgG. IgM antibodies gain access to seminal plasma only when there is a significant inflammatory lesion in the reproductive tract or after vasectomy. It is therefore deduced that IgA affects sperm transport, while both IgG and IgM, which are directed against the head region interfere with gamete interaction i.e. the ability of spermatozoa to fuse with eggs. It has also been reported that *in vitro* exposure of spermatozoa to antisera reduced their egg penetrating ability (Vazquez-Levin et al., 1997).

1.3.5 General Review of how Antibodies Interfere with Fertilisation.

A. Sperm Transport

1. Within semen the binding of immunoglobulins to the surface of spermatozoa will impair their ability to penetrate cervical mucus. Immunoglobulin binding will also lead to an alteration in the motion of sperm within the cervical mucus, resulting in the sperm shaking, vibrating or being rendered completely immobile.

2. On exposure to complement binding, the spermatozoa bound with sperm reactive antibodies within the cervical mucus are subjected to cell damage. The tail directed antibodies that cause complement fixing will promote loss of motility.

3. The sperm that are coated with antibodies which bind complement components, become opsonised thus leading, in theory, to an increase in the rate of phagocytosis of sperm by macrophages in the female reproductive tract.

B. Gamete Interaction

1. Acrosome directed antibodies on the sperm head might plug binding sites for the ZP preventing sperm attachment. Alternatively, acrosome induction by the ZP can be inhibited by ASA (Francavilla et al., 1997a, Zouari and De Almeida, 1993).

2. Complement fixing antibodies, such as IgG or IgM, directed against the sperm head region may impair the ability of the spermatozoon to penetrate eggs, even without a loss of motility, by promoting damage of the acrosomal and plasma membranes.

3. It has also been reported that ASA can induce polyspermy by altering sperm-egg binding (Bronson et al., 1990).

1.4 Established Methods of Detection of Sperm Reactive Antibodies.

Many techniques have been described over the years for antisperm antibody detection in both serum and other body fluids. It has become increasingly apparent that there are two sources of immunoglobulins within the male genital tract. These include those present as transudate from serum and those secreted in the local genital environment. Therefore, circulating sperm-reactive antibodies may not be representative of those ASA present in the semen. This emphasises the need to study further the ejaculate directly rather than solely using serologic tests in the diagnosis of immune-mediated male infertility.

There is presently a lack of a standardized and universally accepted assay for detection of ASA against known antigens. This has been cited many times as the main reason for confusion over the actual role of ASA and their treatment in male infertility (Helmerhorst et al., 1999, Chiu and Chamley, 2004).

1.4.1 Sperm Agglutination: Franklin-Dukes, Kibrik, Tray Agglutination Test, Microagglutination Test.

The agglutination, or sticking together of spermatozoa, is dependent upon the location and spacing of the antigen on the sperm surface to which the specific antibodies are directed. Depending upon the class of immunoglobulin present, the number of epitopes or sites on the antibody molecules that can combine with an antigen varies considerably. The presence of bacteria or amorphous material and debris within semen, as well as non-immunoglobulin proteins in serum, can induce non-immunoglobulin-mediated agglutination, which leads to a false-positive result. Cross linkage between sperm is more likely to occur with large multivalent isotypes, such as IgM and secretory IgA type immunoglobulins.

The complement dependent cytotoxicity assay and the agglutination assays were the first assays to be utilized for detection of ASA. Both assays employed indirect methods for detecting the biological activities of circulating ASA. Multiple comparative studies (Boettcher et al., 1977) indicated that they determine largely the same antibody

specificities but with different sensitivity, which was higher for sperm agglutination techniques, especially the Tray agglutination test (TAT) (Friberg, 1974).

Circulating ASA detected with these techniques ranged from 8.1% to 30.3% in men from infertile couples (Heidenreich et al., 1994, Hargreave et al., 1980, Collins et al., 1993) and at low titres in 2.4% to 10% of fertile men (Heidenreich et al., 1994, Collins et al., 1993). Low titres of sperm agglutinating activity can be due to non-immunological factors (Ingerslev and Hjort, 1979) and represent false positive results. If stricter criteria were applied such as addition of sperm-immobilizing activity (Clarke et al., 1985, Hargreave et al., 1980) or occurrence of sperm-agglutination activity in seminal plasma (Clarke et al., 1985) the numbers presenting with ASA in infertile couples was significantly lower at 4.7% to 7.5%.

1.4.2 Enzyme-Linked Immunosorbant Assay (ELISA).

These tests are based upon a known enzyme being covalently linked to an antiglobulin antibody. The known antiglobulin binds to human immunoglobulins reacting with antigens on the sperm surface. A substrate for that enzyme is then added, and coloured product is measured colourimetrically. The test is simple and basically it quantitatively relates the colour of the enzyme/substrate complex to the amount of immunoglobulins present on the sperm surface. Both whole sperm and sperm membrane extracts fixed to microtitre wells have been used as antibody targets. The ELISA test protocol demands that the sperm be fixed prior to reaction with the test serum and this may lead to denaturation of sperm antigens or membrane damage, resulting in both false-positive and false-negative reactions (Zanchetta and Busolo, 1984, Lynch and Howe, 1987). Extracts from the sperm membrane may in fact not contain the relevant antigens that are associated with the fertilisation process and therefore be of no use in subfertility monitoring or investigations.

1.4.3 Fluorescein-Conjugated Antiglobulins.

This assay identifies antibody location on the spermatazoal surface using fluorescein-conjugated secondary antibodies viewed under a fluorescent microscope (Bohring et al., 2004). Due to its time consuming protocol it is not practical for routine testing but does have a role in determining the sites of antisperm antibody binding.

A fixing procedure involving methanol used in these procedures often results in a high incidence of false-positive reactions. This occurs because the methanol damages the plasma membrane of the spermatozoa and internal antigens are exposed to antiglobulins. Naturally occurring antibodies to these sub-membrane antigens (which sometimes can cross-react with bacterial antigens) are commonly found in men and women and probably play no role in subfertility. With the recent advent of live cell imaging confocal microscopy these limitations could be overcome.

1.4.4 Flow Cytometry.

Flow cytometry has been proposed as an objective method to quantify the IgG and IgA bound to individual viable spermatozoa (Ke et al., 1995, Rasanen et al., 1996, Nicholson et al., 1997). Although some technical limitations have been claimed (Nikolaeva et al., 2000), its widespread use in a clinical setting is becoming accepted. It does not give the regional specificity of the antibody binding however, and would have to be used in conjunction with an appropriate secondary assay.

1.4.5 Radiolabelled Antiglobulin Assays.

Specially radiolabelled antibodies, which are raised against human immunoglobulins, can detect antibodies directly bound to human sperm (Haas et al., 1980, Haas et al., 1982). This method is extremely sensitive and highly specific for the detection of sperm-reactive antibodies when utilised with living sperm as the antibody targets. Unfortunately, there are limitations with this technique since the proportion of sperm in the ejaculate that is antibody-bound cannot be determined. The predominant regional location of binding of each individual class of antibody is also indeterminate.

Therefore, the radiolabelled antiglobin assay represents a less attractive diagnostic tool than the direct binding assays such as the SpermMar assay.

1.4.6 Mixed Agglutination Reaction (MAR).

A washed suspension of Rh-positive human red blood cells (RBCs) sensitised with a human IgG anti-Rh antibody is mixed with drops of the semen to be tested and a xenogenous (e.g. rabbit), anti-human IgG antiserum. If the spermatozoa in the ejaculate are antibody-bound, they will form mixed agglutinates with the RBCs in the presence of the antiglobulin (Jager et al., 1978). The relatively large size of RBC-RBC agglutinates limits the ability of the MAR to determine the regional specificity of antibody binding to the spermatozoa surface or the proportion of spermatozoa antibody-bound.

A commercially available kit, the SpermMar test, is also available (Comhaire et al., 1988) and is available with IgA specificity along with IgG and some data about the regional specificity of binding can be obtained. This assay quick results and is therefore suitable for routine testing in clinical settings.

1.4.7 Immunobead Binding Test (IBT).

This test utilises commercially available micron-sized polyacrylamide spheres, (Immunobeads, Bio-Rad) to which isotype-specific rabbit anti-human antibodies (IgG, IgA and IgM) are covalently bound (Bronson et al., 1984). Spermatozoa washed free of seminal fluid are mixed with a suspension of immunobeads. The method allows one to detect directly immunoglobulins on the surface of living sperm. The region of the sperm surface to which the ASA have bound, the proportion of sperm in the ejaculate that are antibody-bound, and the isotypes of these antibodies can be determined. Because the number of immunoglobulin molecules bound to the sperm surface necessary for bead binding has not been determined, the sensitivity of the assay is unknown.

1.5 Antigens Reacting with Antisperm Antibodies.

The effects of ASA on sperm function, cervical mucus penetration and gamete interaction are probably not universal and are likely to be dependent on their antigenic specificity. Therefore, there is a requirement to identify sperm antigens targeted by naturally occurring ASA and determine their relevance to sperm function and the fertilization process. According to Bronson (1984), "their availability through recombinant deoxyribonucleic acid (DNA) technology should lead to the development of ELISA-based tests that may allow one to identify the specific locus of the fertilization blockade for individual couples".

1.5.1. Direct Identification of Sperm Surface Proteins Reacting with Antisperm Antibodies.

Early studies which identified binding patterns of ASA against sperm antigens using Western blotting (Aitken et al., 1987, de Beer et al., 1993, Paradisi et al., 1996, Parslow et al., 1987) were ineffective as due to the complexity of the patterns, their role in infertility could not be identified. Later, two-dimensional gel electrophoresis followed by mass spectrometric peptide fingerprinting led to the discovery of the identity of sperm antigens reacting with ASA (Auer et al., 1997, Bohring and Krause, 2003, Shetty et al., 2001). These studies however, were unable to distinguish internal from external sperm antigens and therefore their usefulness was negated. Some studies though used vectorial labeling (labeling the sperm surface with iodine or biotin prior to extraction) to identify sperm surface proteins first before identifying ASA reactive bands (Paradowska et al., 2006). This led to a great reduction in the number of reactive antigens identified with a probable increase in false negative findings. It has only very recently that another approach has been adopted, firstly to identify sperm surface proteins and their role in fertility and then showing them to be autoantigenic (Petit et al., 2013).

1.6 Immunotolerence in the Male Reproductive Organs.

Spermatogenesis begins in the male with the onset of puberty, long after the elimination of auto reactive immune cells by the body. This allows for the potential of autoimmune responses by the body upon exposure to the antigenic spermatozoa. In order to overcome this reaction, which would be detrimental to fertility, the testis is a site of immune privilege in the male body (Turek and Lipshultz, 1994). There are several factors that contribute to this special immune status including the blood-tubular barrier, the Fas ligand system and several components of seminal plasma, which are explained in detail below.

1.6.1 The Blood-Testis Barrier.

The nature of the barrier between the blood system and the testis is such that it prevents the entry of many of the cells of the immune system. Evidence of this barrier has come from electron micrograph images which have shown the normal complement of immune cell lineages including macrophages and various lymphocytes in the interstitial spaces between the seminiferous tubules but not within the seminiferous tubules themselves. There are two main components that go to make up this barrier, the myeloid cells that line the outside of the seminiferous tubules and the specialized tight junctions found between the Sertoli cells (Turek and Lipshultz, 1994). This barrier is known to develop gradually in the male with the onset of puberty (Setchell et al., 1990).

Most T-cells within the male reproductive tract are $CD8^+$ suppressor/cytotoxic cells and along with macrophages are located in the epithelium and lamina propria of the rete testis, epididymis and vas deferens (Witkin et al., 1996). As interferon (IFN)-γ-producing activated T-cells induce potent expression of major histocompatibility class (MHC) II molecules on antigen presenting macrophages, several mechanisms exist to inhibit this IFN-γ induced innate immune response. Where the blood-testis barrier is weakest, increased $CD8^+$ suppressor T-cells occur to suppress the immune response to sperm specific antigens (Witkin and Goldstein, 1988). In ASA positive patients, $CD4^+$ T-cells are present in both the genital tract and semen (Witkin and Goldstein, 1988).

1.6.2 The Fas Ligand System in Seminal Plasma.

The Fas ligand (FasL), a membrane bound member of the tumour necrosis factor (TNF) family is expressed on a very narrow range of cell types. Activated T cells express Fas L, which when bound to Fas, induces a signalling cascade that results in the apoptotic death of the T cell; a process known as activation-induced cell death. Fas L is also expressed by cells within immune-privileged tissues such as the testis (Filippini et al., 2001). Here it acts to engage with Fas expressing immune cells that have made their way into the immune-privileged site, resulting in the induction of immune cell apoptosis and the prevention of an immune reaction (O'Connell et al., 2001). The soluble form of Fas L (sFas L) has also being identified as been present in the secretions of the prostate gland and is present in seminal plasma (Liu et al., 1998). Figure 1.6.1 represents the Fas/Fas L system with Fas L-expressing tumour cells inducing apoptosis of immune cells, this is representative of cells in immune-privileged sites such as the testis and sFas L produced by the prostate.

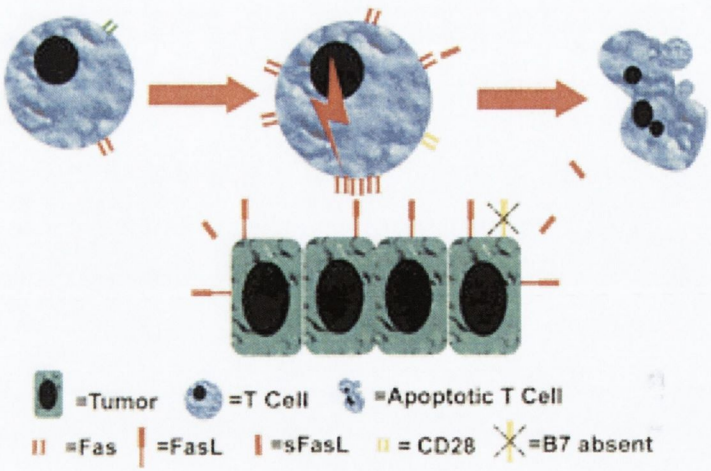


Figure 1.6.1 Constitutive expression of the Fas Ligand helps to maintain a state of immune tolerance in the testis by inducing apoptosis in Fas expressing immune cells. (Diagram taken from O'Connell et al., 2001)

1.7 Immunosuppressive Components in Seminal Plasma.

Seminal plasma is the non-cellular component of semen and is composed of the secretions of numerous glands associated with the male reproductive tract, including the seminal vesicles, the prostate, epididymis, Cowper's gland and the vas deferens.

Seminal plasma contains many components that have been shown to be immunosuppressive, and which are capable of inhibiting the action of most immune cell types including T cells, B cells, Natural Killer (NK) cells, neutrophils and macrophages as well as interfering with antibody production and complement action (James and Hargreave, 1984). Immunosuppression by components of the seminal plasma is particularly important as they accompany the sperm cells into the female reproductive organs during coitus. The female reproductive tract is not a site of immunosuppression and as sperm antigens would be recognised as foreign, there is the potential for the activation of an immune response on exposure to sperm cells, which would be detrimental to fertilisation.

1.7.1 Prostasomes.

The prostasome is a multilaminar vesicle with a diameter of approximately 150nm, which is secreted by the acinar epithelial cell of the human prostate gland and is its function is to fuse with the sperm membrane and enhance the forward motility of the spermatozoa (Ronquist and Brody, 1985). These vesicles have been shown to inhibit mitogen-activated T cell proliferation *in vitro* and to inhibit the phagocytic activity of macrophages (Skibinski et al., 1992). Although their mode of action is unclear, they are most likely the component responsible for the majority of immunosuppression seen in the high molecular weight fractions of seminal plasma. Prostasomes are also able to fuse to sperm at acidic pH and transfer onto the sperm surface complement inhibitors CD46, CD55 and CD59 (Arienti et al., 1997, Carlini et al., 1997, Ronquist et al., 1990).

1.7.2 The Polyamines, Spermine and Spermidine.

These polyamines are present in high concentrations in seminal plasma (Maayan et al., 1995) and when acted upon by polyamine oxidase they are converted to the cytotoxic-aldehyde acrolein and aminopentanal. Spermine oxidase occurs in seminal plasma (Holtta et al., 1975) in high concentrations, though the significance of these reactive aldehyde species in immunosuppression within the male reproductive tract is unclear.

1.7.3 Prostaglandins.

Seminal plasma contains a high concentration of prostaglandins (up to 300μg/ml), particularly those of the E series, PGE_1, PGE_2, 19-0H PGE_1 and 19-0H PGE_2 (Bendvold et al., 1987). Experiments found that the ability of seminal plasma to inhibit NK-cell mediated lysis of target cells could be prevented by treating the seminal plasma with activated charcoal but not by heating. Analysis of different fractions of the seminal plasma showed that the fraction responsible was an acidic lipid fraction, containing prostaglandins (Tarter et al., 1986). Further work showed that prostaglandins also suppress proliferation of lymphocytes. The mechanism of action of these prostaglandins is believed to be associated with their ability to increase the level of the second messenger cyclic adenosine monophosphate (cAMP) inside the cell after binding the EP2 receptor, a G protein coupled receptor on the cell surface and initiating a signaling cascade that leads to the inhibition of the production of interleukin (IL)-2 (Quayle et al., 1989). IL-2 is a cytokine that is important mainly in the stimulation of T cell proliferation, but also in the proliferation of NK cells and B cells.

1.7.4 Zinc.

Seminal plasma contains a high concentration of zinc (over 150μg/ml), which is believed to be important for its immunosuppressive activity. Zinc itself is known to be capable of interfering with many reactions of the immune system, including inhibiting the antigen-stimulated proliferation of lymphocytes (Shivaji et al., 1990), while zinc

protein complexes have also been implicated in inhibiting the attachment, spreading and phagocytic action of macrophages (James and Hargreave, 1984).

1.7.5 Transglutaminases.

Transglutaminases are produced by the prostate gland, the secretions from which are known to have immunosuppressive activity. Treatment of the secretion of the rabbit prostate gland with the transglutaminase inhibitor, neopentyl chloroethyl nitrosourea, dramatically reduced the immunosuppressive activity of the prostate fraction (Mukherjee et al., 1982). A similar effect was seen if the fraction was treated by absorbing it to transglutaminase antibodies or antibodies to uteroglobulin (Mukherjee et al., 1983).

The enzymes are believed to act by causing the cross linking of uteroglobulin found on the spermatozoa to other sperm membrane antigens, and thereby masking potentially antigenic proteins on the surface of the sperm cell from immune cells (Mukherjee et al., 1983).

1.7.6 Fc receptor binding protein.

A 94,000 Dalton Fc receptor binding protein is found in seminal plasma and is believed to exert its immunosuppressive action by preventing the binding of immunoglobins to the Fc receptor which is found on immune cells such as phagocytic cells, NK cells and mast cells. Binding of the C terminus of immunoglobins to the Fc receptor leads to the activation of the effector functions of these immune cells (James and Hargreave, 1984).

1.7.7 Complement Inhibition.

The complement system is a component of the innate immune system that involves the sequential activation of a number of serum and cell surface proteins, and can be activated *via* the classical pathway with antigen-antibody complexes or via the alternate pathway with microbial surfaces.

Both pathways lead to the activation of a multiprotein complex capable of insertion into cell membranes, leading to lysis of the cell. Clusterin, an inhibitor of the complement system, which acts by becoming incorporated into the growing membrane attack complex (MAC) and preventing membrane insertion, is present in seminal plasma at a concentration in the region of approximately 50ng/ml (Salehi et al., 2013) as a soluble form and also on the sperm membrane (O'Bryan et al., 1990). Clusterin has also recently been shown to be a ligand for the dendritic cell (DC) receptor DC-SIGN leading to reduced capture of sperm by DCs (Sabatte et al., 2011). Removal of all clusterin from seminal plasma does not completely reduce DC inhibition leading to the conclusion that other DC-SIGN ligands are yet to be discovered in seminal plasma.

Additional complement inhibitors are found embedded in the membrane of the spermatozoon, including membrane cofactor protein (MCP) and decay accelerating factor (DAF) (Kelly, 1995) and are transferred from the prostasome to the sperm membrane. Finally, complement factor H (CFH) is also present on sperm and in seminal plasma and can disrupt the C3 convertase of the complement cascade (Sarma and Ward, 2011) though its function in seminal plasma is yet to be fully elucidated.

1.8 Significance of the Immunosuppressed State of Male Reproductive Tract.

Immunosuppression by seminal plasma is important for preventing autoimmune responses in men and alloimmune responses in women upon exposure to sperm antigens as discussed earlier. However this immunosuppression also leaves the reproductive organs vulnerable to the development of malignancy and to viral and bacterial infection as it prevents the immune system from dealing with these threats in the same way it does in normal tissues of the body. Many sexually transmitted diseases, such as syphilis and gonorrhoea take advantage of the reduced immune system established by these

immunosuppressive agents. Some anti-microbial activity has been identified in semen, including a zinc component (Kelly, 1995) and in bovine seminal plasma a cationic non-enzymatic protein termed seminalplasmin (Sitaram and Nagaraj, 1995)

Seminalplasmin is a 47-residue peptide first isolated in 1979 (Reddy and Bhargava, 1979) that shows no homology to anti-microbial peptides from other species, such as defensins and magainins (which themselves show little or no homology to each other). In spite of the lack of sequence homology, seminalplasmin is believed to exert its anti-microbial activity through much the same mechanism as these other peptides; by interacting with, and destabilizing the lipopolysaccharide of gram negative bacterial outer membrane *via* its positively charged residues, thus gaining access to the inner membrane through which it forms a pore by inserting itself through its amphipathic helix, leading to the death of the bacteria (Sítaram and Nagaraj, 1995).

Recently, a number of anti-microbial peptides derived from the enzymic breakdown of semenogelin have been identified to be present in the seminal plasma of humans. (Zhao et al., 2008) showed several semenogelin 1 peptide fragments derived from the N-terminus of the protein have anti-bacterial activity against a wide variety of bacteria. A proteolytic fragment from semenogelin 2 has also been shown to have anti-bacterial activity which is zinc- and pH-dependent (Edstrom et al., 2008). Semenogelin fragments have also been shown to have anti-viral activity with a fragment termed SG-1 of semenogelin 1 having anti-HIV activity (Martellini et al., 2009). The proteolytic breakdown of other proteins in seminal plasma has also yielded anti-microbial activity, with prostatic acid phosphatase fragment (PAP248-286; also termed Semen-derived enhancer of viral infection, SEVI) forming fibrils that bind to both gram-positive and gram-negative bacteria in a charge-dependent manner that result in enhanced macrophage phagocytosis of the bacteria (Easterhoff et al., 2013). Many other peptides may contribute to these defenses, including the recently discovered human epididymis protein-4 (HE-4) which is a cross-class protease inhibitor that potentially confers protection against microbial virulence factors of a proteolytic nature (Chhikara et al., 2012).

Various cells of the immune system, including NK cells and cytotoxic T lymphocytes, normally monitor for the development of tumour cells in the body. Both

of these cell types are targets for the immunosuppressive agents of seminal plasma, potentially making the male reproductive tract susceptible to tumourogenesis.

1.9 Interaction of Semen with the Female Reproductive Tract Immune System.

The female reproductive tract is covered by distinct epithelial cell layers and types of mucus, with the ectocervix and vaginal canal having type II mucosa while the upper parts of the tract, the endocervix and uterus having type I mucosa (Iwasaki, 2010). These are inhabited by unique microflora and use distinct innate and adaptive immune regulatory pathways. These mechanisms are required as the female reproductive tract is open to a host of sexually transmitted bacterial, fungal and viral diseases. The lower, non-sterile part of the tract, the vagina and ectocervix, has antimicrobial peptides, Toll-like receptors (TLRs), complement and NK cells which work together to prevent host invasion by pathogens (Horne et al., 2008, King et al., 2003). However, if infection does occur, several cell types are mobilized to the vaginal tissue, including neutrophils, monocytes, plasmacytoid DCs and NK cells (Milligan, 1999, Orange, 2006).

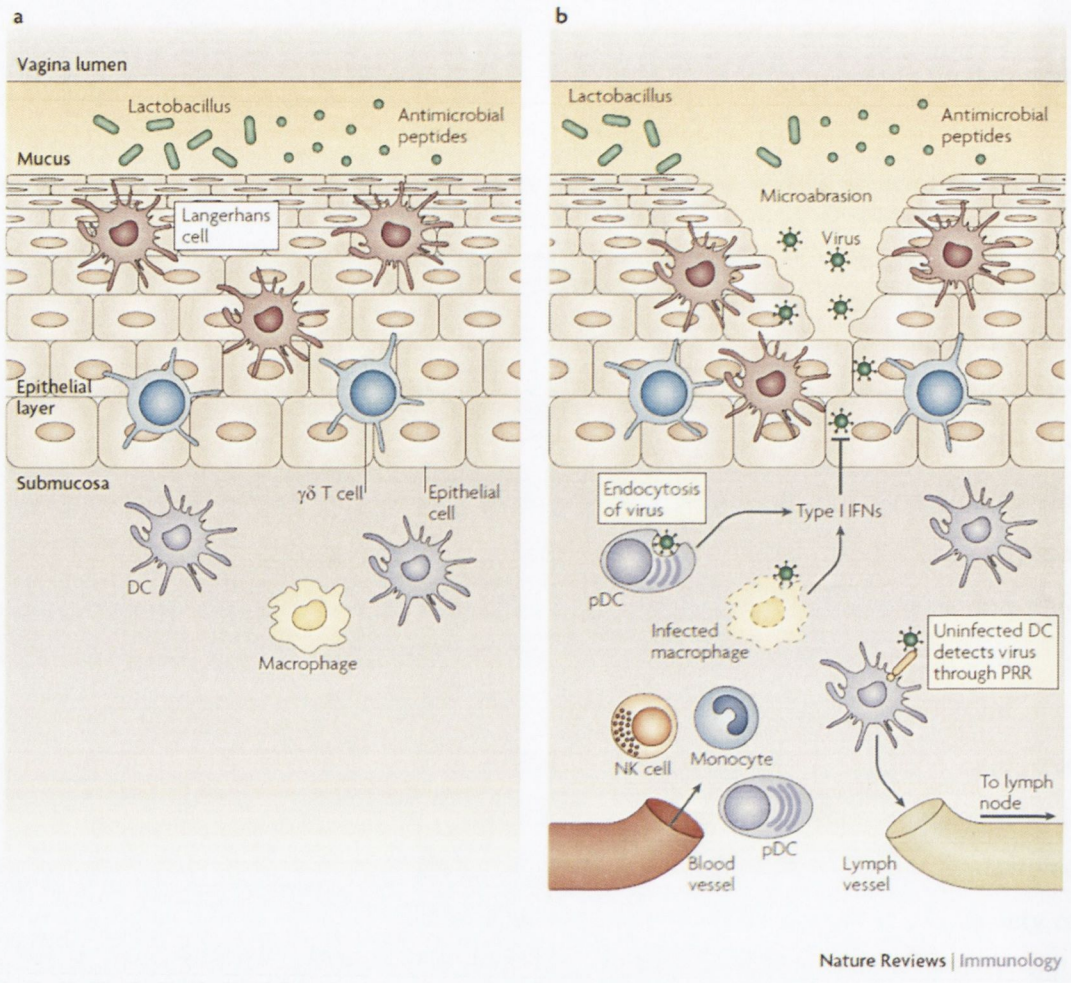


Figure 1.9.1 Innate Defense System of Vaginal Mucosa. Vagina and ectocervix are covered by type II epithelium. Mucus secreted by cervical glands and local epithelial cells provides a barrier to invading pathogens. Lactobacilli constitute the main species of endogenous flora and provide low pH and reactive oxygen species that are toxic to pathogens. **a** | Within and beneath the epithelial layer, there are many innate immune cells, including Langerhans cells, γδ T cells, dendritic cells (DC) and macrophages, that survey the environment for invading microorganisms. **b** | After virus infection, inflammatory responses elicit the recruitment of neutrophils, natural killer (NK) cells, monocytes and plasmacytoid dendritic cells (pDC). These cell types express various pattern recognition receptors (PRRs) to ensure innate detection and initiation of type I interferon (IFN) responses to block further viral spread. (Taken from Iwasaki, 2010).

Different cell types are responsible for immune responses for differing infectious agents, for example intraepithelial γδ T-cells and neutrophils contribute strongly to innate immune protection against vaginal challenge with viruses such as human herpes simplex virus-2 (HSV-2) but have lesser roles with other pathogens (Nishimura et al., 2004). Similar to other mucosal surfaces, multiple pattern recognition receptors (PRRs)

that are expressed by various cell types monitor pathogens that enter the vaginal mucosa. These include TLRs, RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010). Activation of these receptors by different pathogens triggers cytokine, type-1 IFN and antimicrobial peptide release so as to generate effective adaptive immune responses (Iwasaki and Medzhitov, 2010).

It is into this milieu of anti-microbial defenses that sperm are deposited and therefore it is not surprising that sperm and seminal fluid contain factors that modulate the female immune response. As previously described, sperm carry CD46, CD55 and CD59 along with clusterin and CFH to minimize complement attack (Dorus et al., 2012). Other mechanisms of active modulation of the female immune environment also exist.

Prostaglandins of the E (PGE) series are in seminal plasma at extremely high concentrations as previously described. On deposition of semen within the vaginal canal, PGE can act on the epithelia cell of the vagina and ectocervix as well as any infiltrating immune cells. PGE leads to the production of transforming growth factor-β (TGFβ) (Klein-Nulend et al., 1996) and IL-10 (van der Pouw Kraan et al., 1995, Strassmann et al., 1994). TGFβ can also lead to upregulation of PGE in a positive feedback loop (Gilbert et al., 1994), but this is not true for IL-10, which negatively regulates PGE production (Mertz et al., 1994) (summarized in figure 1.9.2).

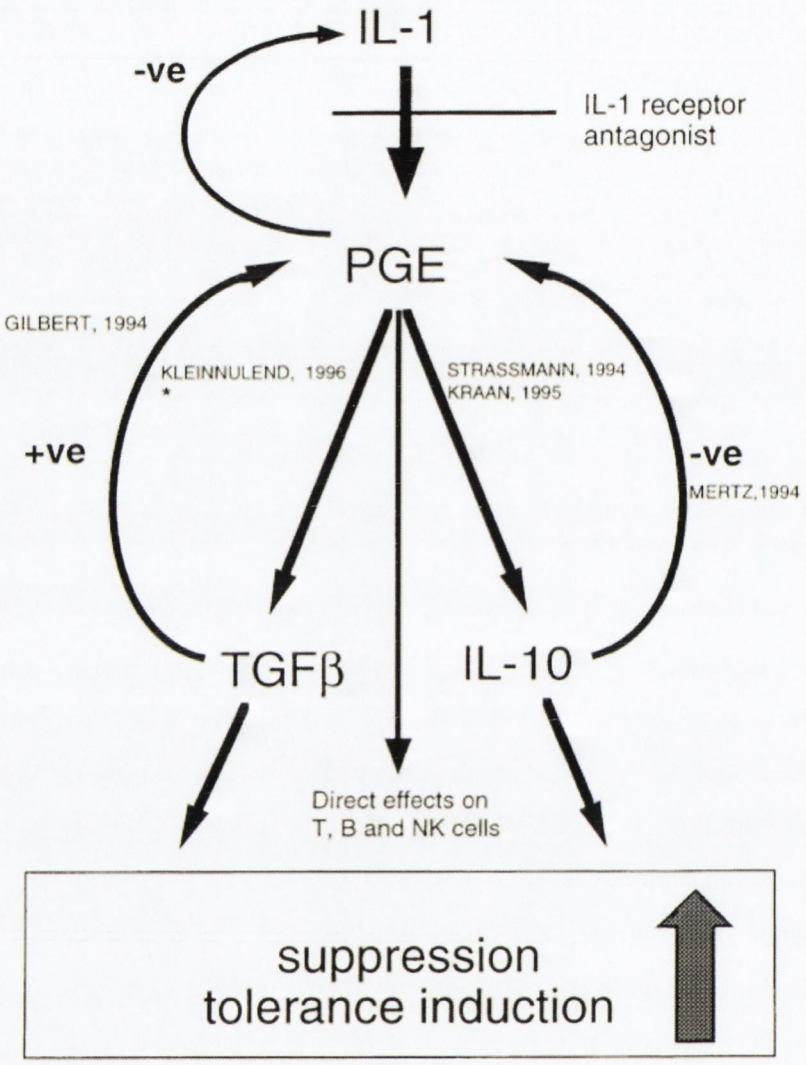


Figure 1.9.2 Control of Suppressive Cytokines by PGE. (Taken from Kelly, 1999).

As IL-12 secretion is also inhibited by PGE (van der Pouw Kraan et al., 1995) along with the upregulation of IL-10, this leads to a shift in IL-10/IL-12 ratios and a shift from a Th1 to a Th2 response. Lately, PGE has also been shown to reduce sperm capture by DCs (Rennemeier et al., 2011). However, in this study they used nearly 3 fold higher levels of PGE than would be present in the seminal plasma, leading to the conclusion that other factors in seminal plasma may also have activity.

TGFβ in seminal plasma and induced by PGE also has a role in inducing T-cell anergy (immune unresponsiveness) within the female reproductive tract through expansion of regulatory T cells (Treg) cells; a subpopulation of T cells that mediate

immune tolerance and protection from auto-immunity (Robertson et al., 2013). Both oestrogen and progesterone induce some expansion of Treg cells in the endometrium (Kallikourdis and Betz, 2007, Arruvito et al., 2007) but expand further with presentation of male antigen post-coitus giving functional tolerance and T-cell anergy prior to embryo implantation (Tafuri et al., 1995, Robertson et al., 1992). Male antigen derived from seminal plasma is taken up by tolerogenic DCs ($CD80^+$, $CD86^+$ and $IL\text{-}4^-$) and presented to Treg cells inducing the $CD4^+$, $CD25^+$, $FOXP3^+$ phenotype of strong immunosuppressive Treg cells (Sato et al., 2003). Tolerogenic DCs also express indoleamine 2,3-dioxygenase (IDO), which directly activates resting Tregs for potent suppressor activity (Sharma et al., 2007) (Figures 1.9.3 and 1.9.4).

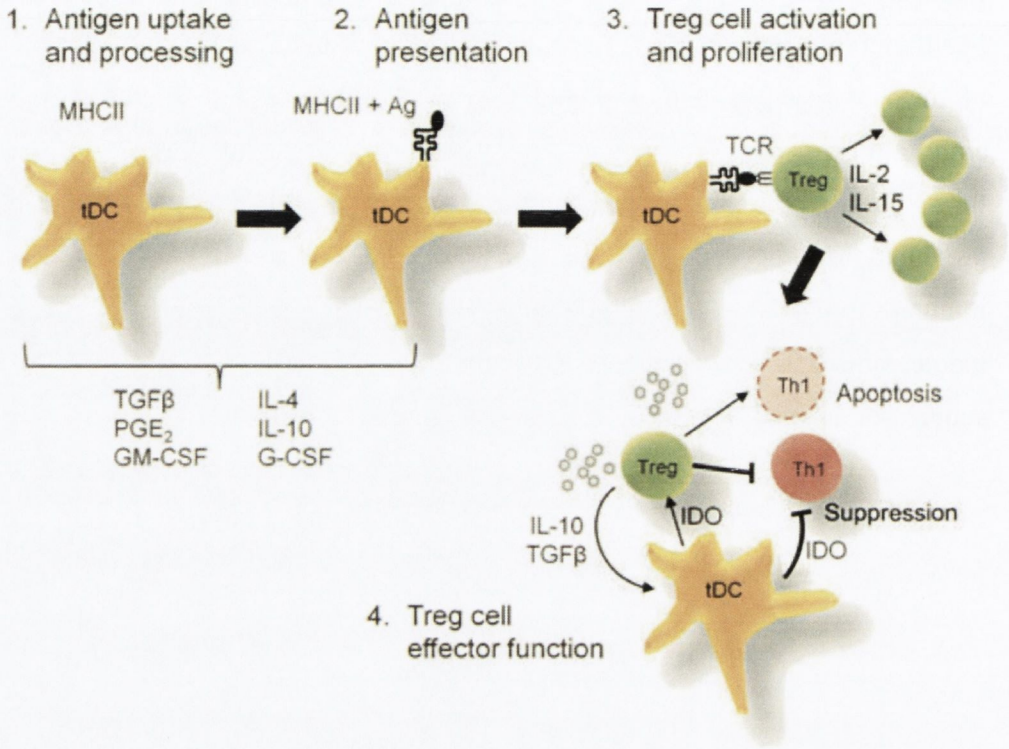


Figure 1.9.3 Schematic Illustration of the Interaction Between Tolerogenic DCs and Treg Cells. After antigen uptake and processing, DCs present antigen fragments on their surface in association with class II MHC molecules, either in peripheral tissues or more often, draining lymph nodes. Treg cells expressing cognate TCRs are activated by the tolerogenic DCs and then proliferate. After export to the peripheral circulation Treg cells are recruited by chemokines and vascular addressins into the designated target tissue. Mature Treg cells secrete IL-10 and TGFβ and induce IDO expression in local DCs to further activate and maintain suppressive function in Treg cells, inhibit Th1 cell proliferation and induce Th1 cell apoptosis. Ag = antigen; DC = dendritic cell; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; IDO = indoleamine 2,3-dioxygenase; IL = interleukin; MHC = major histocompatibility complex; TCR = T-cell receptor; TGF= transforming growth factor. (Taken from Robertson et al., 2013).

These suppressive Treg cells continue into pregnancy and are absolutely essential for maintaining the allogenic conceptus. $CD4^+$, $CD25^+$ Tregs transferred into T-cell

deficient mice were able to prevent rejection of fetuses, whereas if no transfer was performed all pregnancies were aborted (Aluvihare et al., 2004).

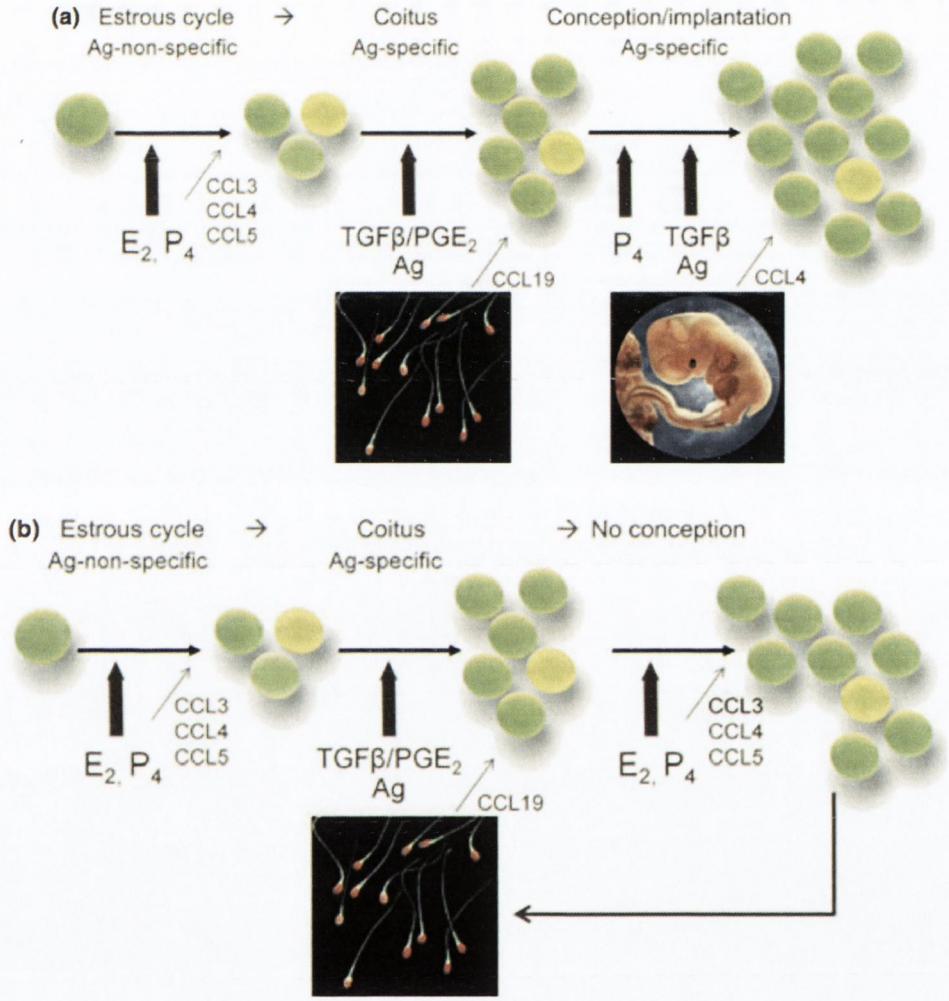


Figure 1.9.4 Illustration of Current Hypothesis of Treg Cell Role in Implantation and Pregnancy Maintenance. At the time of ovulation, elevated estrogen levels cause a systemic expansion in the Treg cell pool, through direct effects on T-cell Foxp3 mRNA expression and elevated uterine expression of chemokines CCL3, CCL4, and CCL5. After ovulation, corpus luteum-derived progesterone may expand Treg populations via direct effects mediated by nuclear progesterone receptors. At coitus, seminal fluid delivers male alloantigen, TGFβ and PGE_2, causing recruitment and maturation of tolerogenic dendritic cells (DCs) into the uterine tissue under the influence of CCL19, and driving further expansion of subsets of antigen-reactive Treg cells in lymph nodes draining the uterus. After conception and embryo implantation, the gestational tissues release alloantigens, immune-deviating cytokines and the chemokine CCL4 to recruit and further expand Treg cell populations (a). In the absence of conception, repeated exposure to seminal fluid might further expand the Treg cell pool (b). E_2 = estrogen; P_4 = progesterone; CCL = chemokine (C-C motif) ligand; Ag = antigen; TGFβ = transforming growth factor-β; PGE_2 = prostaglandin E_2. (Taken from Robertson et al., 2013)

Other factors in seminal plasma also affect female immune response. In porcine models the porcine seminal plasma proteins (spermadhesins) PSP-I and PSP-II form a dimer and regulate post-mating neutrophil infiltration into the uterus by inducing IL-4 production from mast cells which blocks TNFα production from macrophages (Rodriguez-Martinez et al., 2011). Neutrophils are required for removal of immotile and abnormal sperm. In pigs and cattle, neutrophil infiltrates peak at about 3-6 hours in pigs (Rodriguez-Martinez et al., 2010) and approximately 2 hours in cattle (Alghamdi et al., 2009) and is of short duration to remove sperm or any pathogen present and return the endometrium to a normal state ready for implantation.

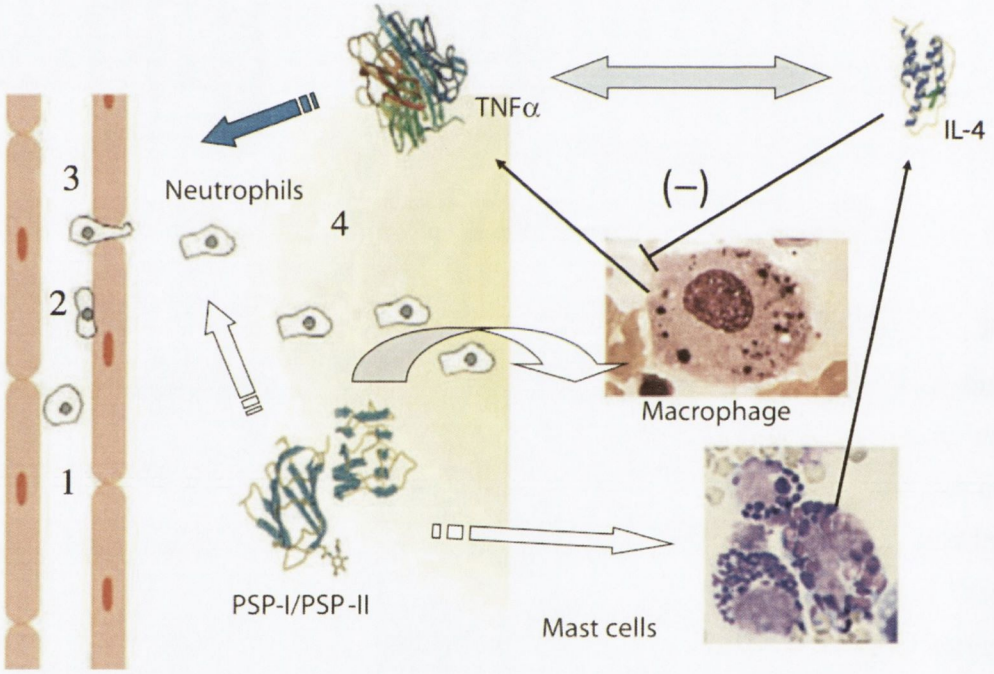


Figure 1.9.5 Cytokine Control by Spermadhesins. The emerging view is that the balance between cytokines released by macrophages (TNFα) and mast cells (IL-4) controls the onset of neutrophil [polymorphonuclear neutrophil granulocyte (PMN)] migration in response to the inflammatory stimuli of porcine seminal plasma proteins I (PSP-I)/porcine seminal plasma proteins II (PSP-II), thereby avoiding excessive PMN recruitment. This regulation seems crucial during situations where an excessive accumulation of PMNs would lead to tissue damage (i.e. in post-mating endometritis) (Taken from Rodriquez-Martinez et al., 2011).

SV-IV in rat also has a range of immunosuppressive and immunomodulatory actions which will be discussed later in chapter 5. Its role in female reproductive tract immune control is as yet unknown but as it can be carried into the uterus on the sperm surface it is likely to have functionality in this environment. As yet, no similar small peptides have been identified in primates/humans.

1.10 Aims of the Project.

When couples enter into a programme of assisted reproduction, they are limited to the number of superovulatory cycles they can receive due to health concerns such as ovarian cancer that may arise from the hormone treatment. When ASA are diagnosed in the male partner it is not known how these may interfere with fertilization as ASA may reduce fertility in a number of ways as previously described. Therefore, identifying the antigens that are targets for ASA and how these antibodies lead to infertility would allow new diagnostic tests to be developed that would determine the best course of treatment for individual couples.

The aims of the project are threefold, firstly to identify novel ASA targets from human sperm membrane extracts and from human seminal plasma along with extracts of mouse reproductive tissues. Secondly, to assign functions to these antigens and clarify how ASA to these antigens may lead to reduced fertility. Thirdly, to determine if the reduced immunosuppressive activity of seminal plasma from ASA patients contributes to the development of ASA or conversely, whether the presence of ASA leads to a reduced state of immunosuppression. This requires the identification of immunosuppressive factors in seminal plasma and their mode of immune modulation.

Chapter 2

Materials and Methods

2.1 Materials

Male Mice (BALB/c and CD1), Female Rats (Wistar) and Female Rabbits (New Zealand) were obtained from the BioResources Unit, Trinity College, Ireland.

Columns, Superdex Peptide 10/300, Superdex 75 10/300 and MonoQ 5/50, along with IPG focusing strips and destreak reagent were purchased from GE Healthcare, Buckinghamshire, UK.

Sequencing grade trypsin and Wizard SV gel and PCR clean up kit were purchased from Promega Corp., Madison, WI, USA.

Medicult IVF medium was purchased from Bio Tipp Ltd, Cahir, Ireland.

Agarose and Nickel-Agarose were purchased from Chromatrin, Dublin, Ireland.

Marvel milk powder was purcha..d from Premier International Foods Ltd, Coolock, Dublin, Ireland.

SpermMar IgG and IgA kits were purchased from FertiPro Ltd, Beernem, Belgium.

Ethanol and Isopropanol HPLC grades were purchased from LabScan Ltd, Sowinskiego, Poland.

Radiochemicals, [^{3}H] Thymidine and [^{14}C] Cholesterol were purchased from Amersham Inc. (now GE Healthcare, see above).

Cobalt chloride and Sodium dihydrogen phosphate were purchased from Riedel de Haen, Seelze, Germany.

PE SiL G and Silica GL20 thin layer chromatography (TLC) plates were purchased from Whatman. (now GE Healthcare, see above).

Ethyl acetate, Acetone and Cyclohexane were purchased from Romil Ltd, Cambridgeshire, UK.

Ecoscint was purchased from National Diagnostics Ltd, Yorkshire, UK.

Acetonitrile was purchased from JT Baker Ltd, Deventer, Holland.

Bradford Reagent was purchased from BioRad, California, USA.

*Nco*I, *Xho*I, *Bam*HI and *Eco*RI were purchased from New England Biolabs, Ipswich, MA, USA.

DNA ladder and unstained protein ladder were purchased from Jena Biosciences, Jena, Germany.

Thiourea, Urea and C7BzO were purchased from Invitrogen Corp, California, USA.

SuperSignal West Pico ECL reagent, pre-stained and unstained protein ladders and Pierce <7kDa cut off filters were purchased from ThermoScientific, through Medical Supply Company, Dublin, Ireland.

HighPure PCR Product Purification kit and HighPure Plasmid mini kit along with WST-1 reagent and Lactate Dehydrogenase (LDH) assay kit were purchased from Roche Diagnostics, Mannheim, Germany.

Caspase 3 fluorogenic substrate (Ac-DEVD-AMC), MOWIOL 4-88 mounting reagent and Protease Inhibitor Cocktail were purchased from Calbiochem Inc, Merck-Millipore, Darmstat, Germany.

Plasmid pET28b, amicon filters (<3kDa and <100kDa) were purchased from Merck-Millipore, Darmstat, Germany.

Streptactin-sepharose and QIAprep Spin MiniPrep kit were purchased from Qiagen Corp., Hilden, Germany.

RPMI 1640 and Dulbecco's Modified Eagle Medium (DMEM) along with penicillin/streptomycin solution and glutamine solution were purchased from Gibco Corp. (Now Invitrogen, see above).

Diethylaminoethanol (DEAE)-sepharose and carboxymethyl (CM)-sepharose were purchased from Pharmacia, Uppsala, Sweden.

Image clone 6775941 CL-C3 was purchased from Source Biosciences, Nottingham, England.

Clone OKT3 anti-CD3 antibodies were purchased from eBioscience, San Diego, CA, USA.

IL-8 and IL-10 ELISA kits along with IgG1 isotype control were purchased from R&D Systems, Minneapolis, USA.

IL-2 was purchased from Chiron Corp., Emmeryville, CA, USA.

CD8α-PeCy7 anti-human antibody and DyLight 594 goat anti-rat IgG were purchased from Biolegend, San Diego, USA.

CD3-Pacific Blue anti-human antibody was purchased from BD Biosciences, Oxford, England.

All other primary and secondary antibodies along with all other chemicals of reagent grade were purchased from Sigma-Aldrich, Poole, Dorset, England.

Plasmid pHis parallel-2 was a gift from Dr. Amir Khan, TCD, Dublin.

2.2 Methods

2.2.1 Semen Analysis and Detection of Antisperm Antibodies.

Semen samples from patients attending the Human Assisted Reproduction Ireland unit in the Rotunda Hospital, Dublin 1, were analysed according to the World Health Organization (WHO) guidelines. Patients were also tested for ASAs bound to spermatozoa using the SpermMar IgG and IgA immunobead tests. Degree of agglutination of the sperm was also noted. Patient cohort consisted of 13 patients who also provided serum samples. Control patient samples were patients attending the clinic who had normal semen parameters.

2.2.2 Immunofluorescence microscopy of spermatozoa.

Spermatozoa from patients with normal semen parameters were centrifuged free of seminal plasma at 600g and washed x3 in phosphate-buffered saline (PBS) and smeared onto clean microscope coverslips and air dried overnight.

A 2% (w/v) paraformaldehyde (PFA) solution in PBS (pH 7.2) was prepared as follows; a 10% (w/v) PFA solution was prepared in distilled water. One part 5 x concentrated PBS and three parts distilled water were added to one part 10% (v/v) PFA solution. Spermatozoa were fixed on the slides by immersion in 2% (w/v) PFA in PBS for 20 min (room temperature). They were then rinsed once in PBS followed by immersion in 5% (w/v) Bovine serum albumin (BSA) in PBS for 1h at room temperature or overnight at 4°C. Slides were then washed in PBS for 10 min and smears were overlaid with a 1/10 dilution of patients sera or seminal plasma in 0.1% (w/v) BSA in PBS and incubated for 2 hr at room temperature. Slides were washed in 0.1% (w/v) BSA in PBS three times for 10 min each.

Finally, a 1/16 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Fc specific) antibody in 0.1% (w/v) BSA in PBS for antigen localisation on spermatozoa, was overlaid on the spermatozoal smears for 90min (37°C). After incubation the coverslips were washed x3 in 0.1% (w/v) BSA in PBS (37°C).

Coverslips were mounted onto slides using MOWIOL 4-88 mounting reagent and coverslips were sealed with nail varnish. The fluorescently labeled samples were observed on a FV1000 Point Scanning Confocal Microscope using a 60x oil immersion objective.

2.2.3 Preparation of Spermatozoal Membranes and Seminal Plasma for Gel Electrophoresis.

Spermatozoa from patients with normal semen parameters were centrifuged free of seminal plasma at 600g and washed x3 in PBS. Spermatozoa were resuspended in 1% (v/v) Triton X-100 in PBS or 1% (v/v) Triton X-114 in PBS and incubated at room temperature with continual agitation for 2 hr. Spermatozoa were removed by centrifugation (14000g) and the Triton X-100 supernatants containing membrane proteins were precipitated by bringing the solution to 10% (w/v) trichloroacetic acid (TCA) and incubating overnight at 4°C. Triton X-114 supernatants were stored on ice for 20 min until the solution went clear and vortexed briefly. The supernatants were brought to 35°C (above the cloud point of Triton X-114) for 5 min and centrifuged at 14000g to form an upper aqueous phase and a lower detergent phase. The upper aqueous phase was removed to a clean tube and brought to 10% (w/v) TCA as before and kept at 4°C overnight. The lower detergent phase was re-extracted by addition of 1ml of 1% (v/v) Triton X-114 in PBS and bringing the solution to 4°C on ice. Phase separation was completed as before and the upper phase discarded. The detergent phase proteins were precipitated by addition of 9 volumes of ice cold acetone and incubation overnight at –20°C.

The Triton X-100 extract and the aqueous phase Triton X-114 extract precipitated overnight in TCA were centrifuged at 100g for 10 sec to pellet precipitated protein. Precipitates were washed x3 in ice cold acetone and after the final wash allowed to air dry for 3 min before addition of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol and 710mM β-mercaptoethanol). The acetone precipitated detergent phase was centrifuged at 100g for 10 sec to pellet precipitated protein. Precipitates were washed x2 in fresh ice cold acetone and after the final wash allowed to air dry for 3 min before addition of SDS-PAGE sample buffer.

Protein precipitates were resuspended in SDS-PAGE sample buffer and boiled for 3 min to fully solubilise proteins. Seminal plasma from the initial step was either stored at –20°C or prepared for SDS-PAGE by addition of 9 volumes ice-cold acetone to precipitate the protein and left overnight at –20°C. The acetone precipitate was centrifuged at 100g for 10 sec to pellet precipitated protein. Precipitates were washed x2 in fresh ice cold acetone and after the final wash allowed to air dry for 3 min before addition of SDS-PAGE sample buffer.

2.2.4 Tris-Glycine SDS-PAGE.

Tris-Glycine electrophoresis was carried out as in Laemmli (1970) using an Atto-mini gel system. Gels were run using constant voltage of 100V. Gels were either stained with Coomassie Brilliant Blue G (0.25% (w/v) in 40% (v/v) Methanol, 10% (v/v) Acetic acid and 50% (v/v) Water) for 2 hr and destained in destain solution (40% (v/v) Methanol, 10% (v/v) Acetic acid and 50% (v/v) Water) or silver stained as described in section 2.5.1 or transferred by semi-dry transfer to polyvinylidene fluoride (PVDF) membranes for western blotting as described in section 2.2.6.

2.2.5 Tris-Tricine SDS-PAGE.

Tris-Tricine electrophoresis was carried out as in Schagger and von Jagow (1987) using an Atto-mini gel system. Gels were run using constant voltage of 100V. Gels were either stained with Coomassie Brilliant Blue G (0.25% (w/v) in 40% (v/v) Methanol, 10% (v/v) Acetic acid and 50% (v/v) Water) for 2 hr and destained in destain solution (40% (v/v) Methanol, 10% (v/v) Acetic acid and 50% (v/v) Water) or silver stained as described in section 2.5.1 or transferred by semi-dry transfer to PVDF membranes for western blotting as described in section 2.2.6.

2.2.6 Western Blotting.

Gels were electrophoretically transferred (blotting buffer- 0.2M glycine, 0.025M Tris, 20% (v/v) methanol, 0.1 % (w/v) SDS) to pre-activated PVDF membranes using a semi-phasing TE70 semi-dry transfer unit run at a constant current of 100mA for 75min. Blots were blocked by incubation in 5% Marvel in PBS with Tween (0.05% w/v) at 4°C overnight or at room temperature for 1 hr. The following antibodies were used as primary and secondary antibodies:

Primary Antibody	Primary Antibody Source	Primary Antibody Dilution Factor	Secondary Antibody	Secondary Antibody Dilution Factor	Detection Method
Patient Derived Sera IgG Fraction	Patients	1:100	Goat anti-Human IgG (Fc specific) HRP conjugate	1:5000	Diaminobenzidine stain
Patient Derived Sera IgA Fraction	Patients	1:100	Goat anti-Human IgA (Whole molecule) HRP conjugate	1:1000	Diaminobenzidine stain
Seminal Plasma	Patients	1:500	Goat anti-Human IgG (Fc specific) HRP conjugate	1:2500	Chemiluminesence
Anti-CRISP	In House Rabbit Inoculation	1:250	Goat anti-Rabbit IgG (Fc specific) HRP conjugate	1:7500	Diaminobenzidine stain
Anti-MVDP	In House Rat Inoculation	1:500	Goat anti-Rat IgG (Whole molecule) HRP conjugate	1:7500	Diaminobenzidine stain
Anti-Semenogelin	In House Rabbit Inoculation	1:350	Goat anti-Rabbit IgG (Fc specific) HRP conjugate	1:5000	Diaminobenzidine stain
Anti-LLR37b	Commercial Sigma-Aldrich	1:1000	Goat anti-Rabbit IgG (Fc specific) HRP conjugate	1:10000	Chemiluminesence

Table 2.1 Antibody concentrations used in western blotting.

The blocked blot was washed three times with Tween (0.05% w/v) in PBS (PBS/Tween) for 5 min per wash. The blots were incubated in primary antibody for 1 hr at room temperature with constant agitation.

After this incubation, the blot was washed six times with PBS/Tween for 1 min per wash. The secondary antibody was then applied at the required dilution for 2 hr at room temperature with constant agitation. After this incubation the blot was washed six times in PBS/tween for 1 min/wash and finally one wash in PBS without tween.

2.2.6.1 Development of Blots using Diaminobenzidine.

To develop the membrane, 5ml diaminobenzidine solution was prepared; 0.08% (w/v) 3,3'-diaminobenzidine, 0.08% (v/v) hydrogen peroxide in 10ml PBS. The membrane was allowed to develop in the diaminobenzidine solution. When adequate staining was achieved blots were washed in copious amounts of dH_2O.

2.2.6.2 Development of Blots using Chemiluminescence.

The Supersignal West Pico Substrate working solution was prepared by mixing equal parts Luminiol/Enhancer Solution and stable Peroxidase Solution as per manufacturer's instructions. The blot was incubated with this solution for approximately 5 min. After draining excess fluid from the blot, it was then placed inside a clear plastic membrane and sealed. Kodak X-Omat film was exposed for differing time periods in the dark, depending on the blot and developed using an autoradiograph developer.

2.2.7 Treatment of Patient Sera Prior to Use in Western Blotting.

2.2.7.1 Concentration of IgG from Patients Sera for Western Blotting.

Patient sera was brought to 45% (w/v) ammonium sulphate by addition of solid ammonium sulphate and mixed until dissolved. This solution was left on ice for 10 min and centrifuged at 14000g. The supernatant was removed and the pellet dissolved in $1/10^{th}$ the volume of original sera using PBS. This fraction contains IgG and was used for all IgG patient sera western blots.

2.2.7.2 Immunoglobulin A (IgA) enrichment from Patient Sera for Western Blotting.

Flow through (unbound material) from the Protein G columns (as in section 2.7.4) was collected and used for western blotting for IgA antigens.

2.2.8 Isolation of Seminal Plasma Antigen:Antibody Complexes using Protein G Agarose chromatography.

Seminal plasma antigen:antibody complexes were isolated using a protein G agarose column. The column was pre-equilibrated using 0.1 M Tris-HCl pH 8. The pH of ASA positive seminal plasma was adjusted by addition of $1/10^{th}$ volume of 1 M Tris-HCl pH 8 and run through the column in 0.1 M Tris-HCl pH 8 followed by 5 column volumes of 0.01 M Tris pH 8. Bound antibody:protein complexes were eluted using 1M glycine pH 3 and neutralized by addition of 50µl/ml of 0.1M Tris-HCl. The column was regenerated with 2M Urea followed by 1M glycine pH2.5. Fractions were assayed for protein absorbance at 280nm.

2.2.9 Detection of Mouse Reproductive Tract Antigens Reacting with Human ASA.

Mice (BALB/c) were sacrificed by cervical dislocation (10 mice) and the caput, corpus and cauda epididymis removed along with the vas deferens and washed in extraction buffer (0.1M Tris-HCl 0.15M NaCl pH 7.5). Each part of the reproductive tract was resuspended in 2ml of extraction buffer containg 20µl protease inhibitor

cocktail and homogenised in a dounce homogeniser. Homogenised tissue was centrifuged at 14000g at 4°C for 10 min. Supernatants were removed and protein precipitated using 9 volumes of ice cold acetone. The acetone precipitate was centrifuged at 100g for 10 sec to pellet precipitated protein. Precipitates were washed x2 in fresh ice cold acetone and after the final wash allowed to air dry for 3 min before addition of SDS-PAGE sample buffer. Protein precipitates were resuspended in sample buffer and boiled for 3 min to fully solubilise proteins.

2.2.10 Preparation of Mouse Reproductive Tissues for Two-Dimensional (2-D) Gel Elecrophoresis.

Mouse reproductive tissues removed as before (section 2.2.9) were homogenised in 5mM Tris (pH 9) and centrifuged as before (section 2.2.9) and supernatants precipitated using 9 volumes of ice cold acetone. The acetone precipitate was centrifuged at 100g for 10 sec to pellet precipitated protein. Precipitates were washed x2 in fresh ice cold acetone and after the final wash allowed to air dry overnight before being re-dissolved in 2-D separation buffer (7M Urea, 2M Thiourea and 1% (w/v) C7BzO detergent). Insoluble material was removed by centrifugation at 14000g at 4°C and the resulting supernatant reduced and alkylated. This was achieved by addition of tributylphosphine to a final concentration of 5mM and incubation at room temperature for 1 hr. Following this iodoacetamide was added to a final concentration of 15mM and again incubated for 1 hr at room temperature. Remaining iodoacetamide was quenched by addition of further tributylphosphine to a final concentration of 15mM. Supernatants were either used fresh or stored at –20°C.

2.2.11 Two-Dimensional (2-D) Gel Electrophoresis.

Samples (as prepared in section 2.2.10) were first mixed with IPG destreak reagent (140µl sample + 10µl destreak reagent) and used to rehydrate a 7cm pH 4-7 IPG strip. Rehydration was allowed to continue for approximately 1 hr and strips were loaded onto a BioRad Protean II isoelectric focusing (IEF) machine as per manufacturer's instructions. Focusing was started at 300V increasing to 5000V over a 3-4 hr period. A total of 12000V.hr were applied to each strip. Focused strips were washed x2 in nanopure water and placed onto a pre-set 15% Tris-glycine resolving gel

(as in section 2.2.4) and sealed with a 1% (w/v) agarose gel in 125mM Tris-HCl pH6.8) and run at 50V constant voltage until dye front entered the resolving gel. Voltage was then increased to 100V until finished. Gels were stained in Coomassie Brillian blue G as described in section 2.2.4.

2.2.12 Trypsinisation of 2-D protein spots for MALDI-TOF Mass Spectrometry.

Spots on 2-D gels of interest were excised with a sterile scalpel in a laminar flow hood. Gel slices were washed x2 in 50mM ammonium bicarbonate, 30% (v/v) actetonitrile for 30 min. Gel slices were briefly washed with 100mM ammonium bicarbonate followed by two washes of 20 min each in 50mM ammonium bicarbonate, 50% (v/v) acetonitrile with one final wash in 100% acetonitrile for 15 min. Gel slices were then desiccated in a speedy-vac.

Pierce sequencing grade trypsin (20µg) was dissolved in 180µl 25mM ammonium bicarbonate and 20µl 1% (w/v) n-octylglucoside. Dessicated gel slices were allowed to re-swell in 3µl of trypsin solution for 1 hr after which a further 10µl of buffer (25mM ammonium bicarbonate with 1% (w/v) n-octylglucoside) was added and incubated overnight at 37°C. The tubes were then centrifuged and any free liquid removed to a clean tube, the gel slice was then immersed in 40µl of acetonitrile:water:trifluoroacetic acid (66.6:33.3:0.1) and the samples sonicated for 30 min in a sonicating water bath. Tubes were centrifuged again and liquid removed to corresponding tube as before and the process repeated. The pooled supernatants were brought to complete dryness on a speedy-vac and dissolved in 5% (v/v) formic acid in water and sent for MALTI-TOF analysis.

2.2.13 Purification of Mouse Vas Deferens Protein (MVDP) from Vas Deferens Homogenate.

2.2.13.1 Ammonium Sulphate Fractionation of Vas Deferens Homogenate.

Calculated amounts of solid ammonium sulphate were gradually added to a measured volume of homogenate to achieve a given percentage saturation of ammonium sulphate between 0-100% (Table 2.2) with continuous stirring. Once the ammonium sulphate had dissolved, the solution was kept on ice for 15 min and then centrifuged at 15,000g for 10 min. Resulting supernatant was removed, measured and the procedure above was repeated. The pellets were resuspended in 1ml of buffer (20mM Tris-HCl, 5mM NaCl and 5mM β-mercaptoethanol, pH 7.4) and an aliquot prepared for analysis on SDS-PAGE by acetone precipitation as per section 2.2.3. The precipitated samples were resuspended with SDS-PAGE sample buffer and run on a 15% SDS-PAGE as in 2.2.4.

Percentage Saturation (%)	Volume of Homogenate (ml)	Weight of $(NH_4)_2SO_4$ added (g)
0-20	3.1 ml	0.3286
20-40	2.9 ml	0.3277
40-60	2.9 ml	0.348
60-80	2.9 ml	0.358
80-100	2.8 ml	0.3892

Table 2.2 Example of ammonium sulphate precipitation by increasing percentage.

2.2.13.2 Dialysis of Ammonium Sulphate Fractions Containing MVDP.

MVDP was identified in the 100% pellet by SDS-PAGE as in section 2.2.13.1. The 100% pellet was dissolved as previously in 1ml of buffer (20mM Tris-HCl, 5mM NaCl and 5mM, β-mercaptoethanol, pH 7.4) and dialysed against 1L of the same buffer (20mM Tris-HCl, 5mM NaCl and 5mM β-mercaptoethanol, pH 7.4). Samples were dialysed at 4°C overnight.

2.2.13.3 Mono-Q Ion Exchange Chromatography of Dialysed 100% Ammonium Sulphate Fraction.

A Mono-Q 5/50 column (Amersham Biosciences) was equilibrated with 20 column volumes of a 20mM Tris-HCl, 5mM NaCl and 5mM β-mercaptoethanol, pH 7.4 buffer. The vas deferens dialysed 100% pellet was applied to the equilibrated column and a gradient of 5-500 mM NaCl was applied. Fractions of 0.5ml were collected and the fractions that corresponded to the peaks obtained from the elution profile were analysed on a 15% SDS-PAGE as described in section 2.2.4.

2.2.13.4 Gel Filtration of Mono-Q Ion Exchange Fractions Containing MVDP.

Fractions that were positive for the 36kDa MVDP from the Mono-Q Column as described in 2.2.13.3 were pooled together and concentrated to 500µl using a Pierce Microconcentrator 7kDa filter. Gel filtration was carried out using a Superdex 75 10/300 column equilibrated with a 0.1M Sodium Phosphate, 0.15M NaCl buffer (pH 6.8). The concentrated MVDP was applied to the Sephadex column (0.5 ml) and fractions (0.5 ml) were collected at a flow rate of 0.8ml/min using 0.1M Sodium Phosphate, 0.15M NaCl buffer (pH 6.8) as running buffer. Fractions corresponding to peaks were analysed by SDS-PAGE as in section 2.2.4.

2.2.14 Structural Characterisation of MVDP.

2.2.14.1 Native PAGE Electrophoresis of MVDP.

To determine the complexity of the purified endogenous MVDP a native PAGE was carried out. A separation buffer (Trizma 1.5M, pH 8.8), stacking buffer (Trizma 0.5M, pH 6.8), 1X running buffer (Table 2.3) and 4X Loading buffer (table 2.4) were prepared. All reagents were free of SDS and β-mercaptoethanol.

1X Running Buffer for Native Gels

Chemical	Volume
Trizma	3 g
Glycine	14.4 g
Distilled H_2O	200 ml

Table 2.3: Composition of Running Buffer for Native Gels.

Loading Buffer (4X concentrated)

Chemical
50 mM Tris pH 6.8
10% Glycerol
0.02% Bromophenol Blue

Table 2.4 Composition of Loading Buffer (4X concentrated) for Native Gels.

9 % Native Gel

Chemical	Separation	Stacking
Acrylamide/Bisacrylamide (29:1)	3 ml	2.8 ml
Distilled H_20	3 ml	1 ml
Buffer (Separation/Stacking)	2.5 ml	1.5 ml
Triton (1% v/v)	960 µl	600 µl
Ammonium Persulphate	100 µl	70 µl
TEMED	30 µl	10 µl
Final Volume	9.63 ml	6.0 ml

Table 2.5 Composition of 9% Native Gel.

A sample of the MVDP purified protein (15µl) was added to 4X loading buffer (5µl). The samples were applied to a 9% native gel and run using an ATTO CrossPower 500 power pack at constant voltage of 60V for the whole duration in the cold room to prevent heat denaturation of protein.

Once the native gel was stained and destained as described in section 2.2.4, the bands of interest were excised, dried in a SpeediVac and rehydrated in SDS-PAGE sample buffer (30µl). These gel pieces were applied to a 15% SDS-PAGE gel and run in the second dimension along with molecular weight standards (20µl) as in section 2.2.4.

2.2.14.2 Investigation of the N-Glycosylation Status of MVDP.

To determine whether MVDP is a glycosylated protein, Glycoprofile™ II, Enzymatic In-Solution N-Deglycosylation Kit (Sigma-Aldrich) was used on a sample of purified MVDP as in section 2.2.13. Removal of N-Glycosylations were carried out as per manufacturer's instructions. In brief, the following additions were made. Ribonuclease B (RNase B)(100µg) (control N-Glycosylated protein) and MVDP (100µg) were added to separate tubes (approximately 90µl each) and 5µl denaturant

solution (2% (w/v) octyl-β-D-glycopyanoside, 100mM β-mercaptoethanol) added. Protein solutions were mixed and heated at 100°C for 10 min and cooled to room temperature. Two aliquots of 45µl each was removed to two fresh tubes and 5µl of a 1X reaction buffer (200mM ammonium bicarbonate added). Peptide-N-Glycosidase (PNGase F) (5µl of 500U/ml solution) was added to one tube of RNase B and MVDP, while 5µl of water was added to control tubes of each protein. Solutions were centrifuged briefly in a benchtop centrifuge at 14,000g and incubated at 37°C for 1hr. The reaction was stopped by heating solutions to 100°C for 10 min.

Once the solutions had cooled, 3X SDS-PAGE sample buffer (25µl) was added to sample tubes (50µl) and analysed using SDS-PAGE as in section 2.2.4 and stained and destained as described in section 2.2.4.

2.2.15 Agarose Gel Electrophoresis of DNA.

1.5% agarose gels were prepared by dissolving agarose (1.5% w/v) by heating in 1X TAE (20mM Tris, 1mM Na EDTA and 10mM Acetic Acid). Ethidium Bromide (1/10,000 dilution of 10 mg/ml stock) was added just prior to casting the gel. DNA was mixed with 10 x DNA loading buffer (33% (v/v) glycerol in 10 x TAE buffer with 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cylanol) and loaded into the gel. Gels were run at a constant 100V until completed and imaged under ultra-violet (UV) light using a UVP Biospectrum.

2.2.16 Cloning and Expression of Recombinant MVDP in *E. coli.*

2.2.16.1 Preparation and Purification of pHis-parallel-2 Vector.

The vector pHis-parallel-2 plasmid was transformed into competent *E. coli* DH5α cells. Briefly, pHis-parallel-2 vector (1μl) was added to competent DH5α cells (30μl) and incubated on ice for 30 min. The mixture was then heated at 42°C for 3 min and placed back on ice for a further 5 min. Cells were allowed to recover by addition of 100μl Luria-Bertani (LB) Broth/Ampicillin (100 μg/ml) and incubation at 37°C for 30 min. The resulting mixture was streaked onto a LB agar/Ampicillin (100 μg/ml) plate and incubated overnight 37°C. One of the resulting colonies was inoculated into LB Broth/Ampicillin (100 μg/ml) (4 ml) and incubated with agitation at 200 rpm at 37°C overnight. This culture was then centrifuged at 2,000g in a Wifug 2000E centrifuge for 10 min at 4°C. The supernatant was removed and the plasmid purified from the cell pellet using a GeneElute Plasmid Mini-Prep Kit as per the manufacturer's instructions.

Purified plasmid was digested with the restriction enzymes *Bam*HI (1μl) and *Eco*RI (1μl). Cleaved products were run on 1% agarose gel in TAE buffer as in section 2.2.15 to determine the effectiveness of the digest and the required cleaved plasmid fragment was excised and purified from the agarose gel slice using the High Pure PCR Product purification kit as per the manufacturer's instructions.

2.2.16.2 Preparation of MVDP Image Clone for Polymerase Chain Reaction (PCR).

MVDP 6775941 CL-C3 image clone was streaked onto LB Agar/kanamycin (30μg/ml) plates and incubated at 37°C overnight. One of the resulting colonies was inoculated into LB Broth/kanamycin (30μg/ml) and incubated with agitation at 200 rpm at 37°C overnight. This culture was centrifuged at 2000g in a Wifug 2000E centrifuge for 10min at 4 °C. The resulting supernatant was removed and the image clone plasmid (pellet) purified using a GeneElute Plasmid Mini-Prep Kit as per the manufacturer's instructions.

2.2.16.3 Preparation of MVDP complementary DNA (cDNA) by PCR.

PCR amplification was carried out to obtain MVDP cDNA incorporating *Bam*HI and *Eco*RI restriction sites using the MVDP image clone as prepared in section 2.2.16.3. This was to allow directional cloning into the pHis-parallel-2 expression vector. The forward primer used for the incorporation of a *Bam*HI site (underlined) was:

5'-CGT GGA TCC GCC ACC TTC GTG GAA CTC AG-3'

The reverse primer used for the incorporation of an *Eco*RI site (underlined) was:

5'-TCG AAT TCT CAG TAT TCC TCG TGG AAA-3'

The PCR reaction mix is shown in Table 2.6 and the cycles used to amplify the MVDP cDNA are shown in Table 2.7.

Chemical	Volume (µl)
Template (MVDP image clone)	1µl
dNTPs	2µl
Taq Buffer with $MgCl_2$(X10)	5µl
Distilled H_2O	39µl
Primers (forward and reverse)	1µl of each
Taq polymerase	1µl

Table 2.6 PCR Reaction mixture for amplification of MVDP cDNA.

Cycle	Temperature	Time
Initial Hot Start	94°C	3 min
Denaturation	94°C	45 sec
Annealing	60°C	45 sec
Elongation	72°C	1 min
Final Elongation	72°C	10 min

Table 2.7 PCR Cycles used in amplification of MVDP cDNA.

The products of PCR were run on a 1% (w/v) agarose gel as in section 2.2.15 to visualise the PCR products.

2.2.16.4 Construction and Purification of Recombinant pHis-parallel-2-MVDP Plasmid.

To remove any excess dNTPs, primers or Taq from the PCR product, PCR purification was carried out on the MVDP cDNA using a High Pure PCR Product Purification Kit as per manufacturer's guidelines. MVDP cDNA purified from 2.3.2 was cleaved using *Bam*HI and *Eco*RI overnight. The resulting cleaved MVDP cDNA was run on a 1% agarose gel and the band of interest was excised and purified as above.

Cleaved MVDP cDNA was ligated using a DNA Ligation Mixture (Takara) according to manufacturer's instructions to the cleaved pHis-parallel-2 vector which was prepared as described in section 2.2.16.1 in a 1:1 ratio. Ligated plasmid mix was transformed into competent *E. coli* DH5α cells by the heat shock method described previously. Cells were then spread onto multiple LB-Agar/Ampicillin (100μg/ml) and incubated overnight at 37 °C.

Resulting colonies from this incubation were inoculated into separate LB-Broth/Ampicillin (100μg/ml) and incubated at 37°C with agitation as in section 2.2.16.1. This culture was then centrifuged as described in section 2.2.16.1 and the plasmid was purified from the resulting pellet using a HighPure Plasmid Mini-Prep kit as per the manufacturer's instructions.

Purified plasmid from each culture was digested with *Bam*HI and *Eco*RI and analysed on 1% agarose gels to confirm insertion of the MVDP gene into the pHis-parallel-2 plasmid vector.

2.2.16.5 Expression of Recombinant MVDP.

Positive MVDP clones as identified and isolated from section 2.2.16.4 were transformed into competent BL21 (DE3) *E.coli* cells using the heat shock method described in section 2.2.16.1 and spread onto LB Agar/Ampicillin (100 μg/ml) and grown overnight at 37 °C. Colonies from theses agar plates were inoculated into 10ml

LB Broth/Ampicillin (100μg/ml) and incubated overnight with agitation at 37°C as starter cultures. This culture was added to LB Broth/Ampicillin (100 μg/ml) (500 ml) in a large 2l flask and placed in a shaking incubator at 37°C and incubated till an OD_{600} of 0.6 was reached.

Gene expression was induced by addition of isopropyl-D-1-thiogalactopyranoside (IPTG) (500μM final concentration) to the culture and incubated for a further 2h with agitation at 170rpm at 37°C. Samples (1ml) were taken at 1 hr intervals from 0-3h and centrifuged at 14000g in a benchtop centrifuge. The resulting pellets were resuspended in 3X SDS-PAGE sample buffer (50μl), lysed by boiling for 2 min and analysed on SDS-PAGE as described in section 2.2.4. The remaining culture was centrifuged at 10,000g for 15 min at 4°C in a Sorvall RC5C centrifuge and resulting cell pellets used fresh or stored at –20°C until used.

2.2.16.6 Purification of Recombinant MVDP.

Pellets achieved from 2.3.4 were resuspended in lysis buffer (50mM Tris, 150mM NaCl, 10mM imidazole, 2mM β-mercaptoethanol, pH 8) (8ml) and sonicated. The sonicated cells were centrifuged at 10,000g for 10 min at 4°C and samples of the supernatant and pellet were retained for analysis.

Nickel-agarose beads (6ml of slurry) was equilibrated in lysis buffer in a chromatography column and lysed cell supernatant passed through. The column was washed with at least 10 column volumes of lysis buffer (or until A_{280} had returned to baseline).

Recombinant MVDP protein was eluted using elution buffer (50mM Tris, 150mM NaCl, 300mM imidazole, 2mM β-mercaptoethanol, pH 8) and fractions (1ml) were collected. To determine which fraction contained the recombinant MVDP samples of each fraction were analysed on 15% SDS-PAGE as described in section 2.2.4. The fractions found to contain the 36kDa MVDP band were concentrated using a 7kDa Pierce concentrator and underwent further purification by fast protein liquid

chromatography (FPLC) gel filtration on a Superdex 75 10/300 column as described in section 2.13.4.

2.2.17 Production of Antisera in Rats to MVDP.

rMVDP was purified as in section 2.2.16.6.

Day 1: 200μg of purified MVDP was emulsified with an equal volume of Freund's complete adjuvant. A female Wistar rat was immunised intradermally at multiple sites along its back.

Day 10: Booster adjuvant was prepared by adding an equal volume of Freund's incomplete adjuvant to 200μg of purified MVDP. The total volume (0.25ml) was injected intramuscularly (right thigh).

Day 28: Booster adjuvant was prepared as in day 10 and injected intramuscularly (left thigh).

Day 42: 8ml of blood was obtained by cardiac puncture.

Whole blood from cardiac puncture was allowed to coagulate at room temperature for 20 min. It was then centrifuged at 17,000g at 4°C for 15 min. The plasma was removed, aliquoted and stored at -20°C.

2.2.18 Assay of Endogenous and Recombinant MVDP Reductase Activity Against Reactive Aldehyde Species.

Kinetic analysis of both recombinant and endogenous MVDP was performed in 100mM sodium phosphate buffer, pH 7. NADPH (100μM) and MVDP (20μg/ml) concentrations were kept constant in all assays. Substrates, 4-hydroxynonenal (HNE) and acrolein, were varied from 1μM to 200μM and initial rates measured by decrease in absorbance at 340nm spectrophotometrically.

2.2.19 Acrolein Reductase Activity of Human Seminal Plasma.

Human seminal plasma was prepared as in section 2.2.3 and assayed for protein content by Bradford assay as in section 2.21. Assays were carried out as in section 2.2.18 with the final concentration of NADPH/NADH in the reaction maintained at 50μM and acrolein at 200μM. The final volume in the cuvette was adjusted to 2ml using buffer and the reaction started by addition of 50μl of seminal plasma. The change in absorbance of NADPH and NADH was measured at 340nm.

2.2.20 Acrolein Reductase Activity of Human Sperm Membrane Extract.

Sperm plasma membrane was isolated using 1% (v/v) Triton X-100 in PBS as in section 2.2.3 and assayed for protein content by Bradford assay as in section 2.21. Assays were carried out as in section 2.2.19 with the final concentration of NADPH/NADH in the reaction maintained at 50μM and acrolein 200μM. The final volume in the cuvette was adjusted to 2ml using buffer and the reaction started by addition of 50μl of sperm membrane extract. The change in absorbance of NADPH and NADH was measured at 340nm.

2.2.21 Detection of MVDP on Mouse and Human Sperm Surfaces by Confocal Microscopy.

Extracted mouse vas deferens tissue was placed in PBS and finely chopped to allow sperm to swim out at room temperature for 1h. Sperm were washed X3 in PBS and resuspended at 10^6 ml^{-1} and 5 x 10^4 sperm overlaid on glass cover slips for 1h to allow them to adhere. Human sperm were prepared as before (section 2.2.2) and allowed to adhere to cover slips as in mouse sperm. Sperm were fixed in 2% (w/v) PFA in PBS for 15 min and washed X3 in PBS/Tween 20. Cover slips were incubated with anti-MVDP antisera diluted 1 in 2000 in 1% (w/v) BSA in PBS/Tween 20 for 2h at room temperature. Cover slips were washed X3 in PBS/Tween and then incubated for 1h in a 1 in 1000 dilution of secondary antibody (DyLight 594 goat anti-rat IgG in 1% BSA in PBS/Tween). Cover slips were washed as before and mounted in MOWIOL 4-88 reagent (Calbiochem) and imaged on a FV1000 Point Scanning Confocal Microscope using a 60x oil immersion objective.

2.2.22 Alleviation of Spermine Toxicity by MVDP.

THP1 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum (FCS), 2mM glutamine, 100 IU penicillin and 100 μg ml^{-1} streptomycin as in section 2.11.4 and plated in a 96 well plate at 1 x 10^5 cells in 200μl per well with or without recombinant MVDP (20μg per well) at 37°C in 5 % CO_2 overnight. Spermine was added in a range of concentrations from 0.01μM to 100μM and incubated overnight. Alamar blue was added as per manufacturer's instructions and incubated for 6 hr at 37°C in 5 % CO_2. Fluorescence was measured at 544nm excitation and 590nm emission.

2.3 Purification of Cysteine Rich Secretory Protein 1 (CRISP1) from Murine Cauda Epididymal Tissue.

CRISP1 was purified as described in Hearty and Carroll (1987) using a two step method: firstly chromatofocusing followed by affinity purification on Procion Red HE3B.

2.3.1 Chromatofocusing of Cauda Epididymal Fluid and Homogenates.

Mouse cauda tissues (15 CD1 mice) were removed as before (section 2.2.9) and homogenised in 5mM Tris (pH 9) and centrifuged as before (section 2.2.9) and supernatants dialysed overnight at 4°C against 5L of 0.025M imidazole buffer pH 7.4. The non-diffusable material was centrifuged at 50,000g for 30min at 4°C and the resulting supernatant applied to a column (27 x 1.5 cm) of chromatofocusing PBE 94 which had previously being equilibrated with imidazole buffer. Non-bound protein was removed by washing column in imidazole buffer until the A_{280} had returned to baseline. Polybuffer 94, pH 5, diluted 8-fold to give 14 column volumes was applied to the matrix to generate a pH gradient. Fractions were collected (1ml) and analysed for the presence of CRISP1 by SDS-PAGE as in section 2.2.4.

2.3.2 Procion Red HE3B sepharose 4B Chromatography of CRISP1 Positive Fractions from Chromatofocusing Column.

Fractions positive for CRISP1 were pooled and dialysed x3 against 1L of 50mM Tris-HCl pH 7.4 and applied to a Procion Red HE3B-sepharose 4B column (10 x 1cm) which was equilibrated in 50mM Tris-HCl pH 7.4. Non-bound protein was removed by washing column in imidazole buffer until the A_{280} had returned to baseline. Bound material was desorbed with a continuous gradient of 0-1M KCl in 50mM Tris-HCl pH 7.4 and collected in 1ml fractions. Fractions were analysed by SDS-PAGE as in section 2.2.4 and positive fractions pooled and dialysed x2 against 1L 50mM Tris-HCl pH 7.4.

2.3.4 Production of Antisera to CRISP1.

CRISP1 was purified as in sections 2.3.1 and 2.3.2.

Day 1: 200µg of purified CRISP1 was emulsified with an equal volume of Freund's complete adjuvant. A female New Zealand Rabbit was immunised intradermally at multiple sites along its back.

Day 14: Booster adjuvant was prepared by emulsifying equal volumes of Freund's incomplete adjuvant and 200µg of purified CRISP1. Booster was injected 6 x 0.25ml subcutaneously and 1 x 0.25ml injected intramuscularly (right thigh).

Day 28: Booster adjuvant was prepared as in day 14 and injected as before except into the left thigh.

Day 42: 25ml of blood was obtained by cardiac puncture.

2.3.5 Superovulation of Mice and Egg Collection.

Superovulation was induced in female CD1 mice by intraperitoneal injection of 5U follicle stimulating hormone (FSH) (Folligon), followed 48h later by administration of 5U human chorionic gonadotrophin (hCG). Ovulation occurred 12-14 hr later and mice sacrificed by cervical dislocation. The oviducts were excised and flushed with PBS into sterile embryo dishes. The ova and cumuli oophori were transferred using a syringe and catheter into further embryo dishes containing 1ml of Medicult IVF

medium pre-equilibrated in 5% CO_2 at 37°C. Between 15-20 ova were placed in each dish ready for sperm insemination.

2.3.6 Preparation of Mouse Sperm for IVF of Mouse Eggs.

Male CD1 mice were sacrificed by cervical dislocation and their vas deferens removed and placed in 1ml of PBS in an embryo dish. Vas deferens were chopped finely with a dissection scissors and incubated at 37°C for 1.5 h. The motile sperm that had swum free of the tissue was harvested and placed in a centrifuge tube and spun at 500g for 5 min. The supernatant was decanted off and sperm were resuspended in the remaining liquid in the centrifuge tube. Sperm were then layered under 1ml of Medicult IVF medium pre-equilibrated in 5% CO_2 at 37°C using a glass pipette for 2h at 37°C. The motile sperm, which had swum into the Midicult medium layer, were removed with a clean glass pipette and counted on a Makler sperm counting chamber. Sperm preparations were now ready for insemination of ova.

2.3.7 Effects of Anti-CRISP1 Antibodies Bound to Sperm on Ova Insemination.

Sperm (2×10^7) prepared as in previous section 2.3.6 were incubated with either 2mg of rabbit anti-CRISP1 or 2mg of antibody derived from pre-immune rabbit serum (antibodies were purified on protein A-agarose as described in section 2.3.5) and incubated at 37°C for 1h. After incubation 5×10^5 sperm were added per egg to the embryo dishes containing mouse ova in Medicult IVF media (section 2.3.5) and incubated in 5% CO_2 at 37°C for 48h. Dishes were viewed under 200x magnification and fertilization scored. Two-cell and four-cell embryos were classified as fertilized whereas ova displaying single pro-nucleii were classified as unfertilized. Ova showing disintegration or other anomalies were excluded from the analysis.

2.4 Identification of Antigens in Seminal Plasma and on the Sperm Surface.

2.4.1 Separation of ASA Reactive Antigens in Seminal Plasma by Chromatofocusing.

Spermatozoa were removed from seminal plasma as described in section 2.2.3. Seminal plasma (10ml) was then dialysed extensively against 3 x 5 L of de-ionised water at 4°C The seminal plasma was then centrifuged at 10,000g for 10 min at 4°C and the supernatant retained. An equal volume of 0.05M imidazole buffer pH 7.8 was added the supernatant and then applied to a chromatofocusing column of PBE 94 (0.8 x 10cm) previously equilibrated with 0.025M imidazole buffer pH 7.8 at 4°C. Polybuffer pH 4 (5ml), diluted 8-fold to give 14 column volumes was run through before the application of the sample so as to avoid exposing the sample to any extremes of pH. Dialysed seminal plasma was applied to the matrix and the remaining diluted polybuffer 94, pH 5 applied to the matrix to generate a pH gradient. Fractions were collected (3ml) and analysed by SDS-PAGE as in section 2.2.4.

2.4.2 Preparation of Antibodies to Seminal Plasma Antigen from Chromatofocusing.

Large preparative 15% SDS-PAGE gels were prepared and samples from the chromatofocusing column (section 2.4.1) were loaded. Gels were run as before and stained with Coomassie Brilliant Blue. Bands of interest were excised and electroeluted on a BioRad Model422 Electroeluter. Electroeluted material was mixed 1:1 with freund's complete adjuvant and the same inoculation procedure undertaken as in section 2.3.4. Blood was obtained as before by cardiac puncture and processed as in section 2.3.4. Electroeluted material was also digested with trypsin and analysed by mass spectrometry as in section 2.2.12.

2.4.3 Pull Down of Sperm Surface Antigens with Protein G.

Sperm were prepared as in section 2.2.3. Sperm were resuspended at 1 x 10^8/ml in PBS and 50µl of ASA positive patient sera or negative control sera as prepared in section 2.2.7.1 was added. Sperm were incubated at 37°C for 1 hr and harvested by

centrifugation at 600g followed by x3 washes in PBS. Sperm were finally resuspended in 1% (v/v) NP-40 in PBS and incubated at room temperature for 1 hr with continuous agitation. Sperm were removed by centrifugation at 13,000g and the supernatant removed to a clean tube. Protein G-sepharose slurry (100µl) was added and incubated for 1 hr with continuous agitation at 4°C. Protein G was sedimented by centrifugation at 600g for 2 min and washed with 1ml of 1% (v/v) NP-40 in PBS followed by 2 washes in PBS. Bound antibody:protein complexes were eluted using 1M glycine pH 3 and neutralized by addition of 50µl/ml of 0.1M Tris-HCl. Eluted material was digested with trypsin and analysed by mass spectrometry as in section 2.2.12.

2.5.1 Silver Staining of SDS-PAGE gels.

SDS-PAGE gels were run as in section 2.2.4. All further steps were performed with constant agitation on an orbital shaker. Gels were removed from glass plates and washed in deionized water for 5 min. Gels were then soaked in ethanol:acetic acid:water (40:10:50) for 1 hr, followed by soaking in ethanol:acetic acid:water (5:5:90) for 2 hr or overnight. Gels were washed in deionized water for 5 min and the soaked in 1% (v/v) glutaraldehyde in 0.5M sodium acetate for 30 min. Gels were washed x3 in deionized water for 5 min and the soaked twice in 0.05% (w/v) 2,7 napthalene-disulphonic acid for 30 min followed by 4x washes in deionized water for 15 min each. Gels were then stained in ammoniacal silver nitrate solution for 30 min. Ammoniacal silver solution was made fresh just before use as follows; to prepare 75ml of solution, 0.6g silver nitrate was dissolved in 3ml water, this was then slowly mixed into a solution containing 16ml water, 1ml concentrated ammonia (25%) and 0.15ml of 10N sodium hydroxide. After the transient brown precipitate had disappeared it was made to volume with water.

After staining, gels were washed x4 in deionized water for 4 min each and developed using a solution of 0.01% (w/v) citric acid and 0.1% (v/v) formaldehyde in water for approx 5 min or until staining is complete. Development was stopped using a solution of 5% (w/v) Tris and 2% (v/v) acetic acid in water.

2.6.1 Purification of Semenogellin I and II using FPLC Heparin Affinity Chromatography.

Semenogelin I and II were purified as described in Malm et al. (1996) with modifications. In brief, ejaculates from healthy volunteers were collected into tubes containing 17ml ice-cold 40mM Tris-HCl pH 9 supplemented with 4M urea, 25mM EDTA, 30mM dithiothreitol (DTT) and 100µl protease inhibitor cocktail (Calbiochem). Tubes were held on ice during gel solubilisation and then sperm were removed by centrifugation at 20,000g for 10min at 4°C. The resulting supernatant (2ml) was applied to a FPLC heparin-sepharose column (1ml packed volume) pre-equilibrated in equilibration buffer (100mM Tris-HCl pH 8.7, 5mM EDTA, 10mM DTT and 100µl/L protease inhibitor cocktail). The column was washed with 10 column volumes of equilibration buffer and bound proteins eluted using a 30 column volume gradient of 0-300mM NaCl in equilibration buffer and 0.5ml fractions collected and analysed by SDS-PAGE as in section 2.2.4.

2.6.1.1 Digestion of Semenogelin 1 and 2 with Prostate Specific Antigen (PSA) and the Effects of Cleavage Products on PBMC Proliferation.

Semenogelin 1 and 2 were isolated as in section 2.6.1. Fractions containg Semenogelin 1 or 2 were pooled and 500µl removed to a clean tube to which 2U of PSA was added and incubated at 37°C. Aliquots were removed at 3, 6 and 24 hr and stored at –20°C until used. Each time point was tested for suppression of mitogen induced proliferation of PBMCs as in section 2.11.1 using 25µl/well of each digest time point.

2.7.1 Preparation of Glycolipids from Spermatozoon Membrane.

Spermatozoa of patients with normal semen parameters were collected by masturbation and allowed liquefy for 30min at 37°C. Spermatozoa were removed from seminal plasma by centrifugation at 600g and washed X3 in PBS. Spermatozoal pellets were extracted in Water:Methanol:Chloroform (3:10.8:5.4) (per gram of pellet) with homogenization and the suspension centrifuged at 2200g for 15 min in a Sorvall RT6000B centrifuge in glass centrifuge tubes. The upper phase was removed and the

pellet re-extracted as before and centrifuged. Upper phases were pooled into a boiling tube and heated to 95°C in a water bath till nearly dry, then frozen at –70°C and remaining solvents removed under vacuum on an Edwards "Modulyo" freeze dryer. The resulting pellet was dissolved in methanol and centrifuged to remove any non-dissolved material. Glycolipid preparations were stored at –70°C until used.

2.7.2 Thin Layer Chromatography of Glycolipids.

Glycolipid preparation (10µl) was spotted on Whatman 250µm PE SiL G chromatography plates with polyacrylic binder and run in solvent containing, Chloroform: Methanol: 0.25% KCl (12:7:1.6) as described by Magnani et al. (1982). The plates were dried in a stream of hot air and used either for orcinol/resorcinol staining or immunostaining.

2.7.3 Orcinol and Resorcinol Staining of TLC Plates.

Staining with orcinol and resorcinol was carried out as in Schnaar and Needham (1994). In brief, dried TLC plates were stained by spraying with orcinol (2% w/v orcinol in 2M H_2SO_4) and heating at 110°C for 30 min. Orcinol stain was stored for one week at room temperature in the dark. For resorcinol staining plates were sprayed with resorcinol reagent (1.2g resorcinol was dissolved in 10ml H_2O and 80 ml conc. HCl with 0.5ml of 0.1M $CuSO_4$ added. Reagent was made to 100ml and stored for at least 4 hr. Resorcinol stained plates were heated to 120°C for 30 min. Resorcinol stain was stored for one week at room temperature in the dark.

2.7.4 Immunoglobulin G purification from Patient Sera for Glycolipid Blotting.

Antibody purification was carried out as in Harlow and Lane (2006). In brief, protein G-sepharose 4B Fast Flow (2ml) was equilibrated in 100mM Tris-HCl (pH 8). Patient sera was adjusted to pH 8 by addition if $1/10^{th}$ volume of 1M Tris-HCl (pH 8) and applied to the column. Column was washed with equilibration buffer and IgG eluted with 100mM Glycine-HCl (pH 3) and 500µl fractions collected into tubes containing 50µl 1M Tris-HCl (pH 8) to restore physiological pH. The immunoglobulin

fractions were identified by absorbance at 280nm (1 OD= 0.8mg/ml). Columns were regenerated using 10 column volumes of 2M Urea followed by 10 column volumes of 100mM glycine (pH 2.5). The columns were re-equilibrated in 100mM Tris-HCl (pH 8).

2.7.5 Immunostaining of Thin Layer Chromatograms.

Glycolipids were run on TLC plates as described previously in section 2.7.2. Plates were blocked in 5% (w/v) Marvel, 1% (w/v) polyvinylpyrollidone (PVP) in PBS at 4°C for 2 hr. Plates were washed once in 1% (w/v) PVP in PBS before addition of primary antibody. A 2mg/ml solution of primary antibody (purified antibodies from patient sera as in section 2.7.4) was prepared in 1% (w/v) PVP (50µl/cm^2 of plate). Primary antibody solution was pipetted onto a glass plate and the TLC plate carefully placed face down into the solution and kept at room temperature in a humid environment for 2 hr. Plates were washed x3 in 1% (w/v) PVP in PBS and then placed face down as previously described into secondary antibody (1/7500 dilution of goat anti-human IgG horseradish peroxidase (HRP) conjugate in 1% (w/v) PVP in PBS) Plates were washed x4 in 1% (w/v) PVP in PBS and reactive bands visualized using substrate buffer (0.4mg/ml diaminobenzidine, 0.004% v/v hydrogen peroxide in PBS) at 30°C until bands appeared.

2.7.6 Direct Enzyme Linked Immunosorbant Assay (ELISA) of ASAs reacting with Glycolipids.

ELISA plates (Immobilon 1 flat bottom plates) were coated with either coated with 40µl of methanol as control or 40µl of glycolipid extract and incubated at 30°C overnight until dry. Wells were blocked in 5% (w/v) Marvel, 1% (w/v) PVP in PBS at 4°C for 3 hr. Wells were washed once with 100µl of 1% (w/v) PVP in PBS before addition of primary antibody. A 2mg/ml solution of primary antibody (purified antibodies from patient sera as in section 2.7.4) was prepared in 2% (w/v) Marvel, 1% (w/v) PVP and 100µl added to each well and incubated overnight at 30°C in a humidified chamber. Wells were washed x3 with 200µl of 1% (w/v) PVP in PBS before addition of secondary antibody. Secondary antibody (1 in 8000 goat anti-human IgG HRP conjugate) was made up in 1% (w/v) PVP in PBS and 100µl added to each well

and incubated at 30°C for 2 hr. Each well was washed x3 with 1% (w/v) PVP in PBS. A substrate solution was prepared (0.4mg/ml o-phenylenediamine in 50mM Na_2HPO_4 and 25mM citric acid (pH4.5). with 0.006% (v/v) hydrogen peroxide) and 100µl added per well and incubated at 30°C for 30min and the reaction stopped with the addition of 50µl of 1.25M sulphuric acid. Plates were read on a 96-well plate reader at 490nm.

2.7.7 Dot Blotting of Glycolipids with ASAs on Immobilon-P (PVDF) Membranes.

Glycolipids were prepared as before in section 2.7.1 and 10µl spotted onto PVDF membrane squares of 2x2cm. Methanol (10µl) was also spotted onto a PVDF square as control. The membranes were dried in a stream of hot air and blocked using 5% (w/v) Marvel, 1% (w/v) PVP in PBS for 2 hr at room temperature. A 2mg/ml solution of primary antibody (purified antibodies from patient sera as in section 2.7.4) was prepared in 2% (w/v) Marvel, 1% (w/v) PVP (50µl/cm^2 of membrane) and pipetted onto a glass plate and the membrane layered face down into the solution. After 2 hr the membranes were washed x6 in 1% (w/v) PVP in PBS and then again layered face down into a solution of secondary antibody (1 in 8000 goat anti-human IgG HRP conjugate in 1% (w/v) PVP in PBS) (50µl/cm^2) for 2 hr at room temperature. Membranes were washed x6 in 1% (w/v) PVP in PBS and incubated face down as before in chemiluminescent substrate solution (approx 2ml) and imaged onto X-Omat scientific imaging film as described in section 2.2.6.2. Dot intensities were analysed by scanning densitometry using the "Gel Works" software package.

2.7.8 Determination of ASA Specificity for Glycolipid Oligosaccharides.

Glycolipid preparations (5µl) as previously described in section 2.7.1 were spotted in triplicate on PVDF (2 x 3.5cm) and dried in a stream of hot air in preparation for enzymic digestion of the oligosaccharides or binding of oligosaccharides with the lectin, concanavalin A (ConA).

2.7.8.1 Neurominadase Treatment of PVDF Bound Glycolipids.

PVDF membranes spotted in triplicate with glycolipids as described in section 2.7.7 were incubated in a solution of 0.02U/ml Neuraminadase in 0.05M sodium acetate buffer (pH 5.5) or in buffer alone for 1.5 hr at 30°C. Membranes were washed x4 in 1% (w/v) PVP in PBS before being blocked, stained and imaged as in section 2.7.7.

2.7.8.2 Galactosidase Treatment of PVDF Bound Glycolipids.

PVDF membranes spotted in triplicate with glycolipids as described in section 2.7.7 were incubated in a solution of 0.02U/ml Galactosidase (derived from *E. freundii*) in 0.05M Tris-HCl buffer (pH 7.3) containing 0.05M NaCl and 1mM $MgCl_2$ or in buffer alone for 1.5 hr at 30°C. Membranes were washed x4 in 1% (w/v) PVP in PBS before being blocked, stained and imaged as in section 2.7.7.

2.7.8.3 Fucosidase Treatment of PVDF Bound Glycolipids.

PVDF membranes spotted in triplicate with glycolipids as described in section 2.7.7 were incubated in a solution of 0.02U/ml Fucosidase in 0.05M potassium phosphate buffer (pH6) with 0.1M NaCl or in buffer alone for 1.5 hr at 30°C. Membranes were washed x4 in 1% (w/v) PVP in PBS before being blocked, stained and imaged as in section 2.7.7.

2.7.8.4 Concanavalin A binding of PVDF Bound Glycolipids.

PVDF membranes spotted in triplicate with glycolipids as described in section 2.7.7 were incubated in a solution of 0.5mg/ml Concanavalin A in 0.01M Tris-HCl buffer (pH7.5) containing 0.15M NaCl, 0.005% (v/v) Tween-20, 1mM $CaCl_2$, 1mM $MgCl_2$ and1mM $MnCl_2$ or in buffer alone for 1.5 hr at 30°C. Membranes were washed x4 in 1% (w/v) PVP in PBS before being blocked, stained and imaged as in section 2.7.7.

2.8.1 Trypan Blue Exclusion Dye as Viability Stain in Cell Culture.

Cells maintained in culture were mixed 1:1 with trypan blue reagent and counted on a haemocytometer (model B.S. 748, Hawksley, England). Cells staining blue were designated as unviable and disregarded.

2.9 Cell Culture and Isolation.

2.9.1 Preparation of Human Peripheral Blood Mononuclear Cells (PBMC).

Whole blood taken from healthy volunteers into sodium heparin tubes was carefully layered onto an equal volume of Histopaque 1077 (1.077 g/ml density) and centrifuged at 500g for 30 min at 20°C. The upper layer of plasma was discarded by aspiration with a pasteur pipette. The opaque central layer, which contains the mononuclear cells, was then transferred to a clean centrifuge tube. RPMI 1640 (40ml) supplemented with 10% FCS, 2mM L-glutamine, 100Iu Penicillin and 100 μg/ml Streptomycin was added and the cell resuspended and the resultant mix centrifuged at 500g for 10 min.

The supernatant was discarded and the pellet resuspended in 10ml supplemented RPMI 1640 and mixed by gentle aspiration. The centrifugation and resuspension procedures were repeated twice and the pellet was finally resuspended in 10ml RPMI 1640 supplemented medium. The cells were counted using the trypan blue exclusion method described in section 2.8.1.

2.9.2 Maintenance of Jurkat Cells in Cell Culture.

Jurkat cells were grown in RPMI 1640 supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100IU Penicillin and 100μg/ml Streptomycin. Cells were incubated in a Forma Scientific incubator at 37°C and 5% CO_2. The cells were split every 72 hr by making a 1:5 dilution of the cells in fresh RPMI 1640 supplemented medium. Cells were counted using trypan blue dye exclusion as described in section 2.8.1.

2.9.3 Maintenance of THP1 Cells in Cell Culture.

The THP1 cells were maintained in culture by the method described in section 2.9.2.

2.9.4 Maintenance of U937 Cells in Cell Culture.

The U937 cells were maintained in culture by the method described in section 2.9.2.

2.9.5 Maintenance of Breast Cancer MCF 7 Cells in Cell Culture.

MCF 7 cells were grown in DMEM supplemented with 2mM L-glutamine, 10% (v/v) FCS and Gentamycin (l00mg/ml). Cells were incubated in a Forma Scientific incubator at 37°C and 5% CO_2. The cells were split every 72 hr and seeded at approximately 1-2 x 10^6 cells/20ml in a 75cm^2 flask.

Prior to splitting, the cells were washed with medium containing no FCS. The adherant MCF 7 cells were removed from the surface of the flasks by incubation with 2ml trypsin (0.05mg/ml)/EDTA for 1-5 min. Following treatment with trypsin, 10ml of supplemented DMEM was added and these cells were then centrifuged at 1000g for 5 min. The supernatant was discarded and the pellet resuspended in 1 ml of supplemented DMEM. Cells were counted using trypan blue dye exclusion as in section 2.8.1.

2.9.6 Maintenance of C6 Glioma Cells in Cell Culture.

The C6 Glioma cells were maintained in culture using the same medium and methods described for MCF7 cells in section 2.9.5.

2.10 Preparation of Human Seminal Plasma for Proliferation Assays.

2.10.1 Preparation of Seminal Plasma for Assay of Proliferation in PBMCs and Cell Lines.

The human seminal plasma used throughout the duration of this experiment was obtained by masturbation from patients with normal sperm parameters or presenting with antisperm antibodies in the Human Assisted Reproduction Ireland fertility clinic, Rotunda Hospital.

The samples were passed through a 20 gauge sterile syringe approximately ten times. The sample was then centrifuged at 5,000g for 15 min, at which point the seminal plasma was removed as supernatant and stored at -20°C for further use.

2.10.2 Preparation of Fractions of Seminal Plasma by Size Exclusion Amicon Filters.

Seminal Plasma as prepared in section 2.10.1 (2ml) was added to an amicon 100kDa concentrator sample resevoir. The samples were spun in a Sorvall MC 12V centrifuge at 3,000g for 4 hr at 4°C. The filtrate was removed and added to an amicon 3kDa concentrator and centrifugation repeated. The retentate from the 100kDa filter was made up to 2ml with PBS, respun in the 100kDa filter and the retentate made up to 2ml in PBS to ensure the >100kDa constituents of the seminal plasma were at original concentrations. The filtrate of the 3kDa filter was stored at –20°C and the retentate made to original volume with PBS and respun as before on the 3kDa filter. The resulting retentate was made to original volume, labeled 3-100kDa seminal plasma, and stored at –20°C until used. This process yielded three seminal plasma fractions, <3kDa seminal plasma, 3-100kDa seminal plasma and >100kDa seminal plasma.

2.10.3 Lipid and Aqueous Phase Extraction of <3kDa fraction of Seminal Plasma.

The <3kDa fraction of human seminal plasma (2ml) as in section 2.10.2 was made up to 5 mls with ethanol. This solution was then acidified to pH 3 with hydrochloric acid and extracted three times with 1ml of ethyl acetate. The ethyl acetate extracts were

then washed with 3 x 1 ml of water until the solution was returned to pH 7 and evaporated to dryness in a water bath at 60°C. The residue was subjected to a three-stage distribution between petroleum ether (3ml) and ethanol-water (2:1). The combined aqueous ethanol phases were evaporated to a small volume, acidified to pH 3 and extracted with ether (Hamberg and Samuelsson, 1966). The resulting extract was then returned to pH 7 and stored at -20°C until used.

2.10.4 Continuous Spectrum of Aqueous and Lipid Phases of the <3kDa fraction of human seminal plasma.

A continuous spectrum of the aqueous and lipid phases of the <3kDa fraction of seminal plasma (section 2.10.3) was performed on a Carey spectrophotometer between 200 and 360nm. The sample was prepared by diluting 50µl of sample in 1 ml of distilled water for the aqueous phase or 1ml of ethanol for the lipid phase in a quartz cuvette.

2.11 PBMC and Cell Line Proliferation Assays.

2.11.1 Suppression of Peripheral Blood Monocytes Cell Proliferation by Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

PBMCs (2 x 10^5 per well) were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100IU Penicillin and 100 µg/ml Streptomycin in the presence of Phytohaemagglutinin (PHA)-P (2.5µg per ml final concentration) in 24 well plates. The lymphocytes were cultured in the presence and absence of hydroxylamine (0.1 mM). The affects of human seminal plasma (0-1.0% v/v) on the proliferation response of the lymphocytes were assessed by incorporation of tritiated thymidine. Cultures were routinely set up in triplicate and before addition of the radioactive proliferation marker the lymphocytes were incubated for 36-48 hr at 37°C in 5% CO_2 in a Forma Scientific incubator.

After the initial period of incubation, the cells were pulsed for 5-6 hr with 1µCi per well of [^{3}H] thymidine (specific activity 5 Ci/mmol). Post pulsing, the cells were

harvested under vacuum on a 12 well manual harvester. The cells were collected on Whatman glass fibre discs and washed extensively with PBS. The filter discs were then placed in translucent plastic scintillation vials. 5 mls of ecoscint scintillation fluid was then added to each vial. The liquid scintillation counting was then carried out in a TriCarb 2100 TR scintillation counter.

2.11.2 Suppression of Jurkat Cell Proliferation by Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

The assay was performed using the method described in section 2.11.1 using Jurkat Lymphoma cells in the absence of PHA.

2.11.3 Suppression of U937 Cell Proliferation by Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

The assay was performed using the method described in section 2.11.1 using U937 cells in the absence of PHA.

2.11.4 Suppression of THP1 Cell Proliferation by Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

The assay was performed using the method described in section 2.11.1 using THP1 cells in the absence of PHA.

2.11.5 Suppression of MCF7 Cell Proliferation by Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

MCF7 cells were cultured and split as in section 2.9.5. The assay was performed using the method described in section 2.11.1 using MCF 7 cells cultured in DMEM supplemented with 2mM L-Glutamine, 10% (v/v) FCS and Gentamycin (l00 mg/ml) in the absence of PHA. Cells were removed from plates using trypsin digestion and harvested as in section 2.11.1.

2.11.6 Suppression of C6 Glioma Cell Proliferation by Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

C6 Glioma cells were cultured and split as in section 2.9.6. The assay was performed using the method described in section 2.9.6 using C6 Glioma cells cultured in DMEM supplemented with 2mM L-Glutamine, 10% (v/v) FCS and Gentamycin (l00 mg/ml) in the absence of PHA. Cells were removed from plates using trypsin digestion and harvested as in section 2.11.1.

2.11.7 Suppression of PBMC, Jurkat, THP1 and U937 Proliferation by Fractionated Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

Cells (2 x 10^5) were plated in supplemented RPMI 1640 as previously described in section 2.11.1 in the presence or absence of 0.1mM hydroxylamine. PBMCs were grown in the presence of 2.5µg/ml PHA-P to induce proliferation. The >100kDa and 3-100kDa fraction were used at ranges of 0-40% original concentration of seminal plasma. The <3kDa fraction was used at 0-1% original concentration of seminal plasma. Cells were treated, harvested and counted as previously described in section 2.11.1.

2.11.8 Suppression PBMC Proliferation by the Aqueous Phase and Organic Phase Separations of <3kDa Fraction of Human Seminal Plasma using [^{3}H] Thymidine Incorporation.

The assay was performed using the method described in section 2.11.1. PBMCs were incubated in 0-1% (original concentration of seminal plasma) of either aqueous or organic phases of the <3kDa fraction of seminal plasma (from section 2.10.3) in the presence of PHA-P (2.5µg/ml). Cells were treated, harvested and counted as previously described in section 2.11.1.

2.12 Further Fractionation of Active <3kDa Seminal Plasma Fraction and Possible Spermine Contamination of Active Fraction.

2.12.1 Further Fractionation of the <3kDa Fraction of Human Seminal Plasma on FPLC Superdex Peptide 10/300GL Column.

Seminal Plasma <3kDa fraction (0.5ml) (as in section 2.10.2) was loaded onto a Superdex Peptide 10/300GL column pre-equilibrated in 50mM Tris-HCl, 150mM NaCl and 1mM β-mercaptoethanol pH 7.5 on an AKTA purifier FPLC and eluted with the same buffer. Flow rate was maintained at 0.5ml/min and 0.5ml fractions were collected for a total of 1.25 column volumes. Absorbance at A_{280} and A_{210} were recorded. Fractions were analysed for anti-proliferative activity with and without 0.1mM hydroxylamine as in section 2.11.1 and for protein content by Tris-Tricine SDS-PAGE as in section 2.2.5.

2.12.2 Determination of Elution Time of Spermine on a FPLC Superdex Peptide 10/300GL Column.

A stock solution of spermine (25mM in water) was diluted to 5mM in PBS and was loaded onto a Superdex Peptide 10/300GL column pre-equilibrated in PBS on an AKTA purifier FPLC and eluted with the same buffer. Flow rate was maintained at 0.5ml/min and 0.5ml fractions were collected for a total of 1.25 column volumes. Absorbance at A_{280} and A_{210} were recorded.

2.12.3 Fractionation of the <3kDa Fraction of Human Seminal Plasma Pre-Treated with Pronase on FPLC Superdex Peptide 10/300GL Column.

Seminal Plasma <3kDa fraction (0.5ml) (as in section 2.10.2) was pre-treated with 10µl of Pronase (10mg/ml in 30mM HEPES and 10mM $CaCl_2$ pH 7.5) overnight at room temperature. A control tube of the <3kDa fraction with only pronase buffer solution added was also made. These were the loaded onto a Superdex Peptide 10/300GL column pre-equilibrated in 30mM HEPES and 10mM $CaCl_2$ pH 7.5 on an AKTA purifier FPLC and eluted with the same buffer. Flow rate was maintained at 0.5ml/min and 0.5ml fractions were collected for a total of 1.25 column volumes.

Absorbance at A_{280} and A_{210} were recorded. Fractions were analysed for anti-proliferative activity with and without 0.1mM hydroxylamine as in section 2.11.1.

2.12.4 Detection of Spermine and Other Polyamines by Thin Layer Chromatography.

Detection of spermine and other polyamines were assayed as in Shohat et al. (1990). In brief, sodium carbonate (0.01mg) was added to each fraction (100µl) from the FPLC peptide columns from sections 2.12.2 and 2.12.3 in an ependorf tube. Dansyl chloride (200µl) (25mg/ml in acetone) was added to each sample and incubated overnight at room temperature in the dark. Toluene (250µl) was added to each sample and vortexed thoroughly and centrifuged for 10 min at 5000g. The upper phase was removed and spotted (50µl) onto Silica GL20 TLC plates and separation achieved using ethyl acetate:cyclohexane (2:3) as mobile phase. The plates were dried under a stream of hot air and visualized under UV light. A stock solution of spermine (25mg/ml) was diluted 1 in 10 in water and underwent the same steps and acted as positive control.

2.13 Determination of Physical Characteristics and Properties of Active Compound in <3kDa FPLC Fractionated Samples.

2.13.1 Effects of Proteolysis on the PBMC Anti-Proliferative Affects of the Active Peptide Fraction from FPLC Gel Filtration Column.

The <3kDa fraction of seminal plasma was applied to a peptide gel filtration column as in section 2.12.1 and the fractions analysed for PBMC anti-proliferative affects as in section 2.11.1. The active fraction was then subjected to proteolysis. Trypsin, trypsin inhibitor and *S. aureus* non-specific protease were made up at 100µg/ml in 50mM Tris-HCl, 150mM NaCl and 5mM $CaCl_2$. The active fraction from FPLC was aliquoted into 50µl amounts in 3 tubes and the following additions made; to tube 1, 1µl trypsin was added, to tube 2, 2µl of trypsin inihibitor follwed by 1µl trypsin and finally to tube 3, 1µl of *S. aureus* non-specific protease. Tubes were incubated overnight at 37°C and tested for anti-proliferative effects on PBMCs as in section 2.11.1.

2.13.2 Determination of the Ionic and Affinity Profiles of the Seminal Plasma Anti-Proliferative Peptide Present in FPLC Fractions.

2.13.2.1 Peptide Binding to CM-Sepharose.

Active fraction (100µl) was mixed with 50µl of CM-sepharose (equilibrated in 50mM Tris-HCl pH 7.5) on ice for 30 min with occasional agitation. The mixture was centrifuged at 500g for 3 min and the supernatant was then tested for activity on PBMC proliferation using the [^{3}H] Thymidine assay as described in section 2.11.1 and a 20 µl sample was run on high cross linked 16% Tris-Tricine SDS-PAGE gel as described in section 2.2.5.

2.13.2.2 Peptide Binding to DEAE-Sepharose.

Active fraction (100µl) was mixed with 50µl of DEAE-sepharose (equilibrated in 50mM Tris-HCl pH 7.5) on ice for 30 min with occasional agitation. The mixture was centrifuged at 500g for 3 min and the supernatant was then tested for activity on PBMC proliferation using the [^{3}H] Thymidine assay as described in section 2.11.1 and a 20 µl sample was run on high cross linked 16% Tris-Tricine SDS-PAGE gel as described in section 2.2.5.

2.13.2.3 Peptide Binding to Nickel-Sepharose.

Active fraction (100µl) was mixed with 50µl of nickel-sepharose (equilibrated in 50mM Tris-HCl pH 7.5) on ice for 30 min with occasional agitation. The mixture was centrifuged at 500g for 3 min and the supernatant was then tested for activity on PBMC proliferation using the [^{3}H] Thymidine assay as described in section 2.11.1 and a 20 µl sample was run on high cross linked 16% Tris-Tricine SDS-PAGE gel as described in section 2.2.5.

2.13.3 Determination of Peptide Fraction Affinity for Divalent Metals Using Murexide.

Murexide (50μM) was made up in 10mM Tris-HCI, pH 7.4. The spectrum of an 800μl sample of this solution was recorded on an Agilent 8453 spectrophotometer from 300nm to 700nm in a 1 ml cuvette. 100μl of a 500μM of nickel chloride was then added to the cuvette and mixed. The spectrum of this solution was then recorded from 300-700nm. 100μl of a 500μM EDTA was then added to the cuvette and mixed. The spectrum was again recorded from 300-700nm.

In a fresh cuvette, the experiment was repeated except in place of 100μl of EDTA, 20μl of active peptide fraction was added and the spectrum was recorded as before from 300-700nm.

These two steps were then repeated for different divalent metal ions, including zinc chloride, cobalt chloride, copper chloride and ferrous sulphate, all using 100μl of a 500μM solution. Aprotinin (50μM) was used in place of the peptide fraction as a negative control.

2.14 Preparation of Cytospin Slides and Rowmanowsky Staining of PBMCs and Immunological Cell Lines Treated with Active Peptide Fraction.

2.14.1 Cell Treatment and Slide Preparation.

Cell lines (Jurkat, THP1 and U937) (2 x 10^5/ml) were treated with 1% (v/v) of active peptide fraction from FPLC of the <3kDa fraction of human seminal plasma (as in section 2.11.1) in the presence of 100μM hydroxylamine. PBMCs (2 x 10^5) were also treated with PHA-P (2.5μg/ml) in the presence of 100μM hydroxylamine. Incubations were carried out for 0-24hr. Cells were pelleted by centrifugation (1200g for 5 min at 4°C) and resuspended in 250μl of cold PBS. Cells were applied to a cytospin and centrifuged onto slides at 1,200g for 6 min.

2.14.2 Romanowsky Staining of Slides.

Slides prepared as in section 2.14.1 were fixed by immersion in methanol. After drying, the slide was transferred to an acidic dye (Eosin Y in phosphate buffer) and stained for 15-30 sec by slowly agitating the slide in the solution. Without rinsing, the slide was transferred to a basic dye (methylene blue in phosphate buffer) and the staining repeated as per the acidic dye. Each slide was rinsed in water and allowed to air dry. Finally each slide was examined using a Heiss light microscope and cells were photographed using an Olympus SLR camera attached to the microcope.

2.15 Effects of Active Peptide Fraction on Cell Cycle and Cell Death.

2.15.1 FACS Analysis of the Effect on Cell Cycle of Immunological Cell Lines and PBMCs of Active Peptide Fraction.

Cell lines (Jurkat, THP1 and U937) (5 x 10^5) were treated with 1% (v/v) of active peptide fraction from FPLC of the <3kDa fraction of human seminal plasma (as in section 2.12.1) in the presence of 100μM hydroxylamine for varying time periods 0-24hr. PBMCs (2 x 10^5) were also treated with 1% (v/v) of active peptide fraction from FPLC of the <3kDa fraction of human seminal plasma (as in section 2.12.1) with and without PHA-P (2.5μg/ml) in the presence of 100μM hydroxylamine and cell cycle analysed at 24 hr.

At designated time points, the cells were spun down at 500g for 5 min in an Eppendorf 5804 Centrifuge. The supernatant was then poured off and the cells were resuspended in 1 ml of ice cold PBS. The cells were then spun again at 500g for 5 min and the supernatant was removed.

The pellet was resuspended in 200μl of ice cold PBS and the volume made up to 2ml with 70% (v/v) ethanol in PBS. These cells were then stored at 4°C until the day they were read. The fixed cells were spun down at 220g for 5 min and the supernatant was removed. The cells were resuspended in 800μl of PBS. RNase A (100μl of a 10mg/ml solution) and propidium iodide (100μl of a 1 mg/ml solution) was added to

each tube. The tubes were then briefly vortexed, covered with foil and incubated at 37°C for 30 min. The samples were then read using a Becton Dickinson FACS Calibur flow cytometer with Cellquest software.

2.15.2 Detection of DNA Laddering in Cells Treated with Active Peptide Fraction.

Active peptide fraction (200ul) was added to either 20ml of PBMCs (3.5 x 105 cells/ ml) or Jurkat cells (3.5 x 105 cells/ ml), giving a concentration of active peptide equivalent of 1% (v/v). Cells were incubated with peptide for the required time (24 or 48 hr) at 37°C and 5% CO_2. Cells were then spun at 500g for 5 min in an Eppendorf 5804 Centrifuge. The supernatant was removed and the cells were gently resuspended in lml of lysis buffer (20mM EDTA, 100mM Tris-HCl pH 8.0, 0.8% (w/v) sodium lauryl sarcosinate) and left for one hr at 37°C. RNase A (50µl of a 10mg/ml solution) was added and the cell were left for 2.5 hr at 37°C.

Proteinase K (300µl of a 20mg/ml solution) was added and the cells were left at 37°C overnight. 45µl of this DNA sample was added to 5µl loading dye (0.09% bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol, 60mM EDTA) and run on a 1.5 % (w/v) agarose gel in l x TBE (90mM Tris, 90mM Boric acid, 2.5mM EDTA) at 55 V for 4 hr using 1 x TBE as running buffer. The gel was then stained in ethidium bromide and visualized under UV light.

2.15.3 Fluorogenic Assay of Caspase 3 Activation with Varying Concentrations of Seminal Peptide Fraction.

Cell lines (Jurkat, THP1 and U937) (2 x 10^6) were incubated in various concentrations of FPLC active peptide fraction (from section 2.12.1)(0-1%) for 24 hr. The cells were then harvested by centrifugation at 500g at 4°C for 5 min. The cells were washed with ice coId PBS and the pellets resuspended in 200µl of harvesting buffer (20mM of HEPES at pH 7.5, 10% (w/v) sucrose, 0.1% (w/v) CHAPS, 2mM DTT, 0.1% (v/v) NP-40, 1mM sodium EDTA, 1mM phenymethylsulfonylfluoride (PMSF), l µg/ml leupeptin and 1µg/ml pepstatin A). The cells were then incubated at 4°C for 10 min, the

samples were then passed up and down through a 21 gauge needle a number of times. The homogenates were then incubated for a further 10 min at 4°C, after which the samples were centrifuged (minifuge) at 15,000g (4°C) for 15 min.

The supernatants were measured for protein concentration using the method described in section 2.20. Ac-DEVD-AMC (final concentration 20mM) and 100µg of protein from each sample were made up to 3 mls with incubation buffer (100mM of HEPES at pH 7.5, 10% sucrose, 0.1% (w/v) CHAPS and 10mM DTT) and incubated for 60 min at room temperature.

Following incubation the fluoresence was determined using a spectrofluorimeter at excitation wavelength 380nm and emission wavelength 460nm. The amount of amino-4-methyl coumarin released was then determined by comparison with a standard curve generated from known concentations of amino-4-methyl coumarin.

2.15.4 Lactate Dehydrogenase (LDH) Cytotoxicity Assay.

Cell lines (Jurkat, THP1 and U937) or PBMCs were seeded down at a concentration of 2 x 10^5/100ul in a 96 well plate. The cells were then treated with varying concentration of seminal plasma peptide fraction (0-1 % v/v). After 48 hr the plate was centrifuged (Sorvall Minifuge) at 600g and 4°C for 5 min. Supernatant (50µl) was removed and to this was added 50µl of substrate mix from the LDH assay kit (lyophilised diaphorase, lactate, and NAD^+) was added, and the plate was left covered at room temperature for 20 min. After this time, 50µl of stop solution (0.1M NaOH) was added to each well. The absorbance of each sample was measured spectrophotometrically at a wavelength of 490nm using a Molecular Devices microplate reader via the application Softmax, version 2.3.

2.15.5 Peptide Activity on Jurkat Cells Using the WST -1 Proliferation Assay.

2.15.5.1 Time Course of Effects on Cell Proliferation by Peptide Fraction Using the WST -1 Assay.

Jurkat cells were seeded down at a concentration of $5x10^4$ cells/100µl in supplemented RPMI 1640 medium in a 96 well plate. Active peptide was added to the cells at a concentration equivalent to 1 % (v/v) of that in seminal plasma. After 1 hr WST-1 reagent (10µl) was added to each well. The absorbance of the samples was read at various time points after this addition at 450nm-650nm using a Molecular Devices microplate reader.

2.15.5.2 Effect of Peptide Fraction on Jurkat cells Pre-treated with Actinomycin D or Cycloheximide.

Jurkat Cells were seeded down at a concentration of 5 x 10^4 cell in 80µl in a 96 well plate. Actinomycin D or cycloheximide (10µl of a 5µg/ml solution) was added to the cells and the cells incubated for 1 hr at 37°C and 5% CO_2. After this time, active peptide fraction was added to give a final concentration of 1 % of that in seminal plasma. The cells were then incubated at 37°C for 1 hr at which time WST-1 reagent (l0µl) was added. The absorbance of the samples was read at various time points after this at 450nm-650nm using a Molecular Devices microplate reader.

2.15.5.3 Effect of Peptide Fraction on Jurkat Cells in Cell Cycle Arrest.

Jurkat cells were seeded down at a concentration of $5x10^4$ cells in 80µl. Mimosine (10µl of a 300µM solution) was added to the cells and they were incubated at 37°C and 5% CO_2 for 24 hr. After this time active peptide fraction was added to give a working concentration of 1 % of that in seminal plasma. The cells were incubated at 37°C for one hr and then WST-1 reagent (10µI) was added. The absorbance of the samples was read at various time points after that at 450nm-650nm using a Molecular Devices microplate reader.

2.15.6 Romanowsky Staining of Jurkat Cells Pre-treated with Actinomycin D, Cyclohexamide, Mimosine or BAPTA-AM Before Addition Of Peptide Fraction.

Jurkat cells (2 x 10^5/ml) were pre-treated with cyclohexamide, actinomycin D and mimosine as described in sections 2.15.5.2 and 2.15.5.3. For BAPTA-AM treatment a 1 in 10 dilution of a 500μM stock was added to the cells and the cells incubated for 1 hr at 37°C and 5% CO_2. After this time, active peptide was added to give a final concentration of 1 % of that in seminal plasma and incubated for 8 hr. Cells were spun onto slides as in section 2.14.1 and stained as in section 2.14.2.

2.15.7 Determination of the Effects of the Active Peptide Fraction on Cytokine Release from PBMCs after Stimulation.

PBMCs were seeded at 1 x 10^6 cells/ml in RPMI 1640 supplemented media and treated with or without 1% (v/v) active peptide fraction for 2 hr prior to the addition of either PHA-P (2.5μg/ml), lipopolysaccharide (LPS) (10ng/ml) or TNFα (25ng/ml). After 24 hr the cells were transferred to tubes and harvested by centrifugation at 13,000g. The supernatant was removed and stored for assay at –20°C. Supernatants were diluted 1 in 5 for IL-8 ELISA and used un-diluted for IL-10. ELISA assays for IL-8 and IL-10 were then performed as per manufacturer's instructions using supplied IL-8 and IL-10 as standards.

2.16 Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) of the Active Peptide Fraction from Gel Filtration FPLC.

RP-HPLC was carried out on a Agilent 1100 HPLC system with an Aquapore C_{18} column (300 x 1.2mm) at a flow rate of 0.2 ml/min. The column was first pre-equilibrated with mobile phase (0.1% (v/v) in water) and then loaded with 100µl of active peptide fraction from the Peptide column FPLC (section 2.12.1). Specific elution of peptides was achieved using a non-linear gradient of mobile phase and solvent B (Acetonitrile:Water:Trifluoroacetic acid 700:200:0.9) as outlined below in table 2.8.

Time (min)	%Solvent B
0	0
5	25
25	75
30	100

Table 2.8 Non-Linear HPLC Elution Gradient

Fractions (0.5ml) were collected and dried in a cytospin and then re-dissolved in 100µl PBS and tested for activity on PBMCs as in section 2.11.2. Active fractions were treated with trypsin as in section 2.2.12 and subjected to mass spectrometric analysis.

2.17 Cloning of Semenogelin Fragments and Rat SV-IV.

The following sequences from Rat SV-IV and human semenogelins 1 and 2 were formulated from known cleavage products of semenogelins (in black) and protease site and strep-tag added (in red). Sequences were reverse translated and ordered from Genescript Corp with an N-terminal *Nco* I site and a C-terminal *Xho* I site and supplied in pUC57 plasmids.

Rat SV-IV

GRKTKEKYSQSEEVVSESFASGPSSGSSDDELVRDKPYGPKVSGGSFGEEASEEI
SSRRSKHISRSSGGSNMEGESSYAKKKRSRFAQDVLNLEVLFQGPWSHPQFEK

SEM1 164-283 (from semenogelin 1)

ERLWVHGLSKEQTSVSGAQKGRKQGGSQSSYVLQTEELVANKQQRETKNSHQ
NKGHYQNVVEVREEHSSKVQTSLCPAHQDKLQHGSKDIFSTQDELLVYNKNQ
HQTKNLNQDQQHGRKALEVLFQGPWSHPQFEK

SEM1 264-377 (from semenogelin 1)

NKNQHQTKNLNQDQQHGRKANKISYQSSSTEERRLHYGENGVQKDVSQSSIYS
QTEEKAQGKSQKQITIPSQEQEHSQKANKISYQSSSTEERRLHYGENGVQKDVS
QRSIYSQLEVLFQGPWSHPQFEK

SEM1 274-358 (from semenogelin 1)
NQDQQHGRKANKISYQSSSTEERRLHYGENGVQKDVSQSSIYSQTEEKAQGKS
QKQITIPSQEQEHSQKANKISYQSSSTEERRLLEVLFQGPWSHPQFEK

SEM1-inhibin92 (from semenogelin 1)

TYHVDANDHDQSRKSQQYDLNALHKTTKSQRHLGGSQQLLHNKQEGRDHDK
SKGHFHRVVIHHKGGKAHRGTQNPSQDQGNSPSGKGISSQYLEVLFQGPWSHP
QFEK

SEM2 (from semenogelin 2)

NKNQHQTKNLSQDQEHGRKAHKISYPSSRTEERQLHHGEKSVQKDVSKGSISIQ
TEEKIHGKSQNQVTIHSQDQEHGHKENKISYQSSSTEERHLNCGEKGIQKGVSK
GSISIQLEVLFQGPWSHPQFEK

2.17.1 Transformation of *E.coli* with Supplied Plasmids Containing Semenogelin and SV-IV.

The pUC57 Plasmids containing semenogelin fragments and SV-IV (2µl) were gently mixed with 20µl of thawed DH5α *E. coli* cells and placed on ice for 30 min. Cells were heat shocked through placing in 42°C bath for 3 mintues. Cells were then placed on ice for a further 5 min. LB broth (100µl) was added to cells and placed at 37°C for 30 min. The cultures were plated out on a plate containing 100 ug/ml ampicillin and incubated overnight at 37°C. An aliquot of pET28b plasmid was also transformed into DH5α cells as above and grown on plates containing 30 µg/ml kanamycin.

2.17.2 Overnight Culture of Semenogelin and SV-IV Containing Plasmids for Plasmid Miniprep.

A single colony was looped from each plate and incubated overnight in 5ml LB broth containg 100 µg/ml ampicillin (except for pET28b which was grown in 30 µg/ml kanamycin) at 37°C at 150 rpm. Cells were then centrifuged at 4000g and the supernatant removed by decanting. DNA minipreps were prepared from the cell pellet using the Qiagen Miniprep QIAprep Spin Miniprep Kit as per manufacterers instructions.

2.17.3 Restriction Enzyme Digestion of Plasmid DNA and Gel Purification of DNA Inserts.

The pET28b and pUC52 vectors containing the sequences of interested were cleaved using 100U each of both *Nco*I and *Xho*I. The samples were left overnight at

room temperature for 24 hr. Plasmids (20µl) were mixed with 10 x DNA loading buffer and electrophoresed on a 1.5% agarose gel as in section 2.2.15. Following electrophoresis the DNA sequences were imaged under UV light and cut out of the agarose The DNA was isolated from the gel with a Wizard SV gel purification kit and an aliquot run on another 1.5% agarose gel to determine yield.

2.17.4 DNA Ligation and Screening of Colonies.

Inserts were mixed in a 1:1 ratio with pET28b and Takara ligation kit added as per manufactures instructions and the samples left to incubate for 15 hr at 16°C. Transformation of the ligated samples was carried out using the previous protocol (section 2.17.1) using DH5α *E.coli*.. Mini-preps were performed on the samples as before in section 2.17.2 and the samples cleaved with the restriction enzymes *Nco*I and *Xho*I and left overnight at room temperature. Finally a 1.5% agarose gel was run to determine the presence of successful cloning of insertions into the pET28b vector DNA.

2.17.5 Test expression of Semenogelin Fragments and SV-IV.

Plasmids containing the required inserts were transformed into BL21 DE *E.coli* as previously described and plated onto LB agar plates containing 30ug/ml kanamycin. Single colonies of each were inoculated into 5ml of LB broth containing 30ug/ml kanamycin and cultured at 37°C until an Optical density (OD 600) reading of 0.6 on a spectrophotometer. A sample (100µl) was removed and centrifuged at 13,000g for two min, supernatant discarded and the pellet frozen. IPTG was added to the cells to a final concentration of 0.5mM. At intervals of 1 hr, 2 hr and 3 hr after IPTG addition samples (100µl) were removed from the incubation and centrifuged at 13,000g for 2 min and the supernatant discarded and the pellet frozen. The remaining culture at the 3 hr time point was centriguged at 4000g for 10 min and the supernatant removed. The pellet was resuspended in 100ul of 50mM Tris, 150mM NaCl, 5mM DTT and cells lysed by sonication. The sonicated suspension was centrifuged at 13,000g and the supernatant retained and an aliquot (10µl) mixed with 3X SDS-PAGE sample buffer. A sample of the pellet was removed and also mixed with 3X SDS-PAGE sample buffer. 1X SDS-PAGE sample buffer (15µl) was added to each of the frozen samples. The pellets were

sonicated to remove DNA contamination. All resulting samples were heated to 99°C for 3 min just before loading onto a 15% SDS-PAGE gel as in section 2.2.4. Test expressions were also carried out at 18°C and also with 1M NDSB in the sonication buffer.

2.17.6 Expression of Semenogelin Fragments and SV-IV and their Affinity Purification.

The BL21 (DE3) cells containing the plasmids were gown in 5ml LB broth containing 30μg/ml kanamycin overnight. These cultures were used to inoculate 500ml of LB broth containing 30μg/ml kanamycin. When the OD_{600} reached 0.2, temperature was reduced to 18°C and shaking continued until the OD_{600} reached 0.6. IPTG was added to the cultures to a final concentration of 0.5mM and the cultures incubated overnight with shaking.

The cells were harvested by centrifugation at 8000g for 10 min. The pellets were resuspended in extration buffer (100mM Tris, 150mM NaCl, 0.5mM $ZnCl_2$ 1M NDSB pH 8) and sonicated. Cell debris was removed by centrifugation at 12,000g for 15 min and the supernatant dialysed against purification buffer (100mM Tris, 150mM NaCl, 0.5mM $ZnCl_2$ 0.25M NDSB pH 8) overnight. The dialysed supernatant was centrifuged at 12,000g for 10 min to remove any precipitant and applied to 2ml of Streptactin sepharose pre-equilibrated in purification buffer. After sample loading the column was washed with 20 column volumes of purification buffer. Peptides were eluted using 2.5mM Desthiobiotin in 100mM Tris, 150mM NaCl, 0.5mM $ZnCl_2$. Fractions (0.5ml) were collected and aliquots (10μl) mixed with 3X sample buffer and purification assessed by SDS-PAGE. Streptactin columns were regenerated as per manufacturer's instructions using 1mM HABA in 100mM Tris, 150mM NaCl, 0.5mM $ZnCl_2$.

2.17.7 FPLC Superdex 75 Size Exclusion Chromatography of the Sem274 Semenogelin Fragment.

Purification by FPLC was performed on an AKTA FPLC (Amersham Biosciences). A Superdex 75 10/300 column was equilibrated in 100mM Tris, 150mM NaCl, 0.5mM $ZnCl_2$ and 0.5ml of Sem274 (from affinity purification section 2.17.6)(concentrated using a Millipore ultra-3 filter) loaded. The column was run at 0.5ml/min and 0.5ml fractions collected. Fractions associated with A280 peaks were prepared for SDS-PAGE analysis by mixing 10µl of fraction with 3X sample buffer.

2.18 Effects of Sem274 and SV-IV on PBMC Expansion and Activation.

PBMCs were prepared as previously described in section 2.9.1 and seeded in supplemented RPMI 1640 at 1 x 10^6/ml into 24 well plates pre-coated with anti-human CD3 antibody (anti-human CD3 was diluted to 1 µg/ml in PBS and used at 1ml/well. Plates were incubated at 4°C for 16 hr before use). PBMCs were treated prior to addition to the plate with either Sem274 or SV-IV (10µg/ml). After 48 hr cells were transferred to a new 24 well plate and IL-2 was added to a final concentration of 20ng/ml. Fresh IL-2 was also added to the same final concentration at 72 and 96 hr. Aliquots (10µl) were removed at 48 and 120 hr incubation and cell numbers assessed by the trypan blue exclusion method (section 2.8.1). The remaining cells at 120 hr were transferred to FACS tubes and harvested by centrifugation at 600g. Cells were washed x2 in 1% (v/v) FCS in PBS and antibodies added (anti-human CD8α-PeCy7 (2µl) and anti-human CD3-Pacific blue (0.5µl)) in each tube to a final volume of 100µl 1% (v/v) FCS in PBS. Cells were incubated at 4°C for 20 min and then washed x2 in 1% (v/v) FCS in PBS. Cells were resuspended in 0.2 ml 1% (v/v) FCS in PBS and counted on a FACS CantoII flow cytometer. Results were analysed using the FlowJo software (Treestar).

2.19 Prostasome Inhibition of PBMC and Jurkat Cell Proliferation and the Requirement for Zinc Dependent ATPase for this Activity.

2.19.1 Preparation of Human Prostasomes by Ultracentrifugation.

Human semen was centrifuged at 600g for 5 min to remove sperm and other cells. The resulting supernatant was removed and diluted 1:1 with 50mM Tris-HCl 150mM NaCl pH 7.6 (Tris-buffered saline, TBS) and centrifuged at 10,000g for 10 min at 4°C. The pellets were discarded and the supernatant was ultracentrifuged in a Beckman TL-100 ultracentrifuge for 2 hr at 105,000g at 4°C. The resulting pellet was resuspended in an equivalent volume of TBS and centrifuged again under the same conditions. The resulting pellet was resuspended in TBS (one-tenth the original volume of semen). Preparations were either used fresh or frozen at –20°C until used.

2.19.2 Effects of Human Prostasomes on the Mitogen Induced Proliferation of PBMCs.

PBMCs (2 x 10^5/well) were cultured in supplemented RPMI 1640 medium in the presence of varying amounts of human prostasomes (0-40% concentration of prostasomes in the original semen sample). PHA-P (2.5μg/ml) was added to induce proliferation. The plates were incubated at 5% CO_2 at 37°C for 48 hr in a Forma Scientific incubator. After the initial incubation period the cells were pulsed with 1μCi/well of [^{3}H] thymidine and returned to the incubator for a further 4-12 hr. After incubation cells were harvested by vacuum onto Whatman glass fibre discs and washed x5 with PBS. The discs were placed in scintillation vials and Ecoscint scintillation fluid (5ml) added and scintillation counting performed on a TriCarb 2100TR scintillation counter. Experiments were repeated in the presence or absence of 100μM hydroxylamine.

2.19.3 Effects of Human Prostasomes on the Proliferation of Jurkat Cells.

This assay was perfomed as in section 2.19.1 with Jurkat cells in place of PBMCs except no PHA was added to induce proliferation.

2.19.4 Assay of Prostasomal ATPase Activity.

The zinc dependent ATPase activity of the prostasome sample was assayed using a stopped enzyme assay system in the presence of zinc chloride (0.01mM final concentration) and adenosine triphosphate (ATP) in varying concentrations (0.5mM-4mM final concentrations). Prostasomal protein content was measured by the Markwell assay (section 2.20) and 120μg protein used in each reaction. Tubes were made up to 1ml with TBS pH 7.6 and incubated for 1 hr at 37°C. TCA was added to a final concentration of 10% (w/v) and incubated at 4°C for 1 hr. The precipitate was removed by centrifugation at 13,000g for 10 min and the supernatant removed for testing. Blanks for each incubation were made by adding TCA prior to addition of prostasomal fraction. Inorganic phospate release in the ATPase assay was then determined by the method of LeBel et al. (1978).

In brief, the supernatant (1ml) was added to 3ml copper acetate (0.25% (w/v) copper sulphate pentahydrate and 4.6% (w/v) sodium acetate pH 4), 0.5ml 5% (w/v) ammonium molybdate and 0.5ml 2% (w/v) Elon in 5% (w/v) sodium sulphite. The tubes were mixed by vortexing and the absorbance read at 700nm using an Unicam Specrophotometer after 7 min.

2.19.5 Inhibition of Prostasomal Zinc Dependent ATPase by Dicyclohexylcarbodiimide (DCCD).

A stock solution of DCCD was prepared at 600μM in ethanol and stored at –20°C until used. Prostasomes (900μg) were incubated with DCCD (3μM final concentration) for 1 hr on ice. A control sample of prostasomes were incubated with an identical amount of ethanol as vehicle control and incubated for 1 hr on ice. Both treated and control prostasomes were then ultracentrifuged as in section 2.19.1 and washed x2 with TBS (pH 7.6) to remove excess DCCD. Varying amounts of both treated and control prostasomes (150μg, 300μg and 450μg) were then assay for ATPase activity as in section 2.19.4 keeping the ATP concentration constant at 2mM.

2.19.6 Effects of DCCD Treatment of Prostasomes on Mitogen Induced Proliferation of PBMC.

Prostasomes were treated with DCCD and ethanol as vehicle control as in section 2.19.5. Prostasome inhibition of mitogen induced proliferation of PBMCs was then assayed as in section 2.19.2 using 2 x 10^5 cells/well. Ethanol alone was used as negative control. Cells were pulsed with [^{3}H] thymidine and harvested and counted as in section 2.19.2.

2.19.7 Determination of Prostasome Uptake by Jurkat Cells Using [^{14}C] Cholesterol as Tracer.

2.19.7.1 Demonstration of Cholesterol uptake by Prostasomes.

Cholesterol-cyclodextrin complex was made as in Klein et al. (1995). In brief, 30mg of cholesterol was dissolved in isopropanol:chloroform (2:1) along with 1μCi [^{14}C] cholesterol and then added to a solution of 1g methyl-β-cyclodextrin in 11ml of PBS which was preheated to 80°C. Once all the cholesterol was dissolved the solution was freeze dried at –80°C overnight.

Half the resulting powder was taken and dissolved in 1ml of water and contained approx 3.98 x 10^{-7} Ci of radioactive cholesterol. The re-dissolved cholesterol complex (1.75 x 10^{-8} Ci) was added to varying amounts of prostasomes (480μg, 600μg and 720μg) and brought to 1ml in TBS (pH 7.6). Prostasomes were then centrifuged at 105,000g for 2 hr followed by resuspension in TBS and re-centrifuged as before. The prostasomes were resuspended in TBS and an aliquot removed, made to a final concentration of 1% (v/v) Triton X-100 and added to a scintillation vial along with 5ml ecoscint scintillation fluid and counted on a TriCarb 2100TR scintillation counter.

The experiment was repeated with prostasomes previously treated with DCCD as in section 2.19.5.

2.19.7.2 The Effects of Inhibition of the Zinc Dependent ATPase on Prostasome Internalization by Jurkat cells using [^{14} C] Cholesterol as Tracer.

Prostasomes treated with DCCD or ethanol as vehicle control (as in section 2.19.5) were labeled with [^{14}C] cholesterol as in section 2.19.7.1. Labeled prostasomes (100μg) were added to Jurkat cells (2 x 10^5/ml) seeded into 1ml RPMI 1640 supplemented medium in a 24 well plate. After 6 hr incubation, cells were transferred into tubes and harvested by centrifugation followed by washing in TBS (pH 7.6) x 3. The final cell pellet after washing was resuspended in 1% (v/v) Triton X-100 in TBS and added to 5 ml Ecoscint scintillation fluid and counted on a TriCarb2100 scintillation counter.

2.20 Markwell Protein Determination.

The amount of protein present in various samples were calculated according to the method of Markwell et al. (1978), a modified version of the Lowry procedure. BSA was used as a standard.

2.21 Bradford Protein Assay.

The amount of protein present in various samples were calculated according to the method of Bradford (1976). BSA or lysozyme were used as standards.

2.22 Assessment of Plasma Membrane Integrity and Acrosome Reaction by Electron Microscopy.

Spermatozoa were pelleted from seminal plasma (1ml) of ASA or normospermic patients and washed three times in PBS as in section 2.2.3. Alternatively, spermatozoa from normospermic patients whose plasma membranes had been extracted using Triton X-100 or Triton X-114, as described in section 2.2.3 or acrosome reacted spermatozoa (as in section 2.23). Pellets were fixed by resuspension in 1ml 3% (v/v) glutaraldehyde in PBS and were kept for 1h at 4°C. Samples were pelleted by centrifugation (15s) and supernatants removed and pellets resuspended in 1ml PBS and stored at 4°C until used.

Samples were washed six times (6-8 min each) in 0.05M potassium phosphate buffer, pH 6.8 and post fixed in 0.5ml of a solution of 50% (v/v) of osmium tetroxide in 0.05M phosphate buffer (pH 6.8) for 90 min. Spermatozoa were then dehydrated by 10min transfers through ascending concentrations of ethanol (30%, 75%, 95% and 100% (v/v)) followed by two washings in 100% (v/v) propylene oxide (15 and 30 min respectively). Samples were embedded in ascending concentrations of Emix resin (50% and twice in 100% (v/v)). 100% resin was poured into pans and samples were removed from tubes and applied to the new resin in the pans. Pans were left at 60°C for 24 h to set. Tissue blocks were mounted on perspex rods, trimmed and sectioned to 0.5-1.0 μm using a glass knife on a Reichet OM U3 ultratome. The sections were mounted on glass slides, stained with 1% (w/v) toludine blue in a 1% (w/v) solution of sodium borate in order to locate the spermatozoa. Thin sections (70 nm) were cut using a diamond knife and mounted on uncoated 70-300 mesh copper-rhodium grids.

These sections were post stained in 2% (w/v) uranyl acetate for 10 min, rinsed in distilled water, and then placed on a drop of Reynolds lead citrate for 5 min, washed again in distilled water and air dried. Heavy metal stained sections were examined using a Hitachi HUI2A electron microscope operated at 100kV with 30 or 50 μm objective apertures.

2.23 Induction of Acrosome Reaction in Human Sperm.

Sperm were washed free of seminal plasma as in section 2.2.3 and were incubated at a final concentration of 10 x 10^6 cells/ml in Medicult IVF medium. Samples were incubated for 30min in an atmosphere of 5% CO_2 at 37°C. Calcium ionophore A23187 (final concentration 10μM) was added to samples, to artificially induce the acrosome reaction (Yanagimachi and Usui, 1974), and returned to the incubator. After 40 min incubation, sperm were removed by centrifugation and prepared for electron microscopy as in section 2.22.

2.24 Statistical Analysis.

Statistical and graphical analyses were carried out using the statistics software PRISM, version 3.02 or in CricketGraph 5. In all cases points on the graph represent the mean of the replicate values and the error bars are a measure of the standard error of the mean.

Chapter 3

Sperm Surface and Seminal Plasma Antigens Reacting with Antisperm Antibodies from both IgG and IgA MAR Positive Patients.

3. 1 Introduction.

3.1.1 Direct Identification of Sperm Surface Proteins.

Immunoblot and immunoprecipitation techniques have been employed in multiple studies to determine the relative molecular masses of antigens on the sperm surface that are recognized by ASA from infertile patients on one-dimensional electrophoresis of sperm extracts (Aitken et al., 1987, Auer et al., 1997, de Beer et al., 1993, Haneji and Koide, 1990, Paradisi et al., 1996, Parslow et al., 1987, Pillai et al., 1996, Shai and Naot, 1992). The number of identified antigens vary across studies but common to several studies were sperm surface antigens of 18/20 kDa, 24/27kDa, 34/36 kDa, 44/45 kDa, 66/69 kDa, 72 kDa and 79/80 kDa have been found which react to both IgG and IgA of from both sera and seminal plasma of infertile patients but not from fertile controls. However, little information has been obtained concerning the clinical relevance of these identified proteins, as characterization did not proceed further in most studies and sperm surface antigens were not identified. Therefore, no relationship could be drawn between revealed immuno-dominant antigenic bands and immunological infertility.

The first identification of a putative antigen associated with infertility was the discovery of anti-galactosyltransferase antibodies in ASA positive patients when compared to control (Humphreys-Beher et al., 1990). Although galactosyltransferase was not confirmed to be present on the sperm surface or in seminal plasma, fertilization studies in the mouse implicate beta-1,4-galactosyltransferase-1 (GalT) as a sperm receptor for oligosaccharide ligands on the zona pellucida glycoprotein, ZP3 (Lyng and Shur, 2007).

The identification of sperm surface antigens *via* two-dimensional (2-D) electrophoresis (currently among the most powerful tools available for the analysis of complex mixture of polypeptides) has proven effective (Shibahara et al., 2002). Following immunoblotting with ASA, the recognized proteins may be sequenced and characterized by mass spectrometry.

Naaby-Hansen et al. (1997) created a 2-D protein database of human sperm proteins as a means of identifying sperm-surface proteins. These authors labeled the sperm membrane with biotin or iodine before extraction and succeeded in identifying 98 dual-labeled sperm-surface proteins. Using this 2-D database, Shetty et al. (2001) identified 6 immuno-dominant surface antigens recognized by ASA positive sera from infertile male and female patients that were possibly relevant to infertility. However, the exact identities and functions of these proteins have yet to be elucidated.

Also using 2-D electrophoresis, (Auer et al., 1997) demonstrated two immuno-dominant protein zones of 37/36 kDa (P36) and 19/18 kDa (PI8) that were recognized by antibodies eluted from the sperm surface of ASA positive patients and comprised a number of unidentified peptides. Polyclonal antibodies, produced in rabbits against both P36 and P18 inhibited human sperm penetration into zona-free hamster oocytes. Rabbit antibodies that were raised to P36 and P18 could detect the corresponding proteins on most sperm heads in methanol-fixed sperm samples but only in acrosome-reacted living spermatozoa by immunofluorescent staining, leading to the conclusion that they are located on the inner acrosomal membrane. As such, the apparent internal localization of the P36 and P18 antigens makes their involvement in the male immuno-infertility doubtful.

Bohring and Krause (2003), again using 2-D electrophoresis identified 18 antigens from sperm preparations that were reactive with seminal plasma from infertile patients with ASA. Six of these proteins, recognized by most samples, showed amino acid sequences that matched those of heat shock proteins HSP70 and HSP70-2, the disulphide isomerase ER60 (an inactive form of caspase-3) and two subunits of the proteasome complex. However, neither their role in sperm function nor their localization on the sperm surface has been determined. Also, although the authors state that they used highly enriched sperm membrane fractions, it is likely that the method

used (ultracentrifugation) would also co-purify high-density protein complexes from the sperm cytoplasm. The identities of each of the antigens would lead to the conclusion therefore, that the method of isolation of the antigens risks contamination of the preparation with large multi-protein complexes and the interpretation of the data necessitates an understanding of where the reactive antigens are localised.

More recently (Chiu et al., 2004), using 2-D electrophoresis, identified a novel sperm protein recognized by serum ASAs from 2 patients who had remained infertile following vasovasostomy, but not from fertile controls. Its amino acid sequence matched those of a hypothetical protein, XP-085564, from the C-type lysozyme/alpha-lactalbumin gene family which they named SPRASA. However, the apparent localization in the inner acrosome membrane, as indicated by immunoflouresence studies with a rabbit polyclonal antibody, again questions its involvement in the male immunological infertility.

The most comprehensive studies to date using 2-D electrophoresis coupled with mass spectrometry have compiled large numbers of antigens reacting with sera from both male and female ASA positive patients as well as antigens reacting with immunoglobulins derived from patient seminal plasma (Bhande and Naz, 2007, Domagala et al., 2007). Again these large-scale studies have used sperm membrane preparations that could be contaminated with both acrosomal and cytoplasmic constituents. Large numbers of metabolic enzymes along with proteosome components, internal sperm structural proteins and mitochondrial matrix proteins were identified as antigenic determinants for ASAs. The (patho)physiological relevance of these antigens without localization studies on the sperm surface is difficult to ascertain.

3.1.2 Identification of Antigens in DNA Expression Libraries.

Recombinant bacteriophage cDNA libraries from testis have been used to identify antigens reactive to ASA from infertile patients (Diekman and Goldberg, 1994, Liang et al., 1994, Liu et al., 1996). However, as sperm accrue many other proteins as they travel through the epididymis and vas deferens and others are processed by glycosidases and proteases the number of relevant identifiable antigens is limited.

One such antigen detected using this method is the BS-17 antigen, which reacted with the serum from an infertile woman with a high titre of sperm agglutinating antibodies. It is localized on the surface of the acrosomal region of human and other mammalian spermatozoa (Wei et al., 1994) and shares a high homology with calpastatin (Wang et al., 1994), whose expression occurs only in spermatids (Wei et al., 1995). Polyclonal antibodies to BS-17 when bound to the surface of human sperm reduced their ability to penetrate zona-free hamster oocytes (Wei et al., 1994) and in artificial insemination experiments in mouse with antibody coated sperm reduced the number of viable embryos present (Wei et al., 1994). As calpastatin is bound to calpain forming an inactive complex, it was hypothesized that anti-BS-17 antibodies could destabilize the calpastatin/calpain complex leading to a premature acrosome reaction (Koide et al., 2000). It has not yet been recorded whether anti-BS-17 antibodies occur in men.

3.1.3 ASA Recognition of Known Antigens Involved in Fertilization.

In an alternative strategy, sperm antigens, which over the last few years have been identified as playing a role in the fertilization process, can be tested for a possible involvement in human immunological infertility, by assessing their reactivity with ASA from infertile patients.

Serum and seminal ASA from several male infertile patients, but not ASA-negative samples, were reactive with Fertility Antigen-1 (FA-1) antigen (Williams et al., 2008, Naz, 1987), which is localized on the post-acrosomal and tail of spermatozoa of various mammalian species including humans and is expressed in the testis (Naz et al., 1984). Antibodies raised to recombinant FA-1 can block sperm-egg binding in mouse IVF experiments, whilst active vaccination with recombinant FA-1 leads to reduced long term fertility in *in vivo* mouse experiments (Naz and Zhu, 1998). Antibodies raised to human form of FA-1 give a dose response inhibition of both capacitation and acrosome reactivity by blocking tyrosine phosphorylation of FA-1 (Naz and Zhu, 2002).

The YLP12 peptide sequence was first discovered by screening a phage display library with solubilized zona pellucida preparations from human eggs, in order to

identify peptide sequences involved in ZP-binding (Naz et al., 2000). This peptide had reactivity to most sera and seminal plasma from ASA-positive infertile men but not to fertile controls. Monovalent Fab' antibodies to the YLP12 peptide blocked human sperm/ZP binding (Naz et al., 2000, Naz et al., 2002) and also inhibited acrosome reactivity and capacitation *in vitro* (Naz and Packianathan, 2000). The antibodies recognize a 50kDa protein, which is located on the surface of the acrosome and tail and a 72kDa protein in testis, thus indicating that it is modified as the sperm transverses the reproductive tract (Naz and Chauhan, 2002).

Sperm Agglutination Antigen-1 (SAGA-1) was first identified using a monoclonal antibody (mAb), H6-3C4, with sperm agglutinating and complement-dependent sperm immobilizing activities, which was immortalized from the lymphocytes of an infertile woman who exhibited a high titre of sperm-immobilizing antibodies (Isojima et al., 1987). It was characterized as a GPI-anchored glycoprotein of epididymal origin, localized on the surface of human spermatozoa and sequencing demonstrated that the SAGA-1 core peptide is identical to that of CD52 lymphocyte antigen, but the N-linked glycosylation, which represents nearly 80% of the weight of the antigen, is male reproductive tract specific (mrtCD52) (Diekman et al., 2000). Monoclonal antibodies raised to mrtCD52 not only display sperm agglutinating properties but also inhibit cervical mucus penetration by sperm and sperm-egg interactions (Diekman et al., 2000).

Far-western blotting was used to determine binding components on the sperm surface for the ZP glycoproteins on the egg (Petit et al., 2013). Though many of the hits revealed glycolytic enzymes notably they found glutathione transferase M3 (GST M3) and voltage dependent anion channel 2 (VDAC2) as binding to ZP proteins as well as reacting to ASA positive patient sera. This agrees well with previous work (Paradowska et al., 2006) that identified GST Mu5 and VDAC2 along with Apo-lactate dehydrogenase isoenzyme C4 in a study looking for conserved mouse sperm surface protein epitopes recognized by ASA patient sera. All three antigens were shown to occur on the sperm surface in vectorial labeling experiments using biotin.

Izumo proteins are expressed on the sperm of all mammals in a large multi protein complex and have been shown to be essential for gamete fusion in the mouse (Ellerman

et al., 2009, Inoue et al., 2005). Recently, ASAs to izumo proteins have been identified in the sera of immunologically infertile women, but not men (Clark and Naz, 2013).

Sperm antigens are not the only antigenic material in the male ejaculate, ASA also recognize prostasomes, organelles secreted by prostatic cells and which can fuse with sperm surface post ejaculation (Allegrucci et al., 2001). Anti-prostasome antibodies caused sperm agglutination and all sera from 20 infertile patients with agglutinating ASA (Allegrucci et al., 2001). More recently, these antigens where identified by means of 2D-electrophoresis and characterized (Carlsson et al., 2004). Prolactin-inducible protein and clusterin were the immunodominant prostasome antigens.

3.1.4 Sperm Surface Glycosylations as Targets for ASA.

The first report of glycolipids on the sperm surface reacting with ASA was that of a monoclonal antibody (H6-3C4) from an infertile woman, which induced complement-dependent sperm immobilising (SI) reactions (Tsuji et al., 1988). It reacted from a Folch's lower and upper phase extraction of ejaculated but not testicular sperm. Treatment of sperm with neuraminadase did not abrogate H6-3C4 binding to the sperm surface indicating the glyco-determinant was not sialyl-i or sialyl-I. The determinant was deduced to be an internally located, repetitive unbranched N-acetyllactosamine structures independent of terminal substitution and that 2→3 sialyllacto*nor*hexosyl-ceramide was the only upper phase glycolipid reacting with the monoclonal. However, binding of H6-3C4 to the lactosaminolipid is of low affinity, leading to the later finding that its true target was the N-glycosylation of mrtCD52 (Diekman et al., 2000).

Later studies using 30 mouse monoclonal antibodies (mABs) made with whole sperm as inoculants (Kurpisz et al., 1989) found that all antibodies bound glycoconjugates and bound common antigens on lymphocytes, erythrocytes and bacteria. The mABs bound to both upper and lower phase glycolipids and treatment with neuraminidase increased mAB binding to the glycolipids in most cases, whereas treatment with β-D-*N*-acetyl-hexosaminadase or α-*N*-acetyl-galactosaminadase decreased binding in most cases. However, treatment with endo-β-galactosidase did not affect mAB binding indicating that the reactive core is not a lactosaminoglycan.

Sulphoglycolipids have also been characterised as targets for ASA (Tsuji et al., 1992). Pre-absorption of sera from women with SI antibodies with sulpatide or seminolipid resulted in decreased sperm immobilization. This suggests that the 3-O-sulphogalactose residue is a major antigen epitope leading to SI antibodies in infertile women. Sulphoglycolipids are implicated in several autoimmune diseases. In Chaga's disease of the nervous system, induced by chronic Trypannosoma Cruzi infection, the antigenic epitope is sulphatide (Acosta et al., 2012). Sulphatide is also implicated in autoimmune hepatitis, systemic lupus erythematosus, Sjögren's syndrome, mixed cryoglobulinemia and idiopathic systemic vasculitis (Alpa et al., 2007) while anti-sulphated glucuronic paragloboside antibodies have been found in Lumbar spinal nerve root hypertrophy in Waldenstöm's macroglobulinemia-associated polyneuropathy (Ito et al., 2009). Interestingly, women with SI antibodies to sulphoglycolipids show no signs of other autoimmune diseases whereas their male counterparts with ASAs showed a positive correlation with other autoimmune diseases (Shiraishi et al., 2009).

3.1.5 Aims of Chapter.

The aim of this chapter is to identify novel antigenic targets for ASA from both patient sera and seminal plasma. A number of approaches were employed 1) Identification of antigens with conserved evolutionary epitopes from the mouse reproductive tract. 2) Isolation and purification of human seminal plasma antigens reacting with ASA. 3) Pull down of human sperm surface antigens using patient sera and identification of antigens by mass spectrometry. 4) To identify whether glycolipids on the sperm surface are targets of ASA.

3.2 Results

3.2.1 ASA Localization on the Sperm Surface, Effects of ASA on Sperm Membrane Integrity and Western Blotting of Sperm Antigens.

Immunoflourescence studies (section 2.2.2) were carried out using sperm from patients with normal semen parameters and staining with either sera or seminal plasma indicated heterogeneity of binding between patients (Figures 3.2.1.1 and 3.2.1.2 with results summarized in Tables 3.2.1.1 and 3.2.1.2). Though all patients sera showed binding to the midpiece of the sperm (13/13) other sperm domains showed relatively different patterns. The acrosome showed binding in 11 of 13 patients (11/13) while the equatorial region 3/13, posterior head 6/13, tail 9/13 and tip of tail 1/13. Sera from normospermic patients gave no staining at the dilutions used for ASA positive sera.

Patient Number	Acrosome	Equatorial Region	Posterior Head	Midpiece	Tail	Tip of Tail
1	+	-	+	+++	++	+
2	+++	-	-	++	+	-
3	++	-	-	++	-	-
4	++	-	+++	+	+++	-
5	+++	++	+	++	+	-
6	+	++	-	++	-	-
7	++	-	-	++	-	-
8	+	-	+	+++	++	-
9	-	-	-	+++	++	-
10	+++	-	++	+	++	-
11	++	+	-	+	+	-
12	+	-	++	++	++	-
13	-	-	-	+++	-	-

Table 3.2.1.1 Immunoflourescent Binding Patterns of ASAs on the Sperm Surface using Primary Antibody Derived from Patient Sera.

When seminal plasma was used as the primary source of IgG for staining, the patterns were similar to that of the sera from the same patients with the midpiece been stained in all patients. However, binding to other regions did differ between antibody sources with binding to the acrosome down to 9/13 patients. The intensities of staining of the acrosomal region were also reduced when seminal plasma was used as the primary source of antibody. The equatorial region along with the tip of tail domain showed no binding in any of the patients when seminal plasma was used, while the posterior head and tail domains both showed only 3/13 patients with seminal antibodies to these regions, a marked decrease to when sera of these patients was used. Seminal plasma from normospermic patients gave no staining at the dilutions used for ASA positive seminal plasma.

Patient Number	Acrosome	Equatorial Region	Posterior Head	Midpiece	Tail	Tip of Tail
1	+	-	-	+++	-	-
2	-	-	-	+++	-	-
3	+	-	-	++	-	-
4	-	-	++	++	-	-
5	-	-	-	+++	-	-
6	+	-	-	+++	-	-
7	+	-	-	+++	-	-
8	+	-	+	+++	++	-
9	+	-	-	+++	+	-
10	+	-	-	+++	++	-
11	++	-	-	+	-	-
12	-	-	++	+++	-	-
13	+	-	-	+++	-	-

Table 3.2.1.2 Immunoflourescent Binding Patterns of ASAs on the Sperm Surface using Primary Antibody Derived from Patient Seminal Plasma.

Electron microscopy of sperm from normospermic and ASA positive patients was carried out as in section 2.22. Normospermic patients showed intact sperm surface

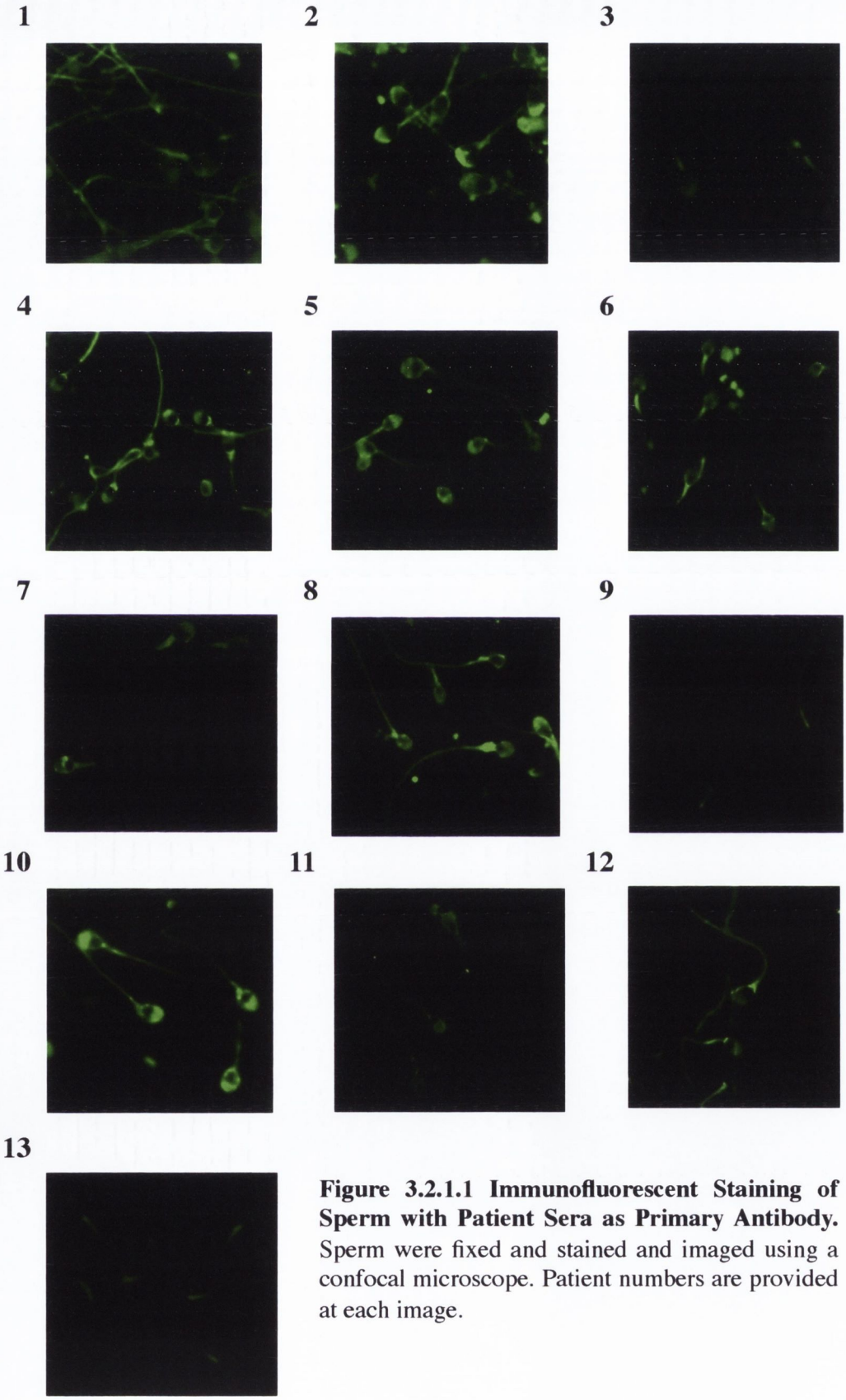


Figure 3.2.1.1 Immunofluorescent Staining of Sperm with Patient Sera as Primary Antibody. Sperm were fixed and stained and imaged using a confocal microscope. Patient numbers are provided at each image.

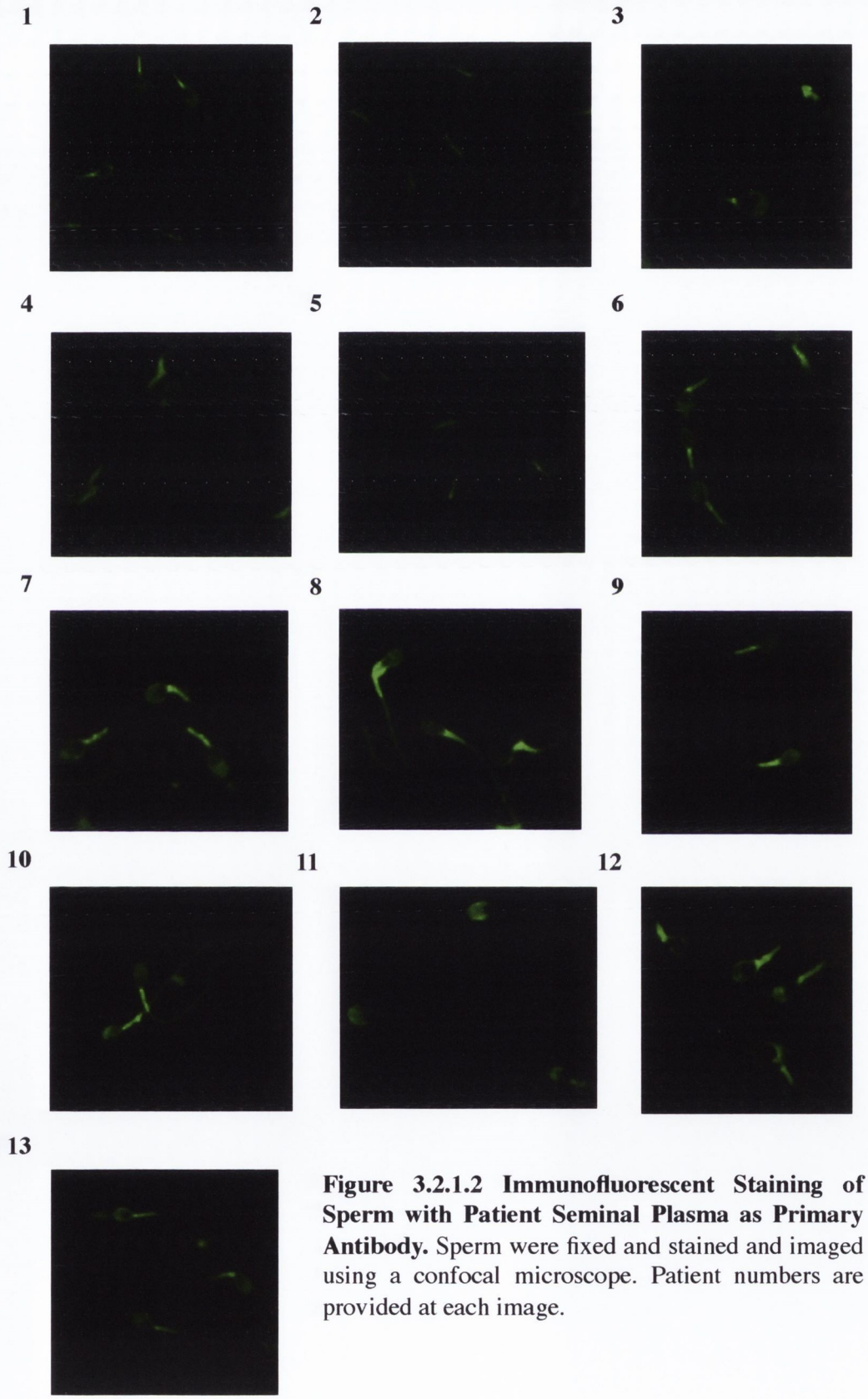


Figure 3.2.1.2 Immunofluorescent Staining of Sperm with Patient Seminal Plasma as Primary Antibody. Sperm were fixed and stained and imaged using a confocal microscope. Patient numbers are provided at each image.

membranes while ASA patients showed disruption of the sperm surface membrane in all patients tested (Figure 3.2.1.3). Sperm membrane extraction with either Triton X-100 or Triton X-114 as in section 2.2.3 followed by electron microscopy of the non-dissolvable material (Figure 3.2.1.4) indicated that detergent membrane extraction removed the outer sperm membrane along with the acrosomal cap structure and inner acrosomal membrane (which can be observed in figure 3.2.1.3B post acrosome reaction).

Western blotting of ASA positive patient sera against detergent extracted sperm membranes (Triton X-100 and Triton X-114) as in section 2.2.6 gave rise to differing binding patterns from patient to patient (Figure 3.2.1.5). However, several antigens of several molecular weights were common to many of the patients (see Table 3.2.1.3) (The molecular weights of antigens are given here as ranges found, e.g. 43/45 meaning 43kDa to 45kDa indicating variability between blots and sperm membrane preparations).

Molecular Mass in kDa of Detected Antigens	# of Patients Reacting with Antigen Species
43/45, 67/68	13
25/26	12
32/33, 35/36, 40/42, 58/60, 120/121	10
16/17	7

Table 3.2.1.3 Antigens Common to Patients with ASA.

The aqueous phase from Triton X-114 separation also showed consistent antibody binding to a range of antigens between approximately 7 and 20 kDa. Blotting of IgG from the sera of a patient reporting with secondary infertility following reversal of vasectomy (vasovasostomy) (Figure 3.2.1.5) indicated that the range of antigens generating an antibody response was unlike those reporting with primary autoimmune infertility, with a range of antigens of between 28 and 32 kDa in Triton X-100 and Triton X-114 detergent phase and 26 and 53 kDa in Triton X-114 aqueous phase extracts.

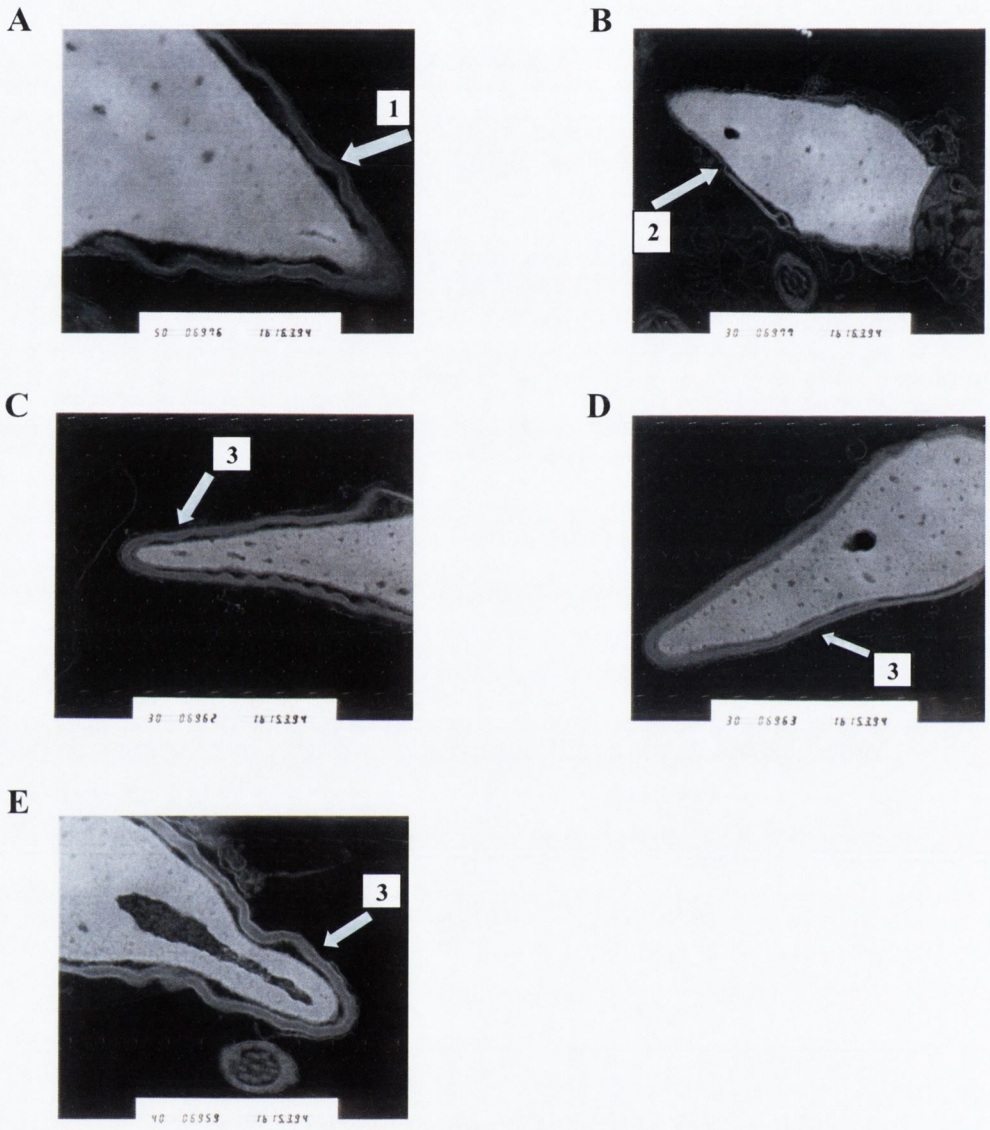


Fig 3.2.1.3 Electron micrographs of spermatozoa from normal and ASA patients. **A.** Electron micrograph of normal human spermatozoa. Arrow 1 indicates the intact outer membrane of the acrosomal cap. **B.** Electron micrograph of normal human spermatozoa which has been induced to undergo acrosome reaction by addition of calcium ionophore A23107. Arrow 2 indicates the loss of the entire acrosomal membrane. **C, D and E.** Electron micrograph of spermatozoa from patients 2, 4 and 11 respectively. Arrow 3 indicates the degeneration of the outer acrosomal membrane in ASA patients.

Triton X-100 **Triton X-114**

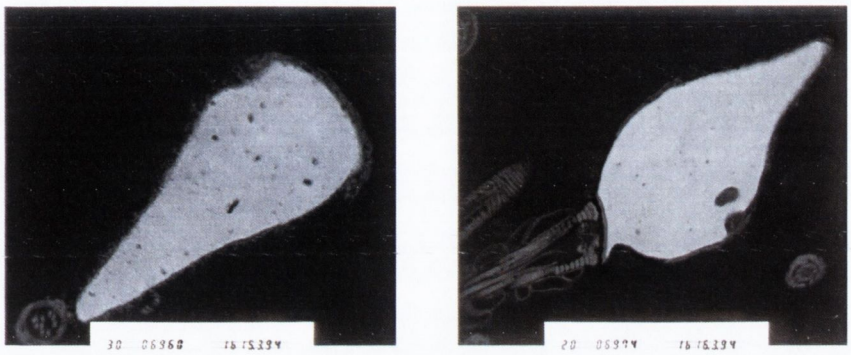


Figure 3.2.1.4 Electron Micrographs of Sperm Extracted with Triton X-100 and Triton X-114. After detergent removal of sperm outer membranes, the residual sperm were prepared for electron microscopy.

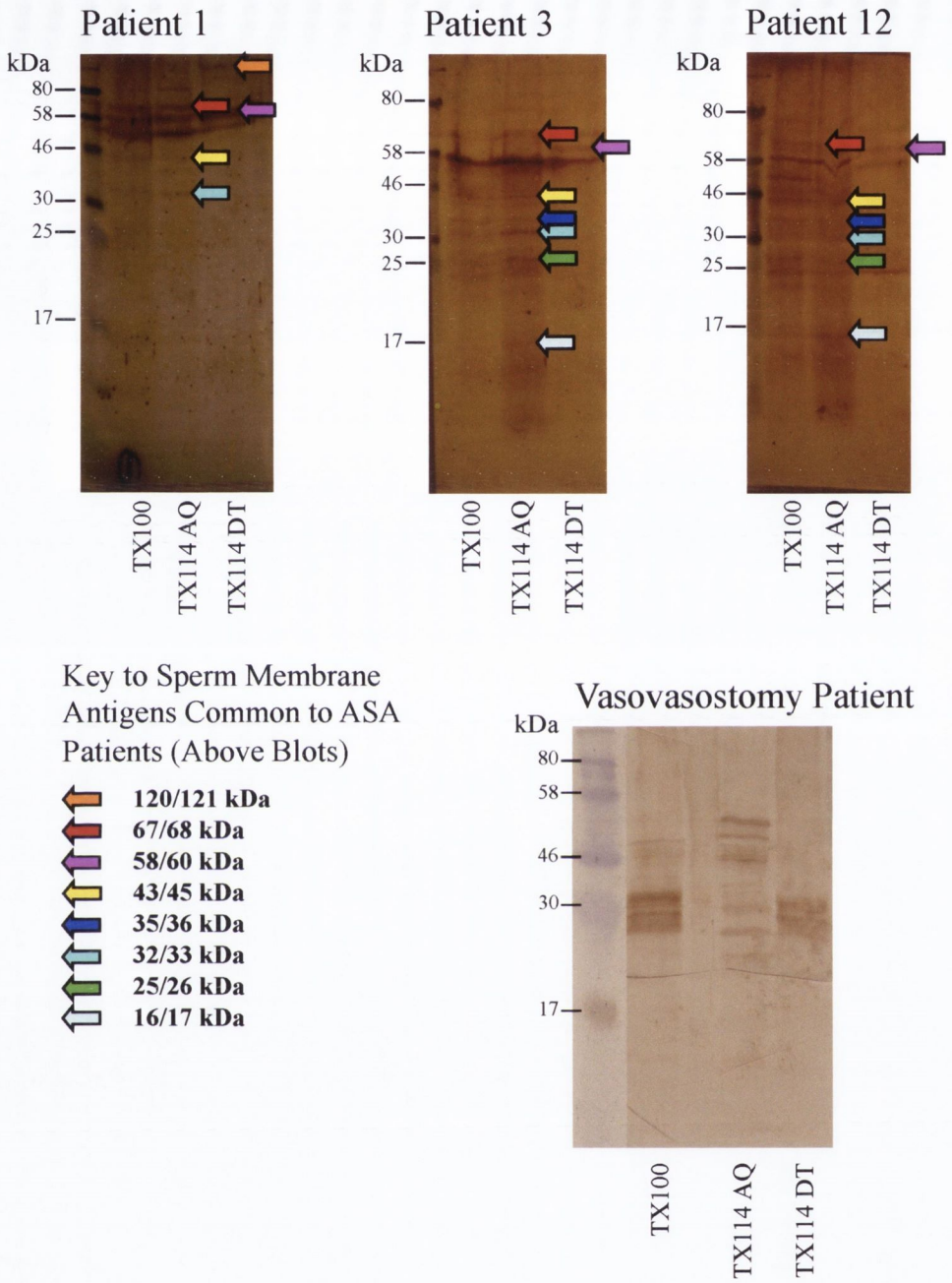


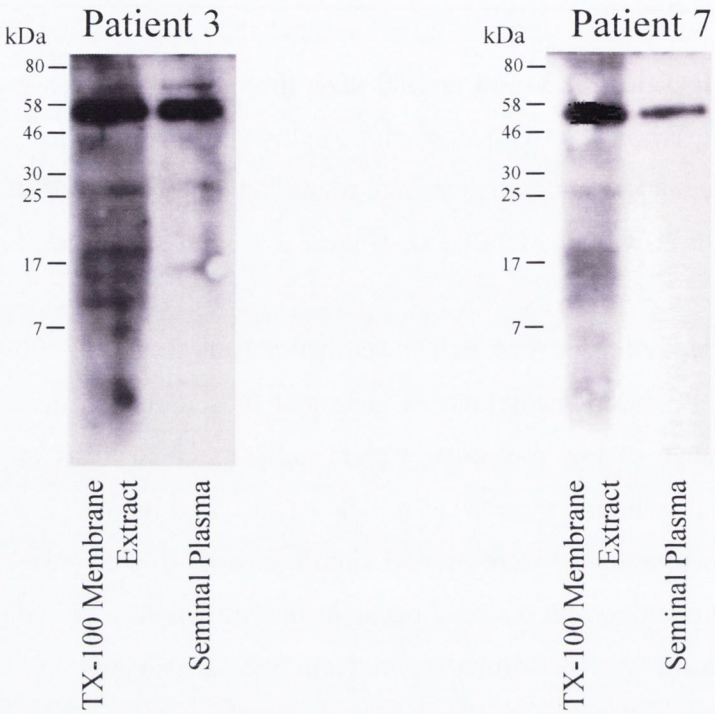
Figure 3.2.1.5 Western Blotting of ASAs from Different Patients Against Sperm Membrane Proteins. Sperm membrane proteins were extracted with either Triton X-100 (TX100) or Triton X-114 and phase separated into aqueous (TX114 AQ) or detergent (TX114 DT) phases. Sperm membrane proteins were separated on a 15% SDS-PAGE gel and transferred to PVDF and blotted using patient sera. Patients 1, 3 and 12 are used as representative blots and are compared to a patient presenting with infertility after vasovasostomy. Antigens common to multiple patients are marked with arrows and are defined in the key below blots. Sperm membrane antigenic targets common to ASA patients are tabulated in Table 3.2.1.3.

Detection of IgA binding antigens in sperm membrane extracts and seminal plasma was carried out using sera denuded of IgG as described in section 2.2.7.2. Though a number of antigens were detected that were similar in size to those that reacted to IgG, ie. 28, 34, 68 and 80 kDa, the binding patterns most resembled those found in the Triton X-114 aqueous phase extractions probed with IgG, in that a number of low molecular weight antigens were found in both Triton X-100 extracts and seminal plasma in the size range of 7-20 kDa (Figure 3.2.1.6A, summarized in Figure 3.2.1.6B).

IgG derived from ASA patient seminal plasma was used to blot for antigens from Triton X-100 sperm membrane extracts and in seminal plasma as in section 2.2.6. Again, a range of low molecular weight antigens (7-20 kDa) in both the membrane extracts and seminal plasma were shown to be present (Figure 3.2.1.7A). That antibodies and antigens were present simultaneously in seminal plasma suggested that antibody:antigen complexes may occur in this milieu so a pull down of ASA patient seminal plasma was performed as in section 2.2.8 using Protein G sepharose. After silver staining of the tris-tricine gel a range of low molecular weight antigens were shown to be present as antigen:antibody complexes in ASA patient seminal plasma (Figure 3.2.1.7B).

Seminal plasma was prepared for separation on a chromatofocusing column as in section 2.4.1. After dialysis and pH adjustment seminal proteins were applied to a chromatofocusing column and eluted with a pH gradient as in section 2.4.1 (Figure 3.2.1.8A). Protein peaks were analysed on a 15% SDS-PAGE gel (Figure 3.2.1.8B) or Western blotted using ASA positive patient seminal plasma (Figure 3.2.1.8C). The unbound protein peak revealed the only reactive antigen to ASAs, at approximately 18 kDa. The reactive band was excised from the gel and prepared for mass spectrometry as in section 2.2.12, and identified as a semenogelin 1 fragment. In order to confirm semenogelin as an ASA target, semenogelin 1 and 2 were purified from seminal plasma as in section 2.6.1 by affinity chromatography on a heparin column (Figure 3.2.1.9A). Eluted proteins were analysed on a SDS-PAGE gel, which showed partial purification of the semenogelins (Figure 3.2.1.9B). The fragment from the chromatofocusing column was also electro-eluted from the gel pieces as in section 2.4.2 and a polyclonal antibody prepared in rabbit as in section 2.4.2. The antibody was used to probe both sperm membrane extracts and seminal plasma (Figure 3.2.1.10A) showing a laddering

A



B

Patient Number	Triton X-100 Sperm Membrane Extract (kDa)	Seminal Plasma (kDa)
3	68, 34, 28, 26, 18, 13, 10, 8, 6	70, 34, 15
7	36, 28, 18, 13, 10, 8, 6	-
9	80, 68, 60, 39, 32, 18, 13	80, 68
12	68, 50, 34, 18, 13,10,8, 6	93, 80, 68, 13, 12

Figure 3.2.1.6 Sera IgA from Patients with ASAs binding to Triton X-100 Extracted Sperm Membrane Proteins and Seminal Plasma Proteins. A. Representative western blots of IgA binding to semen antigens. **B.** Table of antigen molecular weights reacting with IgA ASAs.

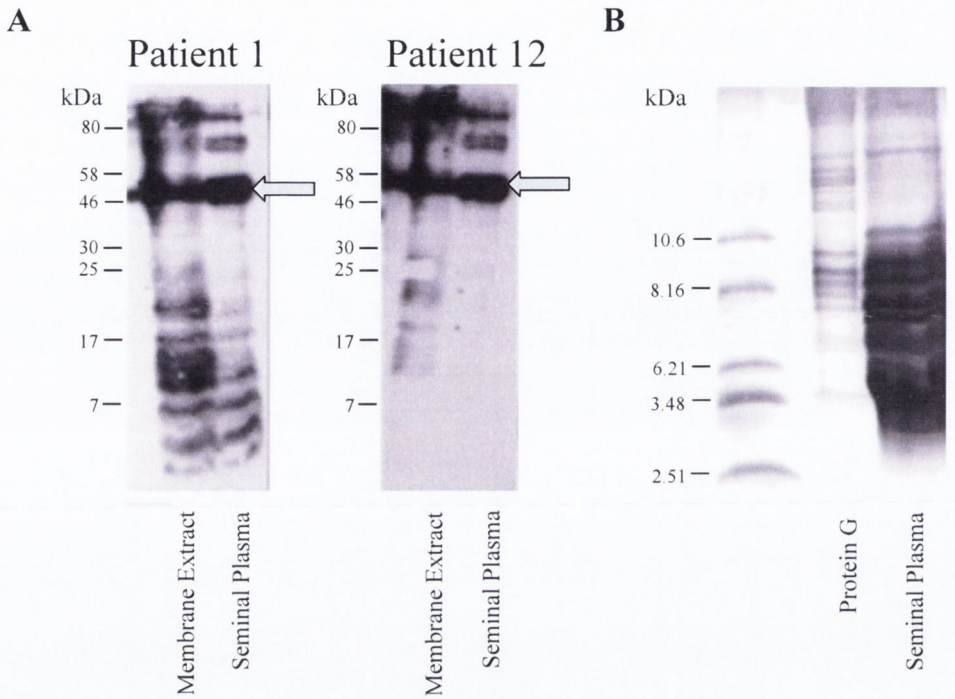


Figure 3.2.1.7 Western Blotting with IgG Present in ASA Patient Seminal Plasma. A. Representative blots from patients 1 and 12 of IgG from seminal plasma binding to antigens in seminal plasma and Triton X-100 sperm membrane extracts. Seminal plasma and membrane extracts (20µg) were run on a 10% tris-tricine gel and transferred to PVDF before incubation with primary and secondary antibodies. Blots were visualised using SuperPico ECL reagent. Arrows indicate presence of IgG heavy chain. **B.** Demonstration of the presence of antibody:antigen complexes in ASA patient seminal plasma. Seminal plasma of Patient 1 was incubated with protein G sepharose and antibody:antigen complexes isolated. Protein G isolates were run on a high cross-linked tris-tricine gel along with 5µg of total seminal plasma protein. Gels were then silver stained to visualise bands.

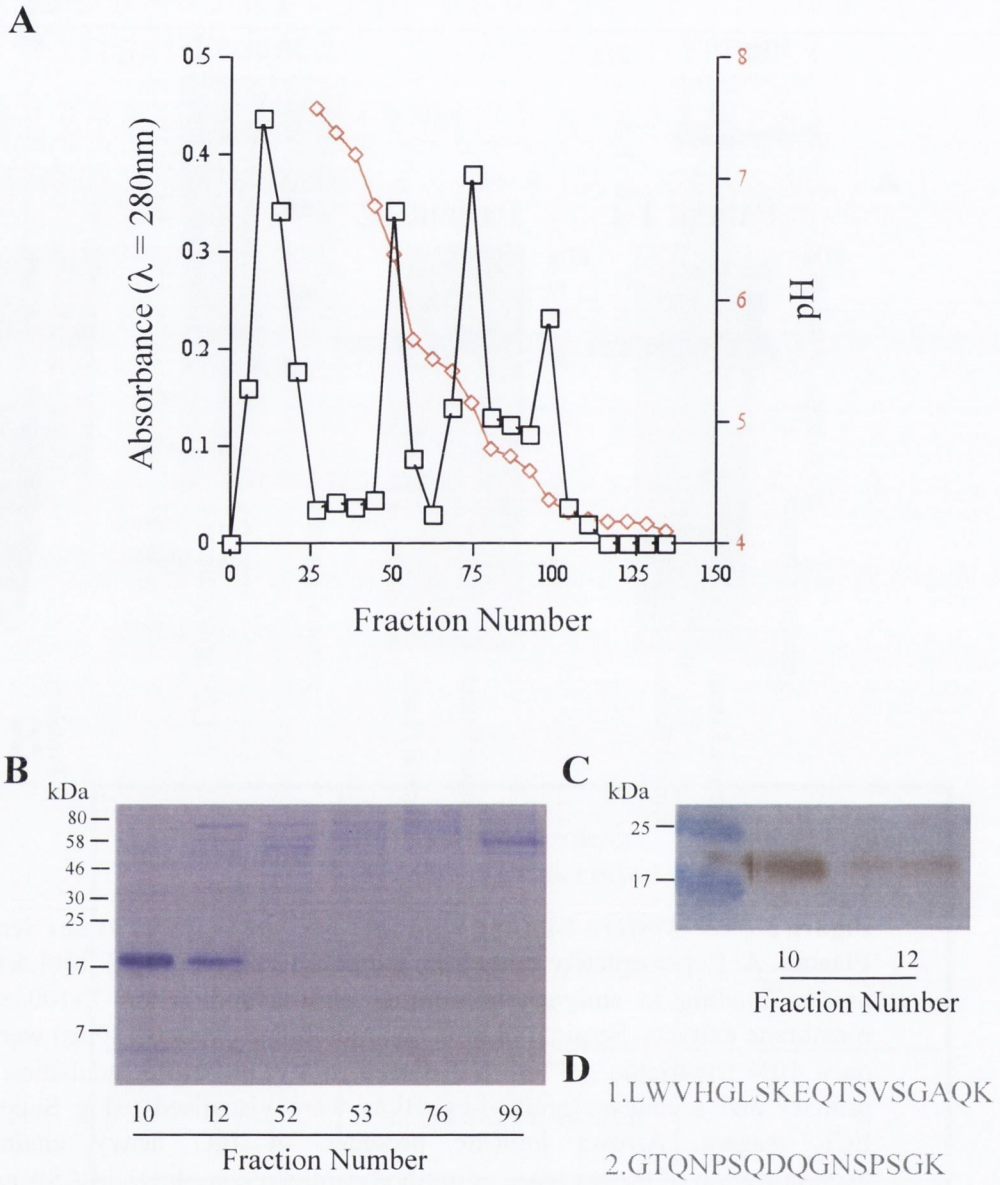


Figure 3.2.1.8 Partial Purification of a ASA Ractive Band from Human Seminal Plasma by Chromatofocusing. A. Human seminal plasma was prepared and applied to a pre-equilibrated chromatofocusing column. Bound protein was eluted using a pH gradient and A_{280} and pH readings recorded. **B.** Aliquots of each peak were precipitated by addition of 9 volumes ice-cold acetone and re-dissolved in SDS-sample buffer and loaded onto a 15% gel and stained with coomassie brilliant blue. **C.** Samples were prepared as in B and loaded onto a 15% gel and transferred to PVDF. The membrane was probed with ASA positive patients sera (Patient 3) and visualised with diaminobenzidine. **D.** Mass spectrometric analysis of reactive band. Identity: semenogelin 1.

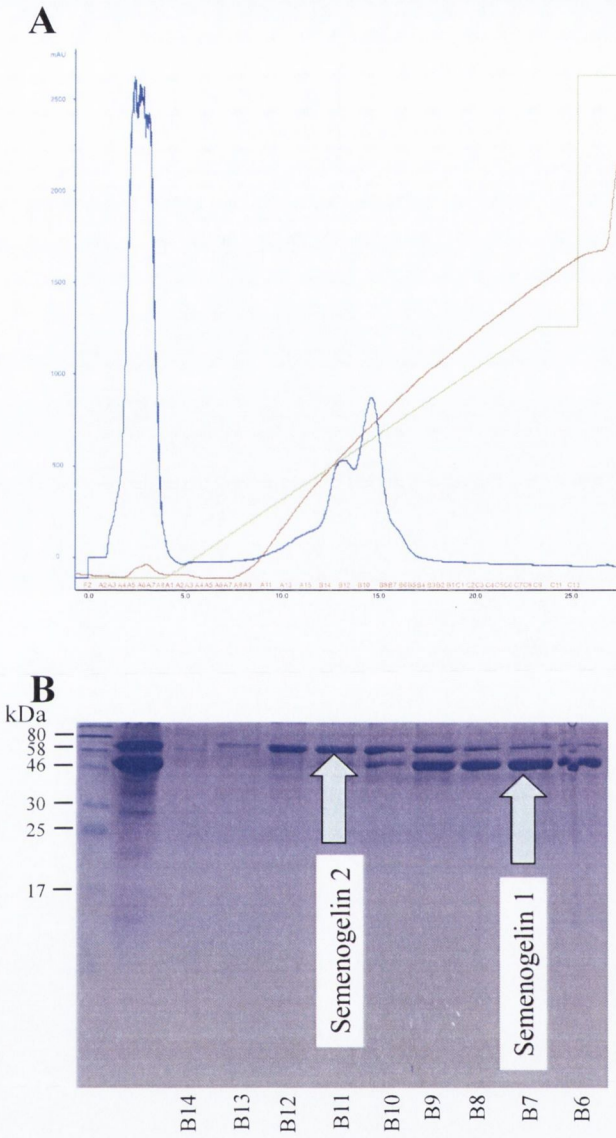


Figure 3.2.1.9 Chromatogram of Semenogelin Purification on Heparin Affinity Matrix. A. Urea treated seminal plasma (2ml) was loaded onto a pre-equilibrated 1ml HiTrap Heparin-sepharose column. Bound protein was eluted using a 0-0.5M NaCl gradient and collected in 0.5ml fractions. **B.** 15% SDS-PAGE gel of fractions from heparin affinity column.

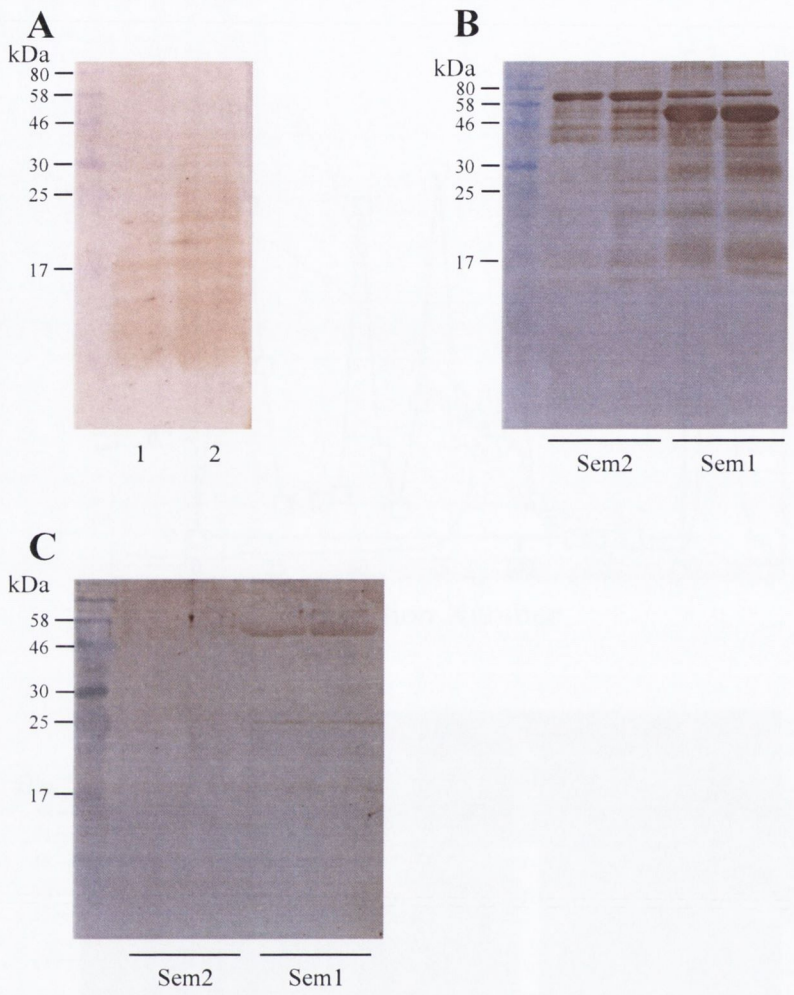


Figure 3.2.1.10 Demonstration of Semenogelin1 Fragment on the Sperm Surface and as a Target of ASAs. A. Triton X-100 sperm membrane extract (20µg Lane 1) and seminal plasma (20µg Lane 2) were run on a 15% SDS-PAGE gel and transferred to PVDF and probed with rabbit anti-semenogelin antibody raised against purified fragment. **B.** Semenogelin 1 and 2 purified on heparin-sepharose were run on a 15% SDS-PAGE gel and transferred to PVDF and probed with rabbit anti-semenogelin antibody raised against purified fragment. **C.** Semenogelin 1 and 2 purified on heparin-sepharose were run on a 15% SDS-PAGE gel and transferred to PVDF and probed with human ASA patient sera (Patient 12). All blots were visualised using diaminobenzidine.

effect for both samples indicating multiple fragmentation pieces of the semenogelins containing the sequence to which the antibody was made. Western blotting with the anti-semenogelin antibody of whole semenogelin 1 and 2 as purified by affinity chromatography showed that the antibody had affinity for both semenogelin 1 and 2 (Figure 3.2.1.10B). However, Western blotting of whole semenogelin 1 and 2 with ASA patient sera revealed that only semenogelin 1 was reactive with ASA antibodies (Figure 3.2.1.10C).

3.2.2 Detection of Evolutionary Conserved Protein Epitopes in the Mouse Reproductive Tract Reacting with Human ASA.

To test whether human ASA reacted with conserved protein epitopes within the mouse reproductive tract, mouse epididymis and vas deferens were dissected out and prepared as in section 2.2.10. SDS-PAGE analysis and Western blotting of these fractions (Figure 3.2.2.1) revealed multiple mouse antigens reacting with the human ASA. The most prominent were at approximately 26 kDa in the cauda epididymis and at around 36 kDa in the vas deferens. These antigens were of particular interest as they corresponded with common antigens displayed previously in blotting of human sperm extracts with ASA (see Table 3.2.1.3). Two-dimensional electrophoresis was performed on both the mouse cauda epididymis and vas deferens extracts as in section 2.2.11 to allow for further separation of these antigens for mass spectrometric analysis (Figure 3.2.2.2 A and B). Western blotting using patient sera was used to confirm reactive spots and the corresponding coomassie stained spots excised and trypsinized and sent for mass spectrometric analysis as in section 2.2.12. The spots were identified as Cysteine Rich Secretory Protein (CRISP1) from the mouse cauda epididymis and Mouse Vas Deferens Protein (MVDP) (AKR1B7) from the mouse vas deferens (Figure 3.2.2.2 C).

3.2.3 Confirmation of CRISP1 as a Target for ASA and the Effects of Anti-CRISP1 Antibodies on Egg Fertilization.

CRISP1 was purified first using a chromatofocusing column followed by affinity chromatography on Procion Red as in sections 2.3.1 and 2.3.2 (Figure 3.2.3.1 A and B) and the purification product analysed by SDS-PAGE (Figure 3.2.3.1 C). The purified CRISP1 was then subjected to Western blotting using ASA positive sera and was

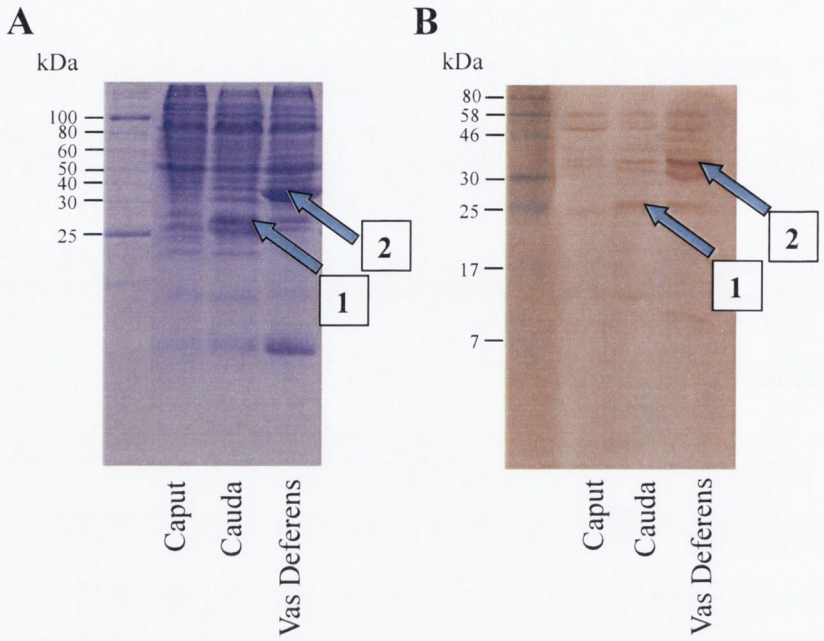
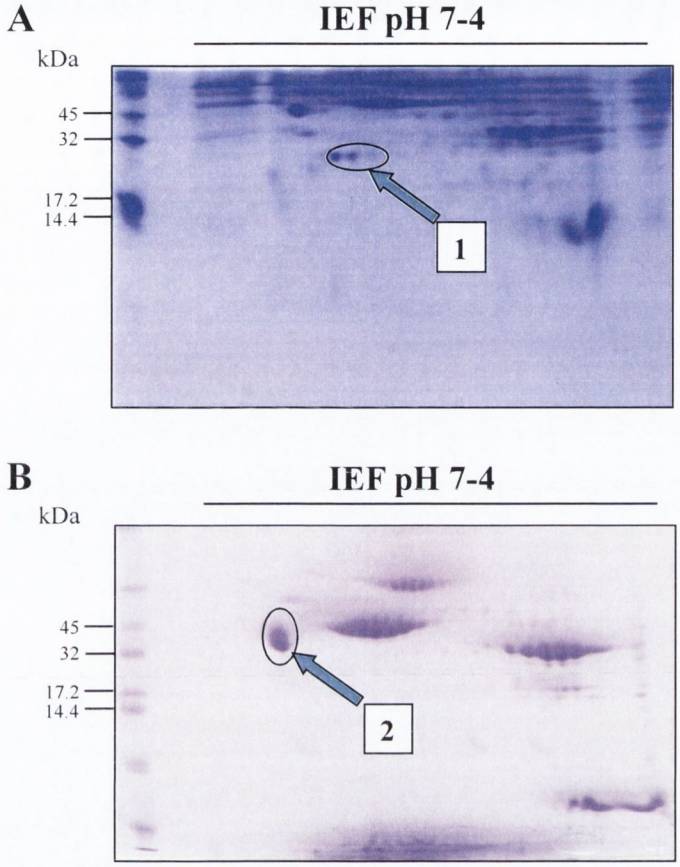


Figure 3.2.2.1 Human Antisperm Antibody Reactivity to Antigens of the Mouse Reproductive Tract. Extracts of mouse reproductive organs, caput and cauda epididymus along with vas deferens were run on a 15% SDS-PAGE gel (20μg/lane) and either stained with coomassie brilliant blue or transferred to PVDF and probed with ASA patient sera (combination of sera from patients 3, 6 and 10). Blots were visualised using diaminobenzidine. Arrows 1 and 2 indicate the main antigenic responses in the cauda epididymus and vas deferens respectively. (Later mass spectrometry identified 1 and 2 to be Crisp1 and MVDP respectively.)



C

Mass Spectrophotometric Analysis of Identified Spots.

Spot 1 Peptides.

1.MSVQEEIVSK

2.MVSPSGSDLLKMEWNYDAQVNAQQWADK

Spot 2 Peptides.

1.MPLVGLGTWK

2.HIDCAYVYHNENEVGEAIQEK

3.STFLDAWEAMEELVDQGLVKALGISNFNHFQIER

4.LIQYCQSKGIAVTAYSPLGSPDRPYAKPEDPVVMEIPK

5.TEEDYPFHEEY

Figure 3.2.2.2 Two-dimensional (2D) Gel Electrophoresis of Mouse Reproductive Tract Tissues and Mass Spectrometric Analysis of Identified Antigens. Mouse cauda epididymus and vas deferens tissue extracts were subjected to denaturing isoelectric focusing on pH 4-7 IPG strips. Focused strips were then loaded on 15% SDS-PAGE gels and when run stained with coomassie brilliant blue. Spots of interest were identified by running identical 2D gels and blotting with ASA positive patient sera. Spots were excised and trypsinised and analysed by MS/MS mass spectrometry. **A.** 2D Electrophoresis of cauda epididymis extract. **B.** 2D Electrophoresis of vas deferens extract. **C.** Peptides identified by mass spectrometry from identified spots.

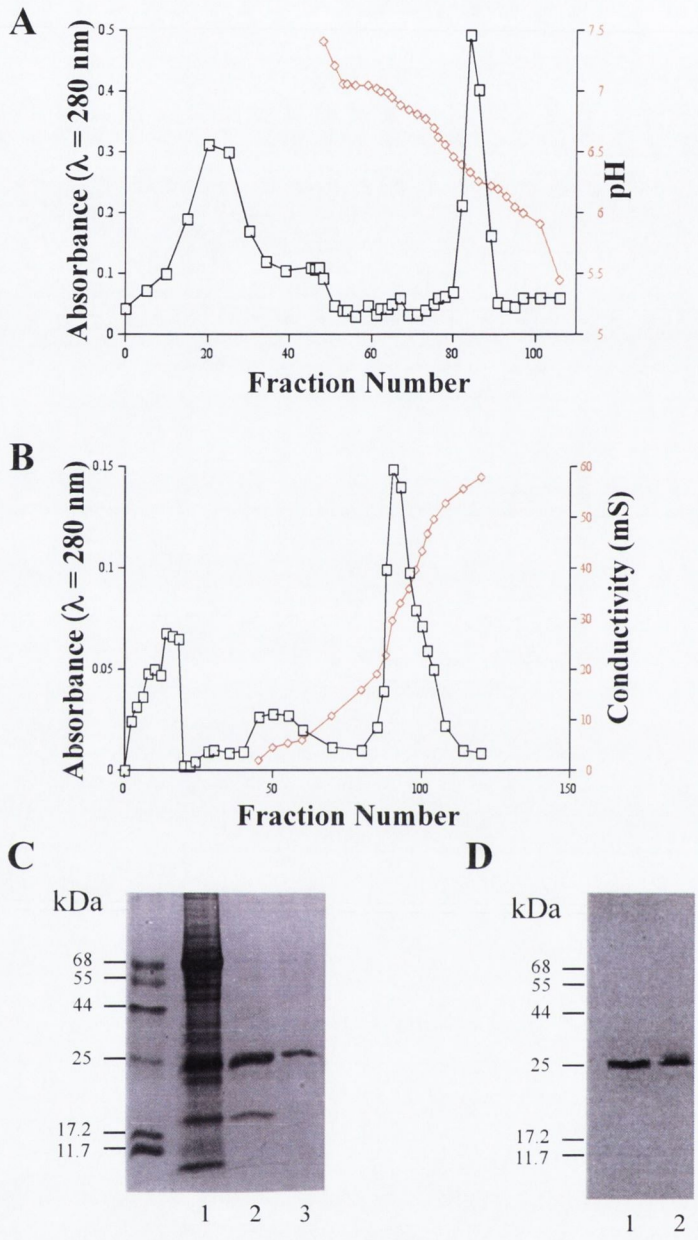
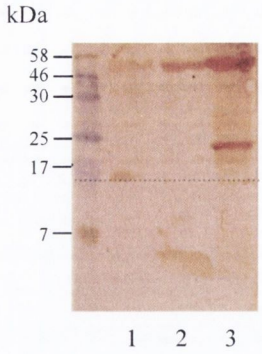


Figure 3.2.3.1 Purification of Mouse CRISP1 from Cauda epididymus and Confirmation of Crisp1 as a Target for Human Antisperm Antibodies. A. Comatofucusing column of mouse cauda fluid. Bound protein is eluted with a pH gradient. **B.** Procion Red HE3B column of the main peak from chromatofocusing column containing CRISP1. Bound protein is eluted with a salt gradient. **C.** SDS-PAGE gel (15%) of cauda epididymal extract (lane 1), main eluted peak from chromatofocusing column (lane 2) and main eluted peak from procion red column (lane 3). **D.** Western blot of main eluted peak from chromatofocusing column (lane 1) and main eluted peak from procion red column (lane 2) using ASA positive sera (Patient 9) as primary antibody.



1. Human Sperm Membrane
2. Human Seminal Plasma
3. Mouse Cauda Epididymal Extract

Figure 3.2.3.2 Anti-CRISP1 Blot of Human Semen Constituents and Mouse Cauda Epididymus. Human Triton X-100 sperm membrane extracts and seminal plasma (20µg each) from normospermic men and mouse cauda epididymus (15µg) were run on a 15% SDS-PAGE gel and transferred to PVDF and probed with anti-CRISP antibody. Blots were visualised using diaminobenzidine.

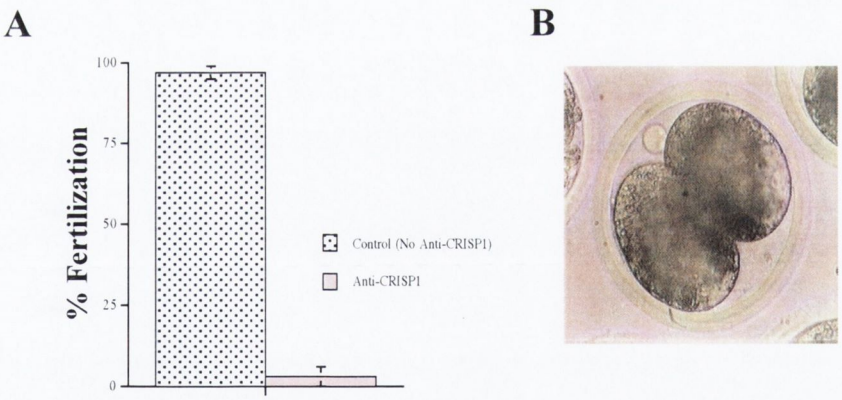


Figure 3.2.3.3 Effects of Anti-CRISP1 Antibody Binding on the Sperm Surface on Fertilization Rate *In Vitro*. A. Mouse sperm derived from the vas deferens were pre-incubated with anti-CRISP1 antibody and then added to a culture plate containing unfertilized eggsderived from superovulated mouse fallopian tubes (5 x 10^4sperm/egg). After 48 hours eggs were assessed for fertilization and 2- and 4-cell embryos classified as fertilised. Control sperm were treated identically except pre-immune rabbit sera used in the place of anti-CRISP1 sera. **B.** Fertilised mouse egg viewed under light microscopy.

confirmed to be a target for ASA (Figure 3.2.3.1 D). Polyclonal antibodies were prepared in a rabbit as described in section 2.3.4 and used to probe a Western blot of human sperm membrane extract, human seminal plasma and mouse cauda epididymis extract (Figure 3.2.3.2). A non-specific band of approximately 55 kDa was noted in all extracts, while as expected the mouse cauda epididymis extract showed a band of around 25 kDa. However, the sperm membrane extract had a reactive band of around 17 kDa while seminal plasma had a reactive band of approximately 6 kDa.

When mouse sperm were pre-incubated with the anti-CRISP1 antibody and incubated with mouse ova in IVF medium as in section 2.3.7, the attachment of anti-CRISP1 antibodies to the sperm surface was found to have a detrimental effect on the fertilization process with less than 1% of eggs being fertilized by sperm pre-incubated with anti-CRISP1 antibodies but having a near 99% fertilization rate when exposed to sperm pre-incubated with the rabbit pre-immune IgG fraction (Figure 3.2.3.3).

3.2.4 MVDP and its Human Orthologue as a Target of Human ASA.

MVDP was purified to homogeneity as described in Chapter 5 and used to inoculate a rat for the purpose of polyclonal antibody production as in section 2.2.17. Purified MVDP was first run on a SDS-PAGE gel and Western blotted using human ASA positive sera. Reactivity of ASA with MVDP was confirmed (Figure 3.2.4.1 A) and the polyclonal antibody generated used to probe a western blot of both purified MVDP and human sperm membrane extract (Figure 3.2.4.1 B). The polyclonal antibody was found to have good specificity for MVDP and also reacted with a band of approximately 35 kDa from human sperm extract, thus confirming the presence of an aldo-keto reductase on the surface of human sperm. Confocal imaging (section 2.2.21) using the polyclonal antibody of both mouse and human sperm indicated that MVDP is mainly localized on the midpiece and tail of mouse sperm, whereas in human, the aldo-keto reductase covers the entire sperm surface of ejaculated sperm with some concentration on the midpiece (Figure 3.2.4.2 A and B). The function of MVDP in the mouse reproductive tract will be further elucidated and discussed in Chapter 4.

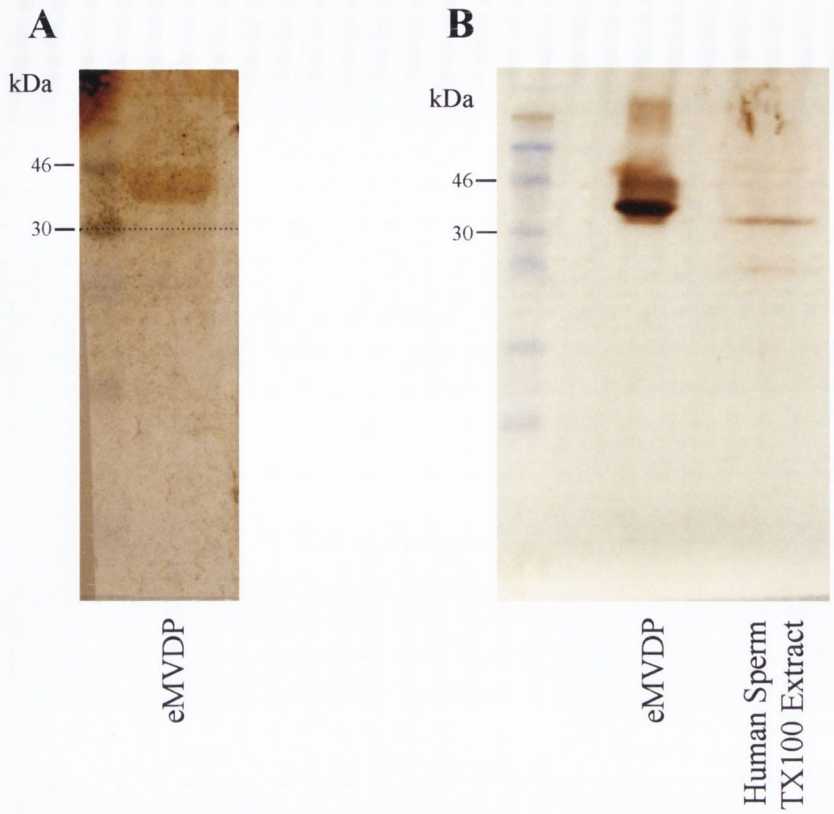


Figure 3.2.4.1 Confirmation of MVDP as a Target for Human ASA and Presence of an Aldo-Keto Reductase on the Human Sperm Surface. A. Purified MVDP (5µg) from mouse vas deferens was loaded onto a 15% SDS-PAGE gel and transferred to PVDF. The blot was probed with ASA positive sera and visualised with diaminobenzidine. **B.** Purified MVDP (5µg) from mouse vas deferens and human Triton X-100 sperm membrane extract (20µg) were loaded onto a 15% SDS-PAGE gel and transferred to PVDF. The blot was probed with anti-MVDP antibody and visualised with diaminobenzidine.

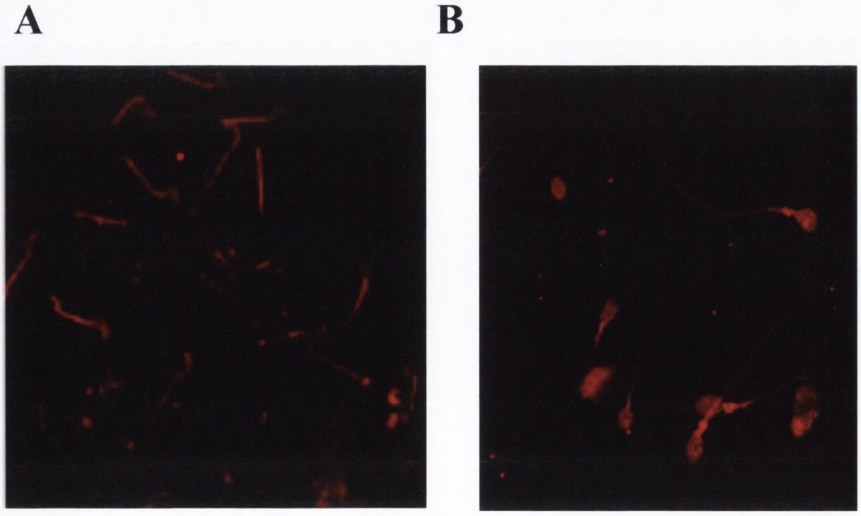


Figure 3.2.4.2 Localization of MVDP and its Human Analogue on the Surface of Human and Mouse Sperm by Confocal Microscopy. A. Localization of MVDP on the mouse sperm surface. **B.** Localization of the human MVDP analogue on the human sperm surface.

3.2.5 Pull Down of Sperm Surface Proteins with ASA.

ASA were absorbed onto the surface of sperm of normospermic patients and the antigen:antibody complexes extracted and prepared for mass spectrometry as described in section 2.4.3 (schematic of protocol in Figure 3.2.5.1).

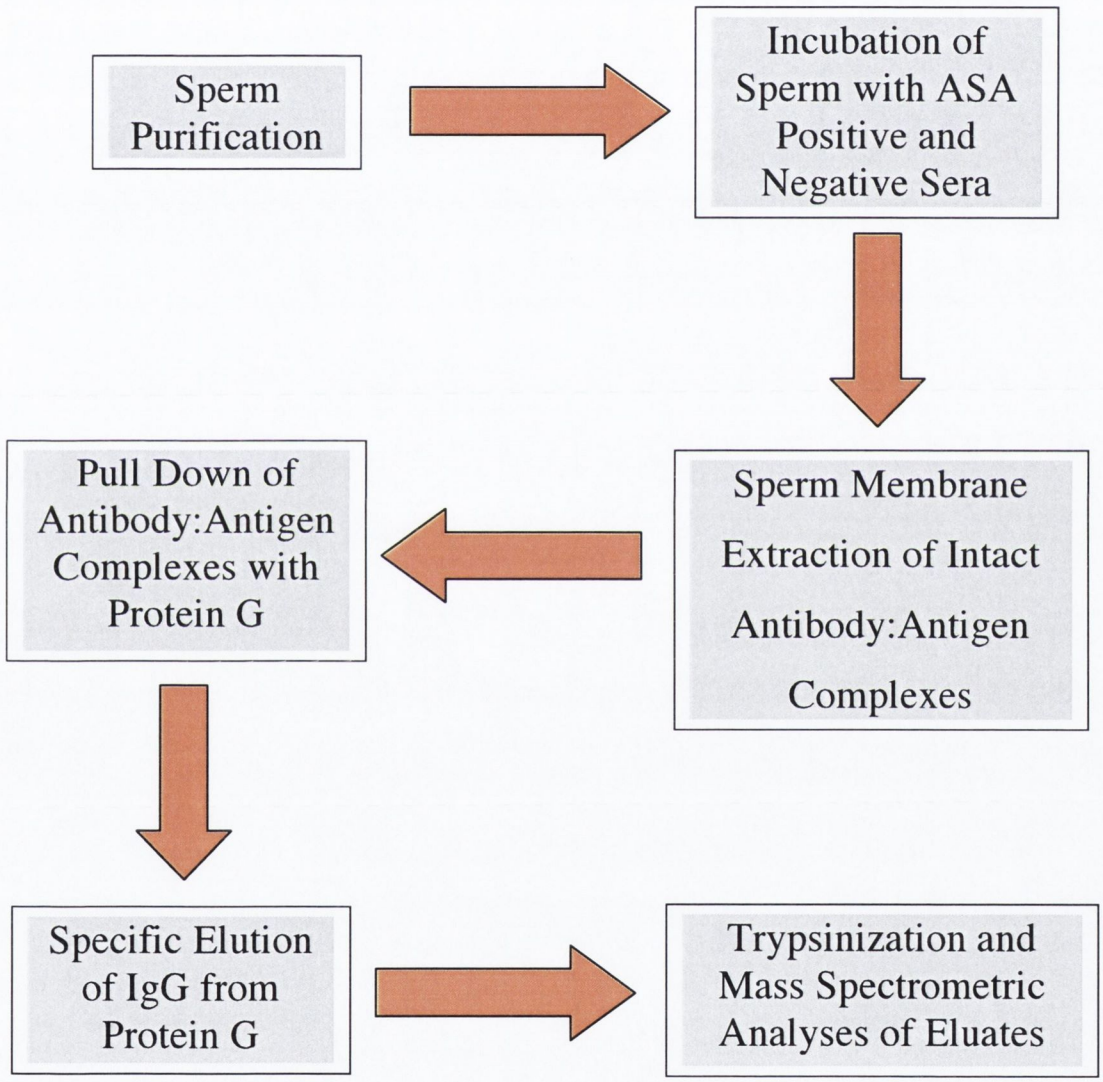


Figure 3.2.5.1 Schematic Representation of Mass Spectrometry Pull Down of Sperm Surface Proteins by ASA.

Mass Spectrometric analyses gave a number of proteins reacting with ASA and from the sperm surface (results tabulated in Table 3.2.5.1).

Protein Identified	Accession Number	# of Peptides Detected	# of Unique Peptides	Predicted Molecular Weight (kDa)
LRRC37B	Q96QE4	7	4	105.6
LRRC37A3	O60309	6	3	180
Radial Spoke Head 1 Homologue	A8MWV0	6	4	30.8
SPACA1	Q9HBV2	2	2	32.1
SPACA4	Q8TDM5	2	1	13
C1orf123	Q9NWV4	3	3	18
S100A8	P05109	2	1	10.8

Table 3.2.5.1 Results of Mass Spectrometric Analyses of ASA Pull Down Experiment.

To confirm mass spectrometry results, Triton X-100 sperm membrane extract, along with Triton X-114 aqueous and detergent phases were western blotted and probed with rabbit anti-LRRC37B antibody as in section 2.2.6. A number of prominent bands were noted in both the Triton X-100 and Triton X-114 detergent phase lanes, with lower levels of binding in the Triton X-114 aqueous phase lane (Figure 3.2.5.2)

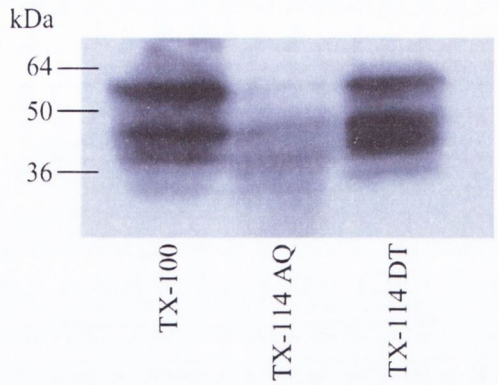


Figure 3.2.5.2 Western Blot of Sperm Membrane Extracts with Anti-LRRC37B Antibody. Sperm extracts (50µg/lane) were loaded onto a 15% SDS-PAGE gel, run at 100V and blotted onto PVDF and probed with anti-LRRC37B antibody.

3.2.6 Sperm Surface Glycolipids as Targets for ASA.

Glycolipids were purified from human sperm as described in section 2.7.1 and separated on TLC plates as in section 2.7.2. Plates were then either stained with orcinol or resorcinol (section 2.7.3) or immunoblotted (section 2.7.5). Orcinol staining detected six glycolipids extracted from the sperm membrane, however, resorcinol only detected three, indicating that these glycolipids had terminal sialic acid residues on their glycosylations (Figure 3.2.6.1 A and B). Immunoblotting using purified IgG from ASA patients (as in section 2.7.4) (Figure 3.2.6.1C) showed a diverse binding pattern of glycolipids with each glycolipid showing binding in at least 4 patients (Results summarized in Table 3.2.6.1). IgG purified from sera of normospermic men gave no glycolipid binding.

Rf Values	Orcinol Staining	Resorcinol Staining	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
0.44	+	+	-	-	+	+	+	+	-	-
0.54	+	-	+	-	+	-	+	+	+	-
0.65	+	+	+	-	+	-	-	+	+	-
0.76	+	-	+	+	+	+	+	+	-	+
0.83	+	-	-	+	-	+	-	+	+	+
0.92	+	+	-	+	-	+	-	-	-	+

Table 3.2.6.1 Detection of Sperm Surface Glycolipids with Orcinol, Resorcinol or by Immunoblotting with ASA Patient Sera.

An ELISA was developed to determine titres of ASA to glycolipids in each individual patient (section 2.7.6). Results showed that levels of ASA anti-glycolipids were of similar levels in most ASA patients and consistently above levels of anti-glycolipid antibodies in normospermic patients. The only divergence from these levels was in patients displaying sperm agglutinating antibodies, who had the highest titres of anti-glycolipid antibodies (Figure 3.2.6.2).

Dot blotting of glycolipids on PVDF followed by cleavage with glycosidases (galactosidase, fucosidase and neuraminadase) or blocking with the lectin, concanavalin A was performed as in section 2.7.8. Dot blots were then subjected to immunoblotting using purified IgG from ASA patients (as in section 2.7.7). Densitometry was performed and the results displayed in Figure 3.2.6.3. Graphing of the results showed that digestion of glycolipids with galactosidase reduced ASA binding by 65% and that blocking with lectin (conA) reduced ASA binding by around 52%. In contrast, cleavage with both neuraminadase and fucosidase both increased ASA binding to glycolipids, by 18% and 7% respectively (Figure 3.2.6.4).

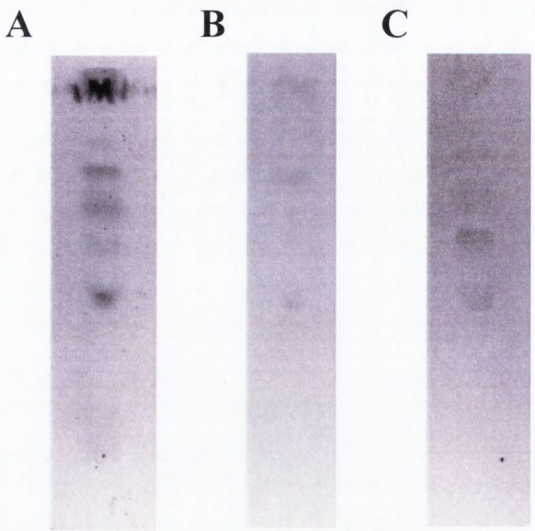


Figure 3.2.6.1 Thin Layer Chromatography (TLC) of Sperm Surface Glycolipids. A. Orcinol staining of TLC separated sperm surface glycolipids. **B.** Resorcinol staining of TLC separated sperm surface glycolipids. **C.** Human ASA patient sera blotting of TLC separated sperm surface glycolipids.

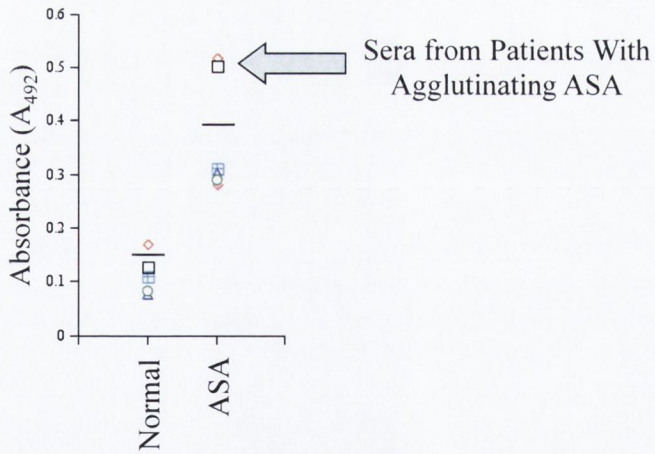


Figure 3.2.6.2 ELISA Based Detection of Human Antisperm Antibodies Reacting with Sperm Surface Glycolipids. Human sperm glycolipids dissolved in methanol were coated onto ELISA plates and probed with IgG from ASA positive and negative patients.IgG was first purified on protein G columns. O-phenylenediamine was used as ELISA substrate and the reaction stopped with sulphuric acid. Absorbance was then read at 492nm.

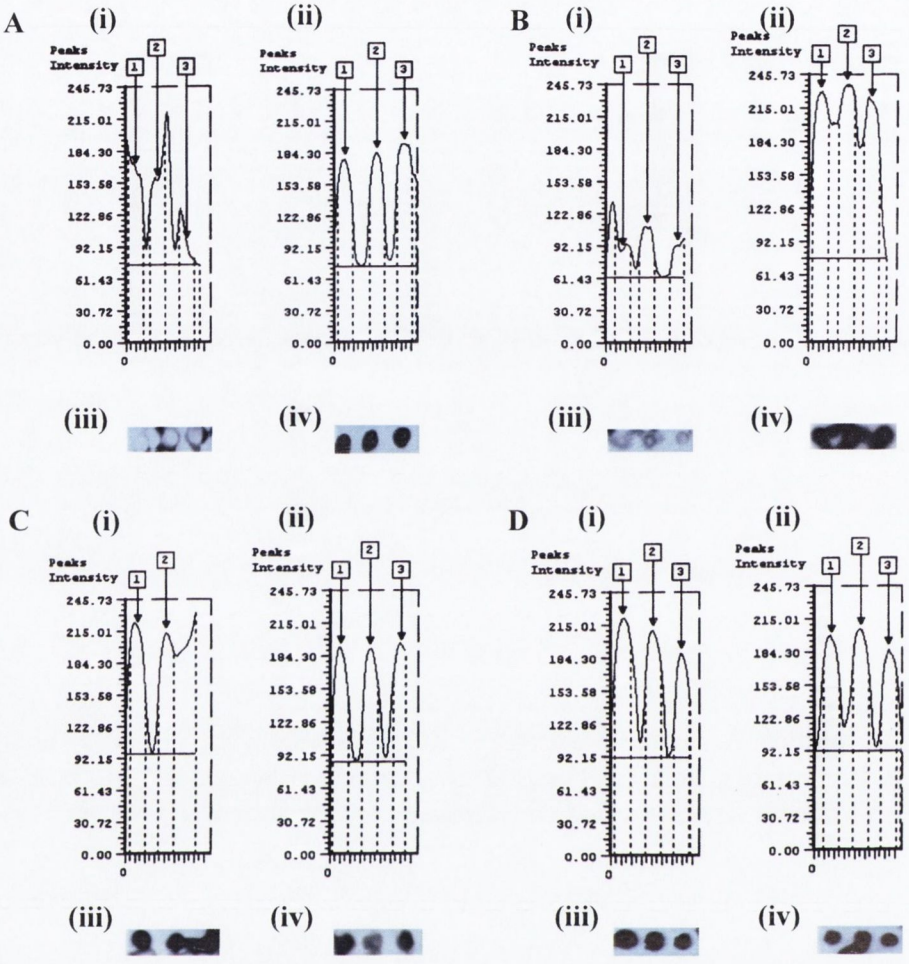


Fig 3.2.6.3 Dot Blotting and Densitometry of ASA Binding to Glycolipids After Cleavage with Glycosidases or Blocking with Lectin. A (i) Densitometric readings of ASA binding to glycolipids first incubated with Concanavalin A **(see A (iii)). A (ii)** Densitometric readings of ASA binding to glycolipids first incubated in Concanavalin A binding buffer **(see A (iv)). B (i)** Densitometric readings of ASA binding to glycolipids first incubated with galactosidase **(see B (iii)). B (ii)** Densitometric readings of ASA binding to glycolipids first incubated in galctosidase cleavage buffer **(see B (iv)). C (i)** Densitometric readings of ASA binding to glycolipids first incubated with fucosidase **(see C (iii)). C (ii)** Densitometric readings of ASA binding to glycolipids first incubated in fucosidase cleavage buffer **(see C (iv)). D (i)** Densitometric readings of ASA binding to glycolipids first incubated with neurominadase **(see D (iii)). D (ii)** Densitometric readings of ASA binding to glycolipids first incubated in neurominadase cleavage buffer **(see D (iv)).**

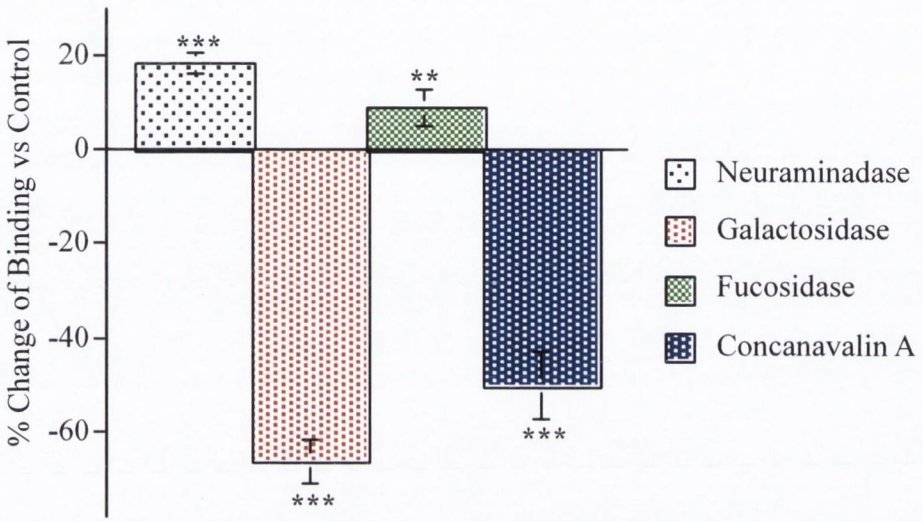


Figure 3.2.6.4 Effects of Glycosidase Treatment and Lectin Binding of Sperm Surface Glycolipids on ASA Reactivity. Glycolipids were spotted (10µl) onto PVDF membranes and subjected to cleavage using the glycosidases, neuraminadase, galactosidase and fucosidase or exposed to the lectin, concanavalin A. After cleavage or lectin binding membranes were probed with purified IgG from patient sera and dot blots visualised using ECL reagent. Densitometry was performed (figure 3.2.6.3) and the results graphed above. Statistical analysis (student t-test) was performed ** $p \leq 0.005$ *** $p \leq 0.001$.

3.3 Discussion.

3.3.1 ASA Localization on the Sperm Surface and Sperm Membrane Damage.

Bohring et al., 2004, performed the only previous comprehensive study on the localization of ASA on the sperm surface. They found that most patients with ASA exhibited binding to the midpiece with serum derived ASA, in good agreement with results presented here (Figure 3.2.1.1). Also, as presented here, they showed lower binding to the acrosome and tail regions of the sperm. No such previous study has been undertaken for seminal plasma derived ASA, but the general binding patterns are similar between sera and seminal plasma from the same patients (Figure 3.2.1.2) with lower levels of both acrosome and tail binding. This may indicate that the titres for these antibodies are lower in seminal plasma or that most of these antibodies are bound to the sperm surface and therefore removed from the seminal plasma on centrifugation of the sperm. It is important to note that in this study the fixation method (2% paraformaldehyde) leaves open the possibility that internal antigens are exposed to ASA binding even in the absence of a permeabilization step. Further controls using antibodies to internal antigens such as tubulin or actin would be advisable to demonstrate whether the sperm outer membrane in compromised during fixation.

Electron micrographs of ASA positive patients when compared to normospermic patients show damage to the outer membrane of the sperm (Figure 3.2.1.3). It has been noted previously that ASA may induce complement activation (D'Cruz et al., 1991). The authors found that complement factors C3 and C5b-9 could both be activated by IgG ASA, which leads to immune injury of human sperm. The previously discovered target protein for ASA, mrt CD52, can interfere with complement activation through binding to C1q (Hardiyanto et al., 2012), though if mrtCD52 is bound by ASA it can be postulated that sperm would be more susceptible to complement mediated injury. As these samples are produced by masturbation, they will not have encountered the acidic environment of the vagina and will not have fused with prostasomes, which carry the complement inhibitors CD46 and CD55. Therefore, complement injury is likely to have occurred either *in vivo* within the reproductive tract of the patient or post-ejaculation during liquefaction of the seminal plasma.

3.3.2 Sperm Surface Antigens Reacting with ASA.

Western blotting of sperm antigens with ASA found a number of common antigens in a high proportion of patients (Table 3.2.1.3). Antigens of 45/47 kDa and 67/68 kDa were found in all patients. These antigens have previously been described in studies by Parslow et al., 1987, Paradisi., et al 1995, Shai and Naot, 1992 and Aitken et al., 1987, but not in de Beer et al., 1992. Each of the other antigenic weights reported here were shown in at least one of the other studies.

Western blotting patterns were relatively similar from patient to patient, however, when serum of a vasovasostomy patient was used as primary antibody (Figure 3.2.1.5) the pattern changed dramatically. After vasectomy the peripheral immune system is exposed to sperm antigens for the first time leading to antibody production, whereas, in patients with primary infertility due to antibodies there is often no prior evidence of injury or infection to the reproductive tract. Therefore, in autoimmune infertility, molecular mimicry may be involved and the "true" antigens for these antibodies may be infectious agents and cross-reactivity with reproductive antigens a byproduct of the immune response. Indeed, cross-reactivity between reproductive tract antigens and bacterial antigens has been observed (Shi et al., 2007).

Western blotting with IgA from patients with ASA showed large numbers of antigens from sperm membrane extracts, many of low molecular weight (Figure 3.2.1.6). In contrast, few antigens for IgA were shown to be present in seminal plasma. The low molecular weight antigens on the sperm surface resemble the blotting for semenogelin (Figure 3.2.1.10) but the absence of these antigens in seminal plasma excludes this possibility. However, blotting of sperm membrane extracts and seminal plasma with IgG from ASA positive seminal plasma also lead to a large number of low molecular weight antigens, though lower amounts of antigen was observed in seminal plasma (Figure 3.2.1.7). Pull down of seminal plasma found that antibody:antigen complexes existed within seminal plasma and the antigens were of very low molecular weight. To identify these antigens, purification techniques were employed (ie chromatofocusing) and an 18 kDa band purified and shown to be a target for ASA (Figure 3.2.1.8). Sequencing of this peptide found it to be a semenogelin 1 fragment. This is the first observation of semenogelin 1 as a target of ASA.

Purification of semenogelin 1 and 2 from seminal plasma (Figure 3.2.1.9) and blotting with the purified proteins with ASA revealed that semenogelin 1 and not semenogelin 2 as a target for ASA (Figure 3.2.1.10). This is unexpected as semenogelin 1 and 2 share a 78% identity. Blotting with the antibody to the 18 kDa reactive band showed that this polyclonal was reactive against both semenogelins and that it existed as multiple differing fragments on the sperm membrane and in seminal plasma. Semenogelin 1 has previously being observed to interact with the sperm membrane through its only cysteine molecule (Cys 239) associating with the protein Eppin on the sperm surface (Wang et al., 2005). The semenogelin 1 fragment (amino acids 164-283) contains the cys239 and is known to act as a sperm motility inhibitor (Mitra et al., 2010). Further cleavage of this molecule by Prostate Specific Antigen (PSA) is required for sperm motility and binding of ASA to this molecule may disrupt induction of sperm motility leading to infertility.

3.3.3 Evolutionary Conserved Mouse Reproductive Tract Protein Epitopes Reacting with ASA.

Previous studies looking for conserved mouse reproductive tract proteins reacting with ASA have exploited two-dimensional gel electrophoresis to separate mouse sperm proteins followed by mass spectrometry (Paradowska et al., 2006). These approaches are also limited as they often reveal internal antigens within the sperm. The approach favoured here is to look for shared antigenic determinants between mouse and human in extracts of epididymis and vas deferens, many of these proteins are later absorbed onto the sperm surface as they transit the reproductive tract.

Western blot analyses of caput and cauda epididymis along with vas deferens showed multiple proteins reacting with ASA. Two proteins, a 26 kDa antigen from cauda epididymis and a 36 kDa protein from vas deferens, were chosen for further investigation as their molecular weights corresponded to antigens previously detected in the western blotting of human sperm extracts and were common to multiple patients (Table 3.2.1.3).

Two-dimensional gel electrophoresis and mass spectrometry were used to identify the antigens (Figure 3.2.2.2). The cauda epididymis 26 kDa protein was identified as CRISP1 and the 36 kDa vas deferens protein identified as MVDP, an aldo-keto reductase, (AKR1B7).

CRISP1 is a member of the CRISP, antigen 5 and Pr-1 (CAP) protein superfamily, which are expressed only in vertebrates and are found mainly in mammalian male reproductive tract and in reptilian venom. CRISPs have 16 conserved cysteines and have two distinct domains, the N-terminal CRISP, antigen 5, Pr-1 (CAP) domain, which contain six cysteines and is approxiamately 20 kDa in size. The other domain is the C-terminal CRISP domain with 10 conserved cysteine residues and is about 6 kDa in size. This domain contains two subsections, a hinge region and an ion channel regulatory (ICR) region (For review see Koppers et al., 2011).

Nomenclature of the CRISP proteins is confusing with human CRISP2 (hCRISP2) having the greatest homology to mouse CRISP1 (mCRISP1) (Koppers et al., 2011), therefore, though mCRISP1 is found to be a target for human ASA in this study (Figure 3.2.3.1), there is a greater likelihood that the true target is hCRISP2. Indeed, Domagala et al., (2007) indicated that hCRISP2 is an antigen for ASA in both vasectomized men and antibodies present in ASA positive seminal plasma but did not find hCRISP1 as an antigen in any other patient group. Western blotting of human sperm membrane extracts and seminal plasma using a polyclonal antibody raised against mCRISP1 showed that reactive bands at around 18 kDa and 6 kDa in sperm membrane extract and seminal plasma respectively (Figure 3.2.3.2), suggesting that hCRISP2 is modified by proteolysis post-ejaculation. As previous peptide mapping experiments of mCRISP1, in this laboratory, showed mCRISP1 to be proteolysed into two similar sized fragments by the activity of chymotrypsin (J. Carroll, personal communication), it seems likely that a seminal plasma protease with chymotrypsin like properties is responsible. As the main chymotrypsin like activity in this compartment is Prostate Specific Antigen (PSA) (Kallikrein 3) it is hypothesized here that PSA is responsible for the cleavage of hCRISP proteins within the ejaculate and that given the molecular weights of the two fragments, that this cleavage occurs at the hinge region of the CRISP protein.

There are multiple lines of evidence that CRISP proteins have a role in sperm-egg interaction at fertilization. Rat protein E (rat CRISP1 homologue) and mCRISP1 associate irreversibly with the sperm outer membrane during epididymal transit (Roberts et al., 2007, Roberts et al., 2003) and remain localized to the region of fusion post-acrosome reaction (Rochwerger and Cuasnicu, 1992). Also, anti-hCRISP1 antisera can significantly reduce the number of sperm penetrating zona free hamster eggs (Ellerman et al., 2006). As demonstated here, anti-mCRISP1 can block *in-vitro* fertilization (Figure 3.2.3.3) almost completely and consequently it can be assumed that patients exhibiting anti-CRISP ASA to have reduced sperm egg interaction with concomitant infertility. As a subset of men with ASA display lowered sperm egg interaction on IVF treatment (Alexander, 1990) it can be assumed that this subset possess ASA to CRISP proteins or other sperm egg interacting proteins at sufficient titres to prevent fertilization.

MVDP (AKR1B7), an aldo-keto reductase, was purified and shown to be a target for ASA (Figure 3.2.4.1), blots with five other patient sera confirmed MVDP to be a target in multiple patients with ASA (results not shown). As MVDP is secreted into the lumen of the vas deferens and that sperm have no known mechanism to internalize proteins, MVDP is likely a sperm surface protein and a "true" target for ASA. Human Aldose Reductase (AKR1B1) has the highest identity among human aldo-keto reductases to MVDP and has been shown to occur on the human sperm membrane (Frenette et al., 2006) and is therefore the likely *in vivo* target of ASA. This is the first report of an aldo-keto reductase as an autoantigen on the sperm surface. MVDP function along with that of its human homologue will be discussed in greater detail in Chapter 4.

3.3.4. Identification of Autoantigens on the Sperm Surface by a Novel Pull Down Method.

Large numbers of previous studies have used two-dimensional gel electrophoresis followed by mass spectrometry to identify sperm antigens targeted by ASA. While this approach is powerful it is also limited, as intracellular antigens are often detected. Studies have identified such diverse proteins as proteome components (C2 and zeta-chain) and ATP synthase beta chain along with many other cytoplasmic and mitochondrial proteins (Bohring and Krause, 2003, Domagala et al., 2007). These internal antigens are highly unlikely to bind ASA *in vivo*, or have a role in autoimmune infertility. However, some studies such as Paradowska et al., (2006), used vectorial labeling of sperm with biotin before extraction to identify antigens present on the sperm surface and to exclude others. This method leads to only small numbers of antigens being detected with a high false negative rate.

The method used here (summarized in Figure 3.2.5.1) binds the ASA to the sperm surface prior to extraction thus removing the requirement to vectorially label the sperm surface. Also, the disassociation of the antibody:antigen complexes from the protein G sepharose using low pH as apposed to SDS sample buffer also reduces the likelihood of non-specifically bound proteins being included in the study.

A total of seven proteins were identified (Table 3.2.5.1), and the process confirmed by Western blotting for one of the antigens, LRRC37B (Figure 3.2.5.2). The distribution of LRRC37B in the Triton X-100 and Triton X-114 detergent phase sperm membrane extracts shows that it has a transmembrane domain as predicted. However, the blot reveals 3 bands of between 40 and 57kDa and not the predicted 105.5kDa in the databases. This is probably due to alternative splice variants of LRRC37B, one of which, C66 SLIT-like protein has been previously shown to be expressed in the testis. LRRC37A3 also has a known testis specific splice variant C114 SLIT-like protein. Both C66 and C114 have previously been implicated in autoimmune infertility (Domagala et al., 2007).

Radial Spoke Head 1 homologue has also been shown previously to elicit ASA responses (Shetty et al., 2007). They show Radial Spoke Head 44 (RSPH 44)(identical

to Radial Spoke Head 1 homologue) to react with ASA and RSPH 44 part of the axonemal core within the sperm tail and also within cilia in the trachea (where it is termed meichroacidin). This internal localization of RSPH 44 should rule it out as a relevant antigen to infertility, but the mouse homologue of RSPH 44, TSGA2, has been shown to have an alternative splice variant which is produced exclusively in the epididymis and coats the sperm during transit, localizing to the anterior acrosome and may play a role in fertilization (Hui et al., 2006).

The sperm acrosome associating proteins SPACA1 and SPACA4 (pseudonyms SAMP32 and SAMP14) have not been shown previously to be autoantigenic. SPACA1 associates with the equatorial segment and inner acrosomal membrane (Hao et al., 2002) whilst SPACA4, a member of the Ly-6/urokinase plasminogen activator receptor (uPAR) superfamily, is localized to the outer and inner acrosomal membranes. SPACA4 is retained on the sperm after acrosome reaction and unwashed sperm displayed a loose association with the plasma membrane (Shetty et al., 2003). Polyclonal antibodies to both SPACA1 and 4 inhibited sperm bind and fusion to zona free hampster eggs, indicating that ASA to these proteins are likely to cause reduced sperm egg binding and contribute to infertility in these individuals.

S100A8 has not previously been shown to elicit an ASA response, but S100A9, to which S100A8 is often associated in a dimer, has (Domagala et al., 2007). The S100A8/S100A9 dimer is a known to be a danger-associated molecular pattern (DAMP) molecule that is pro-innflammatory (Srikrishna, 2012). Its role in fertility and how ASA may affect its function are yet to be elucidated.

C1orf123 is a hypothetical protein not yet described as been present on the sperm surface, again, its role in fertility and how ASA may affect its function are yet to be elucidated.

3.3.5 Glycolipids as Autoantigens for ASA.

As glycosylations have previously been described to contribute to infertility through the generation of female ASA against mrtCD52 and seminolipid (Tsuji et al., 1988, Tsuji et al., 1992), definition of their role in male ASA needed to be clarified. Of the six glycolipids (three of which are gangliosides) found in the Folch's upper phase extraction, all bound ASA from at least three patients tested (Figure 3.2.6.1 and Table 3.2.6.1). Measurement of ASA titres to glycolipids (Figure 3.2.6.2) shows titres in ASA patients are higher than in normospermic patients. Interestingly, the highest titres were all in patients with sperm agglutination in their semen samples, this corresponds with the observation that polyclonals produced in rabbits to sperm glycolipids induce sperm agglutination (Kurpisz et al., 1989).

Results of digestion of the glycosylations with glycosidases (Figure 3.2.6.3 and 3.2.6.4) indicate that ASA are directed to the glycosylation of the glycolipid and not the core sphingosine entity or too any oxidation product of the lipid chain. It can also be concluded from these results that the terminal residues, sialic acid and fucose, are not targets for ASA and mask the inner lactosamine structures which can be cleaved by galactosidase, thus reducing ASA binding.

3.4 Summary.

Using mouse reproductive tract antigens both CRISP1 and MVDP were shown to be targets for ASA. Antibodies to CRISP1 were also demonstrated to interfere with sperm-egg binding and to block fertilization *in vitro*. Antibody-antigen complexes were demonstrated to occur in seminal plasma and that the target in the seminal plasma of the auto-antibodies was semenogelin 1 and not semenogelin 2. A mass spectrometric approach to the identification of sperm surface led to the discovery of seven sperm proteins previously not associated with ASA. Finally, sperm surface glycolipids were shown to be targets of ASA.

Chapter 4

Role of Mouse Vas Deferens Protein (MVDP, AKR1B7) in the Reproductive Tract.

4.1 Introduction.

The Aldo-keto Reductase (AKR) super-family consists of over 125 members in 14 sub-families and are found in bacteria, fungi, plants and animals. AKRs have an important role in the reductive detoxification of aliphatic and aromatic aldehydes into their corresponding alcohols in a NADPH-dependent manner (Jez and Penning, 2001). MVDP (AKR1B7) is a member of this family and has been shown to be expressed in high amounts in vas deferens under the regulatory control of the androgen receptor (Taragnat et al., 1988). It is also expressed in the adrenal cortex where it is regulated by adrenocorticotropic hormone (Aigueperse et al., 1999, Lau et al., 1995), in the ovary where it is expressed upon luteinizing hormone stimulation (Brockstedt et al., 2000) and in ovarian theca cells on application of human chorionic gonadotrophin (Baumann et al., 2007), as well as in lower amounts in the intestine, eye, kidney and liver. Xenobiotic insult leads to MVDP expression in some of these tissues under the transcriptional control of the nuclear factors, liver X receptor (Volle et al., 2004), pregnane X receptor and constitutive androstane receptor (Liu et al., 2009) and farnesoid X receptor (Ge et al., 2011).

The major substrates described to date for MVDP are isocaproaldehyde and 4-hydroxy-2-nonenal (HNE) (Martinez et al., 2001). These are reactive aldehydes whose rapid detoxification and removal are necessary for cell survival. Isocaproaldehyde is produced as a by-product of cholesterol side-chain cleavage during steroidogenesis and therefore the likely primary function of MVDP in both the adrenal cortex and ovarian theca is the detoxification of isocaproaldehyde. However, as the vas deferens is not a known site of steroidgenesis, it is unlikely that the high level of expression of MVDP at this site is functionally related to the detoxification of isocaproaldehyde. Baumann et al., (2007) has hypothesised that the likely substrate for MVDP at this

location was HNE. HNE is produced as a result of polyunsaturated fatty acid peroxidation by reactive oxygen species (Keller et al., 1997). As the membranes of spermatozoa being transported by the vas deferens contain higher proportions of polyunsaturated fatty acids than other cell types, MVDP may have a protective role against lipid peroxidation. However, monoclonal antibodies to protein adducts of the lipid peroxidation product malondialdehyde showed only weak histological staining in different regions of the rat reproductive tract indicating that lipid peroxidation is limited in these tissues (Iuchi et al., 2004). Another monoclonal antibody revealed high levels of staining to the protein adduct of the reactive aldehyde, acrolein.

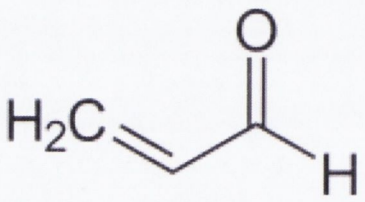


Figure 4.1.1 The chemical structure of acrolein (Propanal).

While acrolein (propanal) is also formed from lipid peroxidation (Uchida, 1999), yields of acrolein are far lower than for other products such as HNE (Spiteller et al., 2001). It is unlikely therefore that lipid peroxidation represents a major source of acrolein in humans (Stevens and Maier, 2008). The main source of acrolein in the reproductive tract is potentially from the catabolism of the polyamines spermine and spermidine, found in high concentrations (2.41mM and 0.14mM respectively) in human semen (Vallely and Rees, 1986), by quinone-dependent copper amine oxidase (diamine oxidase DAO). DAO oxidizes spermine to spermidine and then to putrescine liberating acrolein and ammonia as by-products in stoiciometrically equal amounts (Lee and Sayre, 1998). DAO is found in human semen where high levels of enzyme activity correlate with loss of spermatozoa motility (Holtta et al., 1975) most likely due to the cytotoxicity of acrolein (Maayan et al., 1995). Acrolein is a highly reactive aldehyde mediating a number of adverse effects. It can form strand breakages and adducts with DNA and adducts with lysine, arginine, cysteine and histidine in proteins. The main amino acid target for acrolein adduction is lysine where it forms N$^{\varepsilon}$-(3-formyl-3,4-dihydropiperidino)lysine (FDP-lysine) (Uchida et al., 1998). FDP-

lysine adducts, as mentioned, have been shown to occur in the male reproductive tract of the rat using a monoclonal antibody specific for acrolein-modified protein, with high levels of immunostaining detected in the epithelia of the epididymis, vas deferens, seminal vesicles and prostate gland. In each of these tissues, aldehyde reductase expression, a product of the *AKR1A* gene, co-localised with this staining, but was not associated with the spermatozoa membrane (Iuchi et al., 2004).

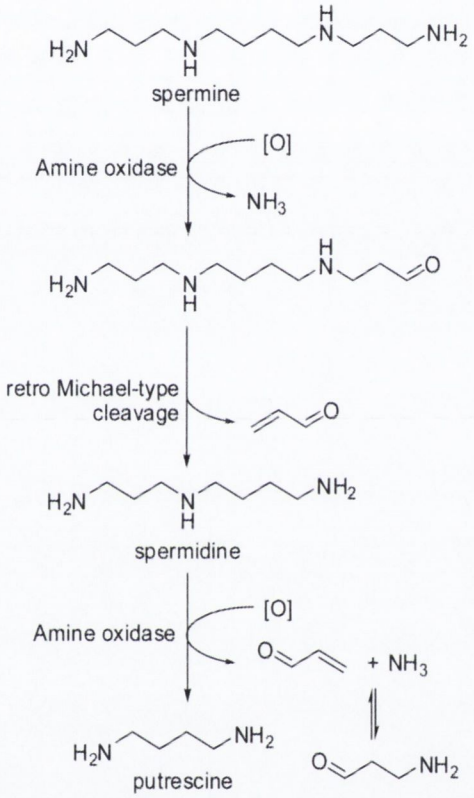


Figure 4.1.2 The catabolic breakdown of spermine by serum amine oxidases. Products are ammonia, acrolein and 3-aminopropanal. The 3-aminopropanal in physiological conditions occurs as an equilibrium with acrolein and ammonia. (Taken from Stevens and Maier, 2008)

The aim of this chapter was to identify a physiological function for MVDP and its human analogue. Acrolein, a highly reactive aldehyde, produced as a byproduct from the catabolism of spermine and other polyamines by polyamine oxidases present in seminal plasma, was shown to be a substrate for MVDP and its human analogue. MVDP and its human analogue are therefore present on the sperm surface to detoxify

acrolein and other reactive aldehydes that would otherwise cause protein adduction and DNA strand breakage, thereby reducing fertility.

4.2 Results.

4.2.1 Ammonium Sulphate Precipitation of Vas Deferens Homogenate.

Vas Deferens were excised from mice and homogenised as in section 2.2.9. After centrifugation the resulting supernatant was subjected to ammonium sulphate fractionation in increasing concentrations as described in section 2.2.13.1. SDS-Page gels (Figure 4.2.1A indicated that MVDP was present in the 80% and 100% ammonium sulphate pellets. These fractions were combined and dialysed against MonoQ loading buffer as described in section 2.2.13.2. Analysis after dialysis by SDS-Page (Figure 4.2.1B) of both soluble and precipitated protein during dialysis indicated that a large proportion of the MVDP remained soluble. A second major contaminating band of approximately 14 kDa was noted in the dialysate, this band purified away in subsequent steps and was likely to be myoglobin based on its colour.

4.2.2 FPLC MonoQ Chromatography of Dialysed MVDP Fraction.

Dialysed MVDP as in section 4.2.1 was loaded onto a MonoQ column and the column washed as in section 2.2.13.3 and bound protein was eluted using a 5-500mM NaCl gradient. Figure 4.2.2A is a representative chromatogram of the MonoQ purification step of MVDP. SDS-Page analysis of the fractions from MonoQ chromatography (Figure 4.2.2B) indicated that although MVDP came off over a number of fractions it was mostly concentrated in fraction B7-B5 and these fractions were pooled and concentrated to 0.5ml as in section 2.2.13.4 for further purification.

4.2.3 FPLC Sephadex75 10/30 Chromatography of MVDP.

Concentrated fractions from MonoQ chromatography as described in section 4.2.2 were loaded onto a Sephadex75 10/30 column and run as described in section 2.2.13.4. Figure 4.2.3A is a representative chromatogram of the Sephadex75 10/30 size exclusion

chromatography. SDS-Page analysis (Figure 4.2.3B) indicated that MVDP eluted at fractions C1-C3. Western blotting was used to confirm identity of MVDP (Figure 4.2.3C) using rat anti-MVDP antibodies as previously described in section 2.2.6.

4.2.4 Native gel electrophoresis of purified MVDP.

Purified MVDP (10μg) was run on a native gel as described in section 2.2.14.1 to determine the homogeneity of the purified protein. MVDP was resolved into two isoforms (Figure 4.2.4A). These bands were excised and run on a second dimension 15% SDS-Page gel to confirm they ran as a single weight (Figure 4.2.4B).

4.2.5 Deglycosylation of purified MVDP.

Purified MVDP (10μg) was deglycosylated as described in section 2.2.14.2 using Peptide-N-Glycosidase F (PNGase) cleavage. Ribonuclease A (RNaseA) was used as control. The reduction in molecular weight (Figure 4.2.5) of RNaseA (lane 2) over control RNaseA (lane 1) demonstrates that it is heavily N-glycosylated, however, the PNGase treated and control lanes for MVDP (lanes 3 and 4) show no change in molecular weight indicating no N-glycosylation of MVDP.

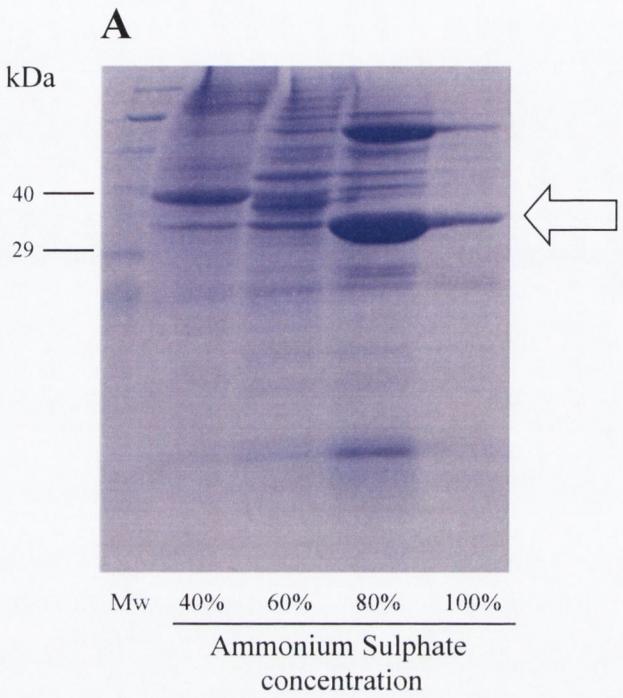


Fig 4.2.1. Fractionation of Vas Deferens homogenate by ammonium sulphate precipitation. **A.** 15% SDS-Page gel of precipitates at increasing ammonium sulphate concentrations and final supernatant. Arrow indicates the position of MVDP. **B.** 15% SDS-Page gel of the 80% and 100% pellet combined and dialysed. Lane 1, Soluble proteins post dialysis. Lane 2, Precipitated proteins post dialysis.

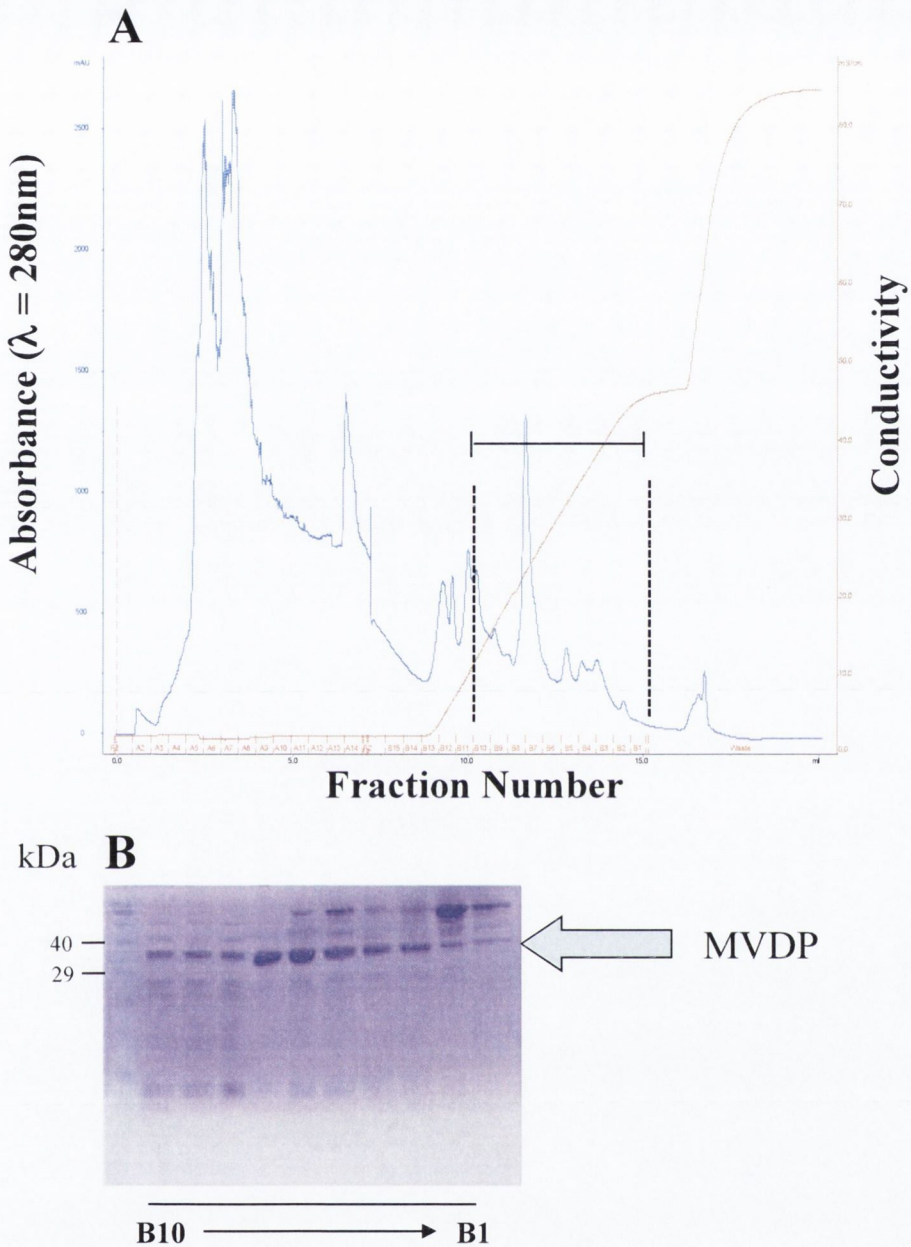


Fig 4.2.2. Purification of MVDP on FPLC MonoQ 5/50 column. **A.** Elution profile of dialysed ammonium sulphate fractions containing MVDP loaded onto a MonoQ 5/50 column. Bound proteins were eluted with a 5-500mM NaCl gradient and collected in 0.5ml fractions. **B.** 15% SDS-Page gel of eluted fractions B10 to B1 from MonoQ 5/50 column.

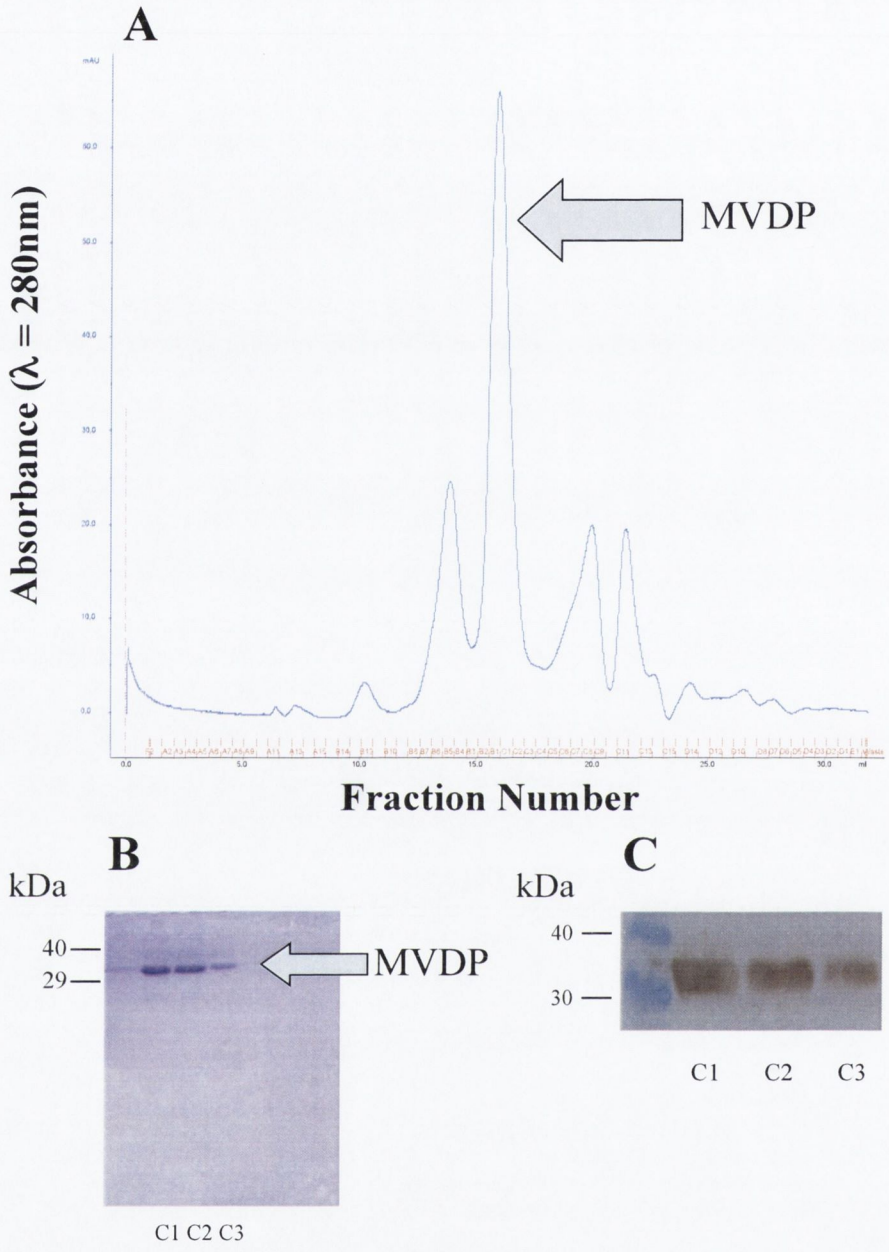


Fig 4.2.3. Purification of MVDP on FPLC Superdex 75 10/300 column. **A.** Elution profile of fractions from MonoQ column containing MVDP loaded onto a Superdex 75 10/300 column. Proteins were collected in 0.5ml fractions. **B.** 15% SDS-Page gel of fractions C1 to C3 from Superdex 75 10/300 column. **C.** Western blot of fractions C1 to C3 from Superdex 75 10/300 column using rat anti-MVDP antibody.

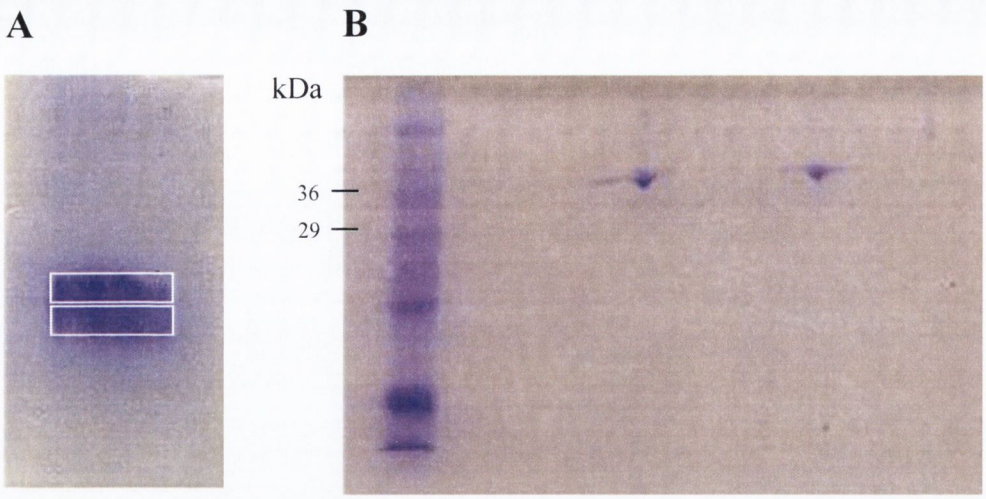


Fig 4.2.4. Isoform detection in purified MVDP. **A.** Native gel of purified MVDP. **B.** 12% SDS-Page gel of top and bottom bands of MVDP from native gel. Gel pieces were excised from native gel, equilibrated in SDS sample buffer and loaded whole onto SDS-Page gel.

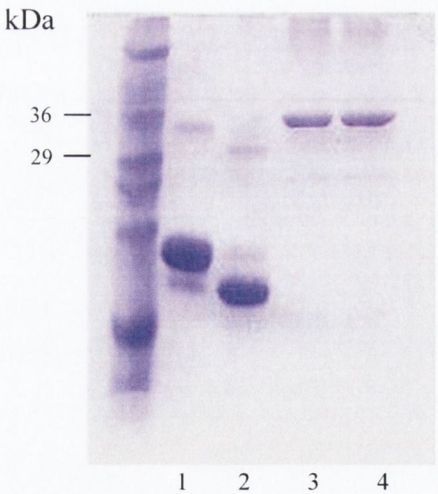


Fig 4.2.5. Deglycosylation of MVDP with PNGase. Lane 1, Control RNase A. Lane 2, RNase A digested with PNGase. Lane 3, Control MVDP. Lane 4, MVDP digested with PNGase.

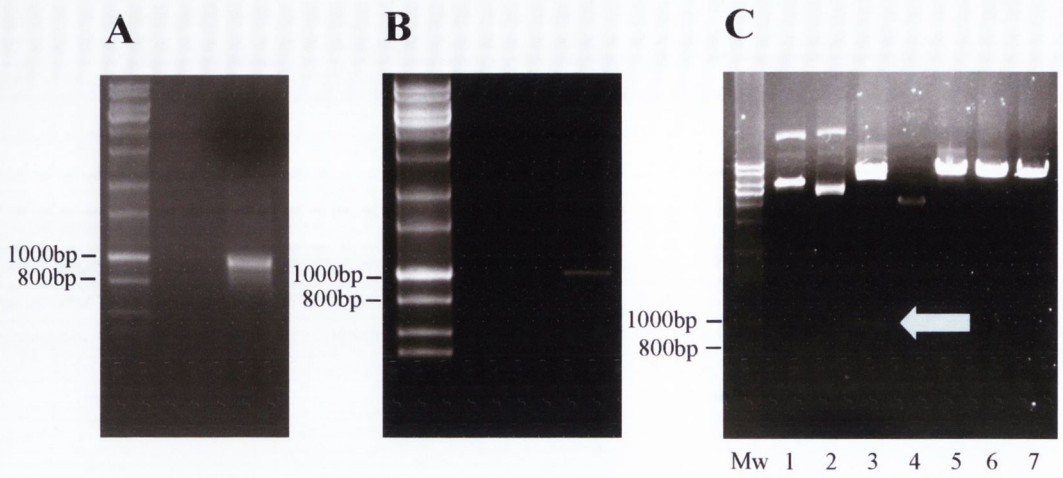
4.3 Cloning and expression of recombinant MVDP.

4.3.1 Cloning of MVDP into the pHis-parallel-2 Vector and Test Expression of rMVDP.

PCR was performed as in section 2.2.16.3 using IMAGE clone 6775941 CL-C3 as template. PCR product was run on a 1.2% agarose gel as in section 2.2.15 and stained with ethidium bromide giving a band of approximately 1000 bp, the correct size for the MVDP gene (Figure 4.3.1A). The product was purified using the Roche HiPure PCR purification spin columns as in section 2.2.16.4 and the purified PCR product subjected to restriction enzyme cleavage with *Bam*H1 and *Eco*R1 as in section 2.2.16.4 and re-purified on a Roche HiPure PCR purification spin column and run on a 1.2% agarose gel as before (Figure 4.3.1B). The pHis-parallel-2 vector was also cleaved with restriction enzymes and purified on a Roche HiPure PCR purification spin column as in section 2.2.16.4. Vector and PCR product were added together in a 1:1 ratio and ligated using the Takara ligation kit as in section 2.2.16.4. Ligation product was transformed in *E. coli* DH5α cells and plated on agar plates with ampicillin as in section 2.2.16.4. Colonies were grown and plasmids isolated using the Roche HiPure plasmid isolation kit (section 2.2.16.4) and the plasmids subjected to restriction enzyme cleavage. Cleaved plasmids were run on a 1.2% agarose gel as before (Figure 4.3.1C). Only lane 3 indicated the presence of the PCR insert.

The plasmid containing the MVDP insert was transformed into *E. coli* BL21 cells as in section 2.2.16.5. Test expressions in 4ml of LB broth for two and three hours post induction with IPTG were carried out (Figure 4.3.1D). Un-induced cells (lane 1) displayed no rMVDP production, while both the two and three hour inductions (lanes 2 and 3 respectively) showed large amounts of rMVDP expression.

Testing of solubility and purification (Figure 4.3.1E) were carried out in 1L of LB broth as in section 2.2.16.5. Cells were harvested by centrigugation, resuspended and sonicated as in section 2.2.16.5. Whole lysate (Lane 1) and supernatant of lysate (Lane 2) indicated that the MVDP partitioned into the soluble phase. The pellet of the whole lysate showed the presence of a minor amount of MVDP (lane 3). The soluble phase was passed through a nickel-sepharose column as in section 2.2.16.6. The unbound



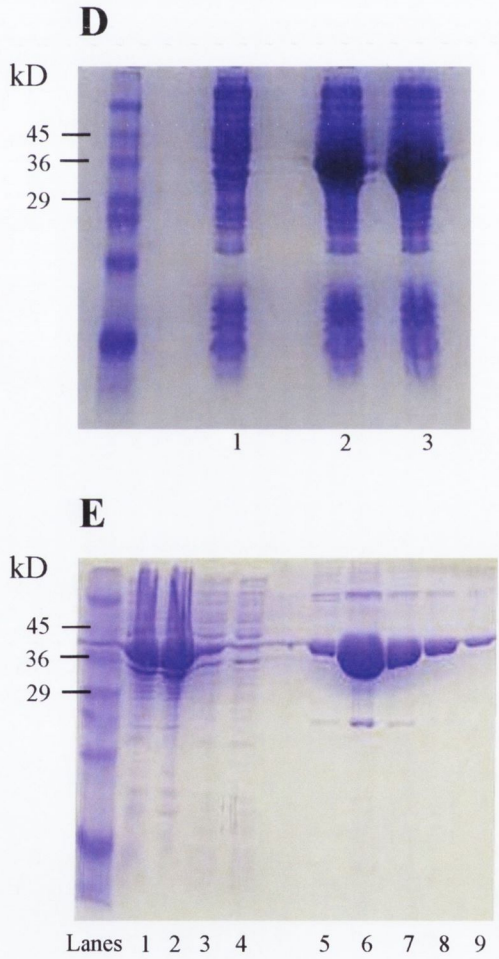


Fig 4.3.1. Cloning and expression of MVDP. **A.** PCR of cDNA of MVDP. **B.** PCR product after cleavage with restriction enzymes and purification. **C.** Ligation of PCR product into pHis-parallel 2 expression vector. Lanes 1 and 2 are uncleaved vector control. Lanes 3 to 7 are purified plasmids from DH5α *E. coli* colonies transformed with ligation mix. Arrow indicates clone positive for inclusion of MVDP PCR product. **D.** Test expression of rMVDP in BL21 *E. coli*. Lane 1, uninduced *E. coli*. Lane 2, 2 hour induction with IPTG. Lane 3, 3 hour induction with IPTG. **E.** Partial purification of rMVDP on nickel-sepharose. Lane 1, Whole lysate after sonication of BL21 *E. coli* with induction by IPTG for 3 hours. Lane 2, Supernatant after centrifugation of whole lysate from lane 1. Lane 3, Pellet after centrifugation of whole lysate from lane 1. Lane 4, Proteins passing straight through nickel-sepharose column. Lanes 5-9, Protein fractions eluted from nickel-sepharose column with 250mM imidazole.

protein fraction was collected (Lane 4) with only a limited amount of rMVDP not binding the nickel-sepharose column. Proteins were eluted from the column using 250mM imidazole as in section 2.2.16.6. Fractions (Lanes 5-9) contained the rMVDP with only minor contamination.

4.3.2 Further Purification of rMVDP by Gel Filtration.

Fractions containing rMVDP from nickel-sepharose chromatography from previous section were concentrated to 0.5ml and run on a FPLC superdex 75 10/300 column as in section 2.13.4. A single peak was obtained (Figure 4.3.2A) and confirmed to be rMVDP by SDS-Page (Figure 4.3.2B).

4.4 Kinetic Analysis of rMVDP and eMVDP.

4.4.1 Analysis of rMVDP and eMVDP with 4-Hydroxynonenal as Substrate.

Kinetic analysis of both rMVDP and eMVDP using HNE as substrate indicated that both forms had the same catalytic profile as previously reported for MVDP from adrenal gland (Martinez et al., 2001)(Figure 4.4.1). The K_m for 4-HNE as substrate was 6.67μM using eMVDP and 12.97μM using rMVDP while the K_{cat} values were 2.21 min^{-1} and 2.17 min^{-1} for eMVDP and rMVDP respectively (see Table 4.4.1).

4.4.2 Analysis of rMVDP and eMVDP with Acrolein as Substrate.

As lipid peroxidation products are not the likely substrate in the reproductive tract, other substrates were investigated. Acrolein, which has previously been shown to form high levels of adducts in the rat reproductive tract (Iuchi et al., 2004), was tested (as in sections 2.2.18 and 2.2.19) and exhibited good substrate qualities (Fig 4.4.2) with a K_m of 17.59μM and k_{cat} of 3.789 min^{-1} for eMVDP while rMVDP had a K_m of 17.81μM and k_{cat} of 1.897 min^{-1} (Table 4.4.1).

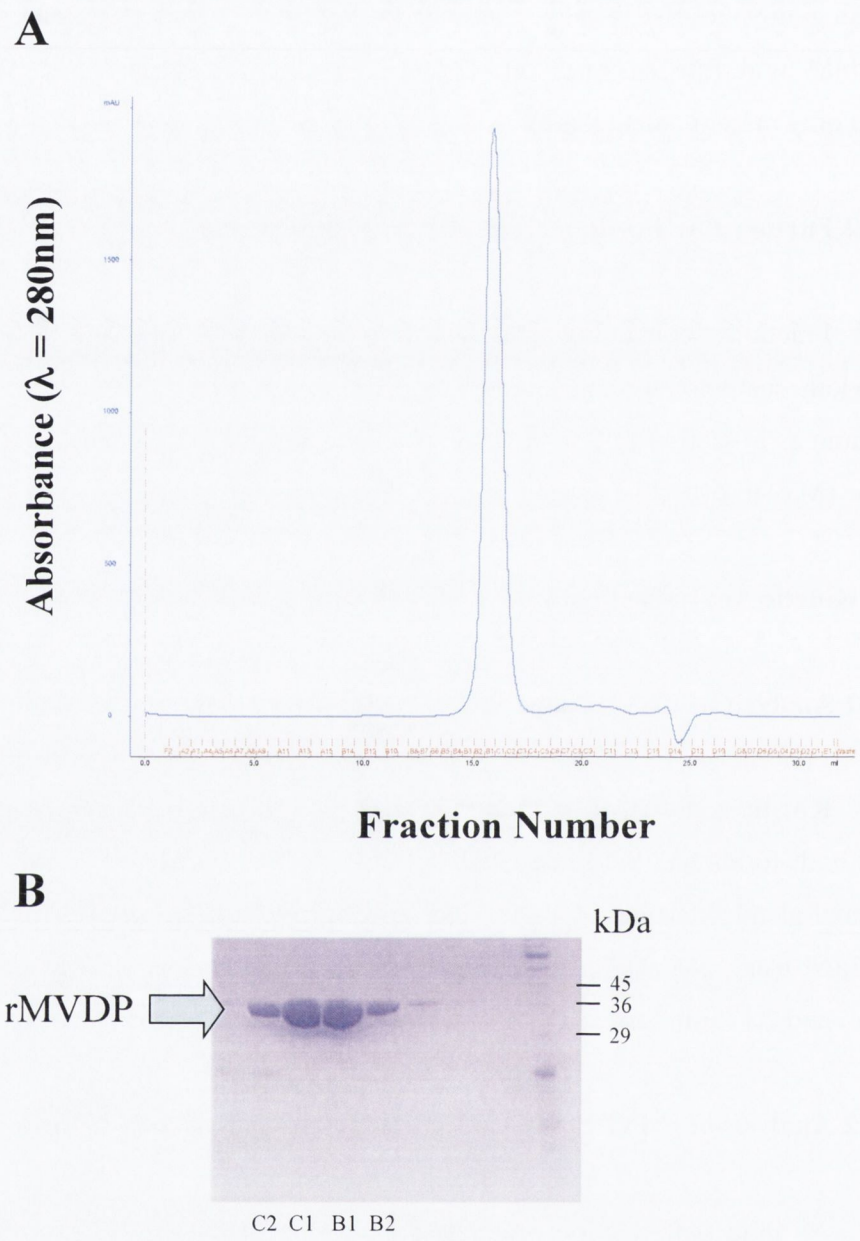


Fig 4.3.2. Purification of rMVDP on FPLC Superdex 75 10/300 column. **A.** Elution profile of fractions from nickel-sepharose column containing rMVDP loaded onto a FPLC Superdex 75 10/300 column. Proteins were collected in 0.5ml fractions. **B.** 15% SDS-Page gel of fractions B1 to C2 from FPLC Superdex 75 10/300 column.

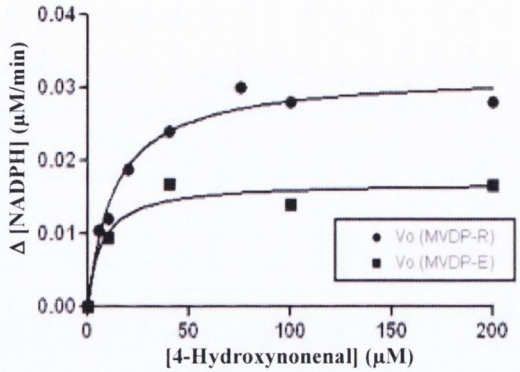


Fig 4.4.1. Michaelis-Menten plots for eMVDP and rMVDP with 4-hydroxynonenal as substrate (0-200μM). NADPH was kept constant at 50μM and 0.25μg/ml and 0.1μg/ml of rMVDP and eMVDP included respectively.

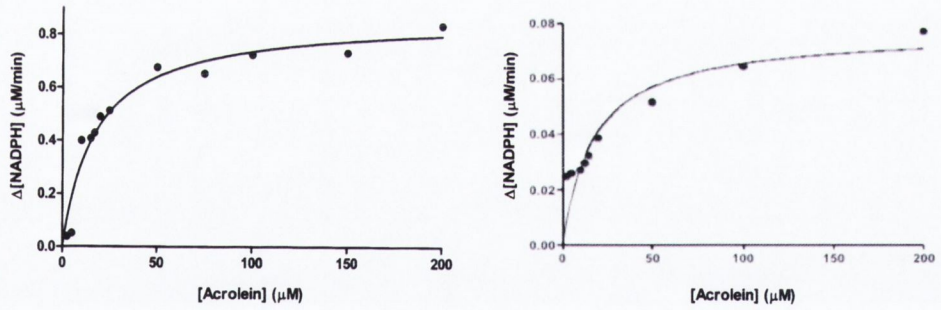


Fig 4.4.2. Michaelis-Menten plots for MVDP with acrolein as substrates for **A** rMVDP (60μg/ml) and **B** eMVDP (0.55μg/ml). NADPH was kept constant at 50μM.

	eMVDP		rMVDP	
Substrate	K_m (μM)	K_{cat} (min^{-1})	K_m (μM)	K_{cat} (min^{-1})
HNE	6.67	2.21	12.97	2.17
Acrolein	17.59	3.789	17.81	1.897

Table 4.4.1. Table of kinetic constants for eMVDP and rMVDP with 4-hyroxynonenal and acrolein as substrates.

4.4.3 Detection of Acrolein Reductive Activity in Human Seminal Extracts.

Human sperm membrane Triton X-100 extract (as in section 2.2.20) also displayed acrolein detoxification with a specific activity of 11.54μmol min^{-1} mg^{-1} while no activity could be detected in seminal plasma (Table 4.4.3).

	Specific Activity of Acrolein Reduction (μmol min^{-1} mg^{-1})
Human Sperm Membrane Extract	11.54
Human Seminal Plasma	Not Detected

4.5 Reduction in Spermine Catabolite Toxicity by rMVDP in a Model System.

Bovine foetal calf serum (FCS) contains an amine oxidase similar to human seminal amine oxidase (DAO), and has been used previously to demonstrate the cytotoxic and immunosuppressive effects of spermine catabolites (Maayan et al., 1995). To demonstrate the ability of MVDP to reduce cytotoxicity on exposure of cells to acrolein, a model system using the monocyte cell line, THP1, was devised as in section 2.2.22 (Figure 4.5.1). The FCS amine oxidase will oxidize spermine thereby releasing acrolein as a reaction product. MVDP increased the IC_{50} for acrolein cytotoxicity in this system from 3.173μM to 14.11μM with 20μg of MVDP present (Table 4.5.1), thus indicating a role for MVDP in protection from spermine oxidation.

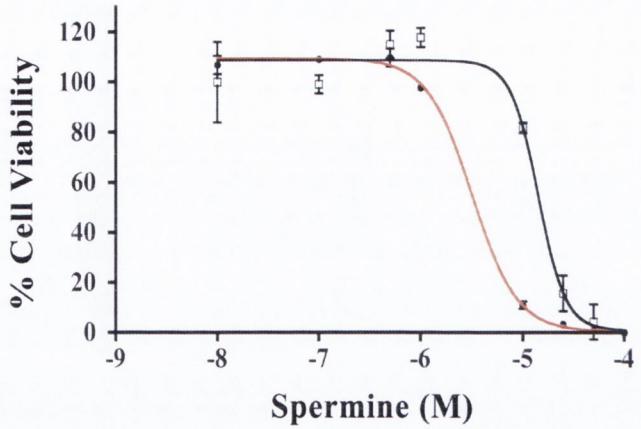


Fig 4.5.1. Reduction of spermine catabolite toxicity by rMVDP. THP1 cells were treated with a range of concentrations of spermine in the presence (--- black line) or absence (--- red line) of 20μg of rMVDP.

	+ MVDP (μM)	-MVDP (μM)
IC_{50}	14.11 +/- 1.63	3.173 +/- 0.76

Table 4.5.1. IC_{50} data for reduction of spermine toxicity by rMVDP in THP1 cells.

4.6 Discussion.

As demonstrated in chapter 3, MVDP has conserved evolutionary epitopes that are recognized by human ASAs. Also, that antibodies to MVDP bind a protein of similar size present on the human sperm surface and confocal microscopy indicates that MVDP in the mouse and its human analogue are distributed over the entire sperm surface. However, the substrate of MVDP in the reproductive tract has not been fully elucidated.

The role of MVDP in the vas deferens has been assumed to be the prevention of toxicity to sperm of lipid peroxidation products and ensuring their continued viability (Baumann et al., 2007). This though can be disputed by the results of Iuchi et al., (2004) who demonstrated limited protein adducts of lipid peroxidation products in the rat reproductive tract. They did however show large levels of FDP-lysine, the protein adduct of acrolein. As acrolein is only a minor byproduct of lipid peroxidation, the major source of acrolein in the reproductive tract is likely to be from the catabolism of the polyamines, spermine and spermidine. Though acrolein concentrations in human seminal plasma have not been measured, basal aldehyde levels have, with normospermic patients having approximately 359μM levels (Maayan et al., 1995). As Iuchi et al., (2004) demonstrated only low levels of lipid peroxidation products but high levels of acrolein adduction it can be deduced that much of the basal aldehyde measured is acrolein.

MVDP knock-out mice (Baumann et al., 2007) displayed no loss of reproductive success, however, they did display a non-significant trend in reduction of vas deferens sperm motility. The decrease in sperm motility is likely as a result of increased acrolein concentration from spermine catabolism as MVDP is no longer present to detoxify this reactive aldehyde, these results would therefore correlate well with the result that increased DAO activity, which would also lead to increased acrolein concentration, reduces sperm motility (Holtta et al., 1975). The function of DAO activity in seminal plasma has not been fully investigated, however, its action in splitting spermine into spermidine, ammonia and acrolein could have two functions. Firstly, spermidine is a substrate for transglutaminase and its role will be discussed further in chapter 5, secondly, ammonia may provide the high pH of seminal plasma (normal range pH 8-

8.6, mean 8.2) (Harraway et al., 2000). The function of this high pH may be to prevent premature prostasome fusion with sperm before they reach the acidic vaginal enviroment.

That the trend in loss of sperm motility that Baumann et al., (2007) noted was not statistically significant and may be due to some redundancy in the system as glutathione S-transferase (GST) which can also detoxify acrolein through conjugation with glutathione (Berhane et al., 1994) is also present on the mouse sperm surface (Paradowska et al., 2006). Interestingly, mouse glutathione S-transferase mu 5 has also been identified as an antigen for human ASA (Paradowska et al., 2006) which leads to the conclusion that ASA positive patients with antibodies to both AKR and GST could be liable to greater acrolein adduction and hence loss of sperm motility. Other human AKRs have been shown to metabolise acrolein (O'Connor et al., 1999) but as yet, have not been demonstrated to be expressed within the reproductive tract.

Sperm can also meet acrolein post-ejaculation within cervical mucus. Cervical mucus contains both DAO and polyamine oxidase (PAO) activity which on contact with semen can oxidize polyamines releasing acrolein. High levels of DAO and PAO in cervical mucus have been linked with cervical carcinoma (Rogers et al., 2002, Fernandez et al., 1995) through the synergistic effect of cytotoxic spermine catabolites and the anti-apoptotic actions of Human Papilloma Virus. Therefore, sperm will require protective enzymes to acrolein both pre and post ejaculation and as demonstrated here, MVDP and its human homolog on the sperm surface could fulfill this role.

The identity of the aldo-keto reductase on the human sperm surface with acrolein detoxification activity is as yet unclear, however AKR1B1, has previously been shown to occur on human sperm. Frenette et al. (2006) demonstrated the expression of AKR1B1 along the entire human epididymis and the presence of AKR1B1 on epididosomes, prostasomes and the sperm surface. They conclude that, as sorbitol dehydrogenase is also present on the sperm surface that AKR1B1 forms the first step of the polyol pathway, the reduction of glucose to sorbitol. Although AKR1B1 is the most structurally related human AKR to MVDP (Cao et al., 1998), MVDP is unable to catalyse the conversion of glucose to sorbitol (Lefrancois-Martinez et al., 1999) suggesting species variation in functionality. Frenette et al., (2006) notes however, that

AKR1B1 is not differentially expressed along the epididymis as in other species (Frenette et al., 2004) and thus it may perform functions other than the regulation of sperm motility through the polyol pathway. AKR1B1 has acrolein detoxification activity, though at a much higher K_m (80μM) than that demonstrated here for MVDP (Kolb et al., 1994).

No acrolein detoxification activity was found in this study (Table 4.4.3) for seminal plasma with prostasomes still present, a discrepancy with Frenette et al., (2006) who as stated previously showed AKR1B1 localization with prostasomes. This may be explained by either AKR1B1 been unable to access its substrates if located within the multi-lamellar prostasomes, though this is unlikely as acrolein can readily cross membranes, or that AKR1B1 concentration in whole seminal plasma containing prostasomes is too low for enzyme activity to be detected. Isolation and solubilization of human prostasomes should resolve whether they contain acrolein reduction activity.

CHAPTER 5

Immunosuppressive Factors in Human Reproduction and Antisperm-antibodies – a Role for Semenogelin.

5.1 Introduction to Immunosuppressive and Immunomodulatory Molecules and Mechanisms of the Male Reproductive Tract.

5.1.1 The Blood Testis Barrier.

During the perinatal period immunological competence is acquired and all antigens being expressed at that time are recognized as "self". Thereafter, any other recognized antigen is treated as "non-self" and an immune response mounted. However, at puberty, germ cells commence meiosis which leads to the expression of unique differentiation molecules found on late pachytene spermatocytes, which persist throughout spermiogenesis. At midspermatid stage further changes to plasma membrane antigens occur and again as the spermatozoa transit the epididymis. Therefore, a state of immune privilege must occur in the testis and along the genital tract to prevent autoimmunity to this array of newly expressed antigens from occurring.

The first line of defense to autoimmunization by these newly formed antigens within in the male reproductive tract is a physical block known as the blood-testis barrier. This barrier forms at puberty, commensurate with the production of sperm antigens. The sertoli cells surrounding the seminiferous tubules expand and their plasma membranes undergo fusion forming an impenetrable barrier to lymphocytes, immunoglobins, complement and cytokines (Witkin et al., 1996). Sertoli cells can also phagocytose degraded sperm cells and residual sperm body parts, which would prove to be antigenic if leaked from the seminiferous tubules (Alexander and Anderson, 1987). However, there is regionally incomplete segregation of immune system and sperm containing compartments of the reproductive tract by the blood-testis barrier, particularly the rete testis, which is exposed to circulating immunoglobins and T-cells and from where soluble reproductive antigens can diffuse out into circulation

(Alexander and Anderson, 1987). Due to this regional permeability of the blood-testis barrier other mechanisms must exist to prevent germ cell immunogenicity.

Another mechanism employed to maintain immune privilege within the testis is the expression of FasL by the sertoli cells (Filippini et al., 2001), which represents a major non-lymphoid source of FasL in the body. Fas is a type I-membrane bound protein which belongs to the tumour necrosis factor (TNF) receptor family, the ligand of which is FasL, a membrane bound TNF family member. The engagement of this receptor with its ligand down-regulates the immune response by induction of apoptosis in the Fas bearing cell, such as lymphocytes, which express Fas (Graves et al., 1998). Therefore, the interaction of the FasL bearing sertoli cells with activated lymphocytes expressing Fas leads to apoptotic cell death of the later, thus maintaining immune privilege (Bellgrau et al., 1995), though there is still confusion whether this occurs in humans or is confined to rodents (Kimmel et al., 2000). The shedding of FasL from membranes by the action of a metalloproteinase gives a product termed soluble FasL (sFasL) (Kayagaki et al., 1995). This form inhibits FasL mediated apopotosis and down-regulates cell killing and is therefore not immunosuppressive (Suda et al., 1997) but has been reported to be anti-inflammatory (Hohlbaum et al., 2000). Though sFasL can be detected in human seminal plasma probably as a result of secretion by the prostate (Liu et al., 1998), studies have yet to link it with any fertility or infertility parameters.

5.1.2 Prostaglandins in the Male Reproductive Tract.

Both immunosuppressive and immunomodulators have been described in human semen. Prostaglandin E (PGE) and 19-OH-PGE are found in semen at up to millimolar quantities (Bendvold et al., 1987). PGE and 19-OH-PGE are potent stimulators of cAMP and thus inhibit lymphocyte proliferation and natural killer cell activity as well as modulating cytokine release from antigen presenting cells (Kelly et al., 1994). They have also been shown to increase production of the immunosuppressive cytokine interleukin-10 (IL-10) and inhibit the release of the inflammatory interleukin-12 (IL-12) when stimulated with lipopolysaccaride (LPS) (van der Pouw Kraan et al., 1995). Prostaglandins extracted from seminal plasma (Denison et al., 1999) have also been demonstrated to increase interleukin-8 (IL-8) production whereas whole seminal plasma

greatly reduced IL-8 production. However, whether their role in seminal plasma is the maintenance of immunotolerance to sperm antigens within the male reproductive tract or prevention of immunologic sperm damage in the female reproductive tract *post coitus* is not yet fully elucidated.

5.2.3 The Prostasome.

Another male factor known to be immunosuppressive is the prostasome. Prostasomes are trilamellar or multilamellar extracellular organelles of about 150nm in diameter (Ronquist and Brody, 1985). They inhibit lymphoproliferation in response to mitogen stimulation and phagocytosis by macrophages (Kelly et al., 1991, Skibinski et al., 1992). Prostasomes have also been shown to fuse with the spermatozoal membrane, though this occurs only at acidic pH and therefore is likely to occur post ejaculation in the vagina (Ronquist et al., 1990). This fusion may represent a means of protecting the sperm as it traverses the female reproductive tract by carrying prostasomal immunosuppressive factors thus modulating female immune response to sperm. Proteins with immunomodulatory properties on the prostasome will be discussed at more length in chapter 6.

5.1.4 Cytokines in the Male Reproductive Tract.

A number of cytokines have also been detected in seminal plasma and may play a role in the prevention of immune attack on the sperm along the male reproductive tract. To date the main cytokines found include transforming growth factor β_1 (TGFβ_1) and TGFβ_2 (Srivastava et al., 1996, Loras et al., 1999), IL-10 (Huleihel et al., 1999, Miller et al., 2002) and activin A (Anderson et al., 1998). In general, either infection or inflammation leads to up-regulation of cytokines, which can lead to either a type 1 (Th1 cell-mediated response) or a type 2 (Th2 humoral response). The Th2 cytokine response, such as IL-10, and the immunosuppressive cytokine TGFβ_1 play an important role in the limitation and resolution of the immune response (MacDonald, 1998). They are also thought to protect against the development of autoimmune disease (Asadullah et al., 2004). Activin A can inhibit the T and B cell activity and opposes the pro-inflammatory cytokines, interleukin-1 (IL-1) and interleukin-6 (IL-6) (Phillips et al.,

2001). TGFβ_1 is at concentrations of between 80 and 200 ng/ml in seminal plasma, however, activated cytokine is at about 3ng/ml, which is still over its IC_{50} value for inhibition of T-lymphocyte function (Nocera and Chu, 1995, Robertson et al., 1997). The enzymes required for activation are present in seminal plasma but due to unfavourable pH conditions are inactive (Chu and Kawinski, 1998). Inhibition of TGFβ_1 with a TGFβ specific antibody led to a decrease in seminal plasma inhibition of mitogen stimulated proliferation of PBMCs (Ochsenkuhn et al., 2006) leading to the conclusion that TGFβ plays an important role in the male reproductive tract in suppressing the immune response to sperm.

5.1.5 Transglutaminases and Their Immunomodulatory Activity.

Transglutaminases have also been implicated in seminal plasma as having a role in suppression of the immune system. Transglutaminases are enzymes that are Ca^{2+}-dependent and catalyse the acyl transfer reaction between glutamine in peptides and primary amine groups (Aeschlimann and Paulsson, 1994). Transglutaminases may use lysine residues either intra-or inter-chain as acyl-acceptor during these reactions or any available primary amine group, such as those found on the polyamines (Tufano et al., 1996) leading to protein:polyamine adducts. This has been shown to occur for the rat seminal vesicle protein SV-IV (Porta et al., 1991). Also, cross bridging by transglutaminase of SV-IV to the sperm surface leads to a decrease in immunogicity of epididymal sperm (Peluso et al., 1994) and in rabbit sperm when uteroglobin is cross linked to the sperm surface by transglutaminase (Mukherjee et al., 1983).

5.1.6 Complement Inhibitors in the Reproductive Tract.

The complement cascade involves a series of enzymatic reactions leading to pore formation in cellular membranes and is activated by several stimuli, the most important being antibody binding to foreign organisms. A number of complement inhibitors have been shown to occur in human seminal plasma mainly on the prostasome and will be discussed further in chapter 6.

5.1.7 Peptides and Proteins with Immunomodulatory Activity.

While no MHC class I proteins have been shown to occur on the sperm surface, β-2-microglobulin (β2m) has been identified on mouse sperm (Dorus et al., 2010) though not on human (Ziegler et al 2002). β2m is expressed on all nucleated non-gamete cells as part of the MHC class I complex and allows discrimination of self from non-self (Carr et al., 1986). This interesting observation though has not yet been followed up and the role of β2m on the sperm membrane is as yet not elucidated.

The defensin family of anti-microbial peptides are extremely well represented in the male reproductive tract across all species. In humans, DEFB 118 (Liu et al., 2001), DEFB 126 (Yudin et al., 2005) and SPAG11 (Ochsendorf, 2008) have all been shown on the sperm surface and in the male reproductive tract. Though their function may be to combat infection and sexually transmitted disease they may have other functions in a reproductive context. DEFB126 is the best characterized and it has been demonstrated that it alters sperm movement through cervical mucus (Tollner et al., 2008) and in rabbits its coating of sperm helps the sperm evade the immune system and thereby traverse the female reproductive tract (Yudin et al., 2005). Indeed, mutations in this defensin have been implicated in subferility in humans (Tollner et al., 2011).

Seminal vesicle proteins of rodents and in particular SV-IV have been shown to have a wide range of immunosuppressive and immunomodulatory functions. This protein and its functions will be discussed further in this chapter.

5.1.8 The Polyamines.

The polyamines, spermine, spermidine and putrescine occur in seminal plasma in milli-molar quantities (Maayan et al., 1995). As diamine oxidase (DAO) is also present, which can catabolise these polyamines, production of the reactive aldehyde, acrolein, occurs in seminal plasma and is cytotoxic in large amounts (Allen and Roberts, 1986). However, the polyamine catabolites do have a immunomodulatory activity, they can inhibit the inducible nitric oxide synthase in monocytes thus affecting their ability to phagocytose and kill invading organisms (Szabo et al., 1994a, Szabo et al., 1994b).

Also, the polyamines may play a part in self tolerance as spermine depletion leads to autoantibody production (Atanassov et al., 1993).

5.1.9 Seminal Vesicle Secretory Protein SV-IV in Rat.

Rat seminal vesicle secretory protein SV-IV is produced in the seminal vesicle in response to androgens and forms part of the rat seminal coagulum as semenogelin 1 and 2 does in human. SV-IV has been shown to have multiple immunological activities in addition to its role in coagulum formation. It has been shown to have both immunosuppressive and anti-inflammatory properties, having the ability to block mitogen stimulation of PBMC and also block the arachidonic acid cascade at the level of Phospolipase A2 (Metafora et al., 1989a). SV-IV can also inhibit the phagocytic and chemotactic properties of neutrophils (Metafora et al., 1989b) and the phagocytic ability of macrophages (Galdiero et al., 1989). Testing *in vivo* demonstrated that SV-IV could also inhibit rat sperm immunogenicity (Peluso et al., 1994) and cell-mediated and humoral responses to alloantigens (Romano-Carratelli et al., 1995, Fuggetta et al., 2008). SV-IV is a substrate for transglutaminase and can be covalently modified by spermine, this modification showed a marked increase in the ability of SV-IV to inhibit mitogen induced PBMC proliferation but had no effect on rat sperm immunogenicity *in vivo* (Porta et al., 1993). SV-IV has also been shown to be covalently modified by phosphorylation on Ser58, a modification that leads to enhanced activation of selenium-dependent glutathione peroxidase (GPX), an enzyme that is known to protect the mammalian spermatozoa from oxidative stress and loss of motility in the female genital tract (Metafora et al., 2001). Finally, SV-IV can control nitric oxide (NO) levels in macrophages. considering the important role played by NO in the process of mammalian reproduction, SV-IV may be involved in the fine tuning of NO concentration in the female genital tract mucosa via an SV-IV-mediated control of iNOS gene expression in local macrophages (Esposito et al., 2002).

5.1.10 Aims of the Chapter.

Previously, Imade et al., (1997) demonstrated loss of immunosuppressive activity in seminal plasma from patients with ASA. This led to the hypothesis that one of the previously described immunosuppressive components of seminal plasma was missing in ASA patients leading to the development of ASA. The aim was to fractionate human seminal plasma from both normospermic and ASA positive patients and identify the missing immunosuppressive component.

5.2 Results.

5.2.1 Suppressive Effects of ASA Positive and Negative Seminal Plasma on Mitogen Induced Proliferation of PBMC's.

PBMCs were isolated as in section 2.9.1 and incubated with 1% v/v seminal plasma either from patients negative for ASAs by MAR test or from patients displaying a positive IgG MAR test. PBMCs were induced to proliferate using PHA as in section 2.11.1 after 1 hour incubation with seminal plasma. Proliferation was measured by ^{3}H-Thymidine incorporation 24 hours post induction of proliferation as in section 2.11.1. The experiment was carried out in the presence (Figure 5.2.1B) or absence of (Figure 5.2.1A) hydroxylamine, an inhibitor of polyamine oxidase, in order to assess the role of spermine and spermidine and their cytotoxic oxidation products in the suppressive activity of seminal plasma. Hydroxylamine has previously been shown (Imade et al., 1997) to inhibit the polyamine oxidase of FCS and prevent the production of cytotoxic end products of polyamine catabolism. Patients seminal plasma not containing ASAs were found to suppress mitogen induction of PBMC proliferation from approximately 16 -17% of control in the absence of hydroxylamine and from approximately 29-29.5% in the presence of hydroxylamine. Thus suggesting that spermine and spermidine catabolites are responsible for just over 10% of the suppressive qualities of ASA negative seminal plasma. In the ASA positive patients however, the figures go from approximately 27-34% of control in the absence of hydroxylamine to approximately 55-61% of control in the presence of hydroxylamine. These figures indicate a contribution of over 33% for polyamine catabolites in the suppressive qualities of ASA positive seminal plasma at the concentration of seminal plasma tested.

5.2.2 Fractionation of Normozoospermic and ASA Positive Seminal Plasma by Molecular Weight Cut-Off Filter Centrifugation.

As seminal plasma has been shown to contain a number of immunomodulatory and immunosuppressive factors (see section 5.1) dissection of the seminal plasma by molecular weight was required to show firstly the levels of suppressive activity in each fraction and at what molecular weight the loss of activity in ASA positive presented so as to determine which immunomodulatory component was missing from these patients.

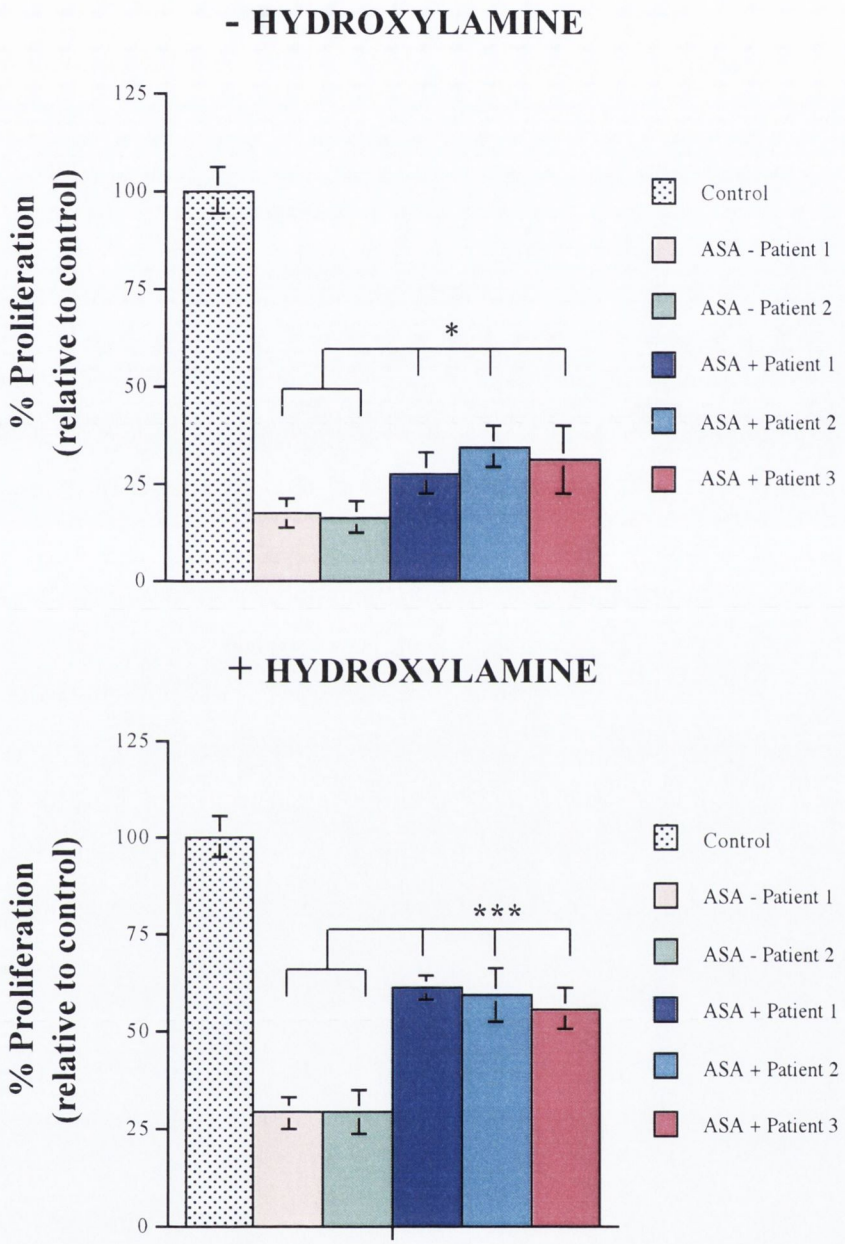


Fig 5.2.1. Suppression of mitogen induced proliferation of PBMCs by Seminal Plasma of Normospermic and ASA Patients. PBMCs (5 x 10^5) were treated with seminal plasma (1% v/v final concentration) from patients with normal semen parameters and patients with ASAs in the presence or absence of 100µM hydroxylamine. Proliferation was induced by addition of 2.5µM PHA-P and measured by the uptake of [^{3}H]-Thymidine. All points represent the mean of triplicates +/- SEM. Statistical values * $p \leq 0.01$ *** $p \leq 0.001$.

Seminal Plasma was passed consecutively through two of amicon cut-off molecular weight filters and washed extensively through these filters, the first at 100kDa and finally at 3kDa as in section 2.10.2. Suppression of mitogen stimulation of PBMCs by the retentate of the 100kDa and 3kDa filters and the flow through of the 3kDa filter were measured by [^{3}H]-Thymidine uptake as in section 2.11.2. The >100kDa fraction (Figure 5.2.2.1B) showed a marked increase in suppressive effects between 25% of seminal plasma (approximately 90% of control) and 40% of seminal plasma (approximately 29% of control). Concentrations of fractions are given as per final concentrations as in seminal plasma. The retentate from the 3kDa filter (3-100kDa fraction) also contained suppressive factors that inhibited mitogen stimulation of PBMCs (Figure 5.2.2.1A) though to a lesser degree than in the >100kDa fraction. Greatest inhibition was noted in the highest dose tested (40% v/v as in seminal plasma) giving a value of approximately 72% of control. The flow through of the 3kDa filter (<3kDa fraction) was tested with and without hydroxylamine as this fraction likely contains the polyamines, spermine and spermidine. It was found to have the highest level of suppressive activity with 1% final concentration as in seminal plasma leading to an inhibition of approximately 95% of control in the absence of hydroxylamine to approximately 23% of control with hydroxylamine present (Figure 5.2.2.2). The level of suppression without hydroxylamine is greater than reported in the previous section (Figure 5.2.1A) and may be due to a differing amount of polyamines in the seminal plasma of this patient.

When ASA positive patients seminal plasma is fractionated as before using the amicon molecular weight cut-off filters and compared to normozoospermic patients seminal plasma (Figure 5.2.2.3) with hydroxylamine present, no differences in suppressive effects on mitogen stimulation of PBMCs can be seen for the >100kDa and 3-100kDa fractions. However, the <3kDa fraction shows a marked difference (approximately 22% of control for normozoospermic <3kDa seminal fraction vs approximately 52% of control for ASA positive <3kDa seminal fraction $p<0.001$). This loss of lymphosuppressive activity for ASA positive patients is similar to that observed in figure 5.2.1 and indicates that the loss in lymphosuppressive activity in ASA positive seminal plasma is caused by the loss of a low molecular weight factor.

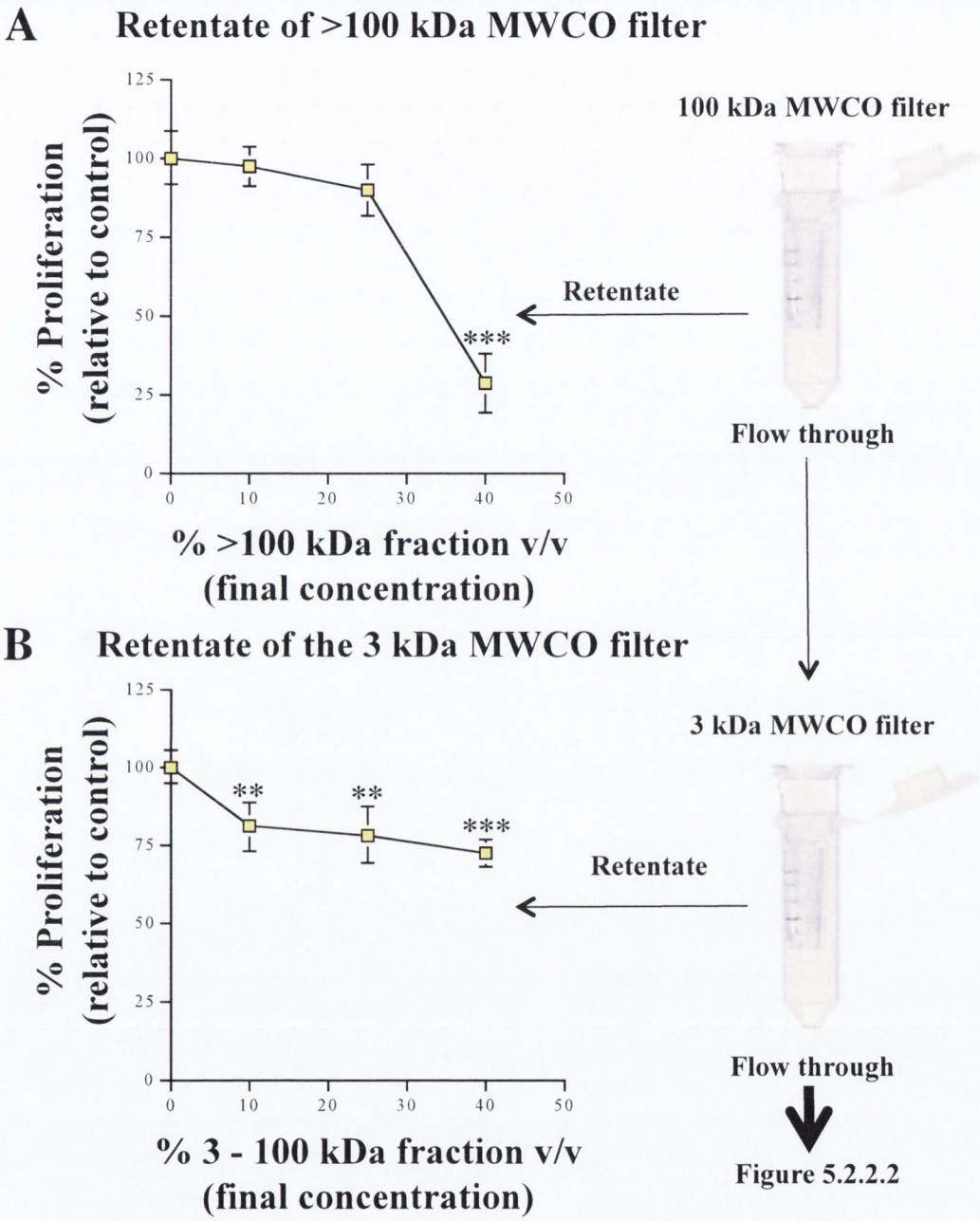


Fig 5.2.2.1. Effects of increasing concentrations of fractions of normal seminal plasma separated by Amicon size-exclusion filters on the mitogen induced proliferation of PBMCs. The effects of increasing concentrations of fractions of normal seminal plasma separated by Amicon size-exclusion filters of 3 and 100 kD (A and B) cut-off filters on the mitogen induced proliferation of PBMCs (5 x 10^5) over 24 hours. Multiple washes on each filter were performed for complete separation of fractions. Proliferation is measured by the uptake of ^{3}H-Thymidine. All points represent the mean of triplicates +/- SEM. Statistical difference is indicated by ** $p < 0.005$ *** $p < 0.001$ vs 0 % seminal plasma fraction.

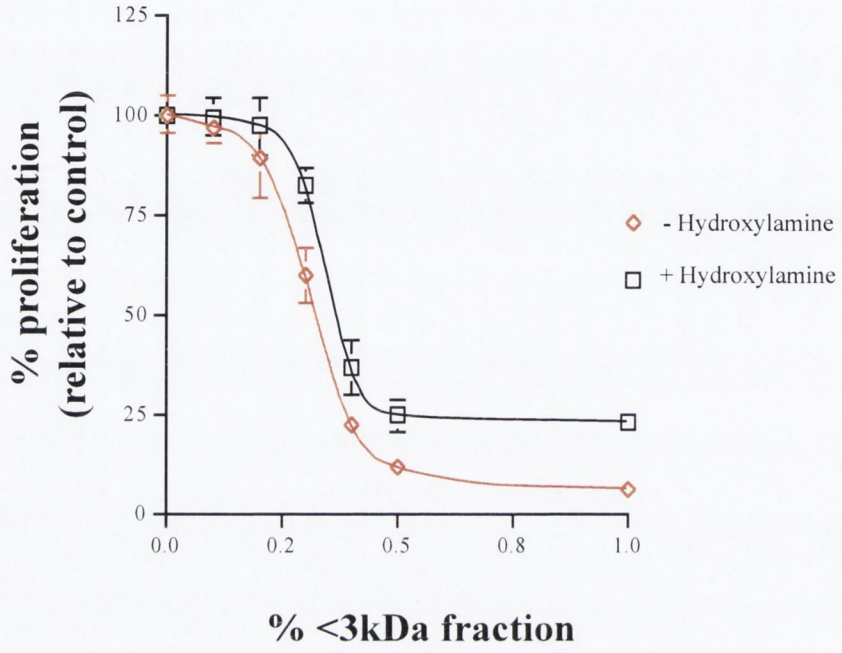


Fig 5.2.2.2. Effects of increasing concentrations of the <3kD fraction of normal seminal plasma on the mitogen induced proliferation of PBMCs. The effects of increasing concentrations of the <3kDa fraction of normal seminal plasma separated by Amicon size-exclusion filter of 3kDa cut-off on the mitogen induced proliferation of PBMCs (5 x 10^5) over 24 hours in the presence or absence of 100μM hyroxylamine. Proliferation is measured by the uptake of [^{3}H]-Thymidine. All points represent the mean of triplicates +/- SEM.

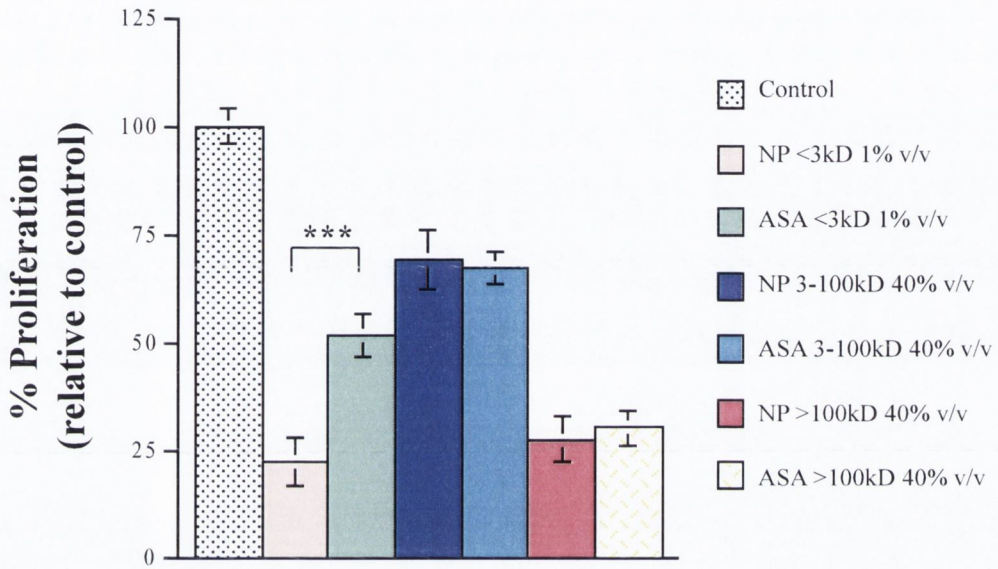


Fig 5.2.2.3. Inhibition of mitogen induced proliferation of PBMCs by Filtration Fractions of Normospermic and ASA Patients Seminal Plasma. Inhibition of mitogen induced proliferation of PBMCs (5 x 10^5) by seminal plasma fractions derived from amicon filtration from patients with normal semen parameters (NP) and from ASA patients. The <3kDa fraction was used at a final concentration of 1% v/v while all other fractions were used at a final concentration of 40% v/v. Incorporation of [^{3}H]-Thymidine was used to measure proliferation and all incubation times were 24 hours. All points represent the mean of triplicates +/- SEM. Statistical differences are indicated by *** $p < 0.001$.

5.2.3 Organic Phase Separation of the <3kDa Seminal Plasma Fraction from Normozoospermic Patients.

As seminal plasma had previously been indicated to contain prostaglandins of the E series (PGE and 19-OH-PGE), organic phase separation previously described to extract prostaglandins (Hamberg and Samuelsson, 1966) was performed as in section 2.10.3 to demonstrate whether the <3kDa fraction's activity was due to the presence of PGE and 19-OH-PGE. A continuous UV/Visible spectra of both the aqueous and organic phases were performed as in section 2.10.4 (Figure 5.2.3A and B respectively). The spectrum of the aqueous phase shows a peak at 280nm, which is indicative of protein being present in this fraction. The organic phase shows a plateau of absorbance from 220-290nm along with a peak at 200nm. Lymphosuppressive activity was measured as before (section 2.11.1) and in the presence of hydroxylamine (Figure 5.2.3C). At a final concentration of 1% v/v the aqueous phase of the <3kDa fraction gave a suppression of approximately 27% of untreated control values whereas the organic phase of the <3kDa fraction gave a value of approximately 83% of untreated control.

5.2.4 Fractionation of the <3kDa Fraction on a FPLC Peptide Column.

To further separate the low molecular weight activity found in the <3kDa fraction, this fraction was applied to a Superdex Peptide 10/300GL column as in section 2.12.1. The elution was followed at two wavelengths (210nm and 280nm) and by conductance. The elution profile (Figure 5.2.4A) showed a large number of peaks for the 210nm wavelength with fewer peaks of lower intensity for the 280nm wavelength. Proteins absorb at 280nm if they contain the amino acids tyrosine, tryptophan or cysteine, therefore, it can be assumed that many of the peptides present in this fraction do not contain these amino acids. Two large peaks of absorbance at 280nm occur at fraction 39 (C7) and fractions 43-45 (C11-C13). The peak at fraction 39 (C7) is of very low molecular weight (approximately 200 daltons) and may represent a prostaglandin peak. The other peak at fractions 43-45 (C11-C13) is after all material should have exited the column, the retention on the column of this residual material indicates that it is likely lipophilic in nature, and its retardation on the column is likely due to hydrophobic interactions with the sepharose. High cross-linked Tris-Tricine SDS-gel electrophoresis

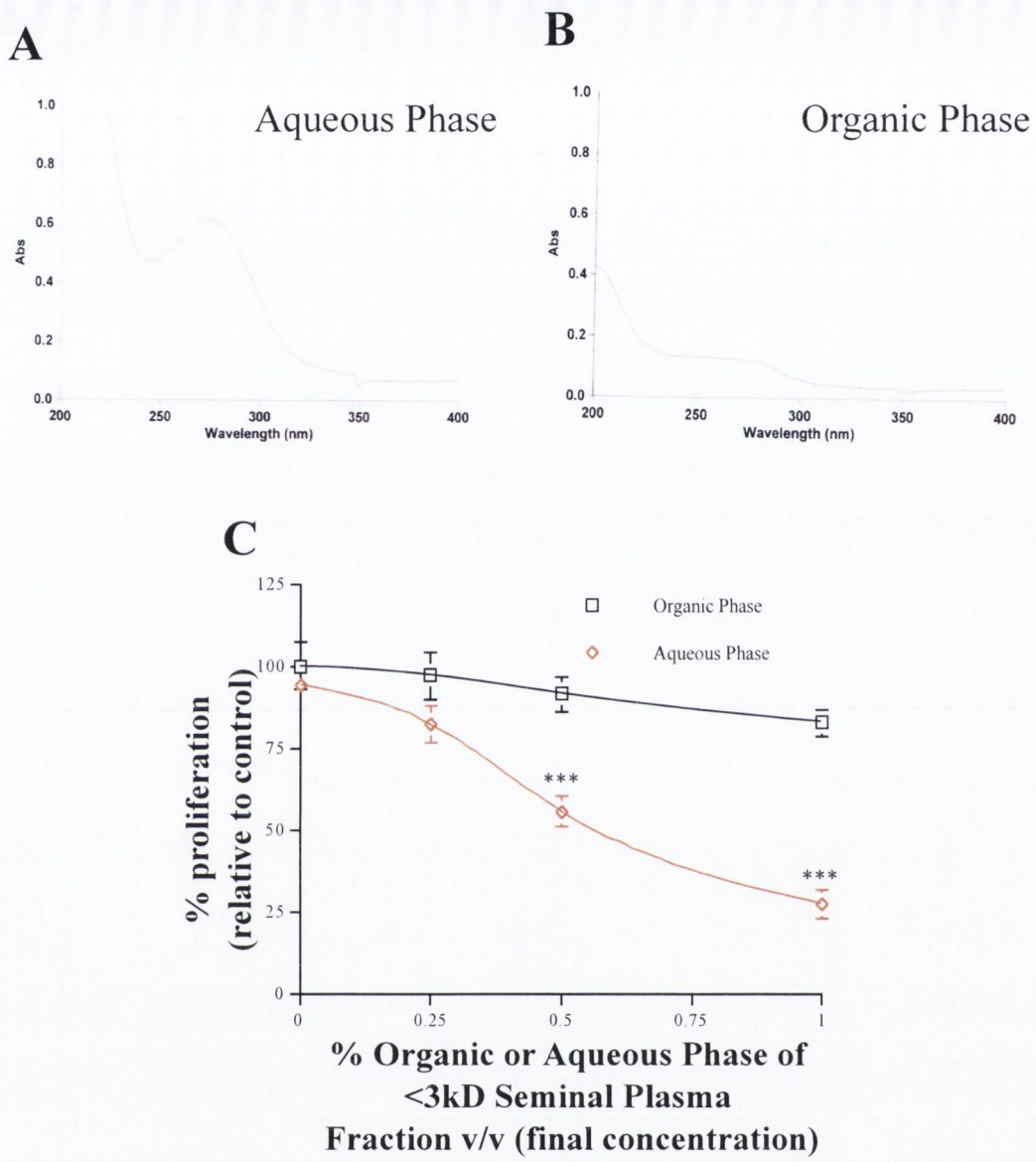


Fig 5.2.3. Properties of Aqueous and Organic Phases of Seminal Plasma After Phase Separation. A. Continuous UV-Vis spectrum of the aqueous phase of the <3kDa seminal plasma fraction post phase separation. **B.** Continuous UV-Vis spectrum of the organic phase of the <3kDa seminal plasma fraction post phase separation. **C.** The effects of increasing concentrations of the aqueous (red line) and organic phase (black line) separations of the <3kD fraction of normal seminal plasma on mitogen induced proliferation of PBMCs (5 x 10^5) in the presence of 100μM hydroxylamine. Incorporation of [^{3}H]-Thymidine was used to measure proliferation and all incubation times were 24 hours. All points represent the mean of triplicates +/- SEM. Statistical difference is indicated by *** $p < 0.001$.

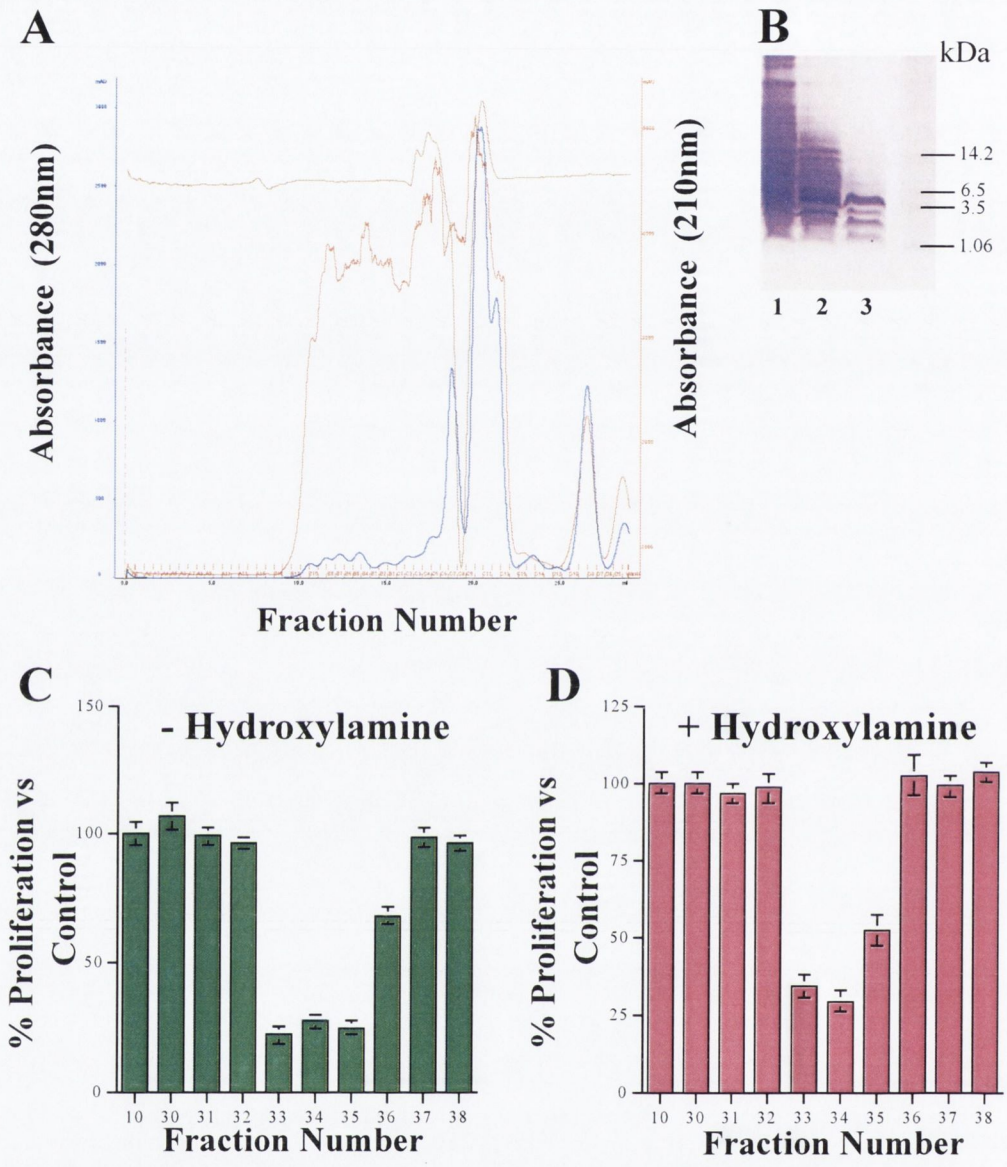


Fig 5.2.4. Further Purification of the <3kDa Fraction by Gel Filtration. A. Gel filtration of 0.5ml of <3kD fraction on FPLC Peptide Column 10/300GL. Blue line (--) represents absorbance at 280nm. Red line (--) absorbance at 210nm and brown line (--) conductance. **B.** High cross-linked 16.5% Tris-tricine SDS-Page gel. Lane 1. Whole Seminal Plasma. Lane 2. <3kDa fraction after filtration. Lane 3. Fraction 33 from Peptide Column. **C and D.** Inhibition of proliferation of Jurkat cells (5 x 10^5) by fractions collected from FPLC Peptide Column 10/300GL of gel filtration of <3kD seminal plasma fraction with hydroxylamine (D) and without (C). Fraction 10 (A10) which is before the void volume on the FPLC Peptide Column 10/300GL was used as control and its value set to 100% proliferation. All incubations were 24 hours and cell proliferation measured by WST-1 chromographic reagent. All points represent the mean of triplicates +/- SEM.

(as in section 2.2.5) (Figure 5.2.4B) of the <3kDa fraction showed that the filter allowed through peptides of up to approximately 8kDa (Lane 2) and that the first active fraction (fraction 33 / C1) (Lane 3) contained a mix of peptides of 2-4kDa.

Lymphosuppressive activity in the presence and absence of hydroxylamine on Jurkat proliferation (as in section 2.11.2) showed activity to be present in fractions 33-36 (C1-C4) in the absence of hydroxylamine (Figure 5.2.4C) and in fractions 33-35 (C1-C3) in the presence of hydroxylamine (Figure 5.2.4D). This indicated that the polyamines, spermine and spermidine, co-eluted in the active fraction but that their cytotoxicity in the presence of FCS were not responsible for the anti-proliferative effects on the Jurkat cells. However, as the molecular weights of spermine and spermidine are so low, this result leaves open the possibility that the polyamines are complexed with peptides and may in this complex exert anti-proliferative effects.

5.2.5 Investigation of Possible Polyamine:Peptide Complexes in the <3kDa Fraction.

To determine whether the polyamine content of the <3kDa fraction eluted at a higher molecular weight than expected due to complexes being formed with peptides, pronase digestion of the <3kDa fraction was carried out. The pronase digested <3kDa fraction was then subjected to gel filtration on the Superdex Peptide 10/300GL column as in section 2.12.3. The resulting chromatogram (Figure 5.2.5A) demonstrates that most of the peptides within the fraction have been fully digested as evidenced by the loss of both A_{280} and A_{210} peaks from the void fraction (fraction 22 (B12)) to fraction 31 (B2). The absorbance at 210nm though rises from fraction 32 (B2) and peaks similar to those obtained previously in the non-pronase treated <3kDa fraction (Figure 5.2.4A) were obtained for fractions 33 (C1) onwards. This indicates that as spermine and spermidine do not absorb strongly at 210nm that most of this absorbance is peptide in origin. Also, this peak corresponds with a rise in conductance, further evidence of highly charged molecules, probably polyamines, associated with these fractions. It can be concluded therefore, that the polyamines do occur as peptide:polyamine complexes and that these complexes protect the peptides from pronase cleavage. The A_{280} peaks previously shown in undigested <3kDa fractionation (Figure 5.2.4A) at fractions 39 (C7) and 43-45 (C11-C13) are unaffected by pronase cleavage and this lends further

weight to the assumption that these peaks are not protein in nature and are likely prostaglandin in origin.

Anti-proliferative activity for the fractions from the FPLC Peptide column of pronase treated <3kDa was tested on Jurkat cells as in section 2.11.2. In the presence of hydroxylamine (Figure 5.2.5C) no activity was found while in the absence of hydroxylamine (Figure 5.2.5B) activity was found in fractions 33-36 (C1-C4) which can be deduced to be the cytotoxic catabolites of polyamine oxidation. Therefore, pronase treatment of the <3kDa fraction did not shift the elution profile of the polyamines but did completely abrogate the lymphosuppressive activity of this fraction.

5.2.6 Determination of the Elution Profile of Spermine by FPLC Peptide Column Size Exclusion Chromatography and its Detection by Dansylation.

A solution of spermine was passed down the Superdex Peptide 10/300GL column as in section 2.12.2 using the same elution parameters as during chromatography of the <3kDa fraction. A rise in conductance and a small rise in the A_{210} at fractions 38-40 (C6-C8) (Figure 5.2.6.1) showed that spermine eluted in these fractions. To confirm this observation and compare with the elution profiles of the <3kDa fraction with and without pronase digestion, fractions were dansylated and run on thin layer plates (TLC) as in section 2.12.4. Spermine and spermidine were detected in fractions 33-35 of both the undigested and pronase treated <3kDa fractions (Figure 5.2.6.2A). Fractions 36-37 of the pronase treated sample also displayed other amine containing compounds of higher mobility on TLC than spermine, which were not detected in the undigested <3kDa sample. One explanation for this is that the pronase digestion was carried out at 37°C overnight allowing degradation of the spermine by DAO to form putrescine and other aminoaldehydes that would also be dansylated and run at higher mobilities in the TLC system being used here. When spermine was run alone on the FPLC Peptide column it eluted in fractions 38-40 (C6-C8) (Figure 5.2.6.2B).

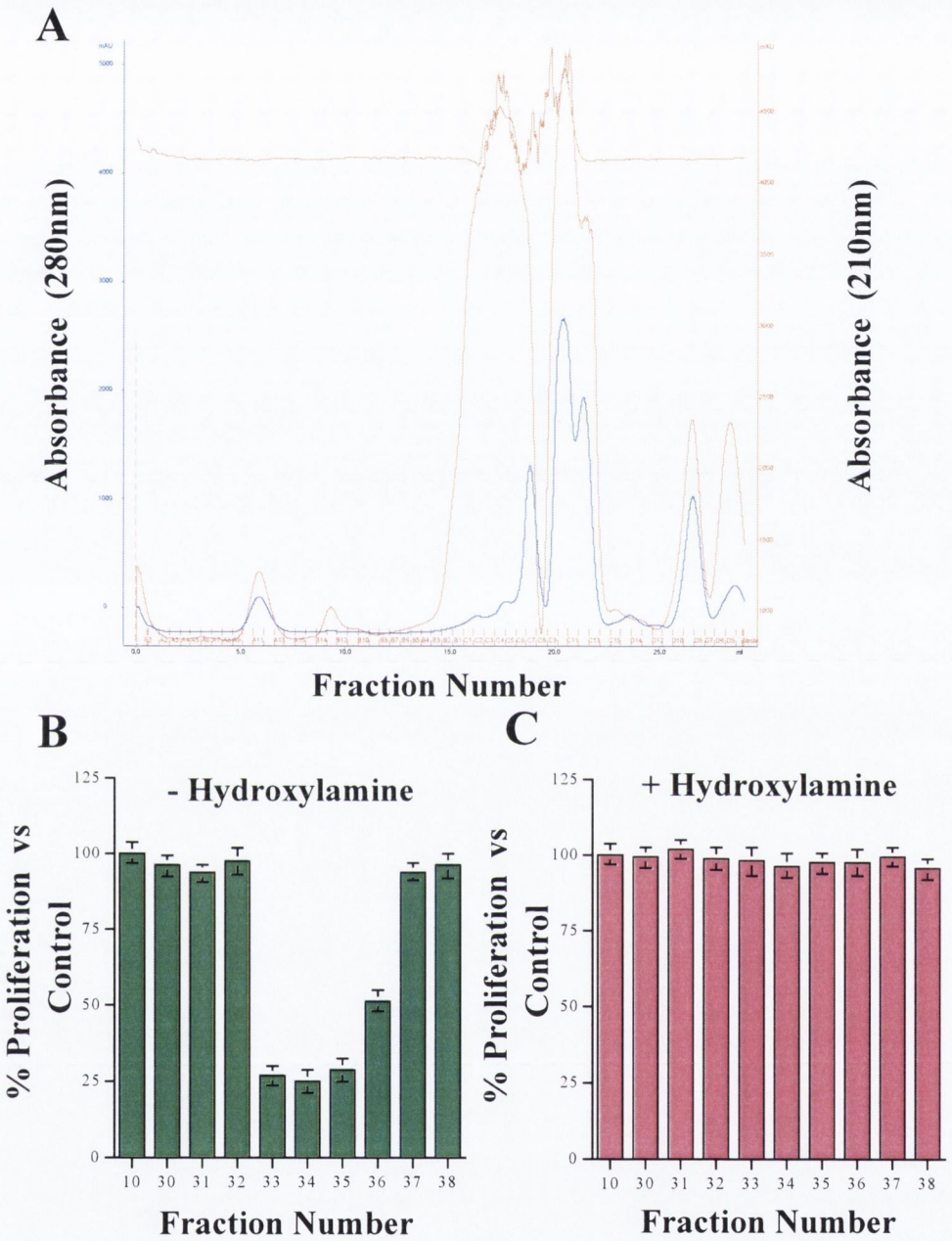


Fig 5.2.5. Gel Filtration and Activities of Pronase Digested <3kDa Fraction. A. Gel filtration of 0.5ml of <3kD fraction treated with Pronase on FPLC Peptide Column 10/300GL. Blue line (--) represents absorbance at 280nm. Red line (--) absorbance at 210nm and brown line (--) conductance. **B and C.** Inhibition of proliferation of Jurkat cells (5 x 10^5) by fractions collected from FPLC Peptide Column 10/300GL of gel filtration of <3kD seminal plasma fraction incubated with Pronase in the absence (B) or presence (C) of hydroxylamine. Fraction 10 which is before the void volume on the HPLC peptide Column 10/300GL was used as control and its value set to 100% proliferation. All incubations were 24 hours and cell proliferation measured by WST-1 chromographic reagent. All points represent the mean of triplicates +/- SEM.

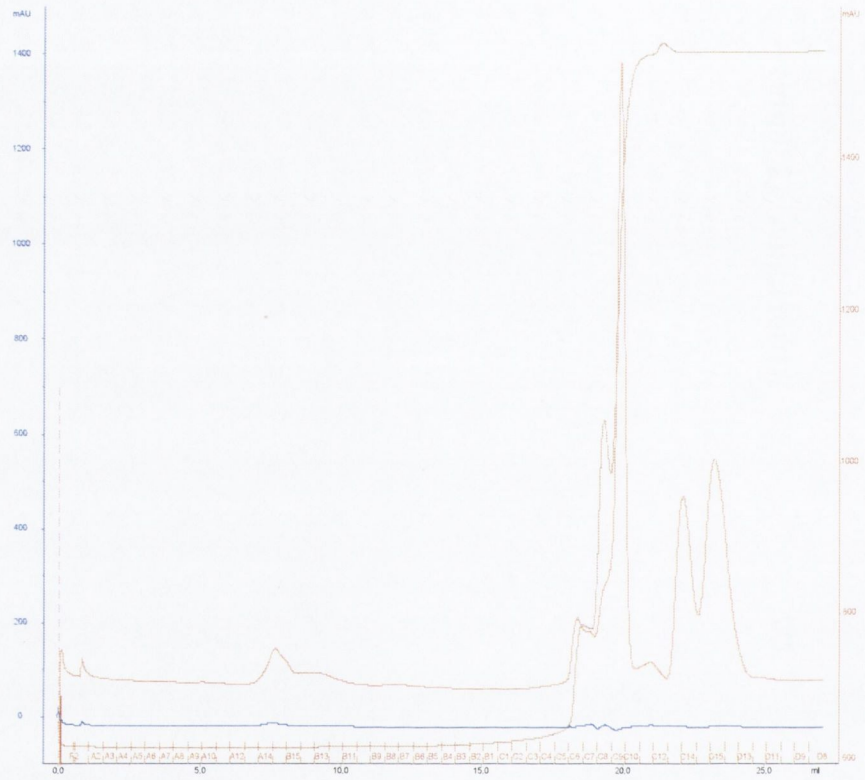


Fig 5.2.6.1. Elution Characteristics of Spermine on Gel Filtration. Gel filtration of 0.5ml of 25mM Spermine on FPLC Peptide Column 10/300GL. Blue line (--) represents absorbance at 280nm. Red line (--) absorbance at 210nm and brown line (--) conductance.

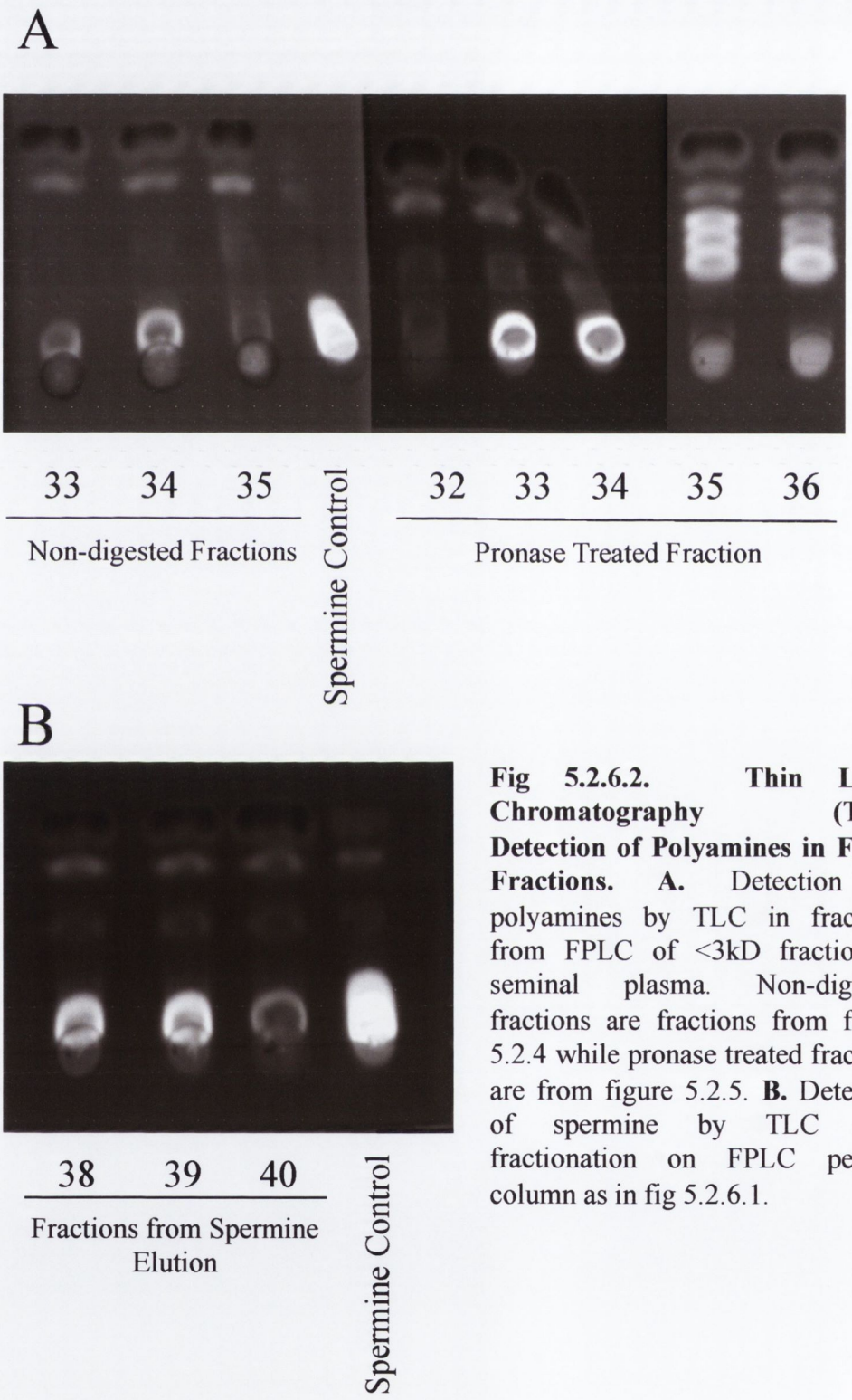


Fig 5.2.6.2. Thin Layer Chromatography (TLC) Detection of Polyamines in FPLC Fractions. A. Detection of polyamines by TLC in fractions from FPLC of <3kD fraction of seminal plasma. Non-digested fractions are fractions from figure 5.2.4 while pronase treated fractions are from figure 5.2.5. **B.** Detection of spermine by TLC after fractionation on FPLC peptide column as in fig 5.2.6.1.

5.2.7 Further characterization of the Fractionated <3kDa Fraction.

5.2.7.1 Proteolysis of the <3kDa Fraction Following Size Exclusion Chromatography.

As it had been previously indicated (Figure 5.2.3A and C) that the main lymphosuppressive activity resided in the aqueous phase of the <3kDa fraction and that its uv-visible spectrum resembled that of protein and could be removed by digestion with pronase (Figure 5.2.5C). To confirm the peptide nature of the lymphosuppressive activity proteolysis was carried out with other proteases with differing substrate profiles. Proteolysis was carried out as in section 2.13.1 using either trypsin or *S. aureus* endopeptidase and trypsin with trypsin inhibitor added as control. The <3kDa fraction was incubated overnight with each of the proteases or protease with inhibitor and lymphosuppressive activity tested as in section 2.11.1. Both the untreated <3kDa fraction and the <3kDa fraction treated with trypsin with trypsin inhibitor displayed full lymphosuppressive activity whereas <3kDa fraction treated with trypsin or the *S. aureus* endopeptidase lost all activity compared to control (Figure 5.2.7.1).

5.2.7.2 Characterization of Peptide Charge and Metal Binding of the Active Constituent of the <3kDa Fraction Following Size Exclusion Chromatography.

Estimation of the charge characteristics of the peptide was performed by pull-down of the <3kDa active fraction with either DEAE-sepharose (a positively charged ion-exchange resin) or with CM-sepharose (a negatively charged ion-exchange resin) with unconjugated sepharose as control as described in sections 2.13.2.1 and 2.13.2.2. The DEAE-sepharose pull down had no effect on the activity of the <3kDa fraction whereas the CM-sepharose removed all inhibitory material (Figure 5.2.7.2A) and therefore the charge on the peptide is positive (cationic). Incubation and subsequent pull-down of the <3kDa fraction with nickel-sepharose as in section 2.13.2.3 also removed all inhibitory material from the fraction (Figure 5.2.7.2A) and indicated that the inhibitory peptide has affinity for nickel. SDS-Page electrophoresis of each of the supernatants after pull-down (Figure 5.2.7.2B) showed that DEAE-sepharose pull-down left the <3kDa fraction with multiple peptides still present, while both CM-sepharose and Nickel-sepharose removed the majority or all peptides present in the fraction.

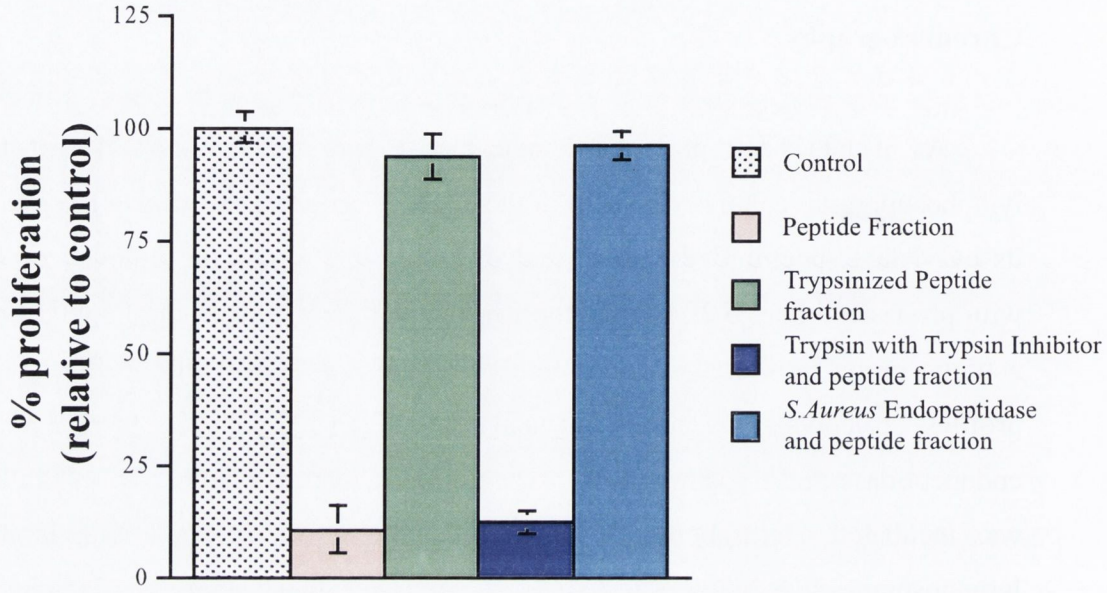


Fig 5.2.7.1. Effects of digestion of the peptide fraction with proteases on the mitogen induced proliferation of PBMCs. (5 x 10^5). Peptide fraction was incubated overnight with either 1µg/20µl trypsin or 1µg/20µl trypsin with 2µg/20µl soybean trypsin inhibitor or 1µg/20µl *s. aureus* non specific protease. PBMCs (5 x 10^5) were treated with 1% v/v final concentration of peptide and digested fractions. Incorporation of [^{3}H]-Thymidine was used to measure proliferation and all incubation times were 24 hours. All points represent the mean of triplicates +/- SEM.

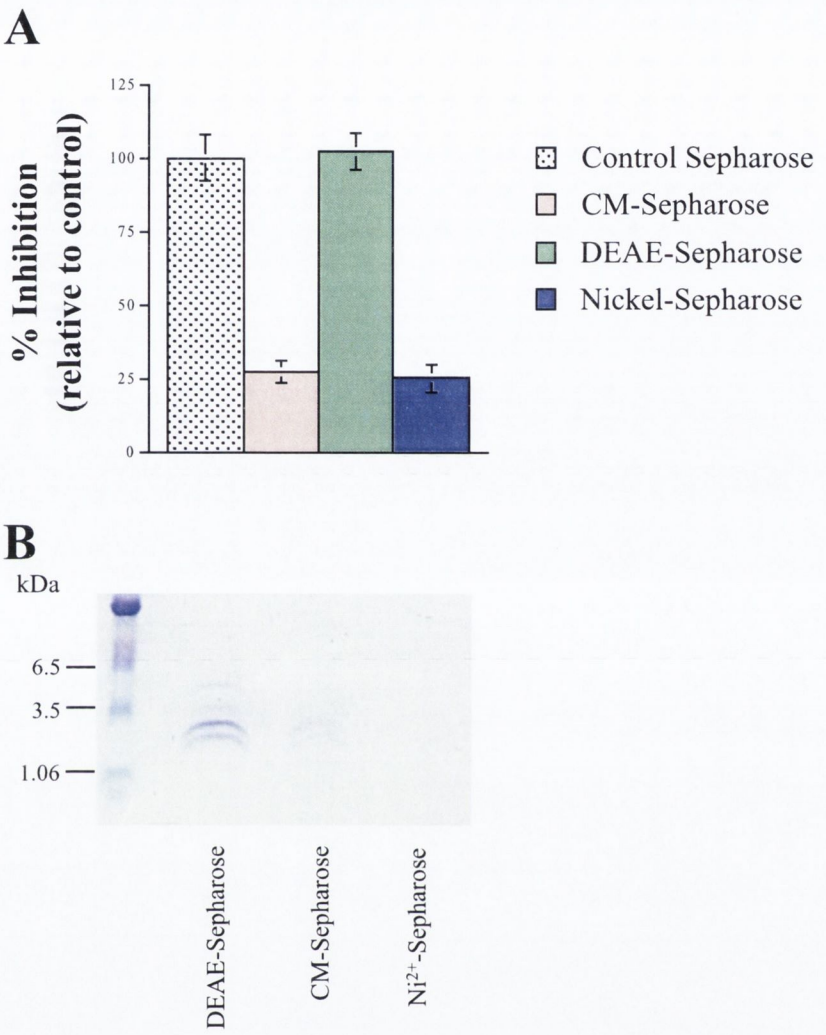


Fig 5.2.7.2. Determination of Active Peptide Binding Characteristics. A. Pull down of activity of peptide fraction with differing substituted sepharoses. Peptide fraction (100µl) was incubated on ice for 1 hour with constant agitation with 20µl of either un-substituted sepharose, CM-sepharose, DEAE-sepharose or nickel-sepharose. The sepharose was spun down and the supernatant added to PBMCs (5 x 10^5) to a 1% v/v final concentrations and mitogen stimulated for 24 hours. Incorporation of ^{3}H-Thymidine was used to measure proliferation. All points represent the mean of triplicates +/- SEM. **B.** High cross linked 16.5% Tris-Tricine SDS-Page gel of the peptide active fraction after pull down with either CM-sepharose, DEAE-sepharose or nickel-sepharose.

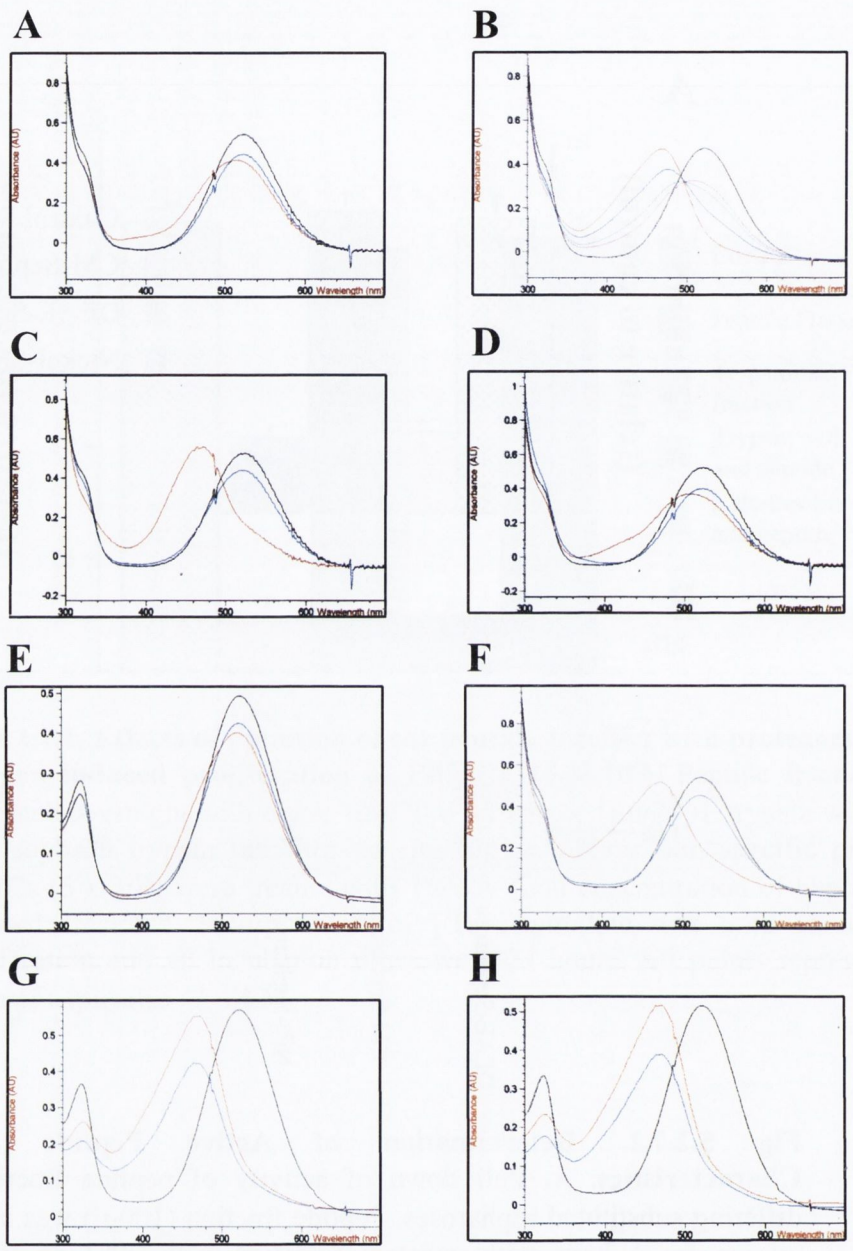


Fig 5.2.7.3. Investigation of the peptide fraction binding of divalent metal ions using murexide. A. Changes in the spectrum of 50μM murexide (black line) after the sequential addition of 50μM cobalt chloride (red line) and 20μl of active peptide fraction (blue line). **B.** Changes in the spectrum of 50μM murexide (black line) after the sequential addition of 50μM nickel chloride (red line), 20μl of active peptide fraction (blue line), an additional 20μl of active peptide (green line) and a further 20μl of active peptide (purple line). **C.** Changes in the spectrum of 50μM murexide (black line) after the sequential addition of 50μM copper chloride (red line) and 20μl of active peptide fraction (blue line).**D.** Changes in the spectrum of 50μM murexide (black line) after the sequential addition of 50μM ferrous sulfate (red line) and 20μl of active peptide fraction (blue line).**E.** Changes in the spectrum of 50μM murexide (black line) after the sequential addition of 50μM zinc chloride (red line) and 20μl of active peptide fraction (blue line). **F.** Changes in the spectrum of murexide (black line) after the sequential addition of 50μM nickel chloride (red line) and 50μM EDTA (blue line). **G.** Changes in the spectrum of murexide (black line) after the sequential addition of 50μM copper chloride (red line) and 50μM (350μl) aprotinin (blue line). **H.** Changes in the spectrum of 50μM murexide (black line) after the sequential addition of 50μM copper chloride (red line) and 350μl Tris-HCl (blue line).

To demonstrate whether the binding of the inhibitory peptide to nickel was shared by other divalent metal ions the <3kDa fraction was tested for chelation of metals using the murexide spectrophotometric assay as in section 2.13.3. Murexide on binding metals shifts its absorption peak in the visible spectrum. Since murexide binding of metals is extremely weak, the addition of any other substance capable of chelating metals removes the metal bound to murexide so restoring its original spectrum. The <3kDa fraction was tested with the divalent metal ions, cobalt, nickel, copper, iron and zinc (Figure 5.2.7.3A-E). All metals tested were able to be bound by the <3kDa fraction, although multiple additions of the fraction were required to remove nickel from murexide (Figure 5.2.7.3B) indicating that it does not bind nickel strongly. To demonstrate the veracity of the system EDTA was used as positive control (Figure 5.2.7.3F) and aprotinin, a small peptide protease inhibitor known not to bind divalent metal ions, as negative control (Figure 5.2.7.3G) along with buffer control (Figure 5.2.7.3H).

5.3 Mechanisms of Action of the <3kDa Fraction.

5.3.1 Comparison of Activity of the <3kDa Fraction in PBMCs vs Cell Lines of Immunological and Non-Immunological Origin.

Anti-proliferative activity of the <3kDa fraction against each of the cell lines of immunological origin, Jurkats (T-cells), U937s and THP1s (monocytes), was demonstrated in the presence and absence of hydroxylamine (Figure 5.3.1.1) as in sections 2.11.2, 2.11.3 and 2.11.4. However, cells of non-immunological origin, MCF7 (a breast cancer cell line) and C6 Glioma (a glial cell line) only showed minimal effect when treated with <3kDa fraction (Figure 5.3.1.2) as in sections 2.11.5 and 2.11.6. In the absence of hydroxylamine MCF7 and C6Glioma cells were inhibited by approximately 21% and 23% respectively compared to control showing that they were less effected by the cytotoxicity of polyamine catabolites than the cells of immune origin. In the presence of hydroxylamine cell growth of the MCF7 and C6Glioma cells were approximately 87% and 107% respectively compared with control. Neither of these figures is statistically significant and leads the conclusion that the active peptide has no effect on cell lines of non-immunological origin.

Determination of the EC_{50} values from the growth inhibition curves (Table 5.3.1.1) pointed to a difference in toxicity by polyamine catabolites on the differing cell lines. Both Jurkat and U937 cells had much lower EC_{50} values in the absence of hydroxylamine indicating that they were more susceptible to polyamine catabolite toxicity. Both their EC_{50} values rose significantly on the addition of hydroxylamine to the assay by 67% and 116% for Jurkat and U937 cells respectively. The EC_{50} value for PBMCs rose by just 16% in contrast and may be due the mix of cell types within this isolate some of which will be more or less susceptible to spermine catabolite toxicity and may also be influenced by the fact that many of the cells present are not actively dividing and are in resting mode. This compares almost exactly with the change in ID_{50} measured by Imade et al (1999) for the effects of seminal plasma with and without hydroxylamine on mitogen stimulation of rat thymocytes.

	EC_{50} (% Seminal Plasma)		
Cell Type	- Hydroxylamine	+ Hydroxylamine	P value
PBMC	0.33 +/-0.02	0.38 +/- 0.04	$P < 0.005$
Jurkat	0.22 +/-0.02	0.37 +/- 0.04	$P < 0.0001$
THP1	0.47 +/- 0.03	0.6 +/- 0.07	$P < 0.0001$
U937	0.22 +/- 0.02	0.47 +/- 0.03	$P < 0.0001$

Table 5.3.1.1 EC_{50} determinations for each of the cell lines for the anti-proliferative activity of the <3kDa fraction in the presence and absence of hydroxylamine. EC_{50} were calculated from the graphs in figure 5.3.1.1 and statistical analysis using a two-way Anova test was carried our in Prism.

Vital staining of cells after treatment with <3kDa fraction with methylene blue and eosin Y after spinning onto slides as in section 2.14.2 showed morphological changes in each of the cell lines tested. Jurkats and U937s (Figure 5.3.1.3) and THP1s and PBMCs (Figure 5.3.1.4) each showed an enlarging of the cells on treatment with <3kDa fraction, though at differing time points. U937s were the quickest to respond to treatment with morphological changes occurring at 3 hours incubation with peptide fraction. Jurkats responded within 6 hours with THP1s between 6 and 24 hours. PBMCs displayed morphological changes from the 6 hour time point but only in a limited subset of cells. Arrows labeled 1 on figures 5.3.1.3 and 5.3.1.4 indicate another morphological change seen with addition of peptide fraction to cells, namely the formation of large

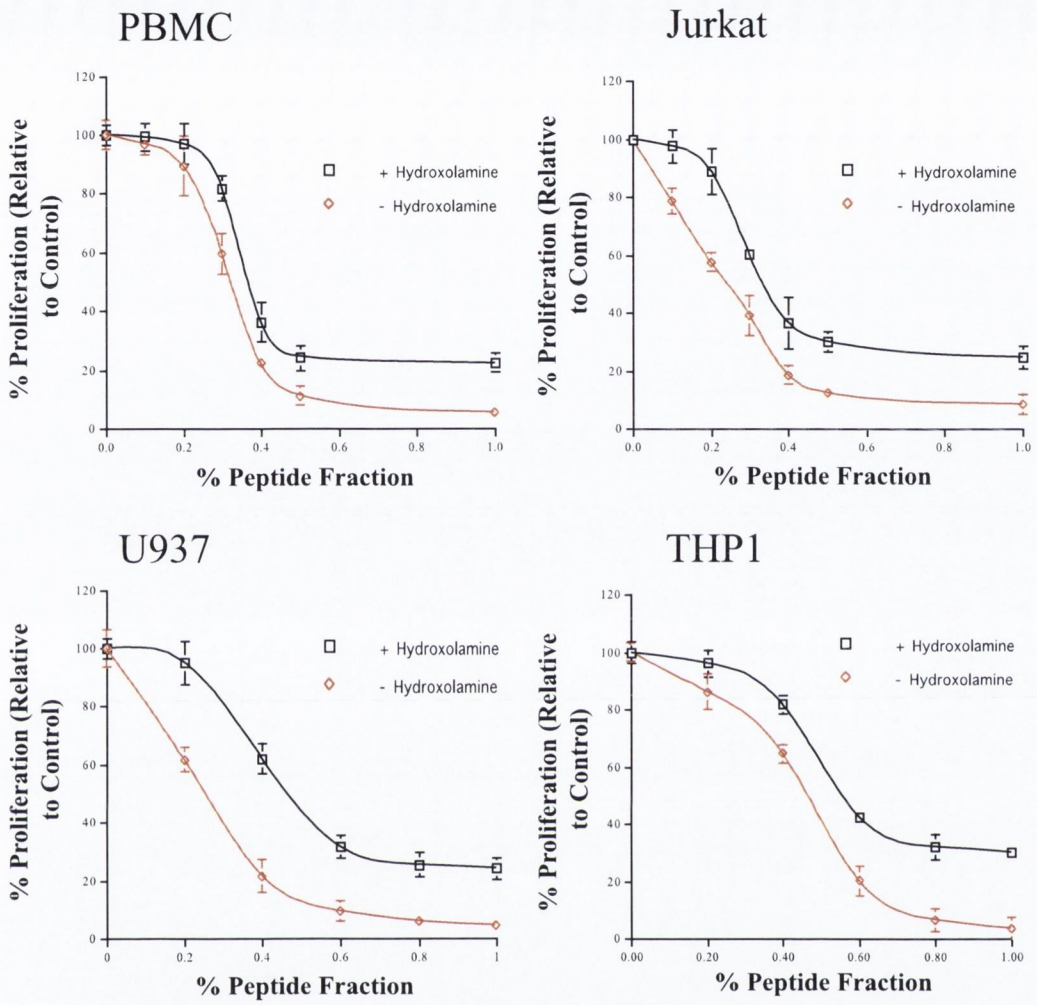
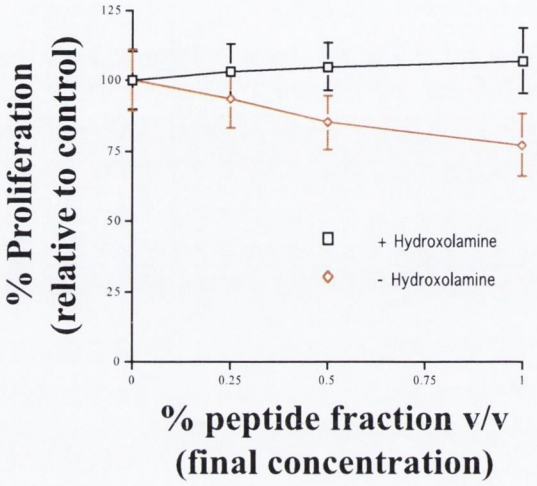


Fig 5.3.1.1. Effects of Peptide Fraction on cell lines of immunological origin. Treatment of cell lines of immunological origin with varying concentrations of <3kD seminal plasma peptide fraction with and without the addition of 100μM hyroxylamine. PBMC (5×10^5) proliferation was induced by mitogen stimulation while all other cell lines (5×10^5) were untreated. Incorporation of [^{3}H]-Thymidine was used to measure proliferation and all incubation times were 24 hours. All points represent the mean of triplicates +/- SEM.

C6 Glioma



MCF7

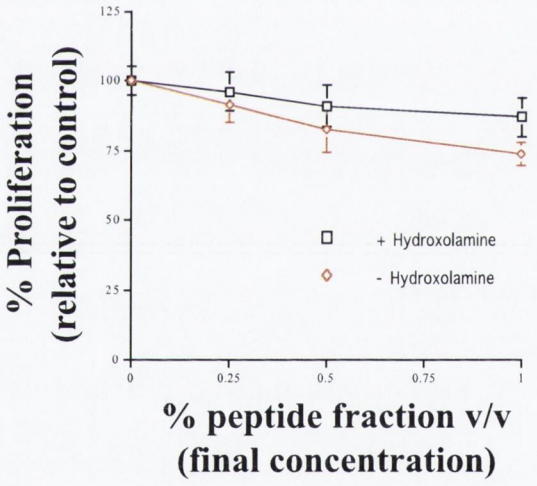


Fig 5.3.1.2. Effects of Peptide Fraction on cell lines of non-immunological origin. Treatment of cell lines of non-immunological origin (5 x 10^5/well) with varying concentrations of <3kD seminal plasma peptide fraction with and without the addition of 100μM hyroxylamine. Incorporation of [^{3}H]-Thymidine was used to measure proliferation and all incubation times were 24 hours. All points represent the mean of triplicates +/- SEM.

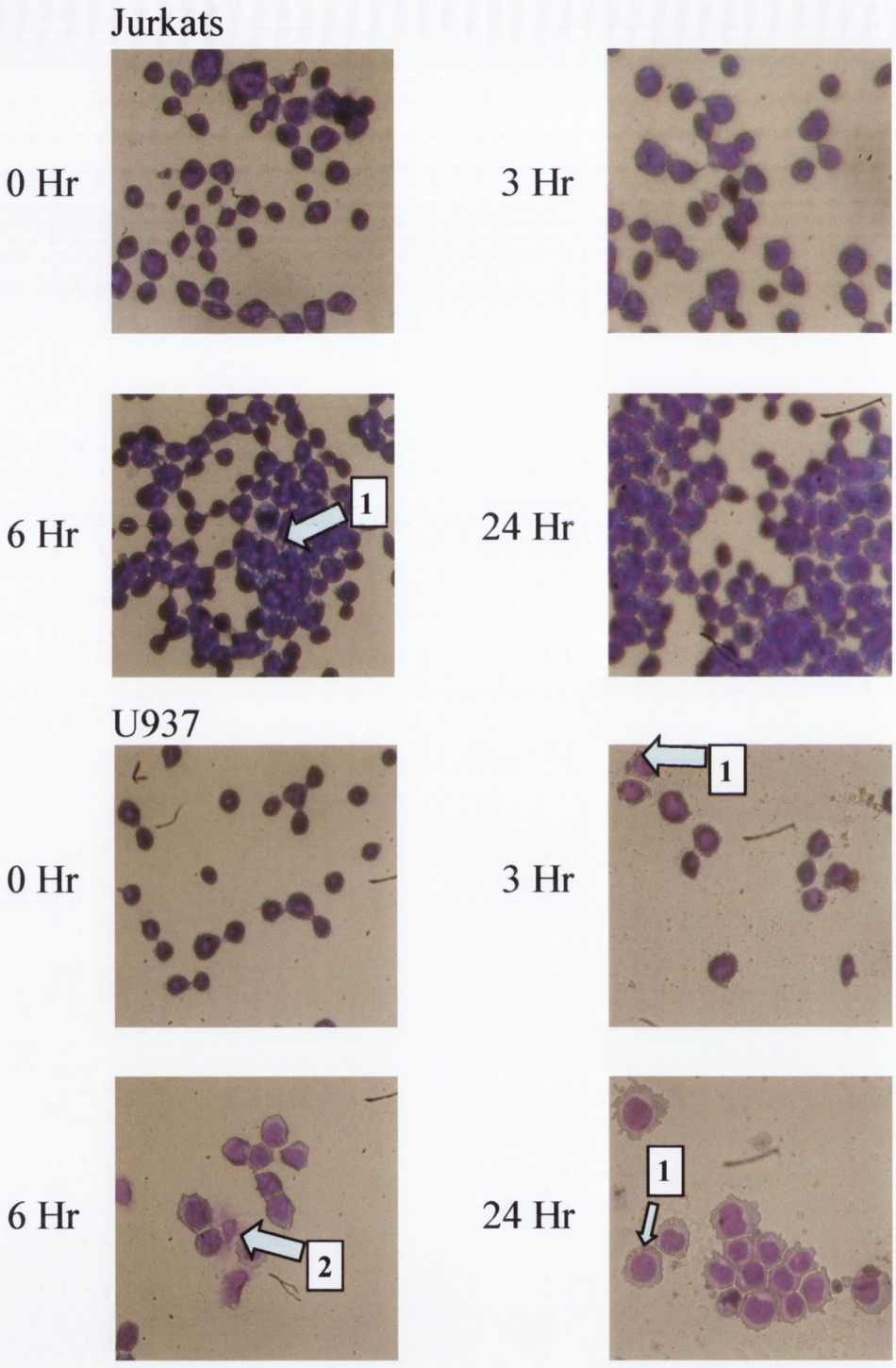


Fig 5.3.1.3. Morphological changes in Jurkat and U937 cell lines on treatment with peptide fraction. Morphological changes in Jurkat and U937 cells on incubation with 1% v/v seminal peptide fraction for differing time periods. All experiments contain 100μM hydroxylamine. Cells were harvested on a cytospin and fixed and stained using Eosin Y and Methylene blue. Images were taken on a 40x light microscope. All images are representative of triplicate experiments. Figures labeled 1 indicated formation of vacuoles. Figures labeled 2 indicate cell death.

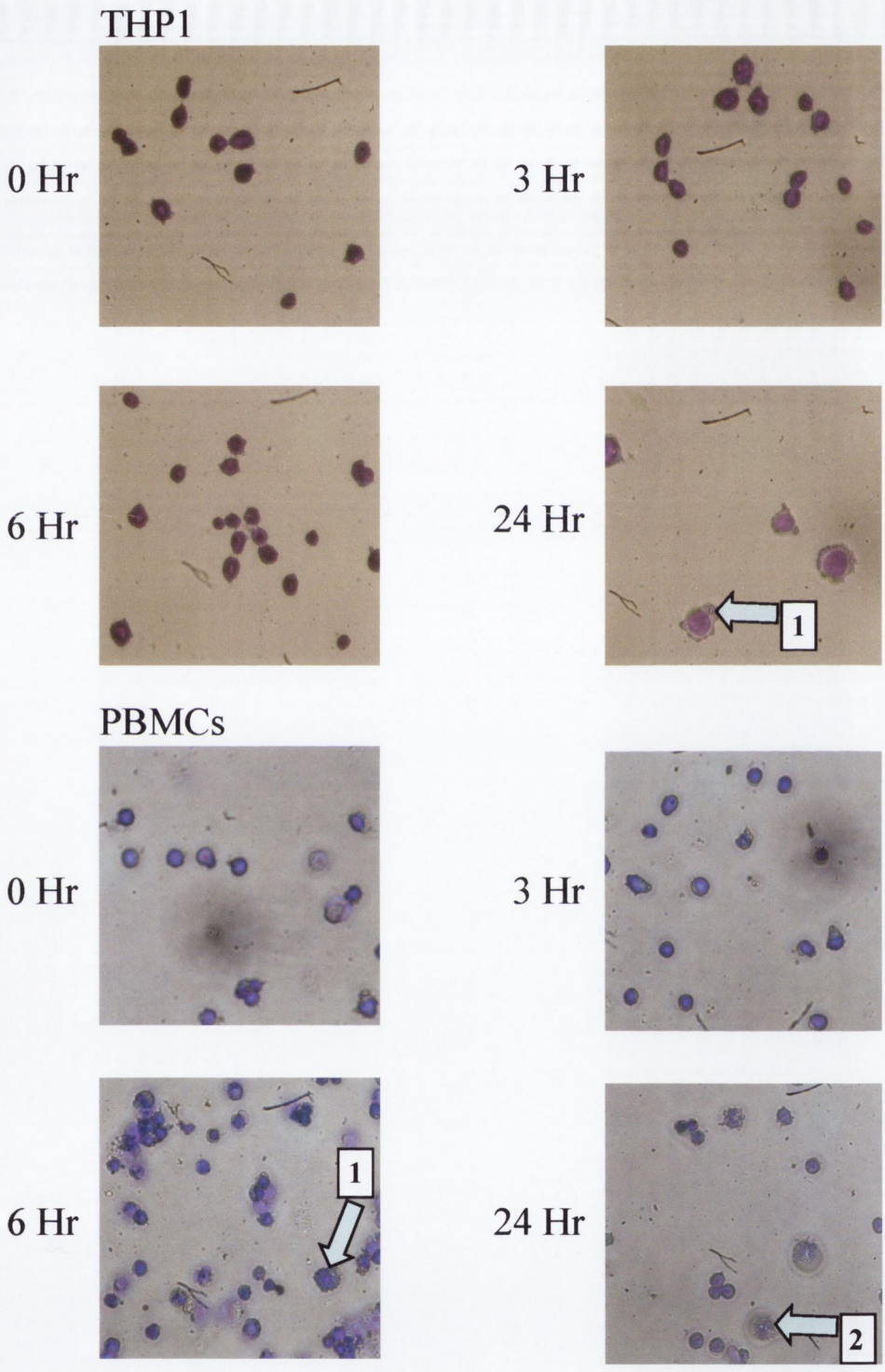


Fig 5.3.1.4. Morphological changes in THP1 cell line and PBMCs on treatment with peptide fraction. Morphological changes in THP1 cells and PBMCs on incubation with 1% v/v seminal peptide fraction for differing time periods. All experiments contain 100μM hydroxylamine. Cells were harvested on a cytospin and fixed and stained using Eosin Y and Methylene blue. Images were taken on a 40x light microscope. All images are representative of triplicate experiments. Figures labeled 1 indicated formation of vacuoles. Figures labeled 2 indicate cell death.

clear vacuoles within the cytoplasmic compartment. These vacuoles are similar in appearance to those formed during autophagic cell death (Li and Vierstra, 2012). Arrows labeled 2 indicate cells with the morphological appearance of primary or secondary necrosis. This was most prevalent in the U937s but occurred in all cell types tested.

5.3.2 FACS Analysis of Cell Cycle in Cell Lines Treated with <3kDa Fraction.

Cell lines were treated with <3kDa fraction for a number of time points and fixed and stained with propidium iodide as in section 2.15.1. PBMCs were analysed at a fixed time point (24 hours) with or without peptide and PHA (for induction of proliferation) as in section 2.15.1. All cell lines (Jurkats, U937s or THP1s) showed an increase over time post treatment with peptide fraction of the Sub-G0 peak indicating breakdown of the DNA. For Jurkats (Figure 5.3.2.1) the Sub-G0 peak increased from 1.83% of cells in control to 15.8% at 6 hours treatment with peptide fraction, while U937s (Figure 5.3.2.2) and THP1s (Figure 5.3.2.3), had increases from 2.28% and 2.47% respectively to 18% and 10.3% respectively at 24 hours post treatment with peptide fraction. However, this occurred at different time points for each of the cell lines, Jurkats having an increase in the Sub-G0 peak at 6 hours incubation with peptide and U937s and THP1s at the 24 hour time point. The Jurkat and THP1 results are in good agreement with cell staining results in the previous section, which showed changes in morphology for the Jurkats and THP1s at 6 and 24 hours respectively. The U937s had morphological changes at 3 hours treatment but only showed DNA fragmentation at 24 hours post treatment. Also interesting to note is the rise in the G2-M peaks in the U937 and THP1 cells (the two monocyte cell lines) from control (19.7% in G2-M phase) to 6 hours post treatment (25% in G2-M phase) for U937s and for THP1s of 19.4% in control to 31.7% after 6 hours of treatment. This would point to a cell cycle arrest in these cell lines at the G2-M phase of the cell cycle on treatment with peptide fraction and after a period of time this arrest leads to cell death and DNA fragmentation (Sub-G0 peak).

The PBMCs untreated with peptide fraction already had higher levels of Sub-G0 peaks (Figure 5.3.2.4) than cultured cell lines presumably due to cellular stress from the isolation process. The Sub-G0 peak rose from 8.94% to 10.4% on stimulation with PHA, though this rise was not significant. The G2-M and S phase cell cycle peaks also

rose from 1.87% to 2.82% for G2-M and 1.27% to 2.30% for S phase. Therefore, stimulation by PHA only induced a small percentage of cells to proliferate within the overall cell population. Addition of the peptide fraction induced a large rise in the Sub-G0 peak to 16.1% without PHA stimulation to 22.1% with PHA stimulation. The G2-M peak also rose to 6.61% without PHA stimulation and to 5.27% with PHA stimulation on treatment with peptide fraction. These figures suggest that the peptide fraction induces cell cycle arrest in the G2-M phase with subsequent cell death (Sub-G0 peak) in PBMCs.

5.3.3 Mechanisms of Cell Death Induced by <3kDa Peptide Fraction.

PBMCs and Jurkat cells were treated with <3kDa fraction as in section 2.15.2 and DNA isolated and run on a 1.5% agarose gel and stained with ethidium bromide as in section 2.2.15. Jurkat cells displayed DNA fragmentation of chromosomal DNA at both 6 and 24 hours treatment with <3kDa fraction (Figure 5.3.3.1). This is as expected from the FACS analysis (Figure 5.3.2.1) of Jurkat cells which showed rises in the sub-G0 peak at these time points. However, PBMCs did not display DNA fragmentation by this method, which is incompatible with the evidence of a Sub-G0 peak in the FACS analysis (Figure 5.3.2.4). This may be due to the lower levels of DNA fragmentation displayed on the FACS analysis for PBMCs as compared to Jurkat cells or for other as yet undefined reasons.

To determine whether the mode of cell death seen in the FACS analysis was due to apoptosis, the levels of caspase 3 activation on treatment with peptide fraction were measured. Each of the cell lines were incubated with increasing concentrations of <3kDa fraction for 6 hours and caspase3 activation measured by addition of caspase 3 substrate, Ac-DEVD-AMC, as in section 2.15.3. Positive controls in each case were cells exposed to UV irradiation and subsequently incubated for 6 hours as in section 2.15.3. Each of the cell lines displayed no activation of caspase 3 (Figure 5.3.3.2) by addition of active peptide fraction indicating that the cell death was not through the conventional caspase cascade and therefore not apoptosis.

If apoptosis is not the form of cell death being induced by the <3kDa fraction then one alternative is necrosis. Lactate dehydrogenase (LDH) release is a commonly used

method of determining necrosis in cell culture and was used here demonstrate whether the <3kDa fraction induced necrosis in the cell lines tested. Each of the cell lines were incubated with 1% v/v <3kDa fraction as before for 24 hours and the cell medium harvested and tested for LDH activity as in section 2.15.4. While Jurkat and U937 cells showed no change in LDH release, PBMCs and THP1 cells did show a significant rise between control and the 1% peptide fraction, though the significance did not rise above $p < 0.05$, and overall levels of LDH were low compared to positive control (Figure 5.3.3.3). This is likely to indicate the occurrence of a secondary necrosis event at the higher concentrations of <3kDa fraction.

Time course analysis of the inhibition of cellular proliferation by the <3kDa peptide fraction of seminal plasma (Figure 5.3.3.4) using WST1 reagent as read out for cell proliferation as in section 2.15.5.1 indicated that at 3 hours post addition of peptide fraction divergence between Jurkat control cells and Jurkat treated cells could be detected. Therefore, the peptide fraction acts quickly on Jurkat cells to limit cell growth.

Pre-treatment of Jurkat cells with mimosine as in section 2.15.5.3 led to a complete abrogation of the effects of the peptide fraction (Figure 5.3.3.5). Mimosine is a plant amino acid that effectively halts cell cycle in G0 phase of the cell cycle. Therefore, it can be surmised that the peptide fraction is only active on cells actively progressing through cell cycle. This complements the data from the FACS analysis indicating a block in cell cycle at the G2-M phase in cells treated with peptide fraction and then subsequent cell death.

To elucidate whether active cell processes are involved in the mechanism of anti-proliferation by the seminal peptide fraction, Jurkat cells were pre-incubated with cyclohexamide and actinomycin D, inhibitors of translation and transcription, respectively (as in section 2.15.5.2). Inhibiting either transcription or translation led to a complete negation of the effects of the seminal peptide fraction (Figure 5.3.3.6). It can be concluded therefore; that the peptide fraction activates cell processes that lead to *de novo* protein production within the cell, which leads to blocks in cellular progression through cell cycle.

Morphological staining of Jurkat cells as before in section 2.14.2 after pre-treatment with cyclohexamide or actinomycin D and treatment for 6 hours with seminal peptide fraction showed that pre-treatment of cells with either compound failed to show the characteristics of treatment with seminal peptide fraction alone; formation of vacuoles within the cytoplasm (Figure 5.3.3.7). Also tested was the compound BAPTA-AM, an intracellular calcium chelator (as in section 2.15.6). BAPTA-AM also fully inhibited the formation of vacuoles within the cytoplasm on peptide fraction treatment of Jurkat cells. This indicates that calcium release from one or more cellular organelles is required for cellular signaling to occur that leads to cell cycle blockage and cell death.

5.3.4 Effects of Active Fraction on Cytokine Release from PBMCs.

PBMCs were plated and treated with PHA-P, TNFα and LPS in the presence or absence of active fraction as described in section 2.15.7. Supernatants were harvested and screened for IL-8 and IL-10 as in section 2.15.7. Active peptide fraction reduced the release of IL-8 dramitically in all treatments tested, whereas IL-10 release was increased in the control treatment and PHA-P treatments only (Figure 5.3.4.1).

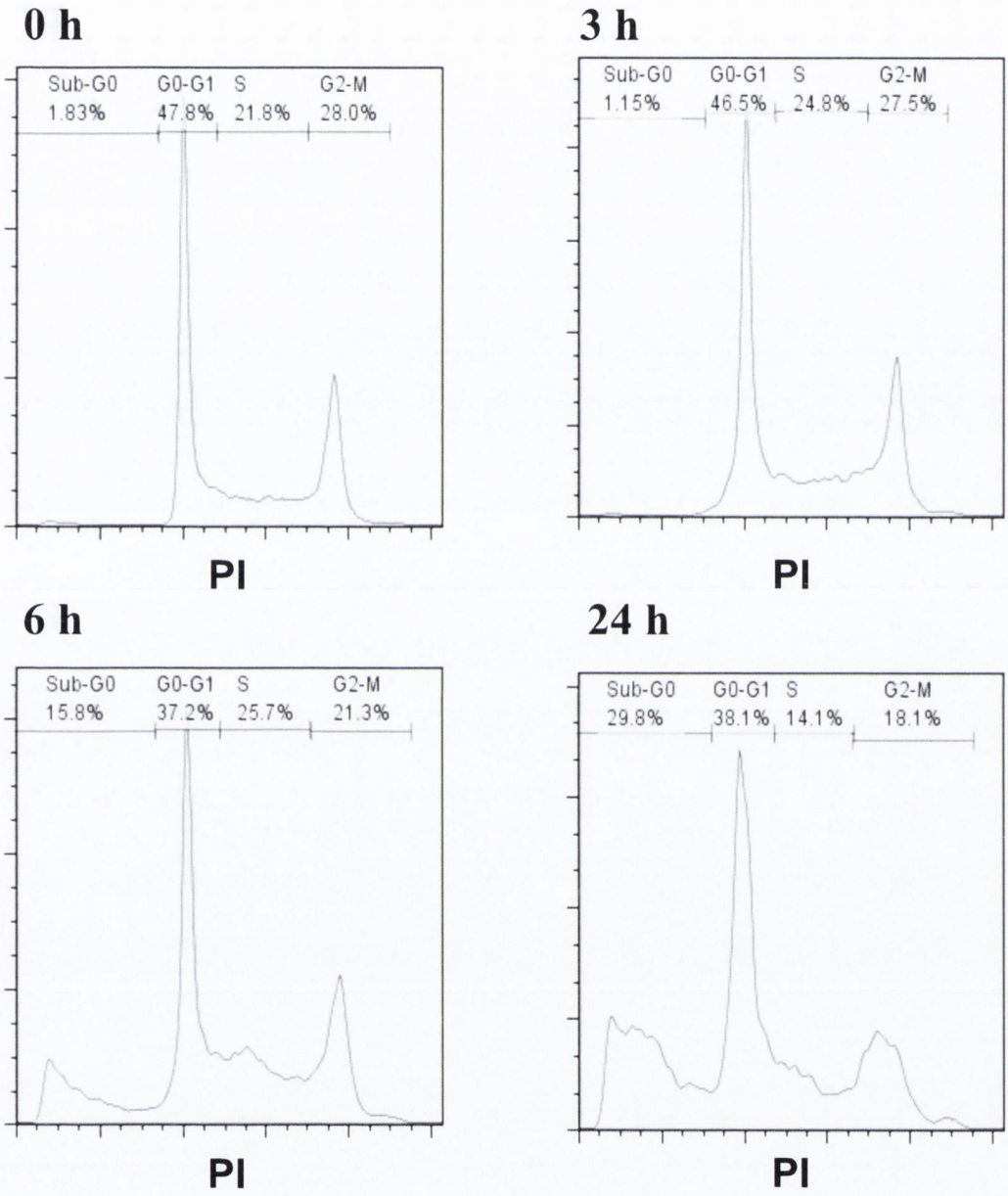


Fig 5.3.2.1. Cell cycle analysis of Jurkat cells incubated for differing time periods with 1% seminal peptide fraction. Cells were fixed and stained with propidium iodide at each time point and analysed by FACS. Each graph is representative of triplicate experiments. All experiments contain 100μM hydroxylamine.

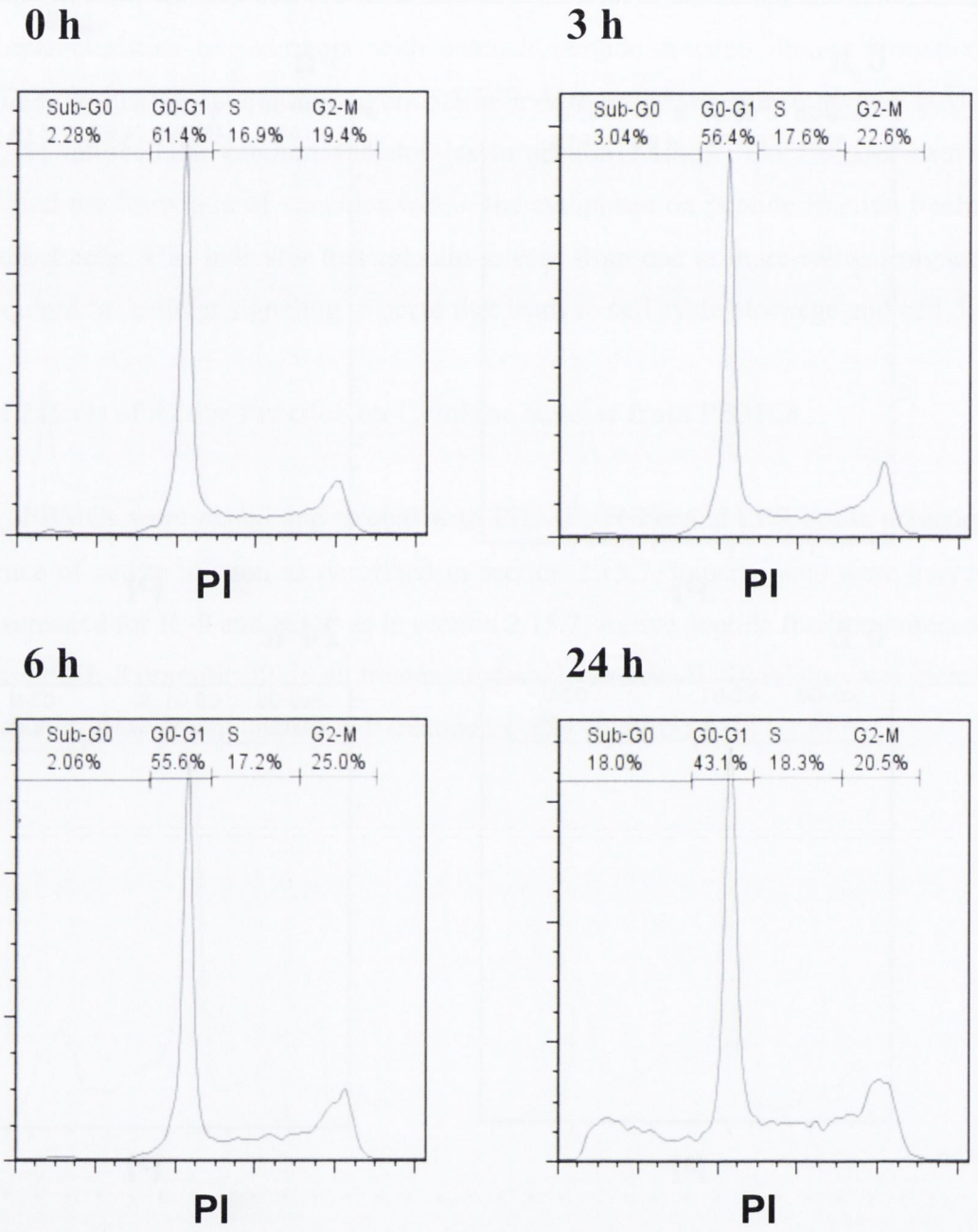


Fig 5.3.2.2. Cell cycle analysis of U937 cells incubated for differing time periods with 1% seminal peptide fraction. Cells were fixed and stained with propidium iodide at each time point and analysed by FACS. Each graph is representative of triplicate experiments. All experiments contain 100μM hydroxylamine.

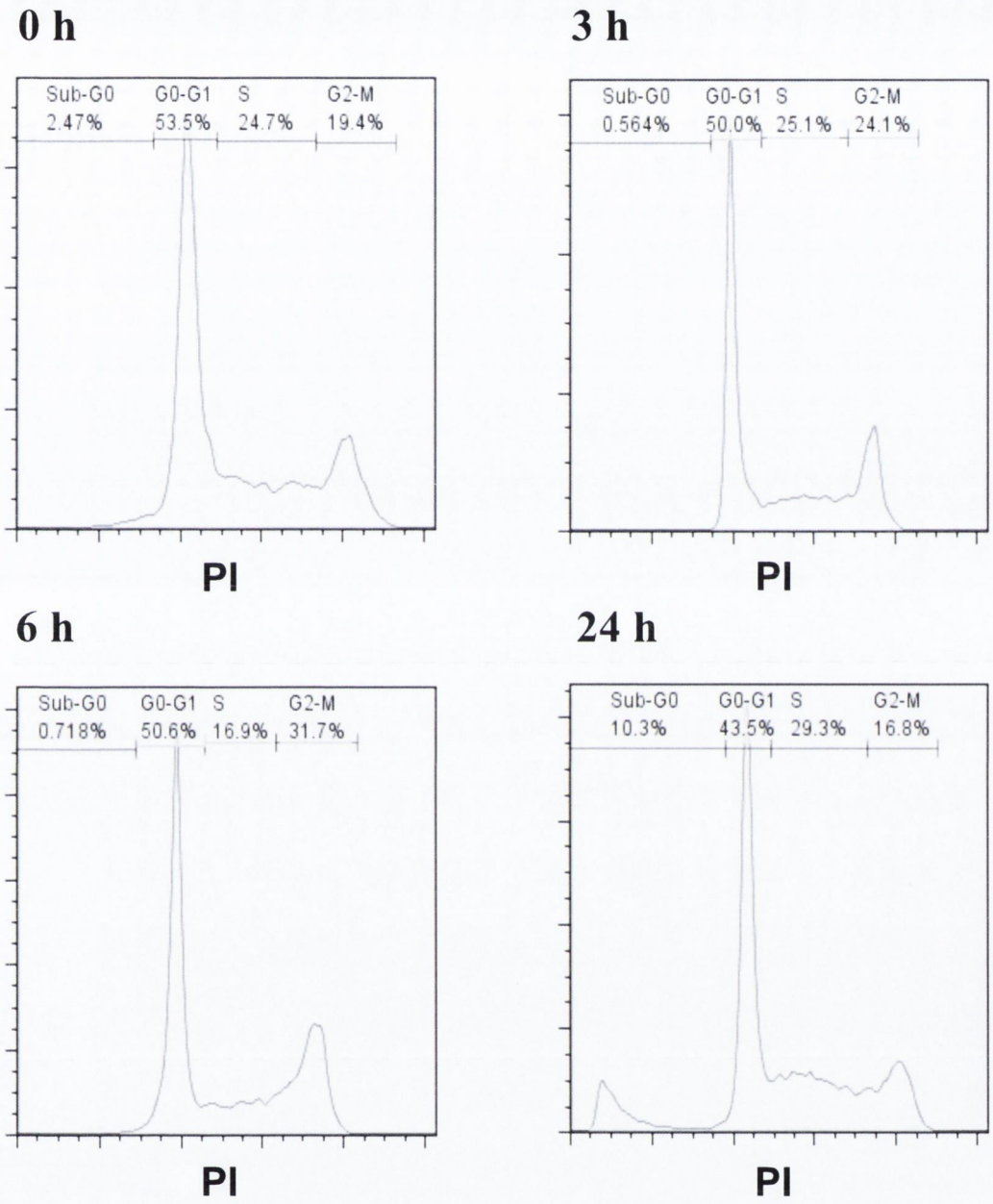


Fig 5.3.2.3. Cell cycle analysis of THP1 cells incubated for differing time periods with 1% seminal peptide fraction. Cells were fixed and stained with propidium iodide at each time point and analysed by FACS. Each graph is representative of triplicate experiments. All experiments contain 100μM hydroxylamine.

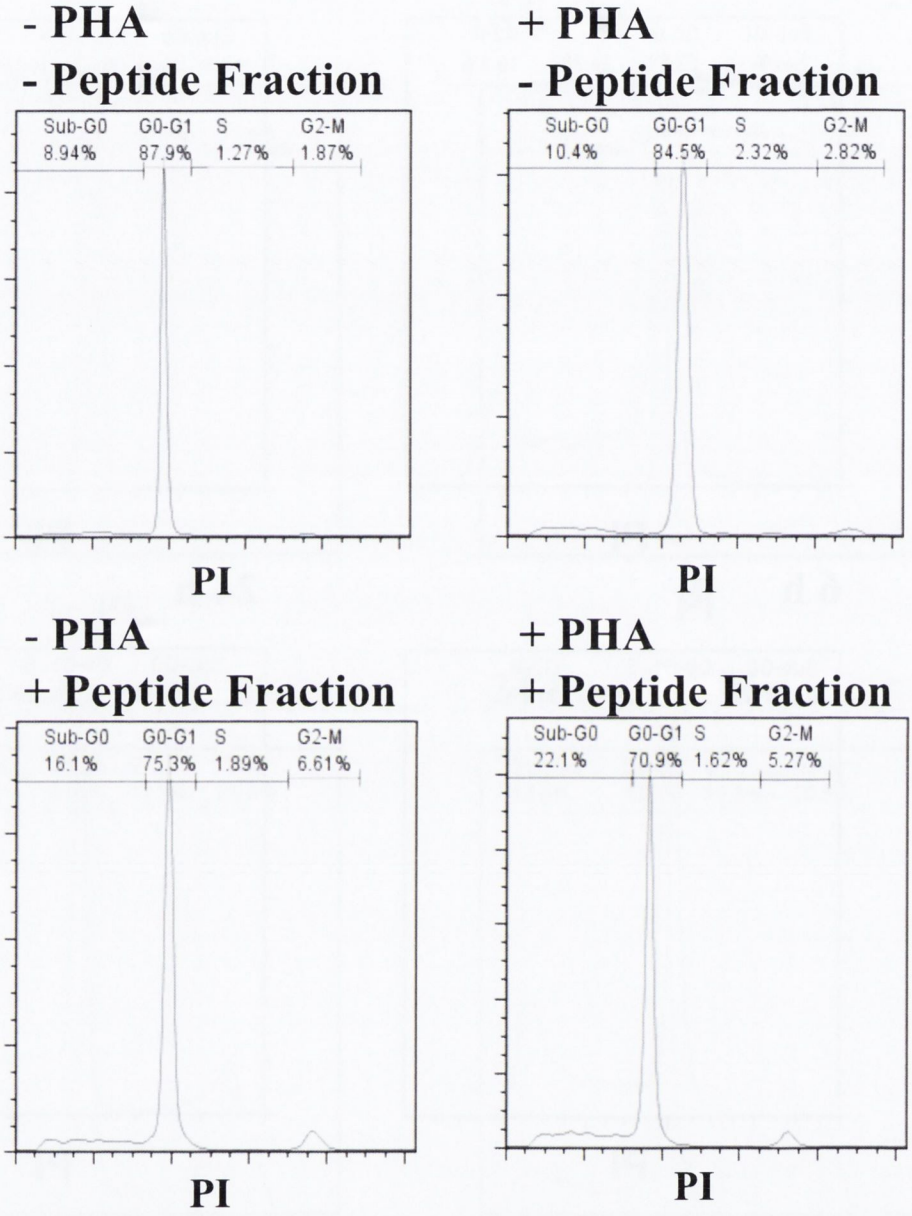


Fig 5.3.2.4. Cell cycle analysis of PBMCs incubated with or without 1% seminal peptide fraction and with and without mitogen stimulation with PHA. Cells were fixed and stained with propidium iodide at 24 hour time point and analysed by FACS. Each graph is representative of triplicate experiments. All experiments contain 100μM hydroxylamine.

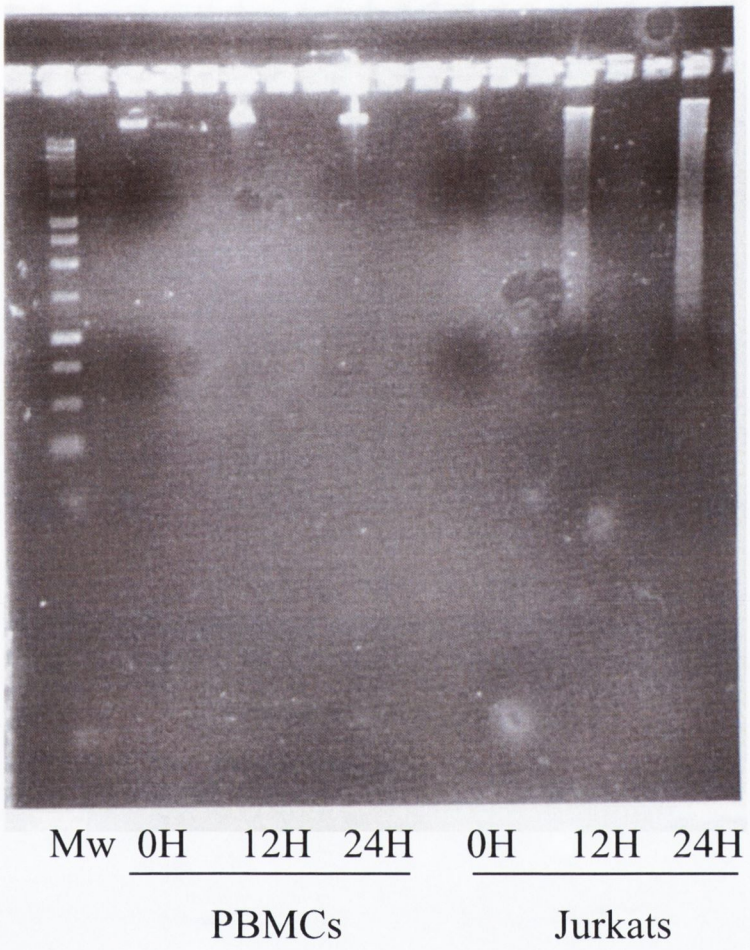


Fig 5.3.3.1. DNA strand breakage in cells in response to treatment with seminal peptide fraction. DNA strand breakage in PBMCs and Jurkat cells at differing time points in response to incubation with 1% v/v seminal peptide fraction. All experiments contain 100μM hydroxylamine. DNA was extracted from incubated cells and run on an 1.5% agarose gel at 150V for 1hr and imaged using ethidium bromide.

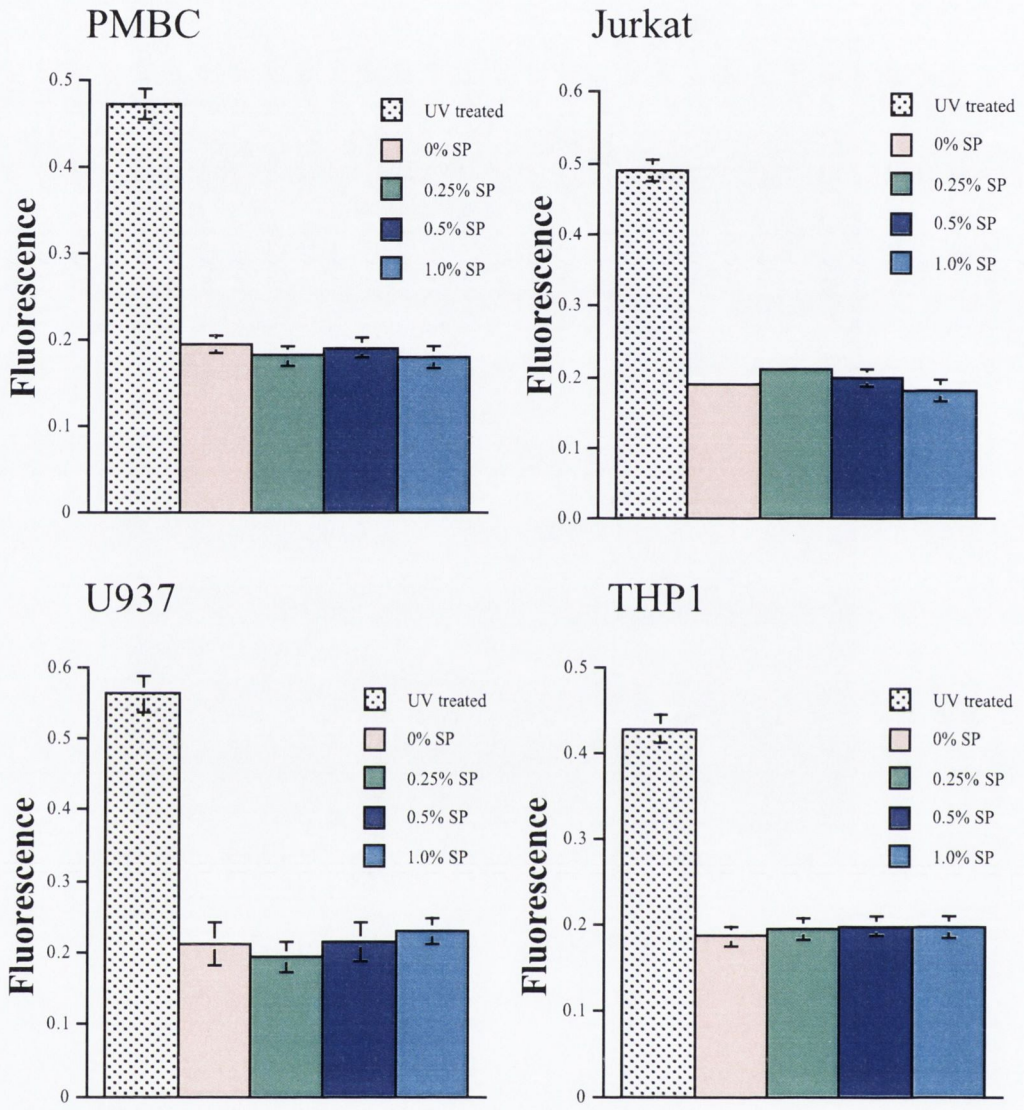


Fig 5.3.3.2. Caspase 3 activation studies. Effect of increasing concentrations of seminal peptide fraction in the presence of 100μM hydroxylamine on caspase 3 activation. Positive controls were UV irradiated cells. All incubations were for 24 hours and each point represents the mean of triplicates +/- SEM.No statistical differences were observed.

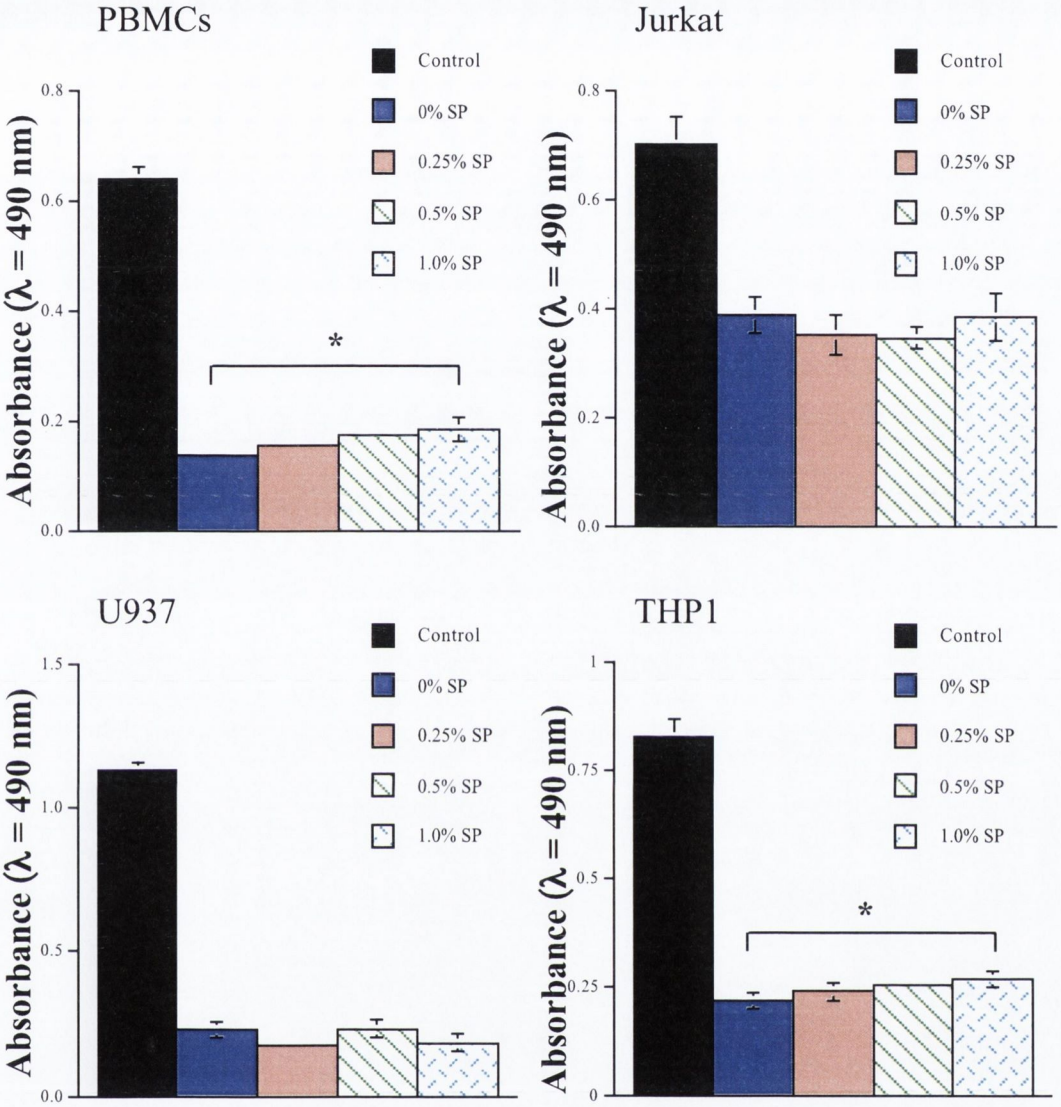


Fig 5.3.3.3. Measurement of necrosis in cell lines treated with seminal peptide fraction. Lactate Dehydrogenase release in PBMCs, Jurkats, U937s and THP1s in response to varying concentrations of seminal plasma peptide fraction. Positive controls are cells subjected to Triton X-100 disruption. All incubations were for 24 hours and each point represents the mean of triplicates +/- SEM. Statistical differences * $p < 0.05$.

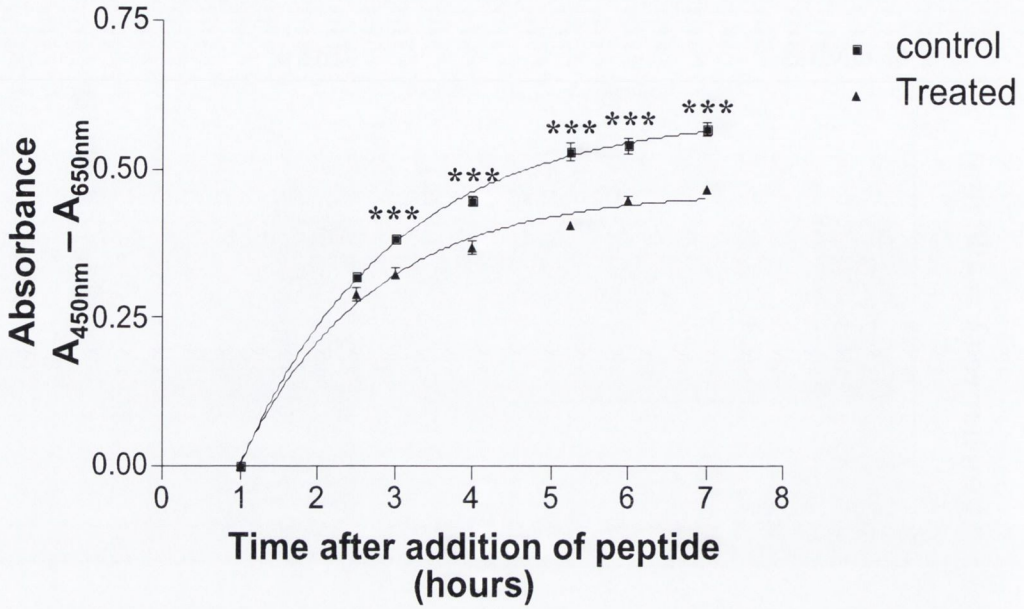


Fig 5.3.3.4. Time Course of cell death of Jurkat cells on incubation with 1% v/v seminal peptide fraction. WST1 colourimetric reagent was added at 1 hour and readings taken at hourly intervals up to 7 hours. Statistical difference using a one-way annova analysis (*** $p < 0.001$) was reached at 3 hours post seminal peptide addition.

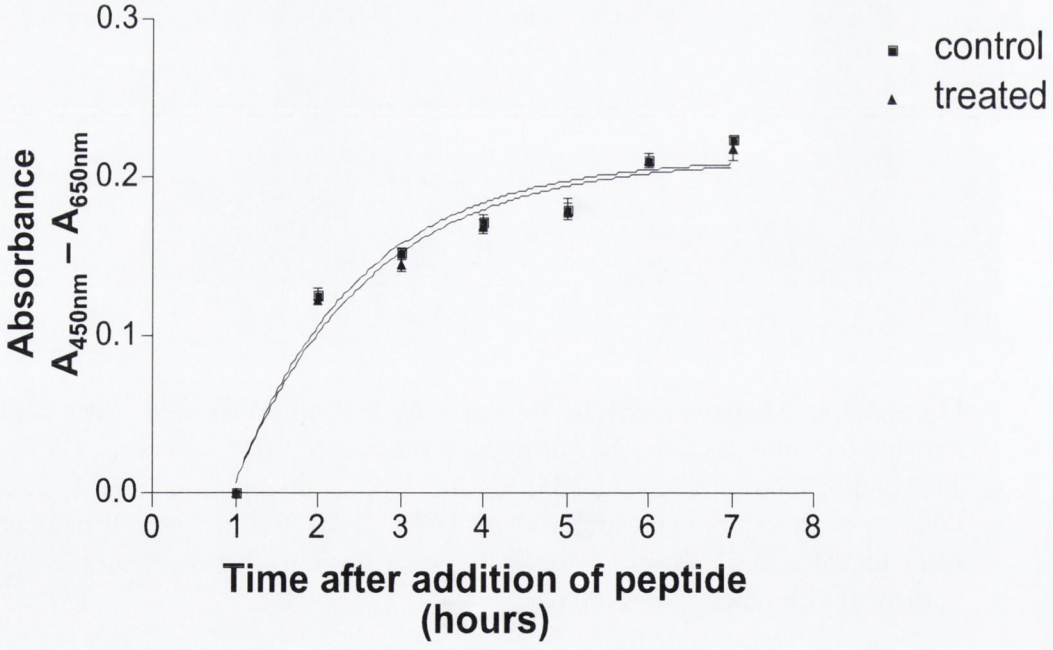


Fig 5.3.3.5. The role of cell cycle in the activity of the <3kDa fraction. Treatment of Jurkat cells with 1% seminal peptide fraction pre-treated overnight with 30μM mimosine to arrest cell cycle in G_0. WST1 colourimetric reagent was added at 1 hour and readings taken at hourly intervals up to 7 hours.

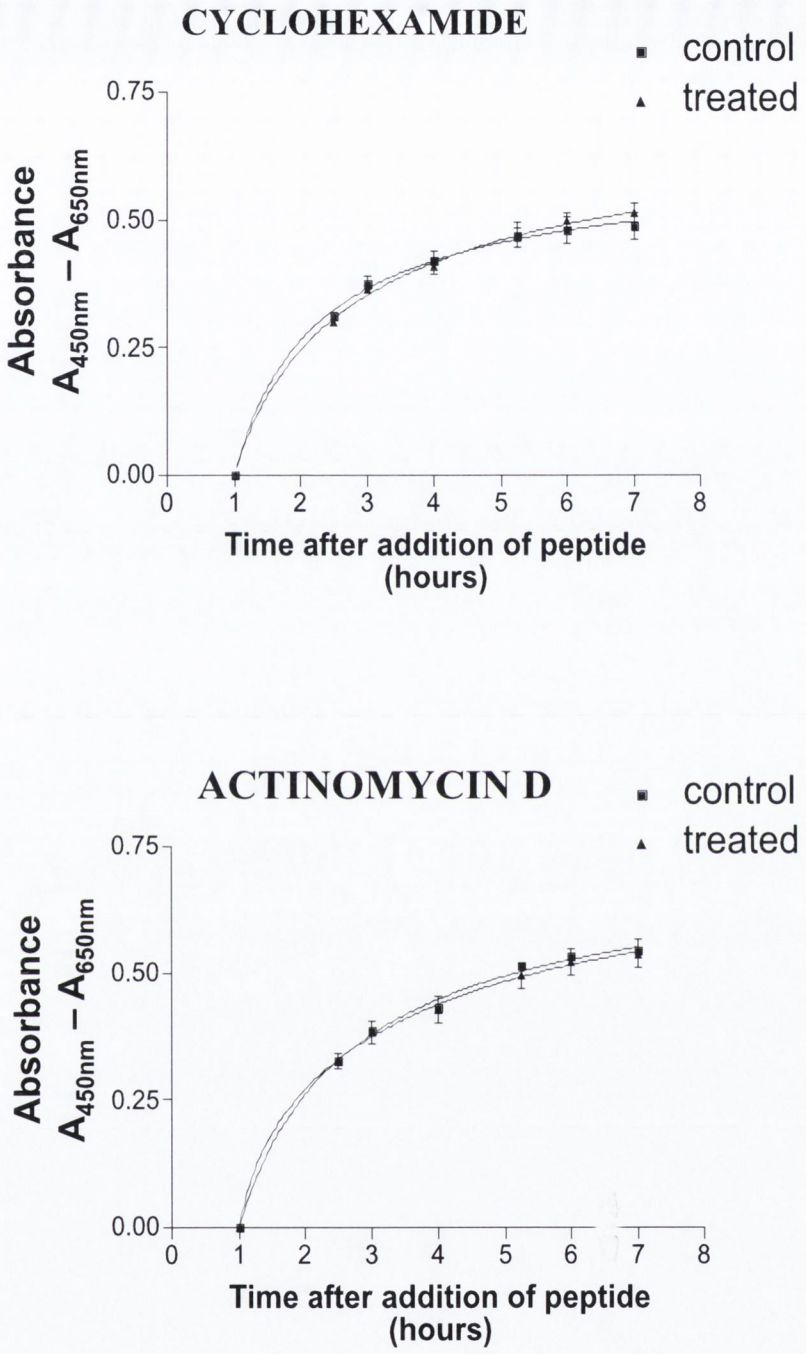
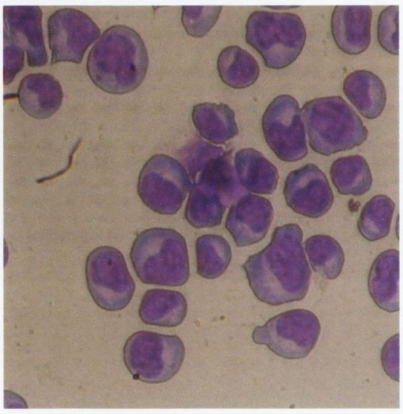
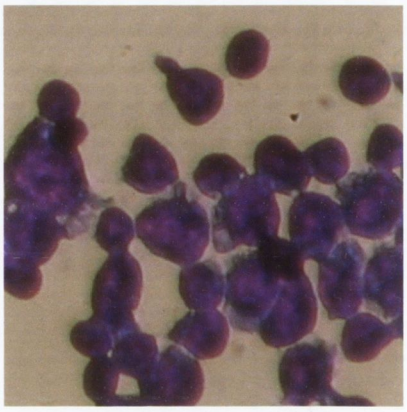


Fig 5.3.3.6. Activity of the <3kDa fraction in the presence of inhibitors of trancription and translation. Treatment of Jurkat cells with 1% (v/v) seminal peptide fraction pre-treated for 1 hour with either with either 0.5µg/ml cyclohexamide or 0.5µg/ml Actinomycin D to arrest translation and transcription respectively. WST1 colourimetric reagent was added at 1 hour post addition of peptide fraction and readings taken at hourly intervals up to 7 hours. All points represent the mean of triplicates +/- SEM. There is no statistical difference between control curves and curves for treated cells.

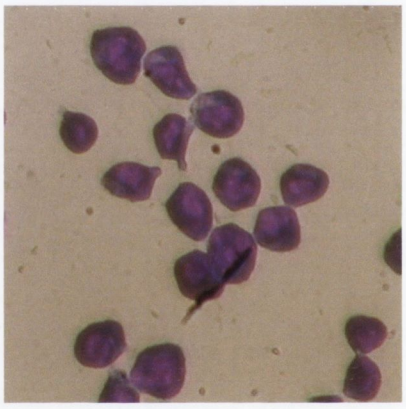
Untreated Control



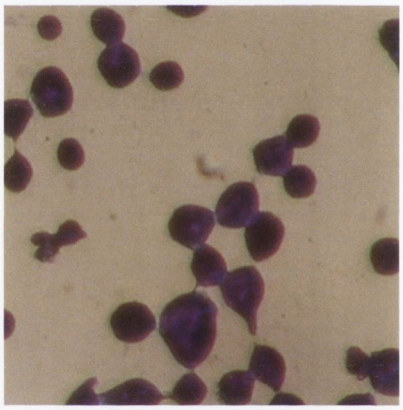
Peptide Treated Control



Cyclohexamide



Actinomycin D



BAPTA-AM

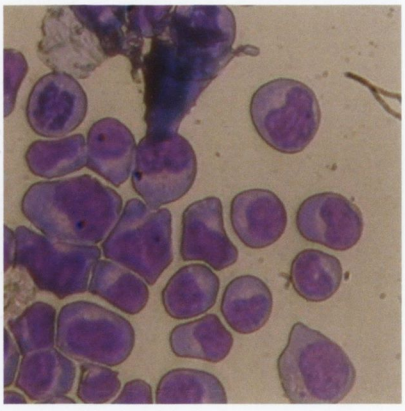


Fig 5.3.3.7. Morphological changes on treatment of Jurkat cells with 1% v/v seminal peptide fraction. Cells were treated for 6 hr with seminal peptide fraction after pre-treatment with 0.5µg/ml cyclohexamide or actinomycin D or 50µM BAPTA-AM for 1 hour.

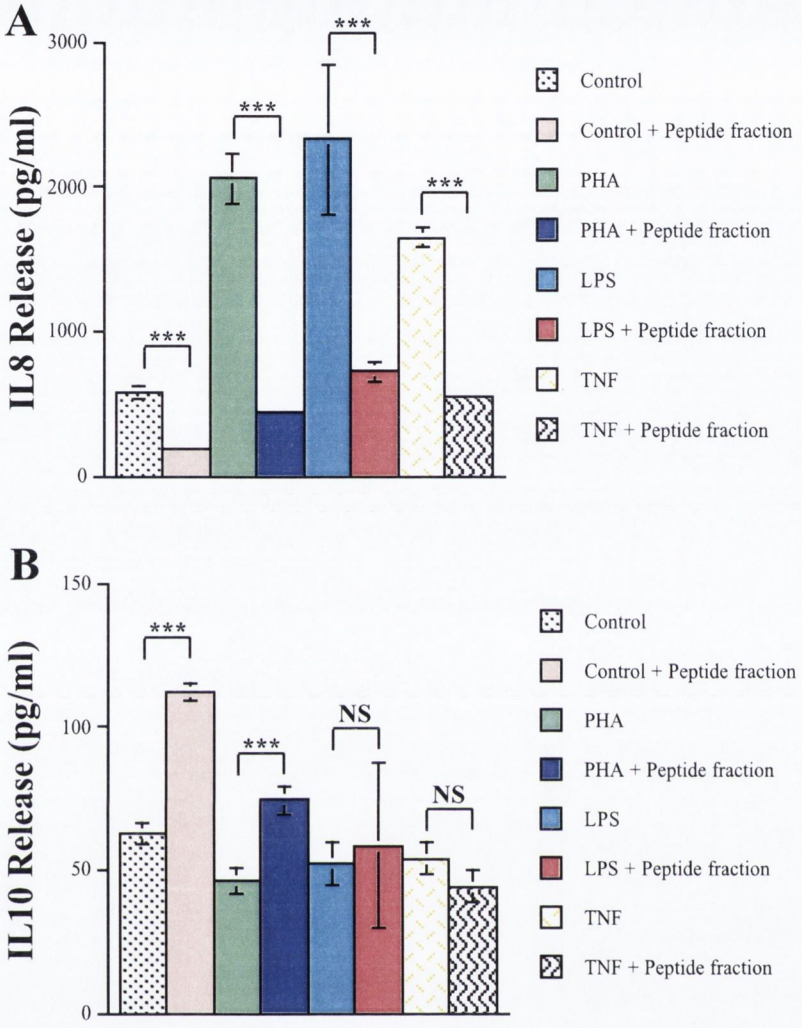


Figure 5.3.4.1 Effect of Seminal Plasma Peptide Fraction on Induction of Interleukin-8 and Interleukin-10 in the Presence or Absence of Stimulatory Molecules, PHA-P, LPS and TNFα. PBMCs (1 x 10^6/ml) were treated with the stimulatory molecules PHA-P (2.5μg/ml), LPS (10ng/ml) or TNFα (25ng/ml) in the presence or absence of 1% (v/v) seminal peptide fraction or with no stimulation in the presence or absence of 1% (v/v) seminal peptide fraction for 24 hours. Cells were removed by centrifugation and supernatants tested for IL-8 and IL-10 by ELISA. All points represent the mean of triplicates +/- SEM. Statistical values *** $p \leq 0.001$, NS not statistically significant..

5.4 Further Purification and Identification of Active Constituent of the <3kDa Fraction.

5.4.1 HPLC C_{18} Reverse Phase Chromatography and Mass Spectrometry of Active Fraction.

The <3kDa peptide fraction was loaded onto a C_{18} reverse phase HPLC column and bound peptides eluted with an acetonitrile gradient as in section 2.16 (Figure 5.4.1A). Fractions were collected in 0.5 ml aliquots and freeze dried as in section 2.16 and tested on Jurkat cells for anti-proliferative qualities with fractions 7 and 8 showing most activity (Figure 5.4.1B). These fractions were combined and subjected to proteolysis with trypsin and analysed by MS/MS. Two separate peptides were detected, both coming from the same region of the major seminal clot forming protein, semenogelin 1 (Figure 5.4.1C). It is worth noting that this partial sequence of semenogelin 1 contains a repeat sequence of SYQSS which may be of importance to the activity of this peptide.

5.4.2 Testing of Semenogelin 1 and 2 and its PSA Cleavage Products for Anti-Proliferative Activity.

Semenogelin 1 and 2 were purified as previously described in section 2.6.1. Both were cleaved for either 3, 6 or 24 hours using PSA protease as section 2.6.1.1. The semenogelins are the natural substrates for this protease in seminal plasma. The whole proteins and cleavage products were tested on mitogen stimulated PBMCs to show anti-proliferative activities (Figure 5.4.2). Semenogelin 1 not cleaved with PSA reduced proliferation to approximately 67% of control, while 3, 6 and 24 hour cleavage with PSA all showed greater inhibition, peaking at 6 hours cleavage with proliferation being approximately 34% of control. There was a slight decrease in inhibition at the 24 hour time point indicating that it may be possible to over proteolyse the protein with subsequent loss of activity. Semenogelin 2 also showed inhibitory qualities, but as in chapter 3 (Figure 3.2.1.9), purity of the semenogelins coming off the heparin column is not complete with cross contamination with each other. Therefore, the anti-proliferative activity may be associated with just semenogelin 1 but semenogelin 2 also shows activity due to the presence of semenogelin 1 in this fraction albeit at much lower

concentrations. However, as the following sequence alignment shows, semenogelin 1 and 2 share a high degree of homology in this area of the genes and therefore activity may not be confined to semenogelin 1 alone.

5.4.3 Sequence Alignment of the Semenogelins with SV-IV of Rat.

The sequence of semenogelin 1 containing the peptides found by mass spectrometry was blasted against the data bases and a homology found to SVS-IV from rat. SV-IV is a small peptide of approximately 9kDa secreted from the seminal vesicle of rat and shown to inhibit the mitogen stimulation of both human and rat PBMCs (Metafora et al., 1989b). Sequence alignment of semenogelin 1 and 2 and SV-IV (Figure 5.4.3) indicate areas of conservation with the highlighted glutamine and lysine being important for SV-IV activity. This glutamine of SV-IV has been shown to be a substrate for the enzyme transglutaminase using the indicated lysine as an acyl-acceptor (Porta et al., 1993). This transglutamination greatly increases its activity in the inhibition of mitogen stimulation of PBMCs (Porta et al., 1993). SV-IV can also use spermidine as an acyl-acceptor giving a spermidine adducted peptide, which also leads to increased activity of the peptide as an immunosuppressant (Porta et al., 1990).

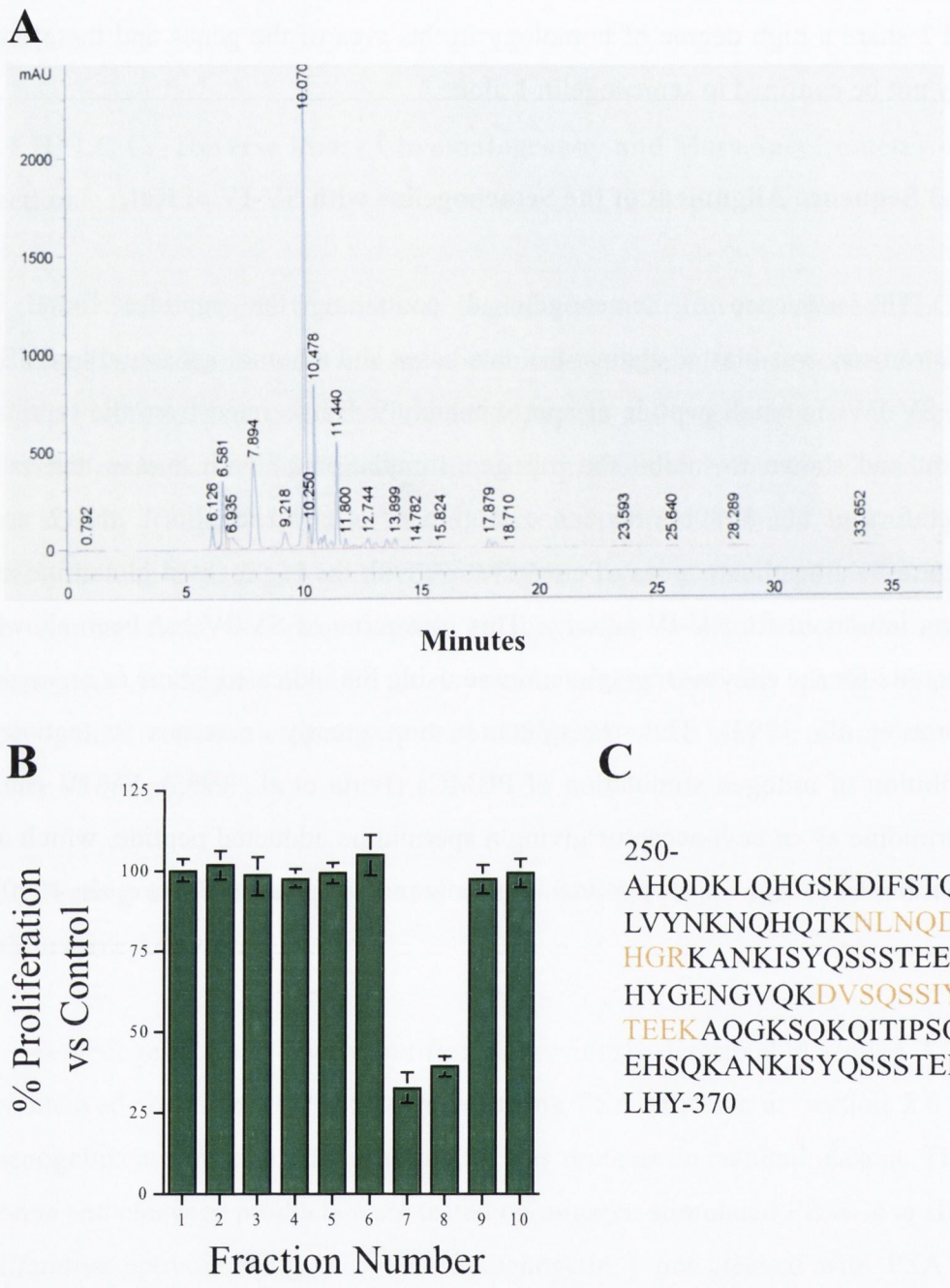


Fig 5.4.1. Further Purification of Active Seminal Peptide by HPLC and Identification by Mass Spectrometry. A. HPLC C_{18} reverse phase chromatography of the <3kDa peptide fraction of seminal plasma. 0.5 ml fractions were collected from 5min into the run. **B.** Fractions from the HPLC chromatography were freeze dried and resuspended in 100µl PBS and tested on Jurkat cells using WST1 reagent to quantify proliferation. **C.** Fractions 7 and 8 were combined and subjected to MS/MS after trypsinization. Peptides colured orange were detected and are shown *in situ* (black) in a segment of the semenogelin 1 protein.

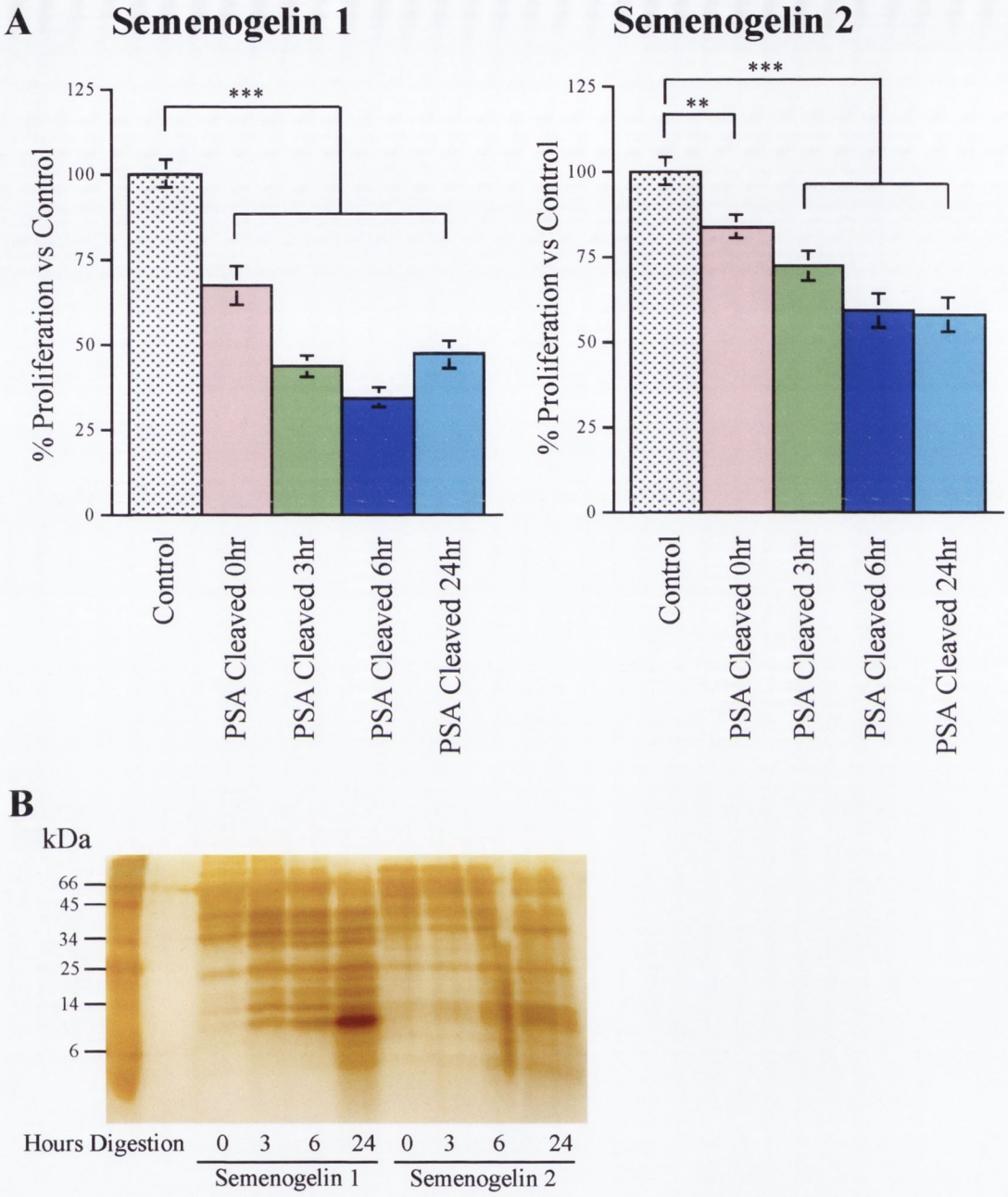


Fig 5.4.2. A. Inhibition of PBMC proliferation by Semenogelin 1 and 2 and their PSA cleavage products. Semenogelin 1 and 2 was purified and 50µg/200µl added to PBMCs (5 x 10^5) either whole or cleaved with PSA for 3, 6 or 24 hours. PBMCs proliferation was induced by mitogen stimulation. Incorporation of [^{3}H]-Thymidine was used to measure proliferation and all incubation times were 24 hours. All points represent the mean of triplicates +/- SEM. Statistical values ** $p \leq 0.005$ *** $p \leq 0.001$. **B.** 15% SDS-PAGE gel of Semenogelin 1 and 2 at specific time points of PSA cleavage. Gels were silver stained.

Fig 5.4.3 Sequence Alignment of Semenogelin 1, Semenogelin 2 and SVS-4 using Multalin. Full length protein sequences for semenogelin 1 and 2 from human and SV-IV were downloaded from Uniprot and aligned using the online alignment tool Multalin. Arrows indicate conserved glutamine and lysine residues between semenogelin 1 and SV-IV that are important in SV-IV for transglutamination.

5.4.4 Cloning of Semenogelin Fragments and SV-IV and Expression Analysis.

Semenogelin fragments and SV-IV were cloned into pet28b as in section 2.17. Plasmids containing the Semenogelin fragments (Figure 5.4.4.1) were transformed into BL21 (DE3) *E. coli* and test expressions carried out as in section 2.17.5. SV-IV, Sem 274, Sem2 and Sem164 all showed good expression while Sem264 and Inhibin92 did not express well (Figure 5.4.4.2A). Solubility testing revealed that SV-IV, Sem274 and Inhibin92 expressed soluble protein whereas all the other clones were mainly insoluble (Figure 5.4.4.2B). Test purifications on Streptactin-sepharose of SV-IV, Sem274 and Inhibin92 gave good yields of protein but Sem2, Sem164 and Sem264 as expected after showing low solubility did not (Figure 5.4.4.3). Sem274 did display a contaminating band and required further purification by gel filtration on a superdex 75 10/300GL column (as in section 2.17.7) (Figure 5.4.4.4) giving pure peptide. Inhibin92 on concentration proved to be unstable in aqueous solution.

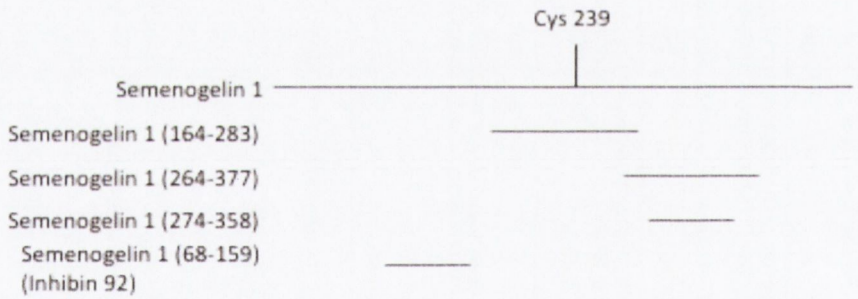


Figure 5.4.3.1 Schematic Representation of Cloned Fragments of Semenogelin 1 Compared to Full Length Semenogelin 1.

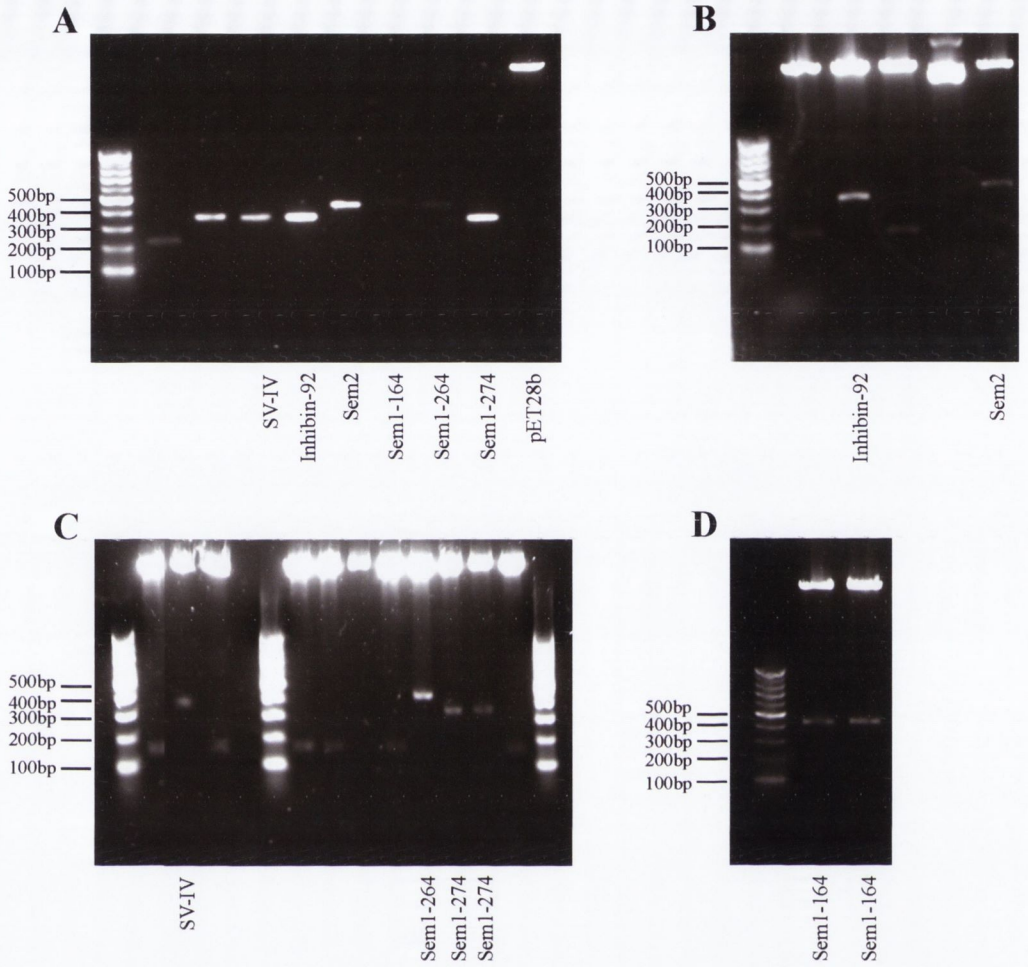


Figure 5.4.4.1 Cloning of Semenogelin Fragments and SV-IV. Synthesized DNA of semenogelin fragments and SV-IV in pUC18 vector were grown in BL21 *E. coli* and vectors isolated and cleaved with restriction enzymes *Nco1* and *Xho1*. Cleavage products were run on a 1.5% agarose gel and the cleaved inserts excised and extracted using a gel purification kit. Expression vector, pET28b, was also grown in BL21 *E. coli*, cleaved as with pUC18 vectors and the linearized plasmid purified from the gel as before. **A.** Purified inserts of semenogelin fragments, SV-IV and pET28b linearized plasmid. **B-D.** Inserts of semenogelin fragments and SV-IV were ligated into pET28b linearized vector. Screening of colonies was performed and positive clones indicated in figures B-D.

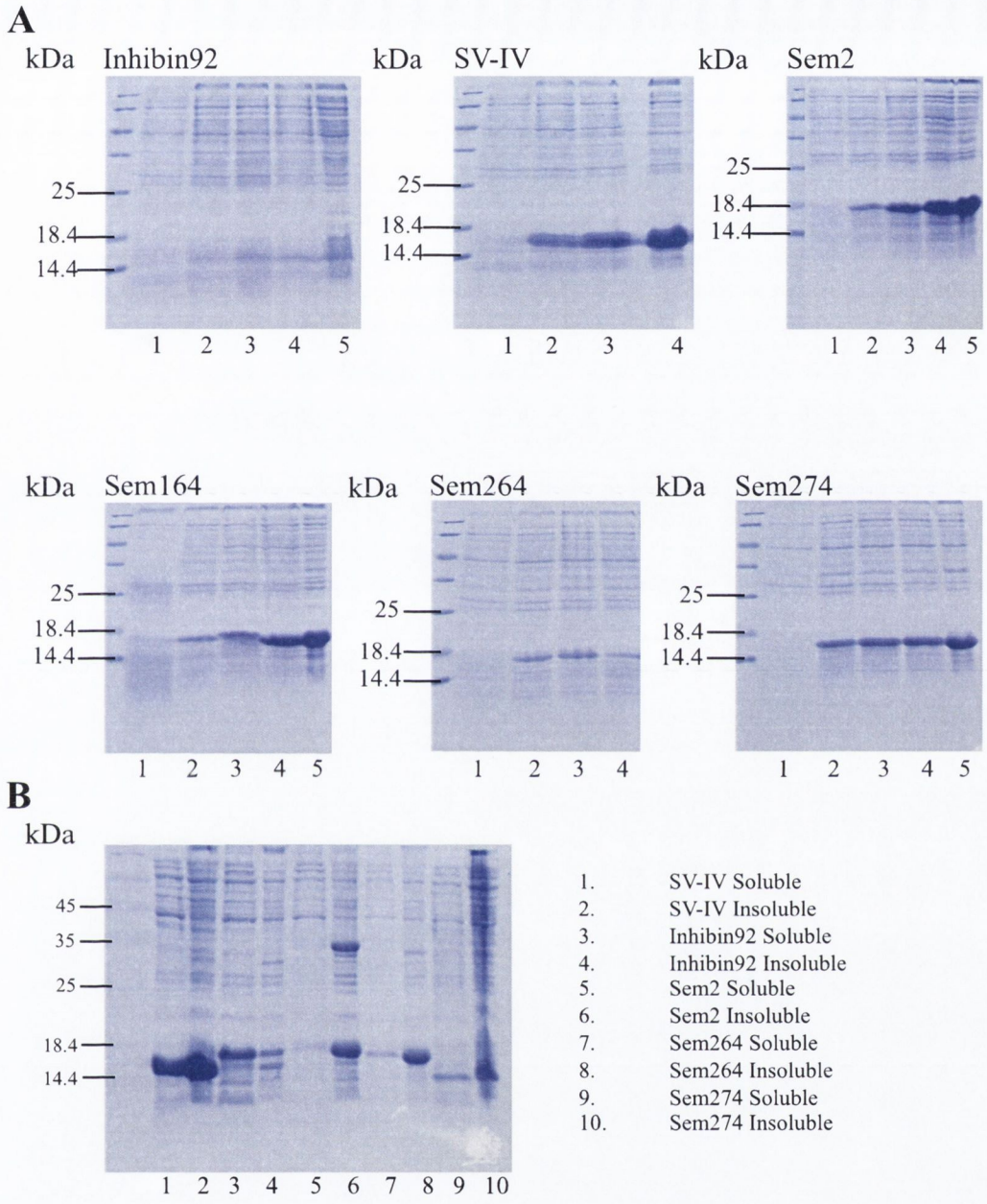


Figure 5.4.4.2 Test Expressions of Semenogelin Fragments and SV-IV and Solubility Testing. A. Semenogelin fragments and SV-IV were grown in BL21 *E. coli* and induced to express by addition of IPTG. Samples were taken prior to induction (lane 1) and at 1 hour intervals up to 4 hours post induction (Lanes 2-5) and run on 15% SDS-PAGE gels. **B.** Semenogelin fragments and SV-IV were grown in BL21 *E. coli* and induced to express by addition of IPTG. Cells were harvested by centrifugation and sonicated. Soluble and insoluble fractions were dissolved in SDS sample buffer and analysed on a 15% SDS-PAGE gel.

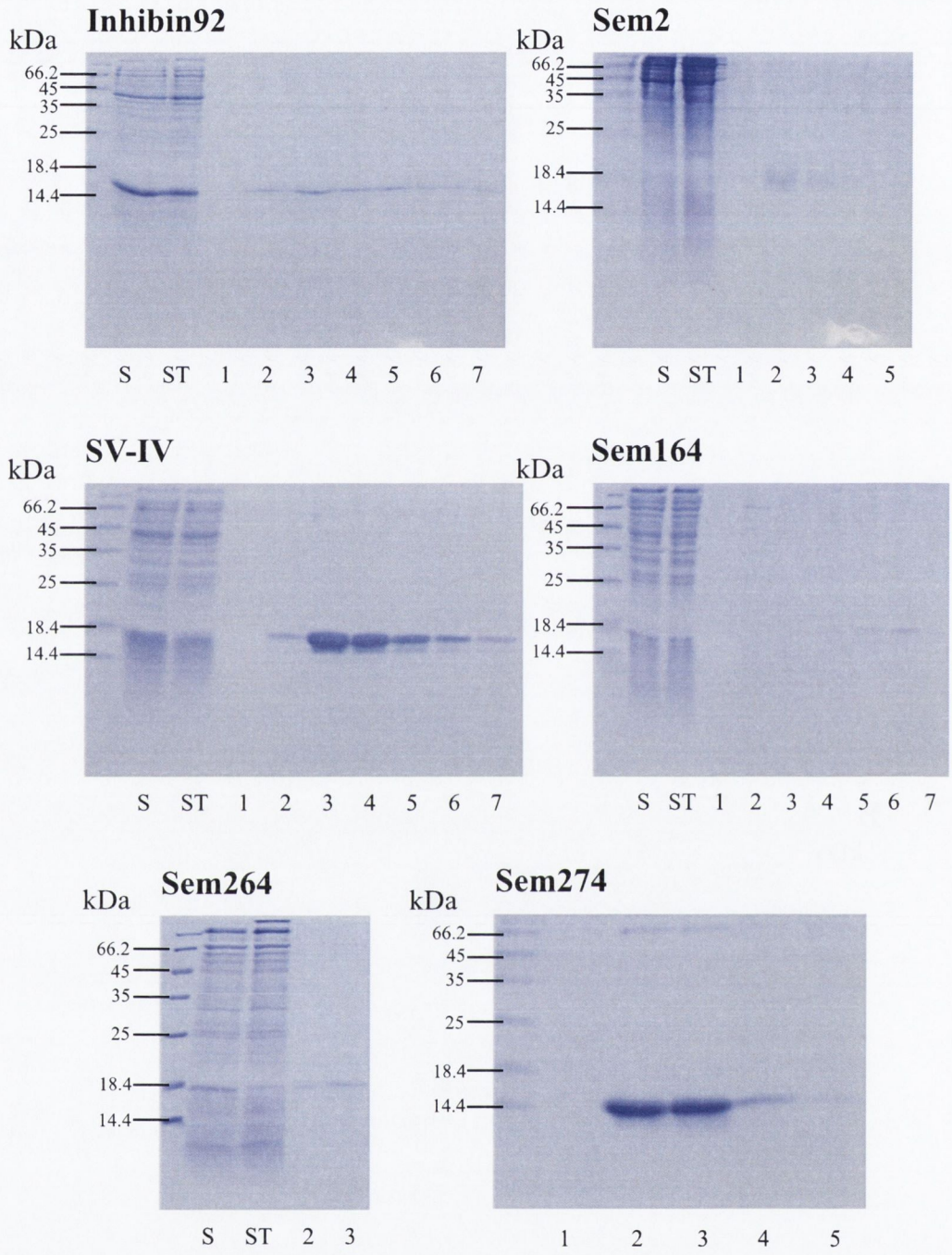


Figure 5.4.4.3 Affinity purification of Recombinant Semenogelin Fragments and SV-IV. Semenogelin fragments and SV-IV cloned into pET28b were grown in LB broth (1L) and expression inducedwith IPTG. Cells were harvested, sonicated and centrifuged and purified on a 1ml Streptactin column using desthiobiotin to elute bound protein and 1ml fractions collected. Legend: S = Supernatant of sonicated *E. coli* after centrifugation. ST = Material that did not bind streptactin column. Numbers indicate fractions collected from columns after elution with desthiobiotin.

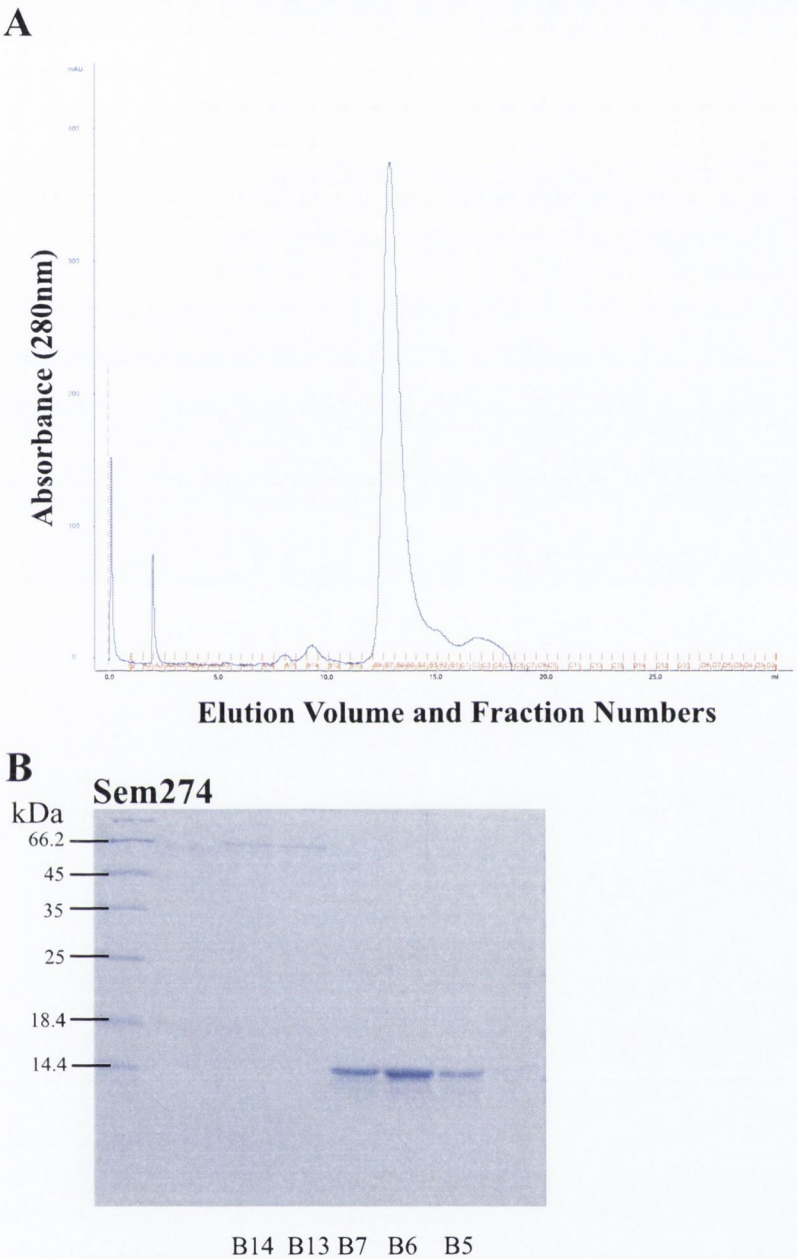


Figure 5.4.4.4 Further Purification of Sem274 by Size Exclusion Chromatography. Sem274 was purified as previously described by affinity purification on streptactin beads. The streptactin column fractions positive for Sem274 were concentrated and applied to a Sephadex G50 column equilibrated in PBS. Fractions (0.5ml) were collected and run on a 15% SDS-PAGE gel for analysis.

5.4.5 Effects of SV-IV and Sem274 on PBMC Proliferation and Activation.

PBMCs were treated with anti-CD3 antibody and IL-2 in the presence or absence of SV-IV as described in section 2.18. Cell numbers were counted by the trypan dye exclusion method as described in section 2.8.1 at 3 and 5 days incubation. At the end of the 5 day incubation cells were stained with anti-CD3-PerCP and anti-CD8-PeCy7 and analysed by flow cytometry as described in section 2.18.

SV-IV and Sem274 prevented PBMC expansion on stimulation with anti-CD3 and IL-2 (Figure 5.4.5.1). Activation of the PBMCs as measured by $CD8^+$ T-cell expansion was also significantly reduced (Figure 5.4.5.2).

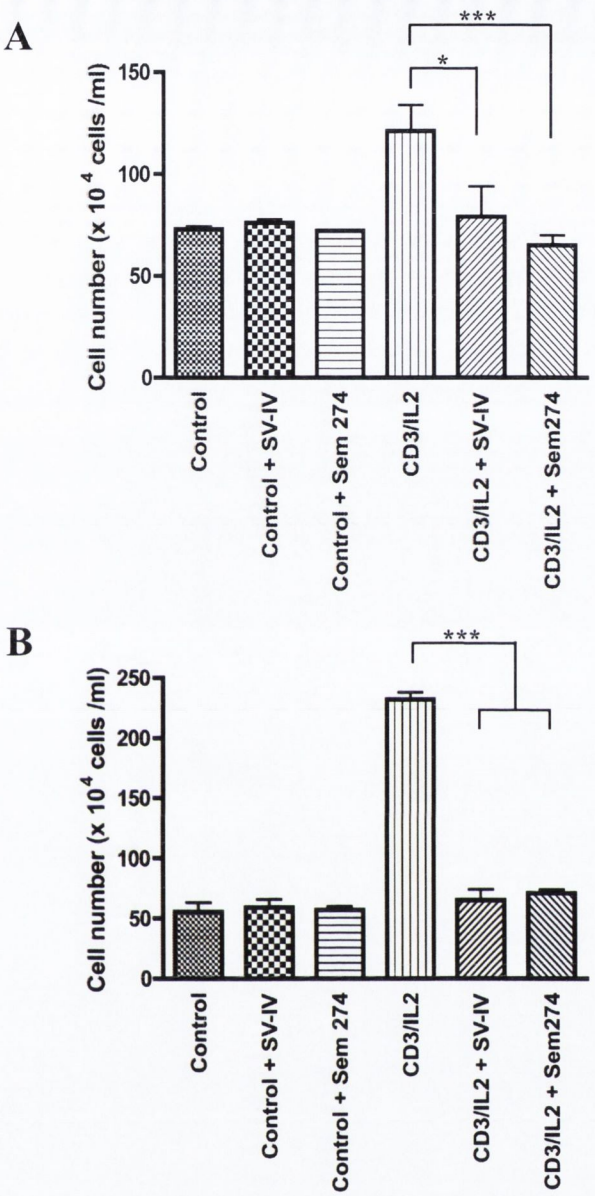


Figure 5.4.5.1 Effects by Sem274 and SV-IV on T-cell Proliferation Induced by anti-CD3 and IL2 Stimulation. PBMCs (1 x 10^6/ml) were either left untreated or treated with 10µg/ml SV-IV or Sem 274 peptides for 2 hours and then divided into either unstimulated or simulated with anti-CD3 antibody and IL2 treatments. Cells were counted at varying intervals by the trypan blue exclusion method to assess proliferative response. **A.** Cell numbers 3 days post induction of proliferation. **B.** Cell numbers 5 days post induction of proliferation. Results shown are the mean of 2 experiments +/- SD. Student t-test *= $p < 0.01$ ***= $p<0.001$.

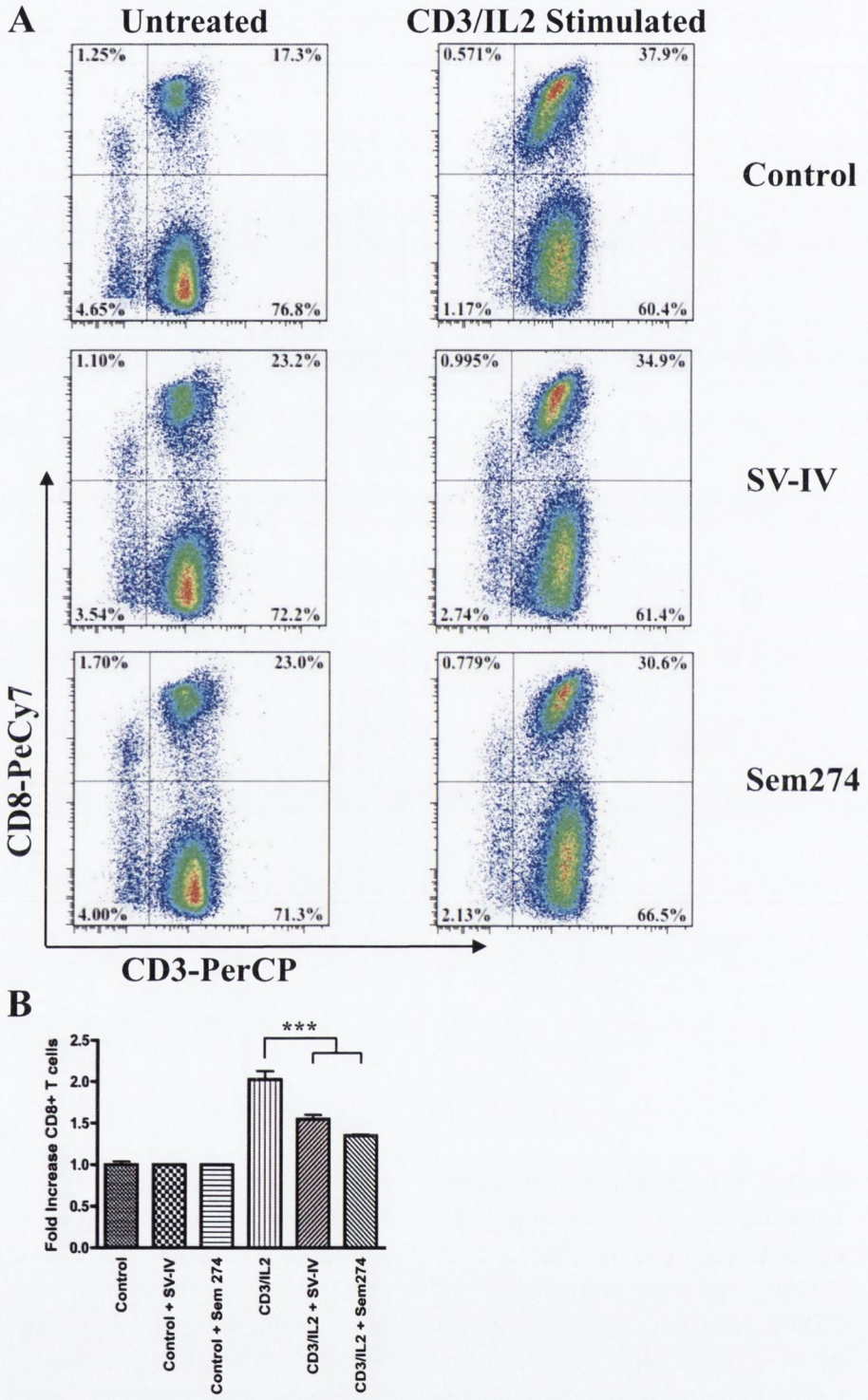


Figure 5.4.5.2 Effects of SV-IV and Sem274 on CD8$^+$ T cell expansion. PBMC pre-treated with either SV-IV or Sem274 were treated with anti-CD3 antibody followed by addition of IL2 to induce T cell expansion. Cells were stained with anti-CD3-PerCP and anti-CD8-PeCy7 antibodies and analysed by flow cytometry. **A.** Representative dot plot analysis of CD3$^+$ CD8$^+$ T cells from one experiment. **B.** Graphic representation of fold increase of CD8+ T-cells normalised to corresponding control. Results shown are the mean of 2 experiments +/- SD. Student t-test ***= $p<0.001$.

5.5 Discussion.

The initial experiments to determine the suppression of lymphocyte proliferation by seminal plasma in patients with or without ASAs were roughly in line with those described by Imade et al., (1999). However, some differences are noted, firstly Imade et al., (1999) showed no difference in inhibition of lymphocyte proliferation between ASA and normozoospermic patients in the absence of hydroxylamine whereas it is indicated here (Figure 5.2.1A and B) that ASA positive patients demonstrated a lower suppression, 27.33-34.29% of control than normozoospermic patients, 16.19-17.14% of control. This result though can be explained by the result of Maayan et al (1995) who indicated that patients positive for ASAs have levels of spermine and basal aldehydes within the range of normozoospermic patients but that further oxidation of the spermine by exogenously added amine oxidase is severely compromised when compared to normal seminal plasma. Therefore, it can be assumed that on addition to culture medium containing polyamine oxidase from FCS that a lower proportion of the spermine present in the seminal plasma of ASA patients is available to the enzyme and thus lower levels of cytotoxic catabolites are formed when compared to normozoospermic patients seminal plasma. In the presence of hydroxylamine the levels of suppression of ASA and normozoospermic patients seminal plasma showed even greater divergence with normal seminal plasma giving an inhibition of proliferation of 28.88-29.15% of control in the normozoospermic patients to 55.42-60.66% of control in the ASA positive patients. This agrees well with the figure indicated by Imade et al., (1999) who showed a reduction in relative activity of around 35-40% for ASA positive seminal plasma versus control with hydroxylamine present.

Fractionation of seminal plasma into three by molecular weight (<3kDa, 3-100kDa and >100kDa) indicated that the greatest suppression of mitogen stimulation of PBMCs occurred in the low molecular weight fraction and was hydroxylamine insensitive. The amounts of both the 3-100kDa and >100kDa fraction required to give suppression levels similar to that of the <3kDa fraction were in excess of 40x greater for the >100kDa fraction and could not be reached at any level by the 3-100kDa fraction (Figures 5.2.2 and 5.2.3).

The >100kDa fraction is likely to contain the trilamellar or multilamellar extracellular organelle, the prostasome. These organelles have previously described as having lymphosuppressive qualities (Ronquist and Brody, 1985) and will be discussed further in chapter 6. The 3-100kDa fraction may have multiple suppressive molecules, most likely cytokines, namely TGFβ and IL-10 among others (see section 5.1). This fraction has the least suppression of the three fractions tested and its importance in the reproductive process may be limited to suppression of the immune response along the male reproductive tract as there is no indication that these Th2 response cytokines can be transferred across the cervical mucus with sperm into the female reproductive tract.

The <3kDa fraction may also contain a number of previously described lymphosuppressive factors. These include the prostaglandins (mainly of the E series), zinc complexes (James and Hargreave, 1984) and the polyamines. Imade et al (1999) showed that dialysis of normal seminal plasma using a 3.5kDa cut-off dialysis bag reduced hydroxylamine insensitive suppressive activity by 50% so indicating that much of the activity is in the low molecular weight range.

Molecular weight fractionation of ASA positive seminal plasma using amicon molecular weight cut-off filters as described (section 2.10.2) showed that the lower lymphosuppressive activity seen in Figure 5.2.1 is attributable to a molecule of a low molecular weight (<3kDa fraction) (Figure 5.2.4). As hydroxylamine is present in this assay, the polyamines, spermine and spermidine, may be discounted as being a contributing factor to this loss of activity. The most likely candidates for the reduced activity in this fraction are therefore the E-series prostaglandins and Zn-complexes.

Further fractionation of the <3kDa fraction by phase separation yielded an aqueous phase with a continuous spectrum indicating the presence of protein in this fraction. The organic phase of the <3kDa fraction produced a more difficult spectrum to interpret with a peak just above 200nm and a broad peak from 230-290nm, this is likely to represent a number of differing prostaglandin species as PGE2 has a known peak at 210nm with another minor peak at 270nm, while 19-OH-PGE2, PGE1 and PGF2 have numerous absorption maxima between 233 and 288nm (Anderson and Speroff, 1971). When inhibition of mitogen stimulation by each fraction was tested the majority of the activity could be found in the aqueous phase. The lower than expected activity in the

organic phase of the <3kDa fraction, expected to contain the prostaglandins, could be due to a number of factors. Firstly, (Quayle et al., 1989) suggested that some of the prostaglandins present in seminal plasma are bound within the protein fraction and therefore, in the system used here, are unlikely to pass through the 3kDa cut off filter, while it is also possible that the lipophilic prostaglandins do not pass readily through the <3kDa cut-off filter. Secondly, (Lieb and Karmali, 1985) suggests that PGE and 19-OH-PGE when assayed at concentrations approximately relating to 1% v/v seminal plasma, do not exert enough lymphosuppression to account for the low molecular weight fraction of seminal plasma.

Fractionation of the <3kDa fraction gave an active fraction of between 2 and 4kDa that corresponded well with previously results (Pitout and Jordaan, 1976, Untergasser et al., 2001). That the lymphosuppressive activity could be neutralized by digestion with pronase (Figure 5.2.5) indicated that the active molecule was a peptide. This conclusion was also drawn by Pitout and Jordaan, (1976), who showed that the active molecule they partially purified and named SLIF (Seminal leukocyte inhibitory factor), reacted with the chlorine gas-starch-iodine reagent and had a pI of between 7.6 and 8 thus indicating a peptide. The co-elution of the polyamines with this fraction could show that the active peptide is complexed with either spermine or spermidine. While pronase cleavage did not alter the elution profile of the polyamines it did remove all lymphosuppressive activity, leading to the conclusion that either the active peptide is not bound to polyamines or if it is complexed to the polyamines, then vital constituent parts of the peptide not bound to the polyamines are cleaved leading to loss of activity. Complexing of polyamines to peptides has received little attention in the literature though it has been shown that spermine and other polyamines can bind to synthetic peptides and promote or stabilize α-helical structures (Tabet et al., 1993). However, it is unlikely that the active peptide described here is complexed to the polyamines as Pitout and Jordaan, (1976) extracted their sample using *n*-butanol phase separation to remove the polyamines and had no loss of activity in the peptide fraction. Another explanation for the polyamines resolving on size exclusion chromatography with a molecular weight of 2-4kDa would be if they occurred as either an aggregate or polymer in seminal plasma, though there is no evidence as yet for this possibility.

Proteolysis with the more specific proteases, trypsin, and *S. aureus* endopeptidase both resulted, as with pronase, in the complete loss of activity of the <3kDa fraction further confirming the nature of the active component as peptide.

Pull down of the peptide with charged matrices showed that the peptide within the <3kDa fraction with lymphosuppressive activity is cationic, which agrees with Pitout and Jordaan, (1976) who showed the pI of the activity at between 7.6 and 8. The peptide activity could also be pulled down by nickel-sepharose (Figure 5.2.7.2) and binding to divalent metals demonstrated (Figure 5.2.7.3). The likely divalent metal that the peptide fraction binds to *in vivo* is zinc, which is found in high concentrations in seminal plasma (Vickram et al., 2013). Zinc has been shown to bind to a wide range of molecular weight molecules in seminal plasma (Kavanagh, 1983) and in particular, zinc binds to the semenogelins, the major clot forming protein of semen and regulate prostate specific antigen activity (Jonsson et al., 2006, Jonsson et al., 2005).

Testing of the peptide fraction for anti-proliferative activity in cell lines of immunological and non-immunological origin indicated that the active peptide only inhibited the growth of cell lines derived from immunological sources (Figures 5.3.1.1 and 5.3.1.2). Interestingly, the non-immunological cell lines, MCF7 and C6Glioma were not as susceptible to the cytotoxic effects of polyamine catabolites when the peptide fraction was used in the absence of hydroxylamine. Untergasser et al., (1999), however, showed anti-proliferative activity and cell death in prostate cell lines with a 2-4 kDa low molecular weight extract of seminal plasma from gel filtration. They also indicated that cell death in these prostate cancer cell lines was caspase 3 independent and that DNA strand breakage occurred. These results are very similar to those shown here, however, they made the assumption that the polyamines would not be eluting in this fraction and therefore did not do these experiments with hydroxylamine present. Therefore, it is possible that prostate cancer cell lines are more susceptible to the effects of the cytotoxic catabolites of the polyamines than the non-immunological cell lines tested here. Untergasser et al., (1999) also demonstrated a rise in cAMP within the prostate cells prior to cell death, which leads to the conclusion that their low molecular weight fraction may contain prostaglandin E2, known to give rise to increases in cAMP (Kelly et al., 1994), so it is difficult to ascertain whether it is the peptide fraction shown

in this study that acts on the prostate cell lines or a combination of polyamine and prostaglandin.

Morphological studies of the activity of the peptide fraction revealed the formation of vacuoles within the cytoplasm of each of the cell lines tested (Figures 5.3.1.3 and 5.3.1.4). These vacuoles resemble morphologically autophagosomes produced during autophagy, a form of cell death (Li and Vierstra, 2012). During autophagy, cellular components are sequestered into forming vesicles, termed phagophores, which eventually mature into complete double-membraned vesicles called autophagosomes. These vesicles traffic the cellular material to late endosomes / lysosomes, where they fuse to form an autolysosome (Mizushima and Kuma, 2008).

The reduction in IL-8 secretion in PBMCs again discounts the possibility of prostaglandin contamination as prostaglandins extracted from seminal plasma have been reported to increase IL-8 levels (Denison et al., 1999). The increase in IL-10 secretion is also important as it could contribute to a shift in IL-12/IL-10 ratios in the female reproductive tract and thus provide a state of immunotolerance *post coitus* and during implantation.

The finding that fragments of semenogelin 1 are present in the active fraction and that cleaved semenogelins are responsible for lymphosuppressive activity is not wholly surprising as several of the cleavage products have previously been described to have biological activity. Three sequences of semenogelin 1 have been shown to have α-inhibin-like activity (sem1 68-159 (Inhibin92), sem1 108-159 (Seminal Basic Protein) and sem1 108-138 (Inhibin31)) (Ramasharma and Li, 1986, Li et al., 1985, Ramasharma and Li, 1987). Also noted is an anti-microbial activity with Sem1 85-113, Sem1 85-130, Sem1 85-131 and Sem1 85-136 (Zhao et al., 2008). Semenogelin peptides have been shown previously to adhere to the sperm surface and are associated with the membrane bound protein eppin (Wang et al., 2005). The semenogelin 1 peptide associated with eppin is the fragment, amino acids 164-283 (not counting the semenogelin 1 signal sequence of 23 amino acids). This peptide includes the only cysteine residue in semenogelin 1 (C239), which if mutated completely negates binding to eppin (Mitra et al 2010). The binding of this peptide of semenogelin 1 to the sperm surface acts as an inhibitor of sperm motility (Mitra et al., 2010). Sperm motility is then

initiated by PSA cleavage of this peptide. Previously it was described in chapter 3 (Figure 3.2.1.10) that a wide range of differing molecular weight peptides derived from the semenogelins are present on the sperm surface, whether all of these are bound to eppin or in other ways to the sperm surface is unclear, though it is possible that some of these may be cross-linked to the sperm surface by transglutamination, as the semenogelins have been shown to be cross-linked by transglutaminase (Peter et al., 1998) and that SV-IV of rat can also be cross linked to the rat epididymal sperm surface by transglutaminase (Peluso et al., 1994) thereby reducing its immunogenicity *in vivo*.

Cleavage of semenogelin 1 with PSA can lead to two peptides previously described (Malm et al., 2000, Robert et al., 1997) that contain the two sequences detected by MS/MS in this study. They are amino acids 241-337 (Sem264) and 251-335 (Sem274) and were also detected by MS/MS from cleavage of semenogelin by PSA *in vitro* (Malm et al., 2000, Robert et al., 1997). The peptide with activity inthis study has an apparent molecular weight of about 2-4kDa by gel filtration. It is possible that these peptides are further processed by other proteases in seminal plasma such as kallikrein 14 or kallikrein 2 previously detected in seminal plasma (Charlesworth et al., 1999, Emami et al., 2008, Lovgren et al., 1999), with both enzymes having a role in seminal clot liquefaction.

The tryptic peptides of semenogelin 1 found in the active fraction from seminal plasma have a homology with the aforementioned SV-IV of rat. SV-IV is a seminal vesicle secreted protein that like semenogelin 1 in humans is part of the seminal coagulum. Rats and other rodents, however, produce multiple seminal vesicle clotting proteins of much lower molecular weight than the semenogelins found in primates. SV-IV has a molecular weight of 9,758 daltons and is cationic and has a number of biological activities. Firstly, it is immunosuppressive, inhibiting both the cell-mediated and humoral response (Romano-Carratelli et al., 1995) and this activity can be greatly enhanced by transglutamination (Porta et al., 1993). Secondly, SV-IV displays anti-inflammatory, anti-phagocytic and anti-chemotactic activities (Ferranti et al., 1997, Galdiero et al., 1989, Ialenti et al., 2001, Mancuso et al., 1996). Thirdly, SV-IV can inhibit both phospholipase A2 (PLA2) and platelet-activating factor (PAF) biosynthetic pathways (Persico et al., 1993, Camussi et al., 1990). Finally, SV-IV can accelerate the coagulation of human blood by specific inhibition of anti-thrombin III activation (Di

Micco et al., 2000, Di Micco et al., 1997, Di Micco et al., 1994). SV-IV and the semenogelins are members of the REST (rapidly evolving substrates for transglutaminase) gene family (Lundwall and Lazure, 1995). This gene family consist of highly conserved 5' and 3' non-coding sequences while the second exon which is undergoing rapid evolution and codes for the protein product that has little conservation but is nevertheless a transglutaminase substrate (Hagstrom et al., 1996). It is therefore likely that the conserved acyl-donor and acceptor in the semenogelin 1 peptide sequence indicated here will be acted on by transglutaminase forming either intra- or inter-molecular bonds, with possible attachment to the sperm using this method. SV-IV is not the only REST family member to be attached to sperm by transglutaminase, uteroglogin in rabbits has also been shown to attach by this method and also to reduce the immunogenicity of epididymal sperm (Mukherjee et al., 1983) as SV-IV does in rats.

Sem274 and SV-IV have been shown here to have the same effects of T-cell proliferation and activation of $CD8^+$ cells (Figures 5.4.5.1 and 5.4.5.2). It is therefore likely that Sem274 is the human homologue of SV-IV. It is yet to be resolved whether Sem274 has the same range of functions attributed to SV-IV. It is clear however, that seminal plasma plays an important role in priming the female reproductive tract for the presence of the allogenic sperm and for implantation. Seminal plasma can interact with the female immune system and modulate the interplay between sperm and female epithelia and secretions of the female tract by acting as a signal carrier (Rodriguez-Martinez et al., 2011). Indeed, boar seminal plasma contains two cationic peptides, Porcine Seminal Plasma Protein I (PSP-I) and PSP-II, which adhere to sperm and effect the cytokine balance between macrophage TNFα and mast cell IL-4 thus controlling the onset of neutrophil migration and so avoiding excessive accumulation of neutrophils and post-mating endometriosis and tissue damage (Assreuy et al., 2002, Assreuy et al., 2003). Seminal plasma has also been shown to protect human sperm from capture by dendritic cells within the uterus (Rennemeier et al., 2011) and to increase the number of regulatory T-cells (Treg) cells in the uterus to prime for implantation (Robertson et al., 2013). The factors in human seminal plasma responsible for these activities are yet to be elucidated and Sem274 would be a strong candidate.

In conclusion, a large proportion of the lymphosuppressive effect of seminal plasma is present in the low molecular weight fraction and peptide in nature, and is

derived from the major seminal clot forming protein, semenogelin 1. In ASA patients much of this activity is lost and as semenogelin 1 has previously been shown to be a target for ASAs, then it can be hypothesized that the ASAs bind to the active semenogelin peptide reducing or eliminating activity. Another, less likely scenario is that incomplete cleavage of the semenogelin by PSA and therefore lower levels of active peptide leads to lower lymphosuppression in the seminal plasma and the formation of ASAs within the male reproductive tract. The lowered level of lymphosuppression as described here also lends credence to the results of (Witkin and Chaudhry, 1989) showing that female partners of men with ASAs are statistically more likely to have ASAs in their serum, probably due to either lowered semenogelin peptide in the seminal plasma and/or attached onto the sperm surface or otherwise the suppressive peptide is being silenced by attachment of ASAs from the male reproductive tract allowing the female to respond immunologically to the highly antigenic sperm either within the cervical mucus or *in utero*.

5.6 Summary.

Seminal plasma from ASA patients was confirmed to have a lower immunosuppressive activity compared to normozoospermic patients. Furthermore, the reduction in activity was linked to the low molecular weight fraction of seminal plasma. The active component was shown to be a peptide, which was cationic in nature and had the ability to bind divalent metal ions. Fractionation of normozoospermic seminal plasma indicated that this activity was due to a proteolytic breakdown product of semenogelin 1 which had sequence similarities with SV-IV, a previously described rodent seminal protein, which also has immunosuppressive activity.

CHAPTER 6

Prostasomal Inhibition of Mitogen Induced Proliferation of PBMCs – a Role for the Prostasome Bound Zn^{++}-Dependent ATPase

6.1 Introduction

6.1.1 Prostasome Structure and Secretion

Seminal plasma contains extracellular organelles termed prostasomes, which are trilaminar or multilaminar vesicles of 40-500nm in diameter, and having a mean diameter of 150nm (Brody et al., 1983). Prostasomes are secreted by the acinar epithelial cells of the prostate gland and contain large amounts of cholesterol, shingomyelin and glycerophospholipid (Arvidson et al., 1989). The ratio of cholesterol to phospholipids is unusually high at 2.0 when compared to other plasma membranes. The polyunsaturated phosphatidylcholines, which are common in the sperm membrane, are rare in the prostasome membrane (Ronquist and Brody, 1985). Prostasomes are believed to be delivered into the glandular lumen by tranlocation of the entire vesicle from the cell interior to the acinar lumen through the plasma membrane. This event has been designated diacytosis (Ronquist and Brody, 1985).

6.1.2 Enzymes of the Prostasomal Membrane

6.1.2.1 γ-Glutamyltransferase

Human seminal plasma contains 200-500 times the γ-Glutamyltransferase activity of human serum (Rosalki and Rowe, 1973) with the prostate being the major source (Verhoeven and Steeno, 1979). The majority of this activity is associated with the prostasome membrane (Lilja and Weiber, 1983).

6.1.2.2 Protein Kinases

Prostasomes contain at least two protein kinases that have been shown to phosphorylate serine and threonine residues of endogenous acceptor proteins in the prostasomal membrane (Stegmayr et al., 1982) the function of which is unknown. Interestingly, prostasome interactions with sperm decrease tyrosine phosphorylation events associated with capacitation (Bechoua et al., 2011, Pons-Rejraji et al., 2011).

6.1.2.3 ATPases

Prostasomes contain two separate ATPases, a calcium and magnesium dependent ATPase which also requires calmodulin and has an apparent K_m of 0.82mM (Ronquist, 1987). The other ATPase is zinc dependent and has an apparent K_m of 0.43mM (Ronquist, 1988) and can be inhibited with vanadate. Latterly, nonstaurated, long-chain fatty acids were demonstrated to inhibit the zinc dependent ATPase (Ronquist and Frithz, 1992). Though the function of prostasome ATPase is not yet elucidated, vectorial transport of calcium and/or zinc across the membrane may be important as both molecules have been revealed to be enriched within the prostasome matrix compared with the surrounding seminal plasma (Ronquist, 1987, Ronquist and Brody, 1985) (see Figure 6.1.1).

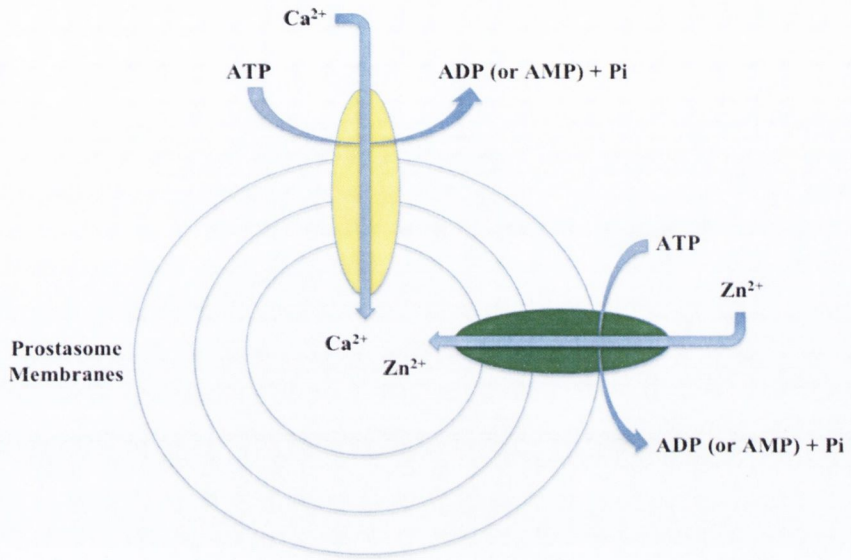


Figure 6.1.1 Schematic Representation of the Current Understanding of ATPase Function on the Prostasome Surface. The Zinc-Dependent ATPase is marked in green and the Calcium/Magnesium ATPase in yellow. Vectorial transport of zinc and calcium from the surrounding milieu using ATP hydrolysis to either ADP or AMP has been demonstrated (Ronquist, 1987, Ronquist and Brody, 1985).

6.1.2.4 Aminopeptidase (CD13)

Aminopeptidase (CD13) is a 150kDa zinc-dependent proteolytic enzyme often used as a marker of prostasomes. (Laurell et al., 1982).

6.1.2.5 Dipeptidyl Peptidase IV (CD26)

Dipeptidyl Peptidase IV is a membrane bound protease which removes dipeptides from peptide chains where the penultimate amino acid is a proline (Yaron and Naider, 1993). It is found specifically in the reproductive tract in prostatic secretions (Wilson et al., 1998) and was first identified on the prostasome by use of a monoclonal antibody (Schrimpf et al., 1999). Dipeptidyl Peptidase IV is involved in the activation of T-cells, along with the regulation of cytokine production and cell

proliferation (Fleischer, 1994, Yaron and Naider, 1993). The Tat (trans-activating) protein of HIV binds to and inhibits Dipeptidyl Peptidase IV while the envelope protein gp120 of HIV has also been shown to effect Dipeptidyl Peptidase IV activity (Subramanyam et al., 1993, Valenzuela et al., 1997). Therefore, prostasomes may bind to and aid transmission of HIV.

6.1.2.6 Neutral Endopeptidase (Enkephalinase) (CD10)

The neutral endopeptidase activity of the prostasome was first identified by mass fingerprint analysis (Renneberg et al., 2001) as the synaptic peptidase, enkephalinase, involved in terminating the actions of the opioid peptides, the enkephalins (Matsas et al., 1983). Its potential substrates include, the tachykinins, the natriuretic peptides, bombesin-like peptides and chemotactic peptides. Its function on the prostasome surface though is yet to be elucidated.

6.1.3 Sperm-Prostasome Interactions

Prostasomes can adhere to and fuse with sperm (Carlini et al., 1997, Matsas et al., 1983, Minelli et al., 1999, Ronquist et al., 1990), the result of which stabilizes the sperm membrane by addition of cholesterol, sphingomyelin and glycerophospholipid thus preventing premature acrosome reaction (Carlini et al., 1997). This fusion has been shown to be dependent on an acidic pH and one or more proteins on the sperm or prostasome surface (Arienti et al., 1997). It is possible that the acidic pH of the vagina overcomes the buffering capacity of seminal fluid allowing fusion to occur. Another fuction of prostasome-sperm fusion may be the transfer of proteins from the prostasome to the sperm, such as CD13 and CD26 previously described, as well as other complement inhibitors (see section 6.1.5). Protein-protein interactions may also play a role in fusion with CD26 and *ecto*-adenosine deaminase on the prostasome and adenosine receptors on the sperm being muted to form a complex (Minelli et al., 1999).

More recently, prostasomes have been shown to contain human chromosomal DNA (Ronquist et al., 2009) and to transfer the DNA to sperm on fusion (Ronquist et al., 2011). Prostasomes are heterogeneous in size as previously described and all sizes

contain chromosomal DNA. Therefore, prostasomes can be affiliated to one DNA-containing exosome family (Ronquist et al., 2012). The function of this DNA and its relevance to fertility are yet to be understood.

6.1.4 Prostasome Influence on Sperm Motility

Addition of prostasomes to washed sperm, increases the proportion of forward motile sperm and sperm have a higher amplitude of lateral head displacement (Fabiani et al., 1994, Fabiani et al., 1995, Stegmayr and Ronquist, 1982). This influence can be attributed to the transfer by prostasomes of progesterone receptors, cyclic adenosine diphosphoribose (CADPR)-synthesising enzymes, ryanodine receptors and other calcium signaling tools to the sperm neck region (Park et al., 2011, Ren, 2011). In the female reproductive tract progesterone induces sperm motility by calcium signaling in the neck region. Progesterone-induced sperm motility relies on cADPR-mediated calcium mobilization through the ryanodine receptors located on acidic calcium stores, followed by calcium entry through store-operated channels. Therefore, the sperm motility enhancement of progesterone is prostasome dependent.

6.1.5 Immunosuppressive Activity of Prostasomes

Prostasomes play a complementary role to the myriad of other immunosuppressive and immunomodulatory molecules of human seminal plasma (as described in detail in Chapter 5). Prostasomes can inhibit both mitogen induced proliferation of PBMCs and phagocytic cell activity (Kelly, 1995, Skibinski et al., 1992). As prostasomes can fuse with sperm as previously described, this immunosuppressant action may be carried throughout the female tract by sperm. The immunosuppressant activity is likely exerted by either phagocytosis of the prostasomes or by prostasome-leukocyte adhesion/fusion (Arienti et al., 1998).

Prostasomes can also contribute to local modulation of the complement system, through CD55 (decay-accelerating factor), CD46 (membrane cofactor protein) and CD59. CD55 and CD46 can inhibit the C3 convertase enzymes while CD59 is an inhibitor of the membrane attach complex (MAC). CD59 has been shown to be

transferred to sperm and may play a role in sperm evading complement attach *in utero* (Rooney et al., 1993).

6.1.6 Antisperm Antibodies and Prostasomes.

Polyclonal chicken anti-prostasome antibodies were shown to induce agglutination of sperm. However, ASA patients displaying anti-prostasome binding did not correlate with sperm agglutination (Allegrucci et al., 2001). How ASA may interfere with prostasome function and whether these ASA contribute to infertility is as yet unknown.

6.1.7 Aims of Chapter.

The aim of this chapter was to investigate the role of the Zn^{++}-dependent ATPase of prostasomes in the immunosuppressant activity of prostasomes.

6.2 Results.

6.2.1 The Inhibitory Effects of Human Prostasomes on Mitogen Induced Proliferation of Human PBMCs and Jukat Cell Proliferation.

Pure preparations of human prostasomes were purified by ultracentrifugation as in section 2.19.1 and added to mitogen stimulated PBMCs to measure reduction of proliferation. Though the degree of inhibition varied between experiments, inhibition was dose dependent and reached maximum levels at between 25% and 40% of prostasomal content (these percentages are given in equivalent prostasome concentrations as found in freshly ejaculated seminal plasma). Inhibition of mitogen stimulation of PBMCs was measured by [^{3}H] Thymidine incorporation (as described in section 2.19.2) with 40% prostasome content as found in seminal plasma leading to an approximately 69% reduction in [^{3}H] Thymidine compared to untreated control as shown in figure 6.2.1.

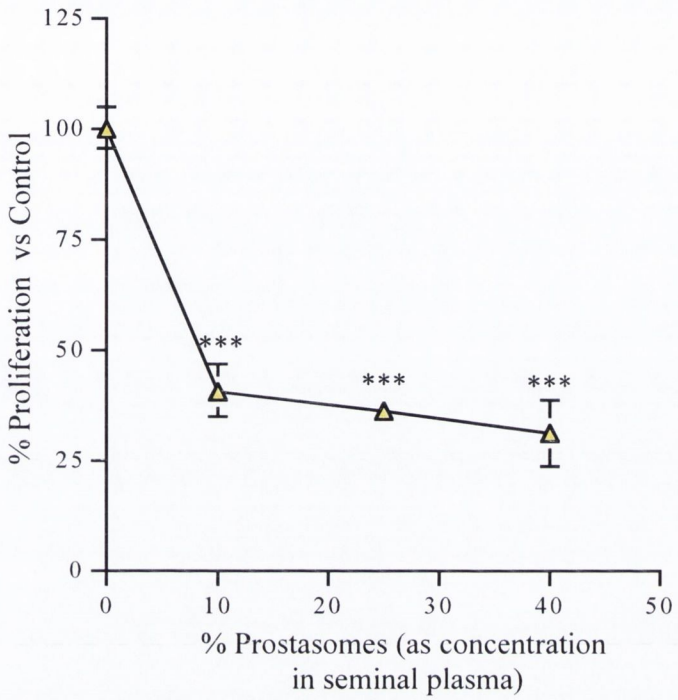


Fig. 6.2.1 The Effect of Increasing Concentrations of Human Prostasomes on the Mitogen Induced Proliferation of Human PBMCs. Percentage prostasomes added represent concentration of prostasomes in seminal plasma. Each point represents the mean of [^{3}H] Thymidine incorporation +/- SEM of triplicate samples. Untreated PBMCs are set to 100% [^{3}H] Thymidine incorporation. Student t-tests were performed *** = $p<0.001$.

The effect of prostasomes on Jurkat proliferation was also measured (as in section 2.19.3). Prostasome content of 40% as found in seminal plasma led to a decrease of 68% in [^{3}H] Thymidine incorporation in proliferating Jurkats (Fig 6.2.2), almost identical to the suppression of mitogen induced proliferation of PBMCs at the same prostasomal content. However, at prostasomal concentration of 10% and 25% yielded reductions in proliferation of Jurkats of 28% and 41% respectively. This compared to reductions in proliferation of PBMCs of 60% and 64% at the same prostasomal concentrations.

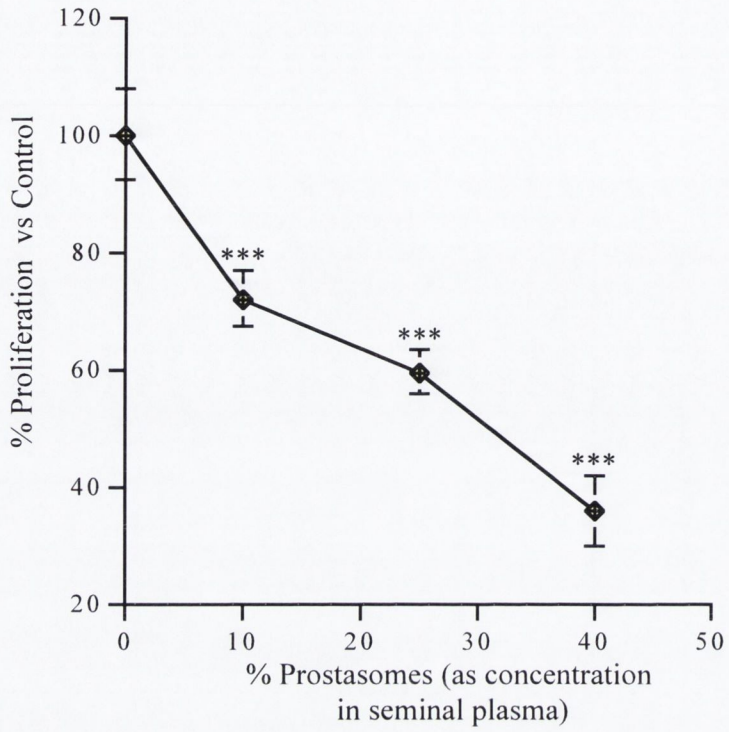


Fig. 6.2.2 The Effect of Increasing Concentrations of Human Prostasomes on the Proliferation of Jurkat Cells. Percentage prostasomes added represent concentration of prostasomes as in seminal plasma. Each point represents the mean of [^{3}H] Thymidine incorporation +/- SEM of triplicate samples. Untreated Jurkat cells are set as 100% [^{3}H] Thymidine incorporation. Student t-tests were performed *** = $p<0.001$.

6.2.2 The Effects of the Polyamine Oxidase Inhibitor, Hydroxylamine, on the Inhibition of Mitogen Induced Proliferation of PBMCs by Prostasomes.

As seminal plasma contains high levels of polyamines such as spermine and spermidine (Maayan et al., 1995) it was required to show that these polyamines were not adhering to or packaged within the prostasomes. To this end hydroxylamine was used to inhibit the polyamine oxidase of the foetal calf serum used in the culture medium (as in section 2.19.2). Figure 6.2.3 shows that inhibition of the polyamine oxidase had no effect on the prostasomal inhibition of mitogen induced proliferation of PBMCs thus indicating that polyamines played no role in this inhibition.

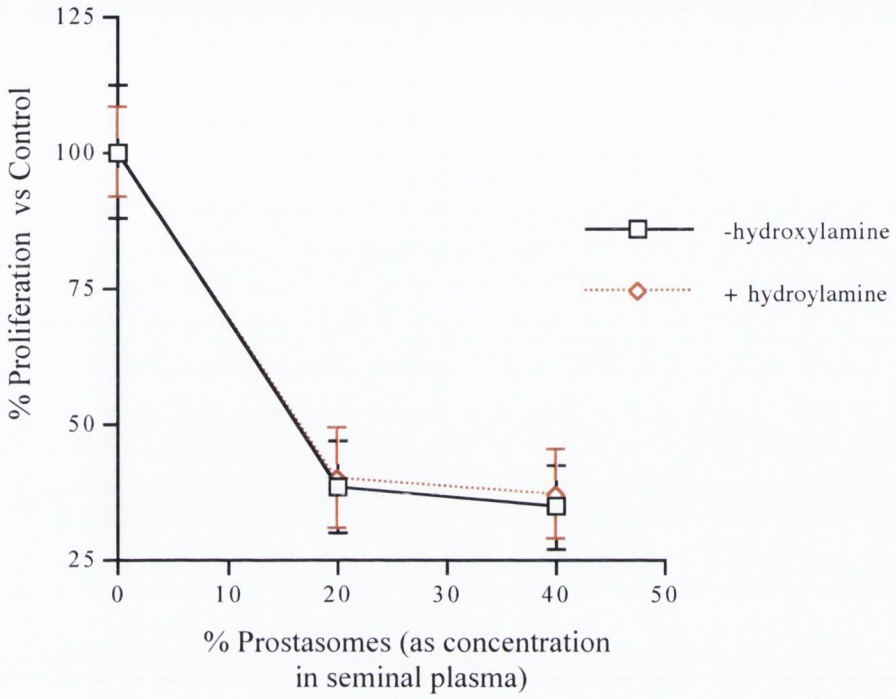


Fig 6.2.3 Comparison of the Effect of Prostasomes on the Mitogen Induced Proliferation of PBMCs in the Presence or Absence of Hydroxylamine. Each point represents the mean of [^{3}H] thymidine incorporation +/- SEM. No statistical difference was noted between the two curves.

6.3 Assay and Inhibition of the Zn^{++}-Dependent Prostasome ATPase

6.3.1 Initial Enzymatic Assay of the Zn^{++}-Dependent Prostasome ATPase

The method of ATPase enzymic assay was modified from (LeBel et al., 1978) as in section 2.19.4. The Zn^{++}-dependent ATPase of prostasomes displayed normal Michaelis-Menten kinetics giving a K_m value of 2.2 +/- 0.2 mM as shown in Fig 6.3.1. Prostasomes were kept constant throughout using 120μg of prostasomes as determined by the Markwell protein assay as described in section 2.20.

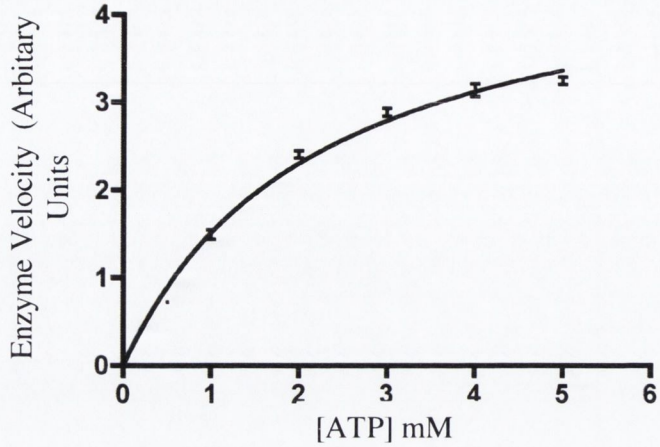


Figure 6.3.1 Michaelis-Menten Curve for Prostasomal Zn^{++}-dependent ATPase with Varying ATP Concentrations. K_m was evaluated at 2.2 +/- 0.2 mM using Prism 5. Inorganic phosphate release from ATP was measured as described in section 2.19.4 in a stopped enzyme assay. Absorbance was read at 700nm and used as enzyme velocity.

6.3.2 Inhibition of the Zn^{++}-Dependent Prostasome ATPase by DCCD.

An inhibitor of ATP hydrolysis by the mammalian ATP synthase, dicyclohexylcarbodiimide (DCCD) was tested on the prostasome Zn^{++}-dependent ATPase (as in section 2.19.5) and shown to be an excellent inhibitor of the Zn^{++}-dependent ATPase of prostasomes (Figure 6.3.2). DCCD (2μM) was shown to completely inhibit Zn^{++}-dependent ATPase activity in a range of prostasome concentrations from 150μg to 450μg total prostasome protein added.

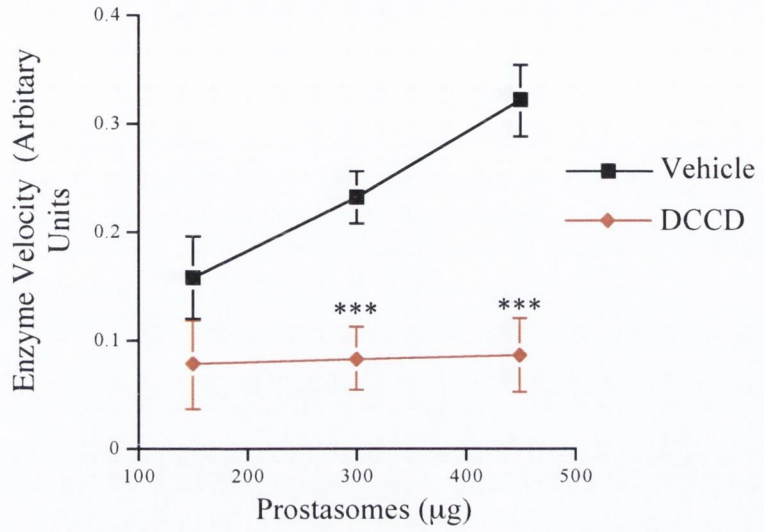


Fig 6.3.2 Inhibition of the Zn^{++}-Dependent ATPase of Prostasomes Using DCCD as inhibitor. Vehicle control used was a comparative amount of ethanol. All assays were performed with 2mM ATP. Inorganic phosphate release was measured as in section 2.19.4 and absorbance was read at 700nm and used as enzyme velocity. Each point is represents the mean of triplicates +/- SEM. Student t-tests were performed *** = $p<0.001$.

6.3.3 The role of the Zn^{++}-dependent prostasome ATPase in inhibition of mitogen induced proliferation of PBMCs.

To ascertain if the prostasome Zn^{++}-dependent ATPase has a role to play in the inhibition of mitogen stimulation of PBMCs by prostasomes, prostasomes were either incubated with 2μM DCCD or in vehicle control (Ethanol) (as in section 2.19.6). DCCD inhibited prostasomes had reduced anti-proliferative effects on PBMCs at each concentration of prostasomes added. At 10%, 25% and 40% prostasomes treated with DCCD, the level of inhibition of proliferation of PBMCs was 19%, 30% and 33% respectively, while vehicle control prostasomes at the same concentration inhibited PBMC proliferation by 46%, 57% and 68% respectively (Figure 6.3.3).

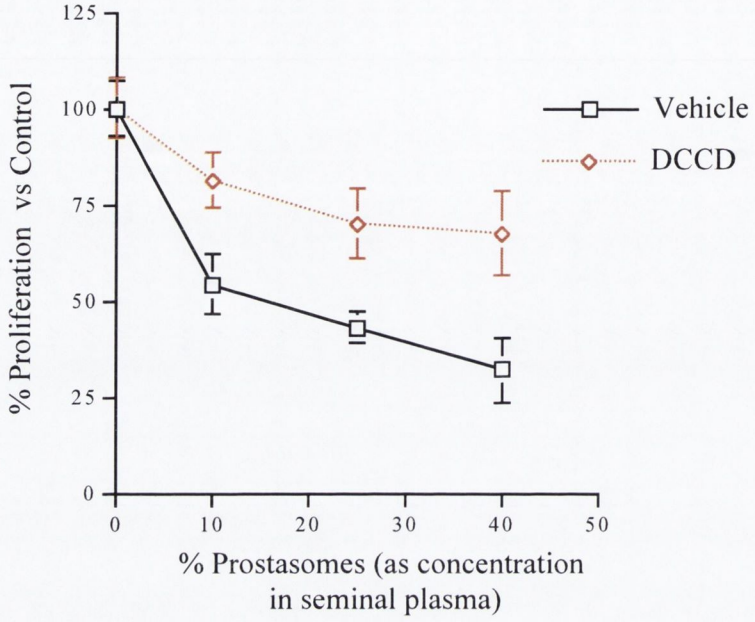


Figure 6.3.3 Effect of Pre-Treatment with DCCD of Prostasomes on the Inhibition of Mitogen Induced Proliferation of PBMCs. Each point represents the mean percentage uptake of [^{3}H] thymidine +/- SEM. Annova two-way analysis indicates a statistical difference of $p<0.001$.

As prostasomes have previously been described to fuse and/or be internalized by lymphocytes in order to suppress their proliferation (Arienti et al., 1998) a method to measure whether the inhibition of the Zn^{++}-dependent ATPase acted at the level of fusion was devised. Cholesterol can be transferred into biological membranes using cyclodextrin as a carrier and this method was used (as described in section 2.19.7.1) to transfer [^{14}C] cholesterol into the membrane of the prostasome. Figure 6.3.4 indicates that uptake of cholesterol in this system by prostasomes is linear for the concentrations of prostasomes used.

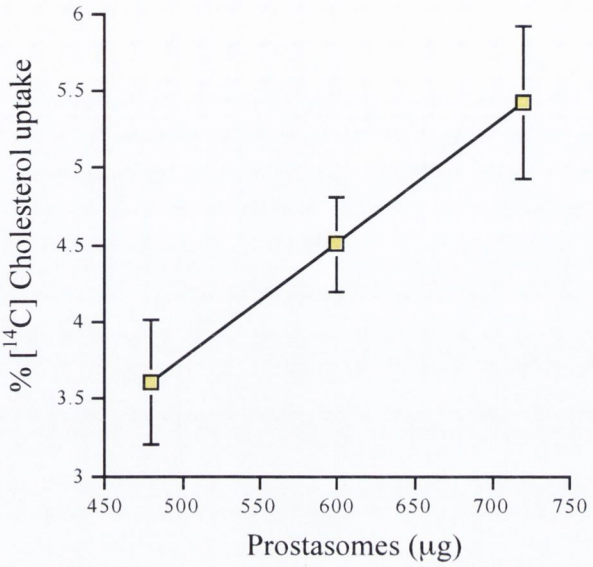


Fig 6.3.4 Uptake of [^{14}C] Cholesterol-Cyclodextrin Complex by Increasing Amounts of Prostasomes. Each point represents the mean of triplicates +/- SEM.

However, when prostasomes that were treated with DCCD were labeled with cholesterol (Figure 6.3.5), it was noted that uptake of cholesterol in the DCCD treated prostasomes was higher than vehicle control prostasomes. This increase was consistent over a number of experiments with uptake almost doubling in each case from approximately 3.57% for vehicle control to 6.75% for DCCD treated prostasomes.

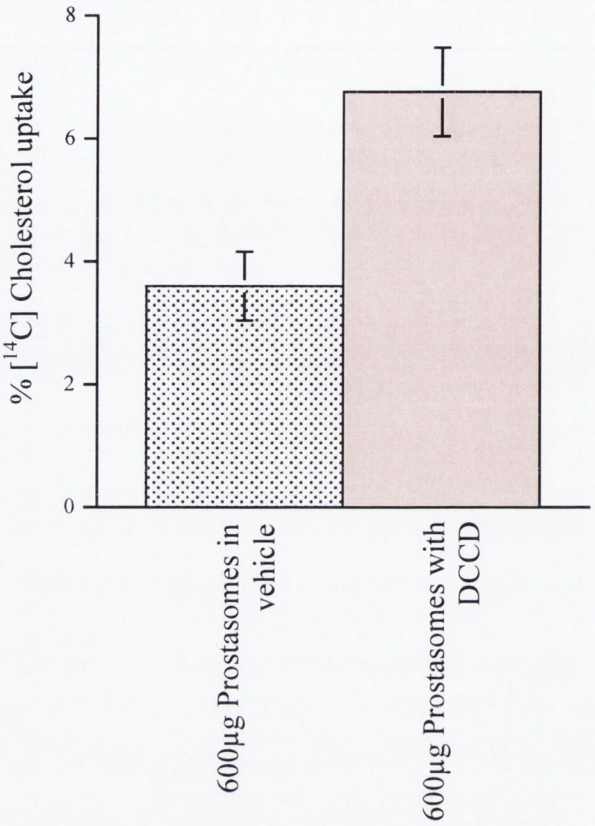


Figure 6.3.5 Uptake of [^{14}C] Cholesterol by Prostasomes Pre-Incubated with either Vehicle or DCCD. Each bar represents the mean of % [^{14}C] cholesterol uptake +/- SEM.

To measure internalization or fusion of the prosatasomes with lymphocytes, a model system using Jurkats was used (as described in section 2.19.7.2). There was significant levels of uptake of vehicle-treated prostasomes by Jurkat cells (82% of total incubated material) while prostasomes treated with DCCD prior to incubation with Jurkats showed low amounts of uptake (12.45% of total incubated prostasomes) (Figure 6.3.6). As DCCD treated prostasomes take up more of the labelled cholesterol the Jurkat cells were treated with equal amounts of prostasomes with and without DCCD and the uptake by Jurkat cells of the radiolabel normalised to total radiolabel added.

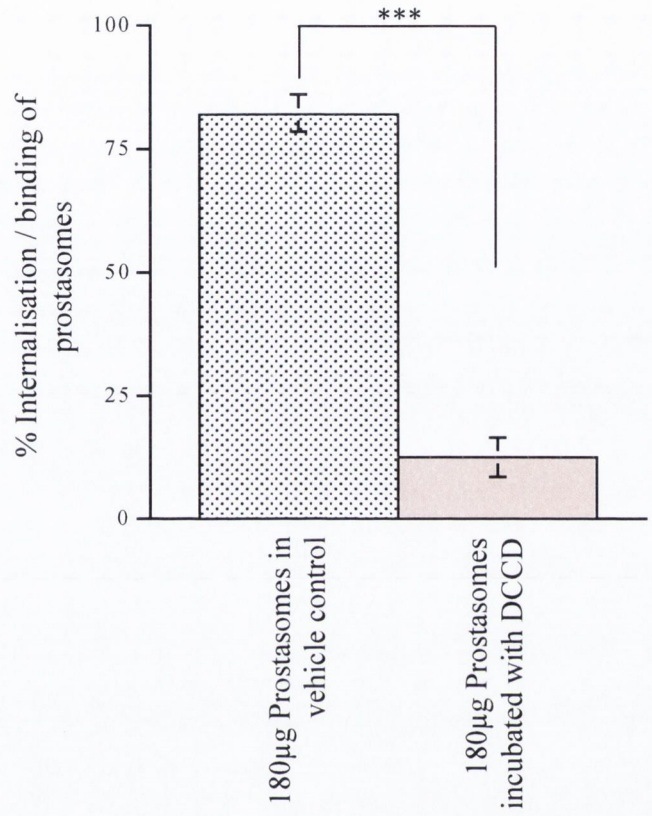


Figure 6.3.6 The Requirement for Functional Zn^{++}-dependent ATPase Activity for the Internalisation and/or Binding of Prostasomes to Jurkat Cells. Each bar represents the mean of dpm counts corrected for differing [^{14}C] Cholesterol uptake between control and DCCD incubated prostasomes in triplicate +/- SEM. A student t-test was performed *** = $p<0.001$.

6.4 Discussion

6.4.1 Inhibition of PBMC and Jurkat Proliferation by Prostasomes

Prostasomes have previously been shown to inhibit mitogen induced PBMC proliferation (Kelly, 1995). The results presented here are in agreement that prostasomes can inhibit this proliferation. It is also demonstrated here that prostasomes have the ability to reduce the proliferation of the Jurkat cells, a transformed T-cell cell line. This indicates that the prostasomes do not act by preventing activation of the PBMCs but rather may affect cell cycle of the leukocytes. The addition of hydroxylamine to the media did not affect the potency of the prostasomes at inhibiting PBMC proliferation indicating that spermine and other polyamines were not contaminating the prostasome preparation; polyamines could potentially lead to spurious anti-proliferative and cytoxic effects through the formation of their toxic oxidiation products as described previously in Chapter 5. This does not preclude, however, that large amounts of polyamines could be packaged within the prostasomes and delivered through fusion with the leucocytes to their cytoplasm. Polyamines such as spermine and spermidine also play a role in T-cell division and activation (Fillingame et al., 1975).

6.4.2 Prostasome Zn^{++}-Dependent ATPase Activity and Inhibition.

The Zn^{++}-dependent ATPase of prostasomes has been characterized previously (K_m of 0.43mM (Ronquist, 1988)) but the K_m reported here of 2.2 +/- 0.2 mM which is significantly higher. The other ATPase on the prostasome surface, the calcium and magnesium dependent ATPase, may have some residual activity and led to the higher reading noted here. DCCD was shown to inhibit the Zn^{++}-dependent ATPase fully.

6.4.3 Role of the Zn^{++}-Dependent ATPase in Prostasomal Inhibition of PBMC and Jurkat Proliferation.

Inhibition of the Zn^{++}-dependent ATPase by DCCD reduced the ability of prostasomes to inhibit mitogen induced proliferation of PBMCs and also inhibited

prostasome binding to Jurkat cells. This indicates that the previously reported binding/fusion of prostasomes (Arienti et al., 1998) to lymphocytes is required for prostasomes to exert their immunosuppressive activity. It also points toward an active, energy consuming event, during the association of prostasome and leukocyte and not a phagocytic event as reported by Skibinski et al., 1992. Recent evidence suggests prostasomes have all the machinery to produce ATP through anaerobic glycolysis from either glucose or fructose and for net ATP production to occur (Ronquist et al., 2013a, Ronquist et al., 2013b). The results presented here indicate that to fuse to leukocytes, prostasomes require an active ATPase enzyme and the production of anaerobically produced ATP might be the fuel required for this process. It would be interesting to show whether the inhibition of this glycolytic pathway could also reduce prostasome immunosuppression or leukocyte binding.

Recent advances in the understanding of immune regulation has indicated that ectoenzymes (an enzyme situated on the outer surface of a cells membrane or in the extracellular space) play important roles in the activation or deactivation of an immune response. CD39 (ectonucleoside triphosphate diphosphohydrolase 1, E-NTPDase 1) that converts ATP to AMP and then CD73 (ecto-5'-nucleosidase, Ecto5'NTase), which converts AMP to adenosine, contribute to down regulation of cytokine release. ATP release occurs in response to infection or other pro-inflammatory stimuli and is either by cell lyses or by non-lytic mechanisms including (i) exocytosis of ATP-containing vesicles, (ii) through nucleotide permeability channels (connexin/pannexin hemichannels, maxi-anion channels, volume-regulated anion channels or $P2X_7$ receptor channels), or (iii) via transport vesicles that deliver proteins to the cell membrane (Antonioli et al., 2013). The extracellular ATP levels can reach between 1.5 and 2μM (Kouzaki et al., 2011) and can up-regulate IL-1β, IL-18, IL-6 and TNFα and a number of other pro-inflammatory cytokines by macrophages and other immune cells (Levesque et al., 2010, Hasko et al., 2011, Petrovic-Djergovic et al., 2012). In the late stages of the response the expression of both CD39 and CD73 are up-regulated and lead to reduction of the ATP halo around the cell and the increase of adenosine. Extracellular adenosine, through interaction with specific receptors can then further reduce activation (Antonioli et al., 2013).

On mating, the female is exposed to non-self antigens (the semen) and presumably this would lead to an ATP release as a danger signal and thus lead to up-regulation of the adjacent immune system. However, this would be detrimental to fertility and it is possible that the prostasome ATPase system is designed to interfere with up-regulation of immune cells through disturbance of the ATP/adenosine ratio in the extracellular space either by hydrolysis of ATP in situ on the prostasomal surface or later after fusion with the immune cell. Both scenarios are possible and need further investigation.

6.5 Summary.

The results presented here indicated that prostasomes can inhibit the mitogen-induced proliferation of PBMCs as well as inhibiting the proliferation of the Jurkat cell line. It was also demonstrated that the inhibition of the Zn^{++}-dependent ATPase on the prostasome could reduce the anti-proliferative effects of prostasomes as well as reduce their ability to fuse/bind to lymphocytes.

CHAPTER 7

General Discussion and Future Work

7.1 Antisperm Antibodies as a Cause of Male Infertility

Antisperm antibodies (ASA) are one of the main causes of infertility, affecting about 1 in 10 men reporting for infertility treatment. It is only on the last 10 years that large numbers of the antigens on the sperm surface that elicit ASA responses are being identified (Francavilla et al., 2007). Significantly, many of the studies do not differentiate between internal and external antigens, nor do they attempt to examine the function of these antigens, or how ASA to these antigens affect fertility. Only very recently have research efforts addressed which sperm-egg binding proteins are antigens for ASA (Petit et al., 2013). A range of other sperm membrane proteins are awaiting characterisation that have vital functions in sperm motility and survival and which are targets for ASA.

Therefore, further research is required to identify which of these antigens occur on the sperm surface and their role in sperm function and fertility. As more of these antigens are identified it may be possible to produce clinical tests, such as ELISAs, that will identify antibody reactive sperm antigens for each individual patient, and the best course of treatment they should undergo. Therefore, the work carried out as part of this thesis in identifying antigenic targets for ASA and their functions is a vital first step in this direction.

Alternatively, as our understanding of these antigens increases it may be possible to produce contraceptive vaccines. To produce a contraceptive vaccine would require the antigen or group of antigens to be expressed solely on the sperm surface and not in any other organ and to be vital to sperm function. Methodology could include induction of sperm agglutination, prevention of sperm progression through cervical mucus,

priming the sperm for immune capture *in utero* or interrupting sperm-egg binding. The mCRISP1 protein identified here as a target for ASA, fulfills these general requirements, as it is restricted to the male reproductive tract and anti-CRISP1 antibodies can inhibit ova fertilization, thus making it a suitable candidate for inclusion in a contraceptive vaccine. Also, the finding that hCRISP is cleaved in seminal plasma, presumably at the hinge region, raises the question of whether hCRISP is involved in innate immunity. The high proportion of cysteine residues in the C-terminal domain of hCRISP bares some resemblance to the anti-microbial defensin proteins, which are also rich in cysteines. Purification and cleavage of CRISP proteins followed by anti-microbial studies should answer this question. This hypothesis is backed by the fact that hCRISP3 is not confined to the reproductive tract but is also found in neutrophil granules (Udby et al., 2002) and that plant PR-1 proteins are produced in response to host challenge (Li et al., 2011).

The elucidation here of semenogelin fragments as immunosuppressant molecules could also lead to contraceptive vaccine development. Natrually occuring ASA to semenogelin has been demonstrated here to reduce the immunosupression associated with seminal plasma, and if this could be induced artificially to reduce fertility, presumably by increased sperm phagocytosis within the female reproductive tract.

MVDP and its human analogue on the sperm surface are unlikely though to fulfill the requirements for a contraceptive vaccine. Its role on the sperm surface as a detoxification enzyme for acrolein is unlikely to allow vaccine development that would reduce sperm viability enough as to render the recipient infertile. It does though have commercial value in both human and animal assisted reproductive settings. As human patients with high levels of spermine and spermine oxidase have been shown to have reduced sperm motility (Holtta et al., 1975), acrolein adduction with concomitant reduced fertility is a real problem. Addition of an acrolein reducing enzyme such as MVDP into the sperm collection media could concievably reduce adduction and preserve sperm motility. This would have applications in both human reproductive clinics and in animal husbandry settings.

Acrolein has also been found to be a causative agent of secondary necrosis after ischaemic heart disease and stroke (Stevens and Maier, 2008). Spermine, leaking from

the hypoxic cells is converted by serum amine oxidases into acrolein and other reactive aldehydes causing necrosis of various tissues on reperfusion. Addition of an acrolein decontamination enzyme to the thrombolytic treatment could reduce this secondary necrosis, thus reducing tissue damage. Mouse reperfusion models using acrolein adducted serum albumin as a marker would establish if this approach has clinical benefits.

The other sperm surface proteins identified here such as LRRC37B are as yet not well enough characterized to establish whether they would be of use in clinical investigations of ASA or in the development of contraceptive vaccines, though the epididymal splice variant of Radial Spoke Head Protein would fulfill the criteria for vaccine development and should be pursued.

7.2 Immunosuppression in the Male Reproductive Tract.

It is demonstrated here that ASA patients have reduced immunosuppressive activity in their seminal plasma and that this activity is associated with a semenogelin 1 cleavage product designated Sem274. Sem274 has homology to SV-IV of rat which has a wide range of immunosuppressive and immunomodulatory functions. As semenogelin 1 has been described here as a target for ASA and that antibody:semenogelin complexes occur in ASA patient seminal plasma, it is therefore likely that the presence of anti-semenogelin 1 antibodies leads to reduced immunosuppression of ASA patients seminal plasma.

The female reproductive tract requires to be primed so as not to attack the sperm which would otherwise be recognized as non-self. A range of changes within the immune regulatory architecture occurs on coitus in response to seminal plasma. Dendritic cells are altered so as not to capture sperm, Treg cells are induced to expand and mast cells secrete IL-4 to reduce neutrophil infiltration (Rodriguez-Martinez et al., 2011). Sem274 is an excellent candidate to fulfill some of these functions in humans. Indeed, Sem274 may have uses outside of reproductive biology and could be used as a therapy in autoimmune disease.

7.3 The Role of the Zn^{++}-Dependent ATPase of Prostasomes in Fertility

The Zn^{++}-dependent ATPase has a function in the immunosuppressive activity of prostasomes. Inhibition of this ATPase led to decreased prostasome-leukocyte interaction and therefore reduced immunosuppressive activity. It is not yet ascertained whether inhibition of the Zn^{++}-dependent ATPase leads to reduced sperm-prostasome interaction, but if so, the Zn^{++}-dependent ATPase could prove to a good candidate for small molecule inhibition, which would have the affect of preventing presentation of crucial molecules from the prostasome to the sperm such as CD55 and progesterone receptors, leaving the sperm open to complement attack and reducing motility.

7.4 Future Work.

7.4.1 Antisperm Antibodies and Their Antigens.

To ascertain the function of the previously described novel ASA binding targets on the sperm surface as well as their expression patterns throughout the body so as to assess them as contraceptive vaccine candidates.

Examination of MVDP and other acrolein detoxification enzymes in maintaining sperm cell viability during assisted reproduction procedures in both human and animal settings.

Investigation of MVDP in reperfusion injury can be carried out in mouse models. As its human homologue aldose reductase has a role in the polyol pathway, converting glucose to sorbitol, it would not have the same clinical use. However, a crystal structure of aldose reductase has been published (Biadene et al., 2007), and the possibility remains that if the MVDP structure was elucidated that aldose reductase could be modified by site directed mutagenises to only have acrolein detoxification activities.

Also, to show whether hCRISPs are proteolytically cleaved and by which protease, and if the C-terminal fragment has anti-microbial activity. It would also be required to show if ASA affected this anti-microbial activity.

7.4.2 Immunosuppressive Effects of Sem274 in the Female Reproductive Tract.

The ability of Sem274 to induce Treg production and cytokine release should be investigated as well as Sem274 effects on dendritic cell phagocytosis and mast cell cytokine release. Mouse models could be used for vaginal insemination of Sem274 and SV-IV to compare effects on the female reproductive tract immune system.

7.4.3 The Zn^{++}-dependent ATPase and Glycolytic Pathway of Prostasomes.

Determination of whether the Zn^{++}-dependent ATPase of prostasomes plays a role in sperm-prostasome fusion is required and if its inhibition reduces or eliminates this fusion. As prostasomes possess a full range of glycolytic enzymes and can produce ATP, it would be interesting to see whether inhibition of this pathway also reduces prostasome-leukocyte interaction and whether the ATP produced by this process is required for the Zn^{++}-dependent ATPase function.

CHAPTER 8

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APPENDIX I

First International Journal of Andrology
ANDROLOGIA

ORIGINAL ARTICLE

Identification of a role for a mouse sperm surface aldo-keto reductase (AKR1B7) and its human analogue in the detoxification of the reactive aldehyde, acrolein

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Keywords
Acrolein—AKR1B7—aldo-keto reductase—detoxification—MVDP

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Summary

Mouse vas deferens protein (AKR1B7), a member of the aldo-keto reductase family, was purified to homogeneity. Antibodies raised to AKR1B7 revealed an aldo-keto reductase on the human sperm surface, while confocal microscopy experiments demonstrated that this enzyme covered the entire human sperm surface and was concentrated on the mid-piece. Further functional characterisation of a recombinant form of AKR1B7 showed that the likely role of AKR1B7 is the reduction of the reactive aldehyde, acrolein, a by-product of spermine catabolism in the reproductive tract. A similar acrolein detoxification activity was displayed by human sperm membrane extracts but was not present in seminal plasma. These results indicate that human sperm possess an aldo-keto reductase on their membrane surface and are thus enzymatically protected against reactive aldehyde species both in the male and female reproductive tract.

Introduction

Mouse vas deferens protein (MVDP, AKR1B7) is a member of the aldo-keto reductase (AKR) super-family, which have an important role in the reductive detoxification of aliphatic and aromatic aldehydes into their corresponding alcohols in a NADPH-dependent manner (Bohren *et al.*, 1989). MVDP is highly expressed in vas deferens under the regulatory control of the androgen receptor (Taragnat *et al.*, 1988). It is also expressed in the adrenal cortex where it is regulated by adrenocorticotrophic hormone (Aigueperse *et al.*, 1999) and in ovarian theca cells on stimulation by human chorionic gonadotrophin (Baumann *et al.*, 2007), as well as in lower amounts in the intestine, eye, kidney and liver.

The major substrates described to date for MVDP are isocaproaldehyde and 4-hydroxy-2-nonenal (HNE) (Martinez *et al.*, 2001). These are reactive aldehydes whose rapid detoxification and removal are necessary for cell survival. Isocaproaldehyde is produced as a by-product of cholesterol side-chain cleavage during steroidogenesis, and therefore, the likely primary function of MVDP in both the adrenal cortex and ovarian theca is the detoxification of isocaproaldehyde. However, as the vas deferens is not a known site of steroidogenesis, it is unlikely that the high level of expression of MVDP at this site is functionally related to the detoxification of isocaproaldehyde. Baumann *et al.* (2007) hypothesised that the likely role for MVDP in the vas deferens is to protect against lipid peroxidation products such as HNE. However, monoclonal antibodies to protein adducts of the lipid peroxidation product malondialdehyde showed only weak histological staining in the rat reproductive tract indicating that lipid peroxidation is limited in these tissues. But a monoclonal antibody to the protein adduct of the reactive aldehyde acrolein revealed high levels of immunostaining in the epithelia of the epididymis, vas deferens and seminal vesicles (Iuchi *et al.*, 2004).

While acrolein (propanal) is also formed from lipid peroxidation, yields of acrolein are far lower than for other products such as HNE (Spiteller *et al.*, 2001). This is unlikely therefore to represent a major source of acrolein in humans (Stevens & Maier, 2008). The main source of acrolein in the reproductive tract is potentially from the catabolism of the polyamines spermine and spermidine, found in high concentrations (2.41 and 0.14 mM, respectively) in human semen (Vallely & Rees, 1986), by diamine oxidase (DAO). DAO oxidises spermine to spermidine and then to putrescine liberating acrolein and ammonia as by-products (Lee & Sayre, 1998). DAO is found in human semen, where high levels of enzyme activity correlate with loss of spermatozoa motility (Holtta

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et al., 1975) most likely due to the cytotoxicity of acrolein (Maayan *et al.*, 1995). Acrolein is a highly reactive aldehyde mediating a number of adverse effects, such as strand breakages in DNA and formation of adducts with lysine forming N^{ε}-(3-formyl-3,4-dihydropiperidino)lysine (FDP-lysine) (Uchida *et al.*, 1998). Therefore, its rapid reduction is a pre-requisite for cell survival.

The aim of this study was to identify a role for MVDP and its human counterpart in the reproductive tract. It is demonstrated here that MVDP can provide protection to sperm by the detoxification of acrolein, pre- and post-ejaculation.

Materials and methods

Subjects and serum

Human spermatozoa and seminal plasma were obtained from patients with normal semen parameters attending the Human Assisted Reproduction Ireland Clinic at the Rotunda Hospital, Dublin.

Mouse tissue extraction

The caput and cauda epididymis and vas deferens of CD1 mice, sacrificed by cervical dislocation, were homogenised in soluble cytoplasmic extraction buffer (ProteoPrep kit, Sigma-Aldrich, Dublin, Ireland) with protease inhibitors (Calbiochem, Darmstadt, Germany), centrifuged at 15 000 ***g*** for 15 min at 4 °C and the supernatant either used fresh or frozen at −80 °C.

MVDP purification

Vas deferens supernatant was ammonium sulphate precipitated in 20% steps from 0 to 100% saturation. The 100% pellet was dissolved in 20 $mmol^{-1}$ Tris, 10 $mmol^{-1}$ NaCl and 5 $mmol^{-1}$ 2-mercaptoethanol, pH 7.4, dialysed against 1 l of the same buffer at 4 °C overnight and loaded onto a MonoQ 5/50 GL column on an AKTA purifier fast protein liquid chromatography (FPLC). Bound proteins were eluted with a NaCl gradient using 20 $mmol^{-1}$ Tris, 1 mol^{-1} NaCl and 5 $mmol^{-1}$ 2-mercaptoethanol, pH 7.4. MVDP positive fractions were pooled and concentrated and the concentrate applied to a Sephadex G200 10/30GL FPLC gel filtation column equilibrated in 20 $mmol^{-1}$ Tris, 50 $mmol^{-1}$ NaCl and 5 $mmol^{-1}$ 2-mercaptoethanol, pH 7.4. MVDP fractions were analysed for purity by SDS-PAGE.

Recombinant MVDP production

The pHis-parallel-2 vector containing full length MVDP was transformed into *Escherichia coli* BL21 cells and grown in LB/ampicillin broth at 37 °C. Recombinant protein expression was induced with Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (500 μmol^{-1}) at an OD_{600} of 0.6. *E. coli* were harvested by centrifugation at 10 000 ***g*** for 10 min at 4 °C after a further 3 h at 37 °C and pellets resuspended in 50 $mmol^{-1}$ Tris, 150 $mmol^{-1}$ NaCl, 10 $mmol^{-1}$ imidazole, 2 $mmol^{-1}$ 2-mercaptoethanol (pH 8) and sonicated. Cell debris was pelleted by centrifugation at 10 000 ***g*** (10 min, 4 °C) and supernatant applied to a 3 ml Nickel-agarose (Chromatrin) column. Bound protein was eluted using 50 $mmol^{-1}$ Tris, 150 $mmol^{-1}$ NaCl, 300 $mmol^{-1}$ imidazole, 2 $mmol^{-1}$ 2-mercaptoethanol (pH 8). The MVDP was concentrated and applied to a gel filtration column as in the previous section.

Human sperm membrane extraction

Human sperm (10^7) were centrifuged (500 ***g***, 5 min) free of seminal plasma and washed X3 in PBS. The sperm pellet was incubated in 1% (v/v) Triton X-100 in PBS with protease inhibitors for 2 h. Cell debris was removed by centrifugation (5000 ***g***, 10 min) and the supernatant stored at −20 °C.

Anti-MVDP antibody production

Purified MVDP (200 μg) was emulsified with Freund's complete adjuvant and injected i.d. into a female Wistar rat on day 1. Boosters in Freund's incomplete adjuvant were injected i.d. on days 10 and 28. Blood was obtained on day 42 by cardiac puncture and serum obtained by centrifugation.

Enzyme assay

Kinetic analysis of both recombinant and endogenous MVDP was performed in 100 $mmol^{-1}$ phosphate buffer, pH 7. NADPH (100 μmol^{-1}), and MVDP (20 $\mu g\ ml^{-1}$) concentrations were kept constant in all assays. Substrates, HNE and acrolein, were varied from 1 to 200 μmol^{-1} and initial rates measured by decrease in absorbance at 340 nm.

Confocal microscopy

Extracted mouse vas deferens tissue was placed in PBS and finely chopped to allow sperm to swim out at room temperature (RT) for 1 h. Sperm were washed X3 in PBS and resuspended at $10^6\ ml^{-1}$ and 5×10^4 sperm overlaid on glass cover slips for 1 h to allow them to adhere. Human sperm were prepared as before and allowed to adhere to cover slips as in mouse sperm. Sperm were fixed in 2% (w/v) paraformaldehyde in PBS for 15 min and washed X3 in PBS/Tween 20. Cover slips were incubated with anti-MVDP antisera diluted 1 in 2000 in

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1% (w/v) BSA in PBS/Tween for 2 h at RT. Cover slips were washed X3 in PBS/Tween and then incubated for 1 h in a 1 in 1000 dilution of secondary antibody, DyLight 594 Goat anti-rat IgG (Biolegend, San Diego, CA, USA) in 1% BSA in PBS/Tween. Cover slips were washed as before and mounted in MOWIOL 4-88 reagent (Calbiochem) and imaged.

Spermine toxicity assay

THP1 cells cultured in RPMI medium supplemented with 10% (v/v) foetal calf serum, 2 $mmol^{-1}$ Glutamine, 100 Iu penicillin and 100 $\mu g\ ml^{-1}$ streptomycin and plated in a 96-well plate at 10^5 cells in 200 µl per well with or without recombinant MVDP (20 µg per well) at 37 °C in 5% CO_2 overnight. Spermine was added in a range of concentrations from 0.01 to 100 μmol^{-1} and incubated overnight. Alamar blue (Invitrogen, Carlsbad, CA, USA) was added as per manufacturer's instructions and incubated for 6 h at 37 °C in 5% CO_2. Fluorescence was measured at 544 nm excitation and 590 nm emission.

Gel electrophoresis

SDS-PAGE was carried out (Laemmli, 1970) and gels stained with Coomassie Brilliant blue or blotted as in the following section.

Western blotting

Gels were transferred to polyvinylidene flouride membrane (PVDF) (Millipore, Cork, Ireland), blocked in 5% fat-free milk and incubated in 1 in 250 rat anti-MVDP sera as primary antibody for 2 h. Blots were washed X3 in PBS/Tween and incubated in 1 in 5000 HRP-conjugated goat anti-rat IgG (Sigma-Aldrich, Dublin, Ireland) for 1 h. Blots were washed and visualised in 0.04% w/v diaminobenzidine, 0.04% v/v Hydrogen Peroxide in PBS.

Protein determination

Protein concentration was assayed (Bradford, 1976) using Bradford Ultra reagent (Expedeon, Cambridgeshire, UK).

Statistical analysis

Statistical analysis (*t*-test) was performed in PRISM 5.0

Results

Mouse vas deferens protein from vas deferens was purified by sequential purification techniques, ammonium sulphate precipitation, MonoQ chromatography and gel filtration chromatography (Fig. 1a) and its identity confirmed by mass spectrometry as MVDP (results not shown). To elucidate the likely human homologue of MVDP, polyclonal rat anti-MVDP antibodies were shown to bind to a 35 kD protein from human sperm membrane (Fig. 1b), indicating the presence of an aldo-keto reductase on the sperm surface.

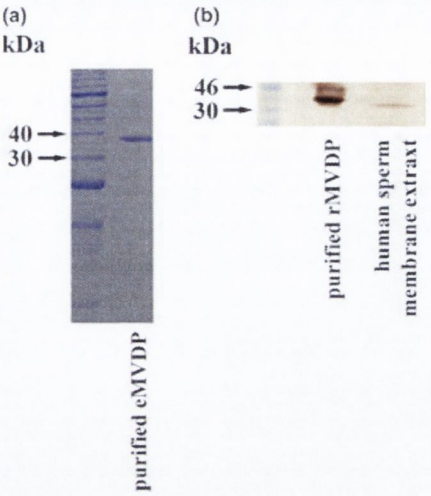

Fig. 1 (a) Coomassie blue stained gel of purification of endogenous Mouse vas deferens protein (MVDP). (b) Blot of rMVDP and human sperm membrane extract with anti-MVDP antibodies.

Kinetic analysis of both rMVDP and eMVDP using HNE as substrate indicated that both forms had the same catalytic profile as previously reported for MVDP from adrenal gland (Martinez *et al.*, 2001). However, as lipid peroxidation products are not the likely substrate in the reproductive tract, other substrates were investigated. Acrolein, which has previously been shown to form high levels of adducts in the rat reproductive tract (Iuchi *et al.*, 2004), was tested and exhibited good substrate qualities (Table 1) with a K_m of 17.81 μmol^{-1} and k_{cat} of 1.897 min^{-1}. Human sperm membrane extract also displayed acrolein detoxification with a specific activity of 11.54 $\mu mol\ min^{-1}\ mg^{-1}$, while no activity could be detected in seminal plasma (Table 2).

Table 1 Kinetic parameters for endogenous and recombinant Mouse vas deferens protein (MVDP) with 4-hydroxy-2-nonenal (HNE) and acrolein as substrates

	eMVDP		rMVDP	
Substrate	K_m (μmol^{-1})	k_{cat} (min^{-1})	K_m (μmol^{-1})	k_{cat} (min^{-1})
HNE	6.67	2.21	12.97	2.17
Acrolein	17.59	3.789	17.81	1.897

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Table 2 Specific activities for human sperm membrane extract and seminal plasma using acolein as substrate

	Specific activity of acrolein reduction ($\mu mol\ min^{-1}\ mg^{-1}$)
Human sperm membrane extract	11.54
Human seminal plasma	Not detected

Values are a mean of triplicate readings.

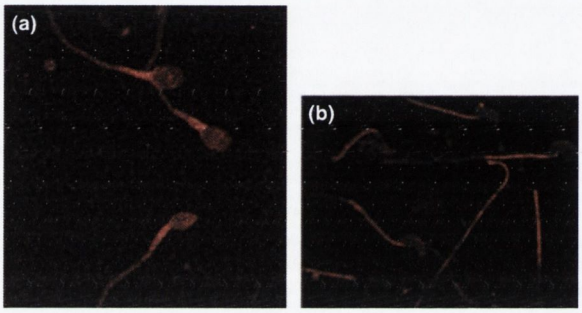

Fig. 2 (a) Confocal microscopy of human sperm stained with anti-Mouse vas deferens protein (MVDP) antibodies. (b) Confocal microscopy of mouse sperm stained with anti-MVDP antibodies.

Polyclonal antibodies raised against MVDP showed reactivity against both mouse and human sperm by confocal microscopy. The mid-piece and tail of mouse sperm reacted specifically with these anti-MVDP antibodies, while the entire surface of human sperm stained positively with increased staining on the mid-piece (Fig. 2).

Bovine foetal calf serum (FCS) contains an amine oxidase similar to human seminal amine oxidase (DAO) and has been used previously to demonstrate the cytotoxic and immunosuppressive effects of spermine catabolites (Maayan *et al.*, 1995). To demonstrate the ability of MVDP to reduce cytotoxicity on exposure of cells to acrolein, a model system using the monocyte cell line, THP1, was devised. The FCS amine oxidase will oxidise spermine, releasing acrolein. MVDP increased the IC_{50} for acrolein cytotoxicity in this system from 3.173 to 14.11 μmol^{-1} with the addition of 20 μg of MVDP (Fig. 3), thus indicating a role for MVDP in protection from spermine oxidation products.

Discussion

The role of MVDP in the vas deferens has been assumed to be the prevention of toxicity to sperm of lipid peroxidation products and ensuring their continued viability (Baumann *et al.*, 2007). This though can be disputed by the results of Iuchi *et al.* (2004) who demonstrated limited protein adducts of lipid peroxidation products in the rat reproductive tract. They did however show large levels of FDP-lysine, the protein adduct of acrolein. As acrolein is only a minor by-product of lipid peroxidation, the major source of acrolein in the reproductive tract is likely to be from the catabolism of the polyamines, spermine and spermidine.

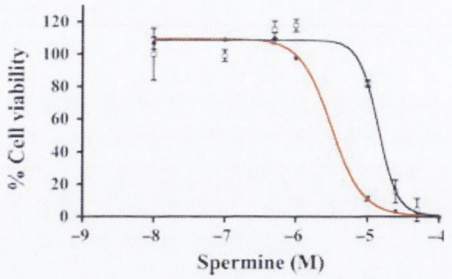

Fig. 3 Reduction in cytotoxicity by spermine catabolites on THP-1 cells by Mouse vas deferens protein (MVDP). IC_{50} increases with MVDP addition from 3.173 ± 0.76 to 14.11 ± 1.63 μmol^{-1}. $P < 0.001$.

Mouse vas deferens protein knock-out mice (Baumann *et al.*, 2007) displayed no loss of reproductive success; however, they did display a nonsignificant trend in reduction of vas deferens sperm motility. The decrease in sperm motility is likely as a result of increased acrolein concentration from spermine catabolism as MVDP is no longer present to detoxify this reactive aldehyde, these results would therefore correlate well with the result that increased DAO activity, which would also lead to increased acrolein concentration, reduces sperm motility (Holtta *et al.*, 1975). That the trend in loss of sperm motility that Baumann *et al.* (2007) noted was not statistically significant may be due to some redundancy in the system as glutathione S-transferase (GST), which can also detoxify acrolein through conjugation with glutathione (Berhane *et al.*, 1994), is also present on the mouse sperm surface. As MVDP has been shown to contain conserved evolutionary epitopes that bind human antisperm antibodies (ASA) (results not shown), it is interesting to note that mouse glutathione S-transferase mu5 has also been identified as an antigen for human ASA (Paradowska *et al.*, 2006), which leads to the conclusion that ASA positive patients with antibodies to both AKR and GST could be liable to greater acrolein adduction and hence loss of sperm motility.

Sperm can also meet acrolein post-ejaculation within cervical mucus. Cervical mucus contains both DAO and polyamine oxidase (PAO) activity which on contact with semen can oxidise polyamines releasing acrolein. High levels of DAO and PAO in cervical mucus have been linked with cervical carcinoma (Fernandez *et al.*, 1995;

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Rogers *et al.*, 2002) through the synergistic effect of cytotoxic spermine catabolites and the anti-apoptotic actions of human papilloma virus. Therefore, sperm will require protective enzymes to acrolein both pre and post-ejaculation and as demonstrated here, MVDP and its human homologue on the sperm surface will fulfil this role.

The identity of the aldo-keto reductase on the human sperm surface with acrolein detoxification activity is as yet unclear; however, AKR1B1 has previously been shown to occur on human sperm. Frenette *et al.* (2006) demonstrated the expression of AKR1B1 along the entire human epididymis and the presence of AKR1B1 on epididosomes, prostosomes and the sperm surface. They conclude that, as sorbitol dehydrogenase is also present on the sperm surface, that AKR1B1 forms the first step of the polyol pathway, the reduction of glucose to sorbitol. Although AKR1B1 is the most structurally related human AKR to MVDP (Cao *et al.*, 1998), MVDP is unable to catalyse the conversion of glucose to sorbitol (Lefrancois-Martinez *et al.*, 1999) suggesting species variation in functionality. Frenette *et al.* (2006) notes, however, that AKR1B1 is not differentially expressed along the epididymis in humans as in other species (Frenette *et al.*, 2004) and thus it may perform functions other than the regulation of sperm motility through the polyol pathway. AKR1B1 has acrolein detoxification activity, though at a much higher K_m (80 μmol^{-1}) than that demonstrated here for MVDP (Kolb *et al.*, 1994). No acrolein detoxification activity was found in this study for seminal plasma with prostosomes still present, a discrepancy with Frenette *et al.* (2006) who as stated showed AKR1B1 localisation with prostosomes. This may be explained by either AKR1B1 being unable to access its substrates if located within the multi-lamellar prostosomes, though this is unlikely as acrolein can readily cross membranes, or that AKR1B1 concentration in whole seminal plasma containing prostosomes is too low for enzyme activity to be detected. Isolation and solubilisation of human prostosomes should resolve whether they contain acrolein reduction activity.

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