Apoptotic Markers in Ejaculated Human Spermatozoa

by

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ABSTRACT

The role of male germ cell death in spermatogenesis is an important one as it removes dysfunctional or genetically damaged germ cells and is necessary to maintain an optimal germ cell to Sertoli cell ratio. The formation of the blood-testis barrier requires the elimination of excessive germ cells and a surge of germ cell apoptosis occurs prior to puberty regulating the ratio of germ cells to Sertoli cells. The aim of this study was to evaluate the presence of four apoptotic markers on sperm from patients with various grades of fertility using flow cytometry. Furthermore, any correlations between the apoptotic marker assays and the standard semen analysis results were identified. This study compares early and late parameters of apoptosis with morphological features in spermatozoa in the same samples.

The three sample groups were identified as: teratozoospermic [G-pattern] (n=26), teratozoospermic [P-pattern] (n=98) and oligoteratozoospermic [P-pattern] (n=36). Standard semen analysis was conducted on the semen samples according to the WHO guidelines. Four apoptotic marker assays using flow cytometry was applied in this study to examine the apoptotic alterations in ejaculate sperm. These assays included the Annexin-V staining for the determination of phosphatidylserine exposure, APO-Direct to identify DNA fragmentation, caspase-3 to detect expression of this active protease during early apoptosis and Fas expression.

For the Annexin-V and caspase-3 assays, statistically significant differences ($P<0.05$) were evident between the three groups. No significant differences ($P>0.05$) were found between the groups with respect to the APO-Direct assay. A significant difference ($P<0.05$) was found when comparing the teratozoospermic [G-pattern] group and the oligoteratozoospermic [P-pattern] group for the Fas assay. A strong positive correlation was evident between

the Fas and the caspase-3 assays in the teratozoospermic [G-pattern] group. For the teratozoospermic [P-pattern group] the following positive correlations existed between the APO-Direct and the Fas assays, APO-Direct and caspase-3 assays and between caspase-3 and Fas assays. The only strong positive correlation was between the caspase-3 and APO-Direct assays in the oligoteratozoospermic [P-pattern] group.

The presence of spermatozoa showing microscopic features resembling apoptosis has been identified in ten human ejaculate samples per sample group. Electron microscopy was used to identify morphological features of apoptosis in these human sperm samples. Classical apoptosis as observed in diploid cells could be identified in sperm and these included: loose fibrillar-microgranular chromatin network, presence of vacuoles in the nuclear chromatin, membranous bodies within the vacuoles of the chromatin, partially disrupted nuclear membranes, plasma membrane protuberances and apoptotic bodies containing cytoplasmic vacuoles and dense masses.

This study has confirmed that semen samples with abnormal semen parameters exhibit the presence of apoptotic markers in sperm. The identification of apoptotic markers on the sperm suggests that abnormalities occur during their developmental process, however, the exact mechanism thereof remains unclear. These findings may suggest that certain apoptotic markers may be an indicator of abnormal sperm function and possibly indicative of male infertility.

DECLARATION

I, the undersigned, hereby declare that "*Apoptotic Markers in Human Ejaculate Sperm"* is my own work and has not previously in its entirety, or in part, been submitted for any degree or examination in any other university. All the resources I have quoted have been indicated and acknowledged by complete references.

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Full Name Dated

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Signed

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3'OH	3'-hydroxyl termini of DNA
A1	Anti-apoptotic member of Bcl family of proteins
Ab	Apoptotic body
ABC 1	vitronectin receptor
ADP	adenosine diphosphate
ah	amorphous sperm head
ANOVA	analysis of variance
Apaf-1	apoptotic protease activation factor-1
AR	acrosome reaction
ATP	adenosine triphosphate
AZF	azoospermic factor
Bad	Pro-apoptotic member of the Bcl family of proteins
Bak	Pro-apoptotic member of the Bcl family of proteins
Bax	Pro-apoptotic member of the Bcl family of proteins
Bcl-2	Apoptosis regulator proteins
Bcl-W	Anti-apoptotic member of Bcl family of proteins
Bcl-X_L	Anti-apoptotic member of Bcl family of proteins
Bid	Pro-apoptotic member of the Bcl family of proteins
Bim	Pro-apoptotic member of the Bcl family of proteins
Bok	Pro-apoptotic member of the Bcl family of proteins
Blk	Pro-apoptotic member of the Bcl family of proteins
BSA	bovine serum albumin
BNIP	Pro-apoptotic member of the Bcl family of proteins
Ca^{2+}	Calcium
CAD	caspase-activated DNase
cAMP	cyclic adenosine monophosphate
CARD	caspase recruitment domain
Caspases	cysteine-aspartate-directed protease

CD 14	class B scavenger receptors on macrophage
CD 36	class B scavenger receptors on macrophage
Ced	*cell death gene*
C. elegans	*Caenorhabditis elegans*
Ces	cell death specification
Ch	chromatin
COMET	modified single cell gel electrophoresis assay
CR	cytoplasmic residue
dADP	2'-deoxyadenosine 5'-diphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
DAZ	gene on Y chromosome of azoospermic men
Dm	dense mass
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependant protein kinase
DNase	Deoxyribonuclease
DPX	distyrene, tricresyl phosphate and xylene mixture
dUTP	deoxyuridine triphosphate
Egl1	Egg-laying defective gene
eh	elongated sperm head
ELISA	enzyme-linked immunosorbant assay
FADD	Fas-associating protein with death domain
FAS	CD95 or APO-1
FasL	Fas ligand
FITC	fluorescein isothiocyanate
FSH	Follicle stimulating hormone
G-pattern	"good" prognosis for fertilization
G-Terato-	teratozoospermic [G-pattern]
HtrA2/Omi	mitochondrial mature serine protease
IAP	inhibitors of apoptosis proteins
ICAD	inhibitor of CAD (caspase-activated DNase)

ICAM	intracellular adhesion molecule 3
ICE	interleukin 1-β-converting enzyme
ICSI	intracytoplasmic sperm injection
IgG	Immunoglobulin G
IVF	*in vitro* fertilisation
kB	kilobytes
kV	kilovolts
lh	large sperm head
LH	Luteinizing hormone
Ma	macrophage
MAR	mixed antiglobulin reaction
Mb	membranous body
Mcl-1	Anti-apoptotic member of Bcl family of proteins
Ml	milliliter
MORT1	death effector domain
n	number
NC	necrotic features
NF-kB	transcription factor
Nm	nanometers
Nuc-1	gene required by phagocytosing host
P	probability
p53	tumor suppressor protein
PARP	DNA repair enzyme
PBS	phosphate buffered saline solution
Phs	Phagosome
PI	propidium iodide
PLC	phospholipase C
P-Oligoterato-	oligoteratozoospermic [P-pattern]
P-pattern	“poor” prognosis for fertilisation
PS	phosphatidylserine

P-Terato-	teratozoospermic [P-pattern]
rh	round sperm head
RIP	receptor interacting protein
RNase	ribonuclease
ROC	receiver operating characteristic
ROS	reactive oxygen species
r_s	Spearman correlation matrix
SCSA	sperm chromatin structure assay
SNARE	N-ethylmaleimide-sensitive factor-attachment protein receptor
SEM	standard error of the mean
sh	small sperm head
Smac/DIABLO	second mitochondria-derived activator of caspase protein
St	spermatid
TdT	terminal deoxynucleotidyl transferase
TNF	tumour necrosis factor
TNFR1	tumour necrosis factor receptor 1
TRADD	death domain-containing protein
TRAIL	TNF-related apoptosis-inducing ligand
TRAF-2	TNF receptor associated factor
TRAMP	TNF receptor-related-apoptosis-mediated protein
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
µl	microliter
V	vacuoles
WHO	World Health Organization
Yq	long arm of Y chromosome
ZP-3	zona pellucida protein 3

CHAPTER 1

General Introduction and Literature review

The main aim of this chapter is to review various aspects of sperm apoptosis and its possible relationship to infertility. Since the context is infertility, it is useful to review selected aspects relating to infertility to provide a sufficient background. Firstly, an overview of the standard semen analysis and the molecular events involved in sperm egg interactions will be presented prior to the review of infertility and apoptosis.

1.1 Standard semen analysis

Semen parameters are used to investigate and establish male fertility. It is a predictive indicator of the functional status of the male hormonal cycle, spermatogenesis and the patency of the reproductive tract. Standardised, descriptive laboratory tests routinely performed on the semen samples according to the WHO guidelines include: sperm volume, pH, sperm concentration, sperm motility (percentage), sperm morphology, speed of forward progression of sperm, agglutination and mixed antibody reaction (WHO, 1999). The quantification of leukocytes and sperm vitality assessments was not performed as part of the standard semen analysis. Only sperm concentration, percentage motile sperm, forward progression and sperm morphology will be considered as primary indicators of infertility, as these collectively are indicators of possible male factor infertility. In addition, the semen sample is analysed for physical parameters during the initial macroscopic examination and these include: liquefaction, appearance, volume, coagulation, viscosity, and pH (WHO, 1999). Following the physical assessment of the semen sample, quantitative and qualitative analysis is performed.

The sperm concentration is determined using the Haemocytometer method and is important for determination of the quantitative aspects of spermatogenesis (Mortimer, 1994; WHO, 1999; Nieschlag, 2001). Sperm motility is routinely assessed on a wet preparation and the forward progression of sperm as well as the percentage of motile sperm is determined (WHO, 1999). The determination of the quantitative motility for each sample requires the calculation of motile sperm and expressed as percentage motility (Coetzee and Menkveld, 1995). Quantification of sperm motility may provide an indication that sperm are able to navigate the barriers in the female reproductive tract that must be overcome to reach the oocyte.

Table 1.1 lists the reference values of semen parameters describing a semen sample with normal parameters. Sperm morphology remains a controversial issue; however, this semen parameter has been identified as the highest predictor of a man's fertilising potential (Ombelet *et al.*, 1997).
Morphological normality of sperm is assessed according to the Tygerberg strict criteria and has been shown to be an important predictor in the outcome of assisted reproduction (Kruger *et al.*, 1986; Van der Merwe *et al.*, 2005).

Any deviations from the reference semen variables (Table 1.1) are described by specific nomenclature (Mortimer, 1994; Coetzee and Menkveld, 1995; WHO, 1999; Nieschlag, 2001). The term normozoospermia describes a normal ejaculate as defined by the reference values outlined in Table 1.1 according to the WHO (1999). The term oligozoospermia describes a sperm count of less than 20 X 10^6 sperm/ml of ejaculate. Asthenozoospermia describes a semen sample with poor motility less than 50% (grades a + b) or less than 25% with progressive motility (grade a). Teratozoospermia refers to an increased number of sperm with abnormal morphology (less than 14% of normal sperm). Oligoteratozoospermia describes a semen sample with a sperm count of less than 10 X 10^6 sperm/ml of ejaculate and an increased

number of sperm with abnormal morphology based on the Tygerberg strict criteria (less than 14% of normal sperm). Oligoasthenoteratozoospermia describes a semen sample with a disturbance in all 3 variables. Van der Merwe *et al.* (2005) concluded that a threshold of <5% normal sperm morphology, a concentration <15 x 10^6/ml, and a motility of <30% (WHO, 1999) should be used as predictive values to identify the subfertile male.

Table 1.1. Reference values of semen parameters according to WHO guidelines (1999).

Semen Parameter	Reference value
Volume	2.0 or more
pH	7.2 or more
Sperm concentration	20 X 10^6 spermatozoa/ml or more
Total sperm count	40 X 10^6 spermatozoa per ejaculate or more
Motility	50% or more motile (grades a + b) or 25% or more with progressive motility (grade a) within 60 minutes of liquefaction
Morphology*	≤15% spermatozoa with normal forms
Vitality	50% or more live
MAR test	Fewer than 50% motile sperm with adherent particles

* Tygerberg strict criteria, Kruger *et al.* (1986)

Two subgroups of sperm morphology have been identified in patients with poor fertilisation rates: a "good" prognosis pattern [G-pattern] with the percentage of normal forms between 5 and 14% and a group with "poor" prognosis pattern [P-pattern] with ≤4% normal forms in the ejaculate (Kruger *et al.*, 1988; Grow and Oehninger, 1995). Both G-pattern and P-pattern groups falls into the teratozoospermic group, however, the G-pattern group has a good prognosis pattern for predicting the outcome of *in vitro* fertilisation (Grow and Oehninger, 1995; Coetzee *et al.*, 1998; Kruger and Coetzee, 1999). The G-pattern teratozoospermic group is considered as the mildly

compromised group with a lesser degree of abnormality. The teratozoospermic [P-pattern] group is considered moderately compromised and the oligoteratozoospermic [P-pattern] group severely compromised. In addition to conventional semen analysis, sperm functional testing such as an acrosome reaction test, sperm penetrating assay, zona-binding assay and sperm chromatin structure assay can be performed as these are valuable in the diagnosis of male infertility. The value of most of these assays is to reflect the biochemical functions of spermatozoa and consequently the capability to fertilise the oocyte (Henkel *et al.,* 2005b).

1.2 Molecular events involved in sperm and egg interactions

In this review, some of the biochemical aspects of human spermatozoa which may be important in the determination of sperm integrity as well as sperm functional potential will be addressed. Furthermore, several aspects of fertilisation will be briefly discussed. The ultimate destination of the fertilising sperm is only reached after a tortuous journey from the testis through the male and female reproductive tract and towards the egg. Recognition of sperm and egg occurs by carbohydrate-protein interactions, which leads to a signalling cascade of events. Two fundamental processes, capacitation and acrosome reaction are important events for sperm-oocyte interactions and will be described.

1. 2.1 Sperm capacitation

Capacitation is a complex series of molecular events in sperm after epididymal maturation and confers on sperm the ability to fertilise an egg (Visconti *et al.,* 2002). During capacitation, sperm acquire hyperactive motility and the ability to undergo the acrosome reaction. Capacitation is accompanied by the modification of membrane characteristics, signal transduction events and enzyme activity (Baldi *et al.*, 2000; Muller, 2000; Visconti *et al.*, 2002). The process of capacitation usually occurs in the female genital tract during the

migration of the sperm to the site of fertilisation that renders the sperm competent for fertilisation of the oocyte (Baldi *et al.*, 2000).

A complex cascade of molecular events occurs during the capacitation process (Baldi *et al.*, 2000; Visconti *et al.*, 2002). The removal of cholesterol from the sperm plasma membrane is an important event for capacitation possibly leading to an increase in the fluidity of the sperm cell membrane. Cholesterol release is the signal that activates membrane signal transduction pathways that lead to capacitation (Baldi *et al.*, 2000). An alteration in membrane fluidity is observed during capacitation, however, the equatorial segment and post-acrosomal region remain totally unaffected (Brucker and Lipford, 1995). Another important feature of capacitation includes ion fluxes that result in an alteration to the sperm membrane potential. An influx of intracellular calcium into the acrosome during capacitation occurs through an ATP-dependent pump (Baldi *et al.*, 2000; Visconti *et al.*, 2002). Intracellular potassium, sodium and chloride ions are modulated during capacitation. The transmembrane movement of bicarbonate ions into sperm could be responsible for the known increase in intracellular pH observed during capacitation (Visconti *et al.,* 2002). Due to the increase in intracellular bicarbonate and calcium ions, an increase in cAMP and adenylyl cyclase stimulation occurs during capacitation. During capacitation, cAMP-dependant kinases are activated, which induce phosphorylation in serine, tyrosine and threonine residues (Baldi *et al.*, 2000; Visconti *et al.*, 2002). The increase in cAMP is important for the phosphorylation of protein kinases involved in the induction of hyperactivation and the acrosome reaction important for sperm capacitation (Visconti *et al.*, 2002). These events result in membrane hyperpolarisation during capacitation, preparing the sperm for the acrosome reaction.

During the final phase of capacitation, sperm develops vigorous movement, termed hyperactivation, which facilitates sperm migration through the female reproductive tract increasing the chance of zona pellucida penetration (Mbizvo *et al*., 1990; Mbizvo, 1995). The molecular events in the flagellum involve the phosphorylation of protein kinases in the fibrous sheath contributing to the changes in tail wave amplitude during hyperactivation (Visconti *et al.*, 2002). These surface changes of the sperm cell during capacitation are important phenomena essential for the fertilisation process.

1.2.2 Acrosome reaction

The acrosome reaction (AR) is an exocytotic process consisting of multiple fusions between the outer acrosomal membrane and overlaying plasma membrane leading to the release of acrosomal enzymes and exposure of the molecules present on the inner acrosomal membrane surface that mediate fusion with oolema (Margalit *et al.*, 1997; Baldi *et al.*, 2000). This process is physiologically induced by ligand zona protein 3 (ZP3)-receptor interaction between the fertilising sperm and the zona pellucida. The most typical changes during the acrosome reaction are fusions and vesiculations between the plasma membrane and the outer acrosomal membrane (Margalit *et al.*, 1997; Baldi *et al.*, 2000). A change in the equatorial region of the plasma membrane of the sperm occurs, allowing it to fuse with the oocyte membrane. Acrosin, a serine protease present in the sperm acrosome liberated during the acrosome reaction is thought to play a major role in the attachment to, and penetration of the zona pellucida by spermatozoa (Margalit *et al.,* 1997; Nakagawa *et al.*, 1997; Honda *et al.*, 2002).

Some of the molecular mechanisms associated with the acrosome reaction include membrane and cytosolic signalling factors activated in response to ZP3 (Baldi *et al.*, 2000; Wassarman *et al.*, 2001). An increase in intracellular calcium precedes or occurs concomitantly with the acrosome reaction in

response to zona pellucida (Brucker and Lipford, 1995). The transient influx of calcium through voltage-activated calcium channels results in the activation of phospholipase C (PLC) through a G protein-mediated pathway (Brucker and Lipford, 1995; Breitbart and Naor, 1999; Baldi *et al.*, 2000; Evans and Florman, 2002). PLC and the transient calcium elevation function in concert to produce persistent calcium entry that drives the acrosome reaction, triggering fusion of the outer acrosome membrane with the plasma membrane and releasing acrosomal contents. The activation of protein kinases during the acrosome reaction also contributes to the fusion of sperm with oocyte (Baldi *et al.*, 2000). A number of soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins located on the apical side of the sperm acrosome region may couple calcium entry to exocytosis (Evans and Florman, 2002). Following penetration of the zona pellucida, sperm adhere to and fuse with the plasma membrane of the egg mediated by adhesion proteins (Evans and Florman, 2002; Henkel *et al*, 2005b). Egg activation occurs after fertilisation and the process of embryonic development is initiated.

1.3 Infertility

The term “infertility” has been described as failure to conceive after one year of unprotected intercourse (Nieschlag, 2001; Leke, 2003). About 15% of couples world-wide remain childless because of infertility (Feng, 2003). Infertility of the male partner is causative in up to two-thirds of all couples unable to conceive spontaneously. Thus, a complete andrological laboratory assessment including a variety of laboratory parameters of the male partner is an important aspect of the overall investigation of a couple and serves as a first line investigation as outlined above.

Semen analysis is not the only determinant of the male fertility status as these standard laboratory tests are merely descriptive of the ejaculate sample. These descriptive tests have limitations in that they fail to provide information

on the functional aspects of sperm regarding the transport of sperm in the female reproductive tract and fertilising capacity of sperm (Mortimer, 2000). Additional information can be derived from sperm functional tests including sperm-mucus interactions, indicative of the ability of sperm to enter the female reproductive tract; the hamster-egg sperm penetration assay; induction of the acrosome reaction to mimic the stimulus of the zona pellucida; the hemizona binding assay and the production of damaging reactive oxygen species (Mortimer, 1994; Henkel *et al*., 20005b; WHO, 1999). At present, sperm function assays are not used as primary tools in the diagnosis of infertility during standard semen analysis procedures as these involve a great deal of equipment and expense. The standard semen analysis is of significant clinical value in the initial diagnosis of male infertility; however, sperm functional testing would provide additional information for the possible achievement of pregnancy.

It remains a possibility that a man could be classified as infertile despite having normal semen analysis or he may be fertile despite an abnormal semen analysis. In response to a dramatically increased demand for infertility investigations, assisted reproductive technology remains an alternative option for couples. The development of assisted reproductive technologies and the lack of reliability of semen analysis in providing prognostic information to predict fertilisation, depends on sperm functional testing as an important diagnostic tool.

1.3.1 Possible causes of male factor infertility

Male infertility has been shown to be associated with genetic disorders; however, in a large proportion of cases no cause can be identified and it is classified as “idiopathic” (Oehninger, 2000; Silber, 2000). Genetic disorders include micro-deletions in the long arm (Yq) of the Y chromosome; which are testis-specific genes important in spermatogenesis (Küpker *et al.*, 1999;

Hargreave, 2000; Minhas and Ripps, 2000; McLachlan and de Kretser, 2001; Feng, 2003). Men with partial deletion of the long arm (Yq) were found to be azoospermic and it was postulated that they possess an azoospermic factor (AZF) on the Yq. Three loci have been identified in this region of the long arm that contains genes of importance to spermatogenesis designated as AZFa, AZFb and AZFc (Aitken, 1999; Namiki, 2000; Aitken and Krausz, 2001). Deletions in AZFa produce the Sertoli cell only syndrome (no germ cells present), AZFb deletions are associated with germ cell arrest at the spermatocyte stage and deletions in AZFc generate arrest at the spermatid developmental stage.

Deletions found on the DAZ gene on the Y chromosome of azoospermic men may also contribute to spermatogenesis impairment (Vogt, 1998; Küpker *et al.*, 1999; Hargreave, 2000; Silber, 2000; Aitken and Krausz, 2001; De Vries *et al.*, 2001; Feng, 2003; Vogt and Fernandes, 2003). The Y chromosome contains a specific set of spermatogenesis genes controlling the process of spermatogenesis also based on the interactive gene network. Mutations of the androgen receptor gene on the X chromosome or mutations in other spermatogenesis genes may be associated with male infertility and poor spermatogenesis (Küpker *et al.*, 1999). Aberrations in sex chromosomes (karyotypes) in the germ cell may be a factor for infertility (Küpker *et al.*, 1999; Hargreave, 2000; Oehninger, 2000; Vogt and Fernandes, 2003).

Infertility is a heterogeneous syndrome in men and it is likely that a multitude of genes are implicated as possible causes of this syndrome. Patients presented with abnormal semen parameters to andrology laboratories should have genetic testing as part of the diagnostic procedure for the infertility assessment as this may provide crucial information regarding the male fertility status. Spermatozoa of infertile men have been reported to possess nuclear aberrations, including abnormal chromatin structure and possible DNA strand

breaks (Sun *et al.*, 1997; Donnelly *et al.*, 2000; Gandini *et al.*, 2000; Irvine *et al.*, 2000; Oehninger, 2000; Zini *et al.*, 2001; Sakkas *et al.*, 2002; Henkel *et al.*, 2004). DNA damage could be attributed to direct oxidative damage (free-radical induced damage) and could be a consequence of apoptosis that could disrupt the functional and genomic integrity of human sperm (Barroso *et al.*, 2000; Oehninger, 2000; Evenson *et al.*, 2002; Oehninger *et al.*, 2003; Moustafa *et al.*, 2004). The excessive generation of reactive oxygen species (ROS) is associated with perioxidative damage to the sperm plasma membrane and the stimulation of DNA fragmentation possibly associated with poor semen quality and male factor infertility (Aitken *et al.*, 1998; Aitken, 1999; Aitken and Krausz, 2001). Another possible theory to explain the origin of DNA damage in sperm is as a result of improper DNA packaging during spermiogenesis (Gorczyca *et al*., 1993; Sailer *et al*., 1995; Manicardi *et al*., 1998; Sakkas *et al*., 1999a and b; Barroso *et al*., 2000; Sakkas *et al*., 2002; Oehninger *et al*., 2003). Another theory that has been proposed is the "abortive apoptosis" theory (Sakkas *et al.*, 1999a and 1999b; Sakkas *et al.*, 2002). Apoptosis in mature sperm is initiated during spermatogenesis in which some cells, earmarked for elimination may escape the removal mechanism contributing to poor quality sperm. "Abortive apoptosis" may fail in the total clearance of sperm earmarked for elimination by apoptosis and therefore ejaculate sperm with apoptotic markers appear to have escaped programmed cell death and reflect the failure of sperm to complete apoptosis.

Three hypotheses exist for possible DNA damage in sperm cells and these include damage during the transition of histone to protamine complex during spermiogenesis (McPherson and Longo, 1993; Sun *et al.*, 1997; Sakkas *et al.*, 1999a; Sakkas *et al.*, 1999b). Sakkas *et al.* (2002) proposed that the presence of DNA damage in sperm is not directly linked to an apoptotic process in spermatozoa, but arises due to problems with the nuclear remodelling process. Sun *et al.* (1997) demonstrated that the exposure to

environmental toxins is positively correlated to DNA damage in sperm. It was found that DNA strand breaks in sperm correlated negatively with semen quality parameters including concentration, morphology and motility (Sun *et al.*, 1997; Sakkas *et al.*, 1999a; Irvine *et al.*, 2000). The assessment of the chromatin status of sperm is important when evaluating the ability of sperm to fertilise the oocyte and should be routinely performed during assisted reproduction.

It is in this light that this study has reviewed the presence of apoptotic markers in human sperm of men attending an infertility clinic in an attempt to outline any possible associations between sperm apoptosis and semen parameters. Sperm apoptosis and assays for selected key apoptotic markers are discussed in section 1.8 of this chapter.

1.4 Cell Death: Necrosis and Apoptosis

Two fundamental, morphologically distinct types of cell death exist differing in their nature and biological significance, namely, necrosis and apoptosis (Samali *et al.*, 1996; Kanduc *et al.*, 2002; Van Cruchten and Van den Broeck, 2002). Figure 1.1 depicts the sequential ultra-structural changes in apoptosis and necrosis. Necrosis will be briefly outlined while the emphasis of this discussion is aimed at the apoptotic process.

1.4.1 Necrosis

Necrosis, also referred to as “accidental” cell death, is a non-physiological or passive type of cell death and usually occurs when cells die from severe and sudden injury, such as ischaemia, sustained hyperthermia, physical or chemical trauma (Wyllie *et al.*, 1980; Cohen, 1993; Samali *et al.*, 1996). This phenomenon refers to a spectrum of morphological changes that follow cell death in living tissue as a result of the progressive degradation action of enzymes on the irreversibly injured cells (Contran *et al.*, 1999). Necrosis

results from two concurrent processes, namely, the enzymatic digestion of the cell and the denaturation of proteins. During necrosis, several changes occur within the cell and the cell rapidly becomes unable to maintain homeostasis (Cohen, 1993; Samali *et al.*, 1996). Refer to Figure 1.1 for the sequential ultra-structural changes in necrosis.

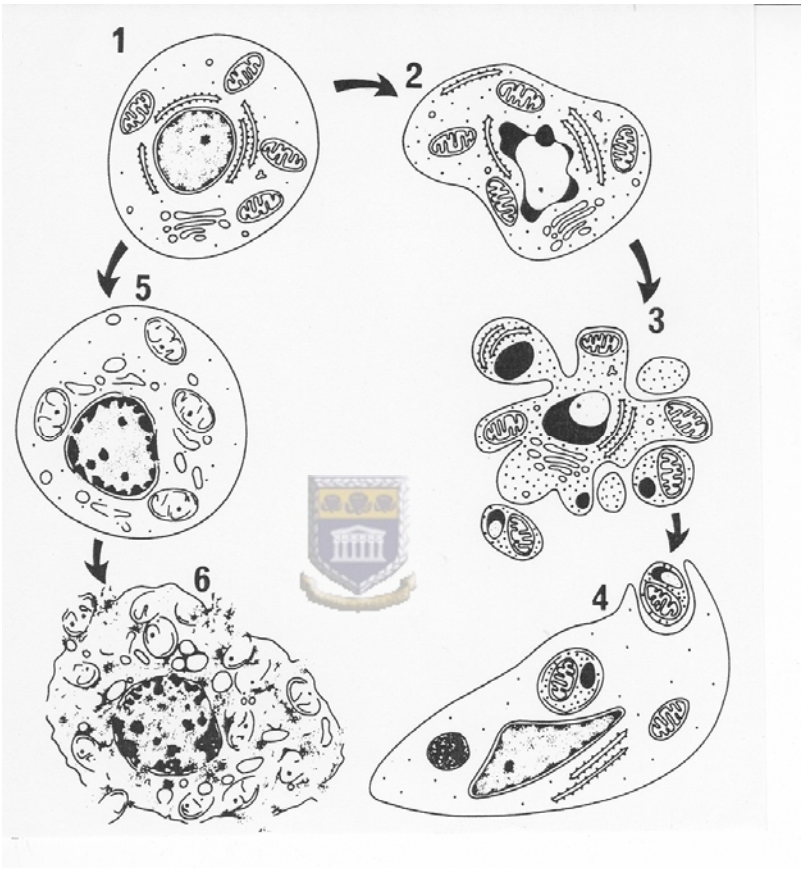


Figure 1.1. Diagram illustrating the sequential ultra-structural changes in apoptosis (right) and necrosis (left). A normal cell is shown at 1. The onset of apoptosis (2) is characterised by compaction and segregation of the chromatin into sharply delineated masses, condensation of the cytoplasm, mild convolution of the nuclear and cellular outlines. Nuclear fragmentation and marked convolution of the cellular surface with the development of protuberances which form apoptotic bodies (3), which are phagocytosed by adjacent cells (4). Signs of early necrosis in an irreversibly injured cell (5) include clumping of chromatin into ill-defined masses and gross swelling of organelles. At a later stage, membranes rupture and the cell disintegrates (6). (Reproduced from Kerr and Harmon, 1991).

Damage of the plasma membrane leads to the loss of calcium, sodium and water balance, which is followed by acidosis and osmotic shock (Bowen *et al.*, 1998). The acidosis leads to the general precipitation of chromatin, which leads to the darkened or 'pyknotic' nucleus. The chromatin initially appears fairly uniformly compacted (*pyknosis*), but with swelling of the nucleus and rupture of its membrane, the marginated chromatin masses may become evident as small discrete masses, *karyorrhexis* (Wyllie *et al.*, 1980; Majno and Joris, 1995). All basophilia are then lost leaving a fairly stained nuclear "ghost" (*karyolysis*) and the swollen cytoplasm also loses its basophilia and cell boundaries become indistinct. The inner and outer compartments of the mitochondria become distended and dense deposits of matrix lipoprotein appear. The dying cells' contents, which include many proteolytic enzymes, are released into the extra-cellular space due to the endoplasmic reticulum and lysosomes, which swell and burst. These digestive enzymes induce further autolytic destruction of the cell, which produces an inflammatory response, which could lead to further tissue damage and eventually scar formation. Specialised phagocytic cells eventually degrade the debris and the process of repair begins.

1.4.2 Apoptosis

The term apoptosis in classical Greek describes the "falling off" or "dropping off" of petals from flowers or leaves from trees, with the connotation of suicide, encapsulates many of the inherent ideas in the apoptosis concept (Kerr *et al.*, 1972). Apoptosis is a genetically controlled active cell death process implicated as being a critical physiological mechanism involved in development and tissue homeostasis (Wilson, 1998; Joza *et al.*, 2002). Apoptosis is a protective mechanism in multi-cellular organisms whereby infected, excessive, potentially dangerous or seriously damaged cells are eliminated or removed (Turk *et al.*, 2002). Cell death by apoptosis differs from necrosis on a morphological basis; however, apoptosis shares certain

common mechanisms with necrotic cell death. In certain circumstances, apoptosis is an inherently programmed event determined by an 'intrinsic clock' specific for the cell type involved (Kerr *et al.*, 1972; Ramachandra and Studzinski, 1995). The apoptotic process appears to play a complementary, but opposite role to mitosis in the regulation of animal cell populations (Kerr *et al.,* 1972). The role of apoptosis in cell population control during development has suggested that there are inherent cellular programmes, which lead the cell to self-destruction, which is fundamental to the normal development of tissues and organisms (Ramachandra and Studzinski, 1995).

Apoptosis comprises of a series of progressive steps that lead desirably to the efficient dismantling of the cell in a manner that minimises the risk of leakage of potentially harmful, cytotoxic intracellular substances into the surrounding micro-environment (Afford and Randhawa, 2000). The sequential steps of the apoptotic pathway have been described by Kerr *et al.* (1972); Wyllie *et al.* (1980); Kerr and Harmon (1991); Majno and Joris (1995); Kanduc *et al.* (2002) and; Van Cruchten and Van den Broeck (2002). Differences in the phenomena during the sequence of events during apoptosis exist, depending on the cell type, and agent or circumstance, which initiates the cell's demise. However, morphological and biochemical similarities exist which suggest that these are variants of the same biological process designed to control the size of cell populations (Ramachandra and Studzinski, 1995). In this review, consideration is given to the broad context of the phenomenon of apoptosis before spermatozoa apoptosis is considered. The initial thrust of this review focuses on the molecular mechanisms of apoptosis as identified from studies in the nematode *Caenorhabditis elegans* and also reviews the physiological and morphological basis of the apoptotic process.

1.4.2.1 The genetic control of apoptosis using Caenorhabditis elegans as a model system

The nematode, *Caenorhabditis elegans* (*C. elegans*) has been used as a model for defining and understanding the core pathway of programmed cell death or apoptosis (Billig *et al.*, 1996; Blanco-Rodrĩguez, 1998; Sinha Hikim and Swerdloff, 1999; Hengartner and Bryant, 2000; Kanduc *et al.*, 2002). The elucidation of apoptosis in this model organism is instrumental to the functions of the cell death genes and the mechanisms by which they affect programmed cell death. The genetic pathway of programmed cell death in the worm serves as a basis for understanding apoptosis in mammalian systems since they have been implemented by the same highly conserved pathway (Verhagen and Vaux, 1999). The genetic control of apoptosis as studied in *C. elegans* is summarised in Figure 1.2. Also shown is the apoptotic pathway as it occurs in *Drosophilia* and mammals.

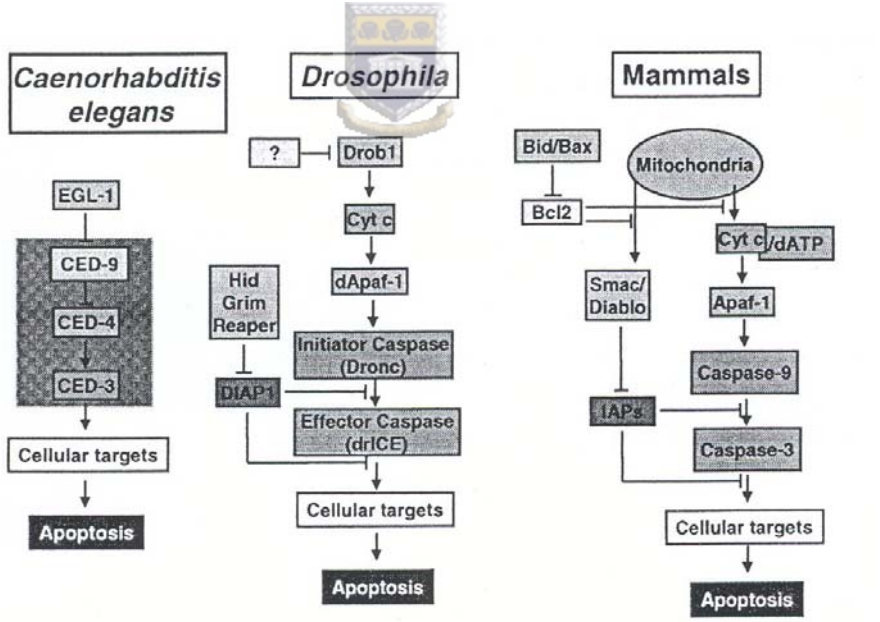


Figure 1.2. Core elements of the apoptotic pathways. The core apoptotic machinery is shown for *Caenorhabditis elegans*, *Drosophilia* and for mammals. The original core of death genes identified in *C. elegans* (*ced-3*, *ced-4*, and *ced-9*) is shown. The apoptotic pathway for *Drosophilia* has not been outlined in the text (Reproduced from LaCasse *et al.*, 2004).

The apoptotic pathway during the development of *C. elegans* is regulated by 14 genes and has been classified into four distinct steps (Bowen *et al.*, 1998; Hengartner and Bryant, 2000). There are genes involved in triggering cell death, genes involved in the execution of the apoptotic process, those required for the recognition and the engulfment of the dying cell and genes involved in the disposal of the remnants of the dying cell. Initiation of apoptosis occurs in cell types committed to the apoptotic fate and is controlled by several genes, which include: *ces-1* and *ces-2* (*ces*=cell death specification). *Ces-1* promotes cell death where *ces-2* inhibits cell death. Another gene, *egl-1* can inhibit entry into the apoptotic process (*egl-1*=egg-laying defective gene). The activities of these genes, which specify cell death will influence the subsequent action of *ced-3* and *ced-4* and dictate the final outcome of life and death. The execution of apoptosis of the selected cells is genetically controlled by *ced-3*, *ced-4* and *ced-9* (*ced*=cell death gene). *Ced-3* and *ced-4* are essential for programmed cell death in *C.elegans* as the execution of apoptosis depends on these genes (Samali *et al.*, 1996; Cohen, 1997; Nuñez *et al.*, 1998; Verhagen and Vaux, 1999; Hengartner and Bryant, 2000; Joza *et al.*, 2002). *Ced-3* is similar to the human interleukin 1β-converting enzyme (ICE), a member of death-inducing cysteine proteases called caspase, which are key effectors in mammalian cell apoptosis (Bowen *et al.*, 1998; Gartner and Hengartner, 1998).

The multiple caspases function together in the proteolytic cascade and are important in the execution of apoptosis. *Ced-9*, inhibit or negate the apoptotic-promoting effects of *ced-3* and *ced-4*. The mammalian *ced-9* homologue is bcl-2 of which several related molecules have been identified (Hale *et al.*, 1996; Harvey and Kumar, 1998; Wilson, 1998). Bcl-2 has several members of the family, of which one group inhibits programmed cell death while the other group promotes cell death (Blanco-Rodrĩguez, 1998; Bowen *et al.*, 1998). Several genes have been identified for the engulfment of the dying cells. One

group of genes consisting of genes *ced- 1*, *ced-6*, *ced-7* or *ced-8* causes major defects while the other group, consisting of *ced-2*, *ced-5* or *ced-10* probably plays a role in the regulation of the recognition of the dying cell by the phagocytosing neighbour (Bowen *et al.*, 1998; Hengartner and Bryant, 2000). Another gene, *Nuc-1* is required in the phagocytosing host for the degradation of DNA.

The caspases (cysteine-aspartate-directed proteases) have been shown as crucial mediators of programmed cell death and function together to form a proteolytic caspase cascade activated by apoptotic stimuli (Cohen, 1997; Bantel *et al.*, 1998; Green and Kroemer, 1998; Harvey and Kumar, 1998; Nuñez *et al.*, 1998; Stennicke and Salvesen 1998; Slee *et al.*, 1999; Joza *et al.*, 2002; Salvesen, 2002; LaCasse *et al.*, 2004; Said *et al.*, 2004). The execution of apoptosis requires the coordinated action of several aspartate-specific cysteine proteases, which are responsible for the cleavage of key enzymatic and structural substrates to facilitate the disassembly of the cell undergoing apoptosis (Cohen, 1997; Nuñez *et al.*, 1998). Some of these proteins involved include: PARP (DNA repair enzyme), DNA-PK (DNA-dependant protein kinase involved in DNA double-strand-break repair process), lamins (major cytoskeletal, structural component of the nucleus), actin and fodrin (membrane-associated cytoskeletal protein related to membrane blebbing) and nuclease (DNA cleavage into internucleosomal fragments). The key elements of the genetic basis of apoptosis described, provides a basis for understanding the pathways initiated during the apoptotic process in mammalian cells.

1.4.2.2 Mechanisms of Apoptosis Stimulation

A cell will undergo apoptosis as a result of information received from its environment interpreted in the context of internal information, such as its cell type, state of maturity and developmental history (Hale *et al.*, 1996). The

pathways to activate apoptosis are different in various cell types; however, the mechanism of death itself may be the same, that is, a final common pathway (Cohen, 1993). A cell first encounters a signal to activate the relevant genetic machinery before it enters into the apoptotic pathway (Afford and Randhawa, 2000). A stimulus, which originates from outside the cell exists which can advance or delay apoptotic cell death. Agents that can penetrate the cell directly, and modulate the apoptotic cascade in the absence of specific cell surface receptors can trigger apoptotic stimulation. These agents include: heat shock, stress factors, free radicals, ultraviolet radiation, numerous drugs and synthetic peptides, toxins and potent lymphocyte enzymes. Two distinct pathways exist for the initiation of apoptosis: extrinsic or receptor-linked apoptosis and intrinsic or mitochondria-mediated apoptosis (Bantel *et al.*, 1998; Joza *et al.*, 2002; Kiechle and Zhang, 2002; Turk *et al.*, 2002; Vaughan *et al.*, 2002; LaCasse *et al.*, 2004). Both of these are the best studied apoptotic-initiating pathways and will be briefly outlined below.

1.4.2.2 (i) Extrinsic or Receptor-Linked apoptotic pathway

The induction of apoptosis occurs via death receptors (cell surface receptors) that transmit apoptosis signals initiated by specific ligands (Green and Kroemer, 1998; Sinha Hikim and Swerdloff, 1999; Slee *et al.*, 1999; Scheider and Tschopp, 2000; Joza *et al.*, 2002; Turke *et al.*, 2002; Sinha Hikim *et al.*, 2003; LaCasse *et al.*, 2004; Fadeel and Orrenius, 2005; Jin and El-Deiry, 2005). This review focuses on apoptosis controlled by cytokines; specifically Fas ligand (FasL) and tumour necrosis factor receptor 1 (TNF) as they have been extensively studied and brief illustrations of these receptor mediated apoptotic pathways will be presented.

The binding of FasL to Fas forms a cluster and a complex beneath the cell membrane with a number of cytosolic proteins to form the active “death domain”, Fas-associating protein with death domain (FADD) or MORT1

(Nagata, 1997; Ormerod, 1998; Nagata, 2000; Joza *et al.,* 2002; Van Cruchten and Van den Broeck, 2002; Wajant, 2002; LaCasse *et al.*, 2004). Fas-induced apoptosis is illustrated in Figure 1.3. This receptor-ligand complex then becomes the recognition molecule for a precursor enzyme, caspase- 8 (FLICE/MACH), which in turn may induce self-activation of the protease domain. The recruitment of caspase- 8 may result in the self-activation of proteolytic activity and trigger the interleukin-1ß-converting enzyme (ICE) protease cascade. Caspase-8 is the major initiator caspase identified in death receptor signalling, while caspase-10 may be involved in initiation (Chen and Wang, 2002). Caspase-2, an initiator caspase has been shown to associate with the cytoplasmic regions of death receptors in response to apoptotic stimuli (Bantel *et al.*, 1998; Joza *et al*., 2002; Said *et al.*, 2004). The activated ICE members can cleave various substrates and cause morphological changes to the cells and nuclei. Presently, 14 known caspase family members exist which form an intracellular proteolytic cascade that modulates many cellular events in the apoptotic pathway (Afford and Randhawa, 2000).

The binding of TNF to TNF receptor (TNFR1) recruits TRADD via interactions between death domains. The death domain of TRADD then recruits FADD/MORT1 in a pathway to activate caspase- 8 and induce apoptosis as shown in Figure 1.3 (Cohen, 1997; Nagata, 1997; Nagata, 2000; Joza *et al.*, 2002; LaCasse *et al.*, 2004). In another pathway, receptor interacting protein (RIP) binds to TRADD and transduces an apoptotic signal through death domain. RIP together with TNF receptor-associated factor (TRAF2) activates NF-kB, which may induce the expression of survival genes.

The Fas receptor and tumour necrosis factor receptor 1 (TNFR1) both trigger apoptosis via FADD (Fas-associating protein with death domain), which results in a sequence of events leading to apoptotic death of the cell. The stimulation of some cell surface molecules, such as Fas ligand (FasL) and

tumour necrosis factor (TNF) can often induce cell death by apoptosis, thereby killing the cell within hours (Williams and Smith, 1993; Nagata, 1997; Nagata, 2000). Other death receptors include: TNF receptor-related-apoptosis-mediated-protein (TRAMP) and two receptors which bind TNF-related apoptosis-inducing ligand (TRAIL), called TRAIL-R1 and TRAIL-R2 (Bantel *et al.*, 1998).

1.4.2.2 (ii) Intrinsic or mitochondria-mediated apoptotic pathway

Mitochondria play an important role in the promotion of apoptosis through the release of cytochrome c (Halestrap *et al.*, 2000). The intrinsic pathway is triggered by stress stimuli, including growth factor deprivation and DNA damage (Schuler and Green, 2001). This pathway involves the release of an extrinsic protein, cytochrome c on the outer surface of the inner mitochondrial membrane from the mitochondria during apoptosis (Bantel *et al.*, 1998; Green and Kroemer, 1998; Nuñez, *et al.*, 1998; Wilson, 1998; Budihardjo *et al.*, 1999; Porter and Jänicke, 1999; Van der Heiden and Thompson, 1999; Joza *et al.*, 2002; Kiechle and Zhang, 2002; Van Cruchten and Van den Broeck, 2002; Sinha Hikim *et al.*, 2003; LaCasse *et al.*, 2004; Fadeel and Orrenius, 2005). The other mitochondrial proteins released from the mitochondrial intermembrane space during apoptosis are Smac/DIABLO and HtrA2/Omi (Joza *et al.*, 2002; Turke *et al.*, 2002; LaCasse *et al.*, 2004). They promote cell death by inactivating inhibitors of apoptosis proteins (IAPs), thereby releasing caspases from inhibition by IAPs.

Cytochrome c is released due to a decrease in the inner mitochondrial transmembrane potential. Caspase-2 engages the mitochondria-dependant apoptotic pathway by inducing the release of cytochrome c and other apoptogenic factors (Smac and HtrA2) in the cell cytoplasm. (Guo *et al*., 2002; Zhivotosky and Orrenius, 2005). Proteins belonging to the Bcl-2 family appear to regulate the membrane permeability to ions and possibly to

cytochrome c (Burlacu, 2003). The regulation of apoptosis by Bcl-2 will be briefly reviewed below. Once released, cytochrome c forms a subunit with other cytosolic protein factors, apoptotic protease activation factor-1 (Apaf-1), procaspase- 9 and either ATP or dATP to activate caspase-3 (Figure 1.3). The action of cytochrome c and dATP causes a conformational structural change of the Apaf-1 protein to form an apoptosome. Caspase-9 is the initiator caspase for mitochondrion-dependant apoptosis (Chen and Wang, 2002). Apaf-1 binds to ATP/dATP and hydrolyses it to ADP or dADP, respectively resulting in the formation of Apaf-1/cytochrome c complex. A conformational change occurs in Apaf-1 and a region, the caspase recruitment domain (CARD) is exposed allowing for the homophilic binding of caspase-9. Thus, this complex recruits and activates procaspase-9 and activated caspase-9 is released from the complex to cleave and activate downstream caspases, caspase-3, -6 and –7.

The induction of apoptosis via the intrinsic or extrinsic apoptotic pathways results in the activation of an initiator caspase, which activates a cascade of events leading to the activation of effector caspases, responsible for the cleavage of key cellular proteins that lead to the typical morphological changes observed in cells undergoing apoptosis. Caspase-8 and caspase-10 are initiator caspases in death receptor-mediated apoptosis, while caspase-9 is the initiator caspase in mitochondrion-dependent apoptosis (Chen and Wang, 2002). These pathways differ in one fundamental aspect: one is “external” in that it is promoted by a series of specific external ligands operating through defined transmembrane receptors; the other is an internal system where activation of the effector enzymes is induced by intracellular changes, involving the mitochondria (Garland, 2000; Joza *et al.*, 2002; Turke *et al.*, 2002). Despite the difference in the initiation manner, the extrinsic and intrinsic pathways merge at the level of caspase-3 and 7 and once activated,

these cleave intracellular targets ultimately leading to the manifestations of apoptosis (Figure 1.3).

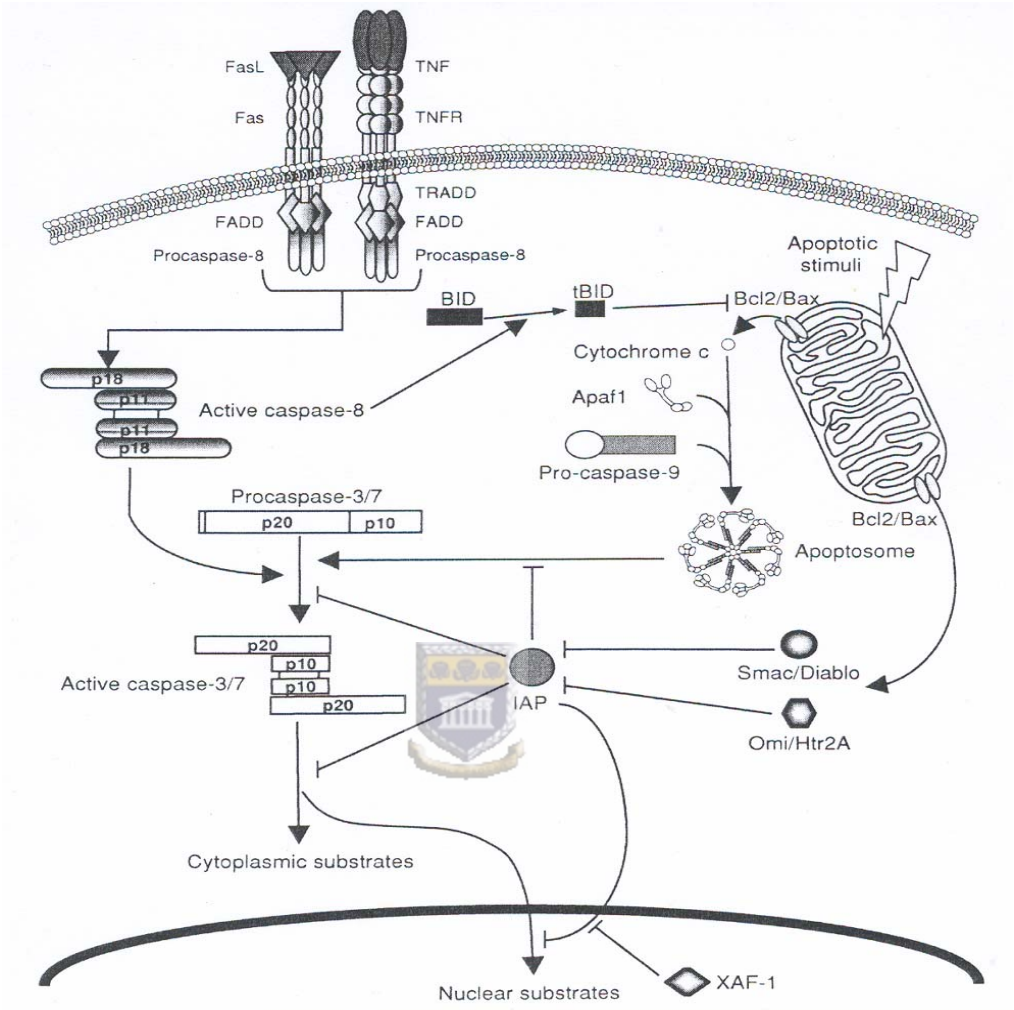


Figure 1.3. Intrinsic and extrinsic apoptotic pathways in mammalian cells. Intracellular stress results in the activation of the mitochondrial, or intrinsic pathway which leads to cytochrome c release, apoptosome formation, and caspase activation. Extracellular ligand binding to death receptors triggers the extrinsic pathways that can either directly result in the activation of the caspases, or require further amplification through the mitochondrial pathway dependant on the cell type. Both apoptotic signalling pathways converge at the level of effector caspases, such as caspase -3 and -7 (Reproduced from LaCasse *et al.*, 2004).

1.5 Morphological and Biochemical Changes during Apoptosis

Apoptosis differs from necrosis in that at the tissue level, little or no inflammation is produced, since shrunken portions of the cell are engulfed by the neighbouring cells, especially macrophages rather than be released into the extracellular fluid (Ramachandra and Studzinski, 1995). Kerr *et al.* (1972) reviewed the distinctive morphological process of apoptosis and the importance as an important basic biological phenomenon. Gross cellular reorganisation at the nuclear, cytoplasmic and cell membrane levels occur during apoptosis.

1.5.1 Nuclear Alterations in Apoptosis

Chromatin condensation and nuclear envelope breakdown occur during apoptosis however, the exact mechanisms are unclear (Hale *et al.*, 1996). Lamins are intermediate filaments that are associated with the inner nuclear envelope and organise the nucleoskeleton to provide a framework for the attachment of chromatin to the nuclear envelope (Hale *et al.*, 1996). During apoptosis lamin disassembly occurs by proteolysis, which may promote the formation of fragments of DNA by allowing the release of matrix attachment regions to give access to the endonucleases. The nucleus shrinks and its chromatin becomes very dense, collapsing into patches, then into crescents in tight apposition to the nuclear envelope, and finally in many cells into one or several dense spheres (Kerr and Harmon, 1991; Cohen, 1993; Ormerod, 1998; Gewies, 2003). The chromatin becomes pyknotic and packed into smooth masses against the nuclear membrane. Wyllie *et al.* (1980) showed that the nuclear outline is abnormally convoluted, becomes grossly indented with discrete nuclear fragments. Accompanying the condensation of chromatin into crescent-shaped caps at the nuclear periphery is nucleoli disintegration and a reduction in nuclear size occurs (Arends *et al.*, 1990). There is a distinct dilation of the nuclear envelope accompanied by nuclear budding (blebbing) and the blebs are often filled with chromatin.

This important characteristic of apoptosis, DNA fragmentation, results from the activation of endonucleases. These endonucleases degrade the chromatin structure into oligonucleozome-sized fragments of ~300 kb and subsequently into smaller DNA pieces of about 50 kb lengths. The ladder pattern produced on agarose gel during apoptotic cell death is the result of cleavage of DNA between the nucleosomes into these nucleosomal fragments. Nagata (2000) has identified a specific DNase (CAD, caspase-activated DNase) that cleaves chromosomal DNA in a caspase-dependant manner when apoptotic stimuli attack the cells. Cells, which are induced to undergo apoptosis, cleave ICAD (inhibitor of CAD) to dissociate the CAD: ICAD complex allowing CAD to cleave chromosomal DNA. DNA fragmentation was initially regarded as a hallmark of apoptosis and a critical process in apoptosis. Bowen *et al.* (1998) demonstrated that nuclear changes of apoptosis occur without endonuclease activation or oligonucleotide production.

1.5.2 Cytoplasmic Alterations in Apoptosis

Concomitant with these early nuclear changes, the cytoplasm shows signs of condensation, microvilli (if present) have disappeared and blunt protuberances have formed on the cell surface (Wyllie *et al.*, 1980; Kerr and Harmon, 1991). Cytoplasmic condensation produces a marked crowding of organelles and translucent cytoplasmic vacuoles are formed. Shrinkage in total cell volume, an increase in cell density, compaction of some cytoplasmic organelles and dilatation of the endoplasmic reticulum accompany morphological changes during apoptosis (Ramachandra and Studzinski, 1995; Gewies, 2003). However, it was shown that the mitochondria maintain their integrity throughout the apoptotic process (Arends *et al.*, 1990; Majno and Joris, 1995; Ramachandra and Studzinski, 1995). Constriction of the nucleus and the cytoplasm into multiple, small membrane-bound apoptotic bodies, also referred to as ‘blebbing’ becomes evident as protuberances on the cell surface. These membrane-bound apoptotic bodies of spherical or ovoid

shapes of various sizes and numbers are either extruded into the lumen from epithelial surfaces or phagocytosed by surrounding phagosomes. Kerr *et al.* (1972) initially presented these ultra-structural features of apoptosis and suggested that it is an active, inherently programmed phenomenon.

One of the cellular modifications during apoptosis in cells occurs in the cytoskeleton where the microtubules and microfilaments are involved (Samali *et al.*, 1996). It has been proposed that actin depolymerization activated by protein kinase C, is essential for the budding process that produces apoptotic bodies (Bowen *et al.*, 1998). Apoptosis is also associated with enhanced transglutaminase activity and in the presence of Ca^{2+}, this enzyme catalyses the reaction which produces a highly cross-linked scaffold of proteins in these apoptotic bodies (Hale *et al.*, 1996; Bowen *et al.*, 1998).

1.5.3 Cell Membrane alterations in apoptosis

There are a number of biochemical changes, which occur in the plasma membrane of apoptotic cells. One of these changes is an alteration of carbohydrates on the plasma membrane of the apoptotic cell, which could play a role in the preferential binding of macrophages to apoptotic cells (Samali *et al.*, 1996; Bowen *et al.*, 1998). Apoptotic cells have a reduced anionic charge, which implies the loss of terminal sialic acid residues, thereby exposing glycoprotein side-chain sugars (Bowen *et al.*, 1998). These cells display cell-surface markers that ensure the swift recognition and removal of phagocytic cells (Hale *et al.*, 1996). Normally the anionic membrane phospholipid phosphatidylserine (PS) is located in the inner plasma membrane, but during apoptosis PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment (Hale *et al.*, 1996). A third mechanism involves the secretion of thrombospondin by the macrophages to form a molecular bridge between the apoptotic plasma membrane and the macrophage membrane (Bowen *et al.*, 1998). These

phenomena are important events in the recognition and removal of apoptotic cells by macrophages.

1.5.4 Phagocytic Recognition of Apoptotic Bodies

The mechanisms in phagocytic recognition involve several molecules on the surface of the apoptotic cell. Ravichandran (2003) has proposed that “eat-me” signals on apoptotic cells serve as markers for phagocytes to specifically recognise and ingest them either by direct or indirect means. The engulfment of apoptotic cells occurs by a two-step process (Rovere-Querini and Dumitriu, 2003). The first step involves recognition of the apoptotic cell by the phagocyte while the second step involves ingestion of the apoptotic cell.

Apoptotic cells have a reduced anionic charge, which implies the loss of terminal sialic acid residues thereby exposing glycoprotein side-chain sugars on the surfaces of the apoptotic bodies (Hale *et al.*, 1996; Bowen *et al.*, 1998). Apoptotic cells also express phosphatidylserine in the outer layers of their plasma membranes following translocation from the inner cell membrane. These cell membrane alterations permit the early recognition of dead cells by macrophages and adjacent cells by phagocytosis. Possible mechanisms involved in phagocytic recognition include several cell surface molecules on the phagocyte and apoptotic cells (Hale *et al.*, 1996; Afford and Randhawa, 2000; Ravichandran, 2003).

One key molecule of the apoptotic cell involved in the recognition process is intracellular adhesion molecule 3 (ICAM) along with anionic phospholipids and phosphatidylserine (PS), which interacts with molecules on the phagocyte. The surface molecules on the phagocyte cell include: class A scavenger receptors macrosialin and ABC 1, the vitronectin receptor, and the class B receptors CD 36 and CD 14. The interaction between phagocyte and apoptotic cell involves the binding of ICAM 3 to CD 14 on the macrophage.

Also, PS attaches to the vitronectin receptor or CD 36 of the phagocyte and possibly occurs due to thrombospondin, which acts as a bridging molecule. The phagocytic recognition of these apoptotic bodies by macrophages is a crucial step for normal tissue homeostasis. Defects in phagocyte recognition of apoptotic cells may lead to the persistence of inflammatory disease where apoptotic cells eventually assume necrotic morphology (Afford and Randhawa, 2000).

1.6 Regulators of Apoptosis

The activation of the apoptosis-signalling pathway occurs in response to various regulatory factors. There are a number of regulators of cell death and the effects appear to depend on the cell type and the stimulus for apoptosis. The bcl-2 family of proteins play an important role in the regulation of apoptosis and a brief overview of some of these regulators will be discussed. Furthermore the regulatory role of p53 during the intrinsic apoptosis pathway will be reviewed.

1.6.1 Bcl-2

A key component of apoptosis activation involves the activation of the caspases that participates in a cascade of events, which ultimately cause disassembly of the cell. The initiation of the caspases proteolytic cascade is regulated by proteins of which the Bcl-2 family plays a pivotal role in the control of apoptosis (Van der Heiden and Thompson, 1999; Joza *et al.*, 2002; Burlacu, 2003).

The Bcl-2 protein is a membrane protease that is localised to the outer mitochondria, endoplasmic reticulum membrane, and nuclear envelope (Van der Heiden and Thompson, 1999; Burlacu, 2003). Eighteen members for the Bcl-2 family have been identified and divided into a subgroup of anti-apoptotic members, such as (Bcl-2, Bcl-X_L, Mcl-1, A1, Bcl-W) and a large group of pro-

apoptotic members, such as (Bax, Bak, Bok, Bad, Bid, Bim, Blk, BNIP and *egl-1*) (Goss *et al.*, 1999; Pepper and Bentley, 2000; Joza *et al.*, 2002; Burlacu, 2003). The balance between the anti-apoptotic and the pro-apoptotic members are critical in determining whether a cell undergoes apoptosis. These proteins regulate apoptosis in part by affecting the mitochondria compartmentalization of cytochrome c (Van der Heiden and Thompson, 1999).

The Bcl-2 family, the most prominent regulators of apoptosis exert their apoptosis-regulatory effects primarily by regulating mitochondrial alterations that precede the activation of caspases and nucleases (Pepper and Bentley, 2000; Joza *et al.*, 2002). The pro-apoptotic bcl-2 proteins are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupt the normal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome c and other pro-apoptotic molecules from the intermembrane space (Goss *et al.*, 1999; Van der Heiden and Thompson, 1999; Burlacu, 2003). On binding to Apaf-1, these mediators activate caspase-9, which triggers the cascade of effector caspases ultimately resulting in irreversible cell death (Figure 1. 3).

1.6.2 p53

Intrinsic stresses such as oncoproteins, DNA damage, hypoxia and survival factor deprivation, can activate the intrinsic apoptotic pathway (Agarwal *et al.*, 1998; Schuler and Green, 2001; Gewies, 2003). p53, a tumour suppressor protein is present in the mitochondria and appears to contribute to the process of apoptosis (Choisy-Rossi *et al.*, 1999; Chang, 2002). p53 is a sensor of cellular stress and is a critical activator of the intrinsic pathway. It initiates apoptosis by transcriptionally activating pro-apoptotic Bcl-2 family members

(bax and bak) and repressing antiapoptotic proteins (bcl-X_L and bcl-2). The regulated release of proapoptotic factors from the mitochondria initiates the release of cytochrome c from the mitochondria. A study by Deng and Wu (2000) found that the subcellular localization of Bax protein plays a key role in deciding the fate of the cell, as the translocation of Bax from the cytosol to the mitochondria is a critical step in p53-mediated apoptosis. The release of cytochrome c from the mitochondria occurs shortly after p53 accumulation on the mitochondrial membrane and induces the formation of the apoptosome. Procaspase-9 is activated, which binds to the apoptosome and further mediates the activation of effector caspases (Schuler and Green, 2001; Gewies, 2003). The execution of p53-mediated apoptosis proceeds through the effector caspases, their mechanism of action depends on the experimental context (Schuler and Green, 2001). Thus, it has been proposed that the intrinsic pathway is necessary for both stress induced and p53-dependant caspase activation.

The p53 gene is highly expressed in the testis and is known to be involved in apoptosis, suggesting that it is one of the major causes of germ cell loss in the testis (Ohta *et al.*, 2003). The findings of a study suggested that p53 mediates spontaneous testicular germ cell apoptosis in mice and failure to remove defective germ cells by this mechanism results in the increased percentages of abnormal sperm and reduced fertility (Yin *et al.*, 1998). It has been postulated that p53 is not active in normal adult human testis, indicating that there are p53-independent mechanisms of spontaneous germ cell apoptosis (Print and Loveland, 2000; Oldereid *et al.*, 2001).

1.7 Germ Cell Apoptosis

Spermatogenesis, the dynamic process of cell differentiation is a precisely controlled and cyclical timed process comprising of proliferation of spermatogonia, meiotic divisions of spermatocytes, and the differentiation of

spermatids into spermatozoa (Blanco-Rodrĩguez, 1998; Blanco-Rodrĩguez and Martĩnez-Garcĩa, 1999; Fujisawa *et al.*, 1999; Print and Loveland, 2000; Kierszenbaum, 2001; Martinčič *et al.*, 2001; Kim *et al.*, 2002; Sinha Hikim *et al.*, 2003). Sertoli cells in close contact with the germ cells facilitate spermatogenesis by providing structural and nutritional support for the neighbouring germ cells. The proliferation and differentiation of germ cells depends on the release of two hormones from the anterior pituitary gland, namely follicle-stimulating hormone (FSH) and luitenizing hormone (LH) (Sassone-Corsi, 1997). The removal of these hormones induces germ cell apoptosis through indirect effects (Print and Loveland, 2000). Spermatogenesis is accompanied by germ cell apoptosis in the seminiferous epithelium and can also be triggered by a variety of stimuli (Billig *et al.*, 1996; Blanco-Rodrĩguez, 1998; Fujisawa *et al.*, 1999; Sinha Hikim and Swerdloff, 1999; Kierszenbaum, 2001; Martinčič *et al.*, 2001; Sinha Hikim *et al.*, 2003).

Kim *et al.* (2002) proposed four possible functional roles for the presence of apoptosis during spermatogenesis. He postulated that germ cell apoptosis is necessary to maintain an optimal germ cell to Sertoli cell ratio, to eliminate any abnormal germ cells, the formation of the blood-testis barrier requires the elimination of excessive germ cells and an apoptotic surge of germ cell apoptosis occurs prior to puberty regulating the ratio of germ cells to Sertoli cells. Approximately 75% of the spermatogonia die in the process of programmed cell death before reaching maturity (Print and Loveland, 2000; Matinčič *et al.*, 2001). A dramatic increase in germ cell apoptosis occurs in some pathological conditions, which include idiopathic infertility in males (Sassone-Corsi, 1997).

It has been shown that spontaneous apoptosis has been observed frequently in spermatocytes, less frequently in spermatogonia and seldom in spermatids (Sasagawa *et al.*, 2001). The presence of apoptosis in human germ cells in the testis specifically in spermatocytes has been reported by Fujisawa *et al.*

(1999). They found that the rate of apoptosis decreased in the testis of infertile men; however, the mechanism remains to be elucidated. A study undertaken by Martinčič *et al.* (2001) determined the presence and frequency of germ cell apoptosis in the human testis from infertile patients while the Sertoli cells were not apoptotic. The exact incidence of male adult germ cell apoptosis remains unclear, since not all degenerating germ cells display the classical morphology of apoptosis (Print and Loveland, 2000; Kierszenbaum, 2001). It has been postulated that androgen withdrawal alters the expression of caspase activity in different cell types of human seminiferous epithelium (Tesarik *et al.*, 2002). The Fas-system has been shown to participate in the regulation of the spontaneous germ cell apoptosis (Richburg, 2000; Riccioli *et al.*, 2003). It has been hypothesized that in the normal state, Sertoli cells express Fas ligand and this triggers apoptosis of a few Fas-positive germ cells revealing a paracrine control between the Sertoli and germ cells (Lee *et al.*, 1997; Lee *et al.*, 1999; Pentikäinen *et al.*, 1999; Kierszenbaum, 2001). They concluded that this death-delivering process ensures testicular homeostasis by the elimination of ill-supported germ cells. The development of mature sperm is the product of a precisely regulated sequence of events in which germ cell proliferation, differentiation, self-renewal and apoptosis is controlled.

1.8 Sperm Apoptosis

The debate regarding sperm apoptosis remains a controversial issue as uncertainty exists as to whether apoptotic sperm are sperm with poor functional activity or in fact indicative of sperm that have failed to complete maturation during spermiogenesis. Several investigations demonstrated the occurrence of possible apoptosis in ejaculated human sperm (Baccetti *et al.*, 1996; Blanc-Layrac *et al.*, 2000; Oosterhuis *et al.*, 2000; Shen *et al.*, 2002; McVicar *et al.*, 2004) and in bull sperm (Anzar *et al.*, 2002). A study conducted by Lachaud *et al.* (2004) examined the ability of human

spermatozoa to initiate apoptosis *in vitro* and their findings suggested that these cells lack the capacity to initiate the apoptotic pathway of cell death.

The ordered sequence of events lead to apoptotic cell death which has been described previously under the headings numbered 1.4.2 and 1.5. The events that characterise the apoptotic cell death can be analysed and quantified by flow cytometric or microscopy based methods. Flow cytometry can measure the typical changes of cells in their morphological and physical properties. Flow cytometry is a method which is able to provide quantitative information regarding cell death in a variety of biological and clinical studies. The flow cytometric-based method is the most objective and statistically robust measure of all tests in the infertility clinic (Evenson *et al.*, 2002). The advantages of this technique include the measurement of apoptosis at the single cell level, the automatic analysis of thousands of cells in a few seconds with precision as well as less subjective and statistically more reliable than microscopic results (Marchetti *et al.*, 2002). The assays described below will be based on studies of spermatogenic cells and will focus on selected key apoptotic events.

1.8.1 Assays that measure DNA fragmentation

The evaluation of DNA integrity in human spermatozoa has been used as a measure to indicate apoptosis in human sperm (Sakkas *et al.*, 1999b; Irvine *et al.*, 2000; Shen *et al.*, 2002). One important biochemical characteristic of apoptotic death is the activation of endogenous endonucleases, which induce numerous breaks following degradation of DNA and chromatin condensation. In a study evaluating sperm of fertile and infertile patients, it was found that apoptotic sperm is present in higher fractions in the infertile population (Baccetti *et al.*, 1996). The authors demonstrated the presence of ultra-structural features typical of the apoptosis models suggested by Kerr *et al.* (1972). Other studies have shown that a negative correlation exists between

the number of sperm with fragmented DNA and abnormal semen parameters (Sun *et al.*, 1997; Sakkas *et al.*, 1999a; Gandini *et al.*, 2000). Gandini *et al.* (2000) reported that a high DNA fragmentation is one of the characteristics of spermatogenetic failure. However, a study by Muratori *et al.* (2000) suggested that no significant relationship exists between DNA breakage and apoptosis. It should be emphasised that DNA fragmentation is not only caused by apoptosis as described under the heading 1.3.1.

A wide range of assays is available for the detection and identification of fragmented DNA in apoptotic cells and has been used to identify apoptotic sperm cells in ejaculate samples either using microscopy-based approaches or flow cytometry. These assays are briefly outlined below. One assay used to quantify DNA damage is the sperm chromatin structure assay as reported by several authors (Sailer *et al.*, 1995; Aravindan *et al.*, 1997; Evenson *et al.*, 1999; Spanò *et al.*, 1999; Barroso *et al.*, 2000; Blanc-Layrac *et al.*, 2000; Spanò *et al.*, 2000; Larson *et al.*, 2001; Zini *et al.*, 2001; Evenson *et al.*, 2002; Giwercman *et al.*, 2003; Larson-Cook *et al.*, 2003; Alvarez *et al.*, 2004; Moustafa *et al.*, 2004). This approach is based on the metachromatic properties of acridine orange to monitor the susceptibility of sperm chromatin DNA to acid-induced denaturation *in situ* (Spanò *et al.*, 1999; Spanò *et al.*, 2000).

Another method which determines sperm DNA fragmentation is the terminal deoxynucleotidyl transferase-mediated dUTP [deoxyuridine triphosphate] nick-end labelling (TUNEL) assay (Sailer *et al.*, 1995; Baccetti *et al.*, 1996; Aravindan *et al.*, 1997; Sun *et al.*, 1997; Lopes *et al.*, 1998; Baccetti *et al.*, 1999; Barrosa *et al.*, 2000; Donnelly *et al.*, 2000; Gandini *et al.*, 2000; Muratori *et al.*, 2000; Oosterhuis *et al.*, 2000; Oldereid *et al.*, 2001; Zini *et al.*, 2001; Anzar *et al.*, 2002; Marchetti *et al.*, 2002; Sakkas *et al.*, 2002; Shen *et al.*, 2002; Benchaib *et al.*, 2003; Henkel *et al.*, 2003; Sakkas *et al.*, 2003;

Henkel *et al.*, 2004; Lachaud *et al.*, 2004; Liu *et al.*, 2004; Marchetti *et al.*, 2004; Henkel *et al.*, 2005b). This approach identifies single- and double-stranded DNA breaks by labelling the free 3'-OH termini using exogenous terminal deoxynucleotidyl transferase (TdT) (Gorczyca *et al.*, 1993; Evenson *et al.*, 2002; Sakkas *et al.*, 2002). The *in situ* nick translation assay quantifies the incorporation of biotinylated dUTP at single-stranded DNA breaks in a reaction catalyzed by the template-dependant enzyme, DNA polymerase (Gorczyca *et al.*, 1993; McKelvey-Martin *et al.*, 1997; Manicardi *et al.*, 1998; Irvine *et al.*, 2000; Evenson *et al.*, 2002).

The modified single cell gel electrophoresis (COMET) assay has been applied in numerous studies (Hughes *et al.*, 1996; Aravindan *et al.*, 1997; McKelvey-Martin *et al.*, 1997; Hughes *et al.*, 1999; Donnelly *et al.*, 2000; Irvine *et al.*, 2000; Chan *et al.*, 2001; Donnelly *et al.*, 2001; Larson *et al.*, 2001; Morris *et al.*, 2002; Sakkas *et al.*, 2002; McVicar *et al.*, 2004). The COMET assay typically measures single and double-stranded DNA breaks in individual mammalian cells (McKelvey-Martin *et al.*, 1993; Evenson *et al.*, 2002). The enzyme-linked immunosorbant assay (ELISA) has been applied in a study to detect single strand breaks immunochemically in cells by Hughes *et al.* (1999). This assay quantifies the histone-complexed DNA fragments.

1.8.2 Assays that measure plasma membrane alterations

Apoptosis induces changes at the plasma membrane level including changes in the permeability and alterations in cell membrane lipids. These changes in the cell surface have been described previously under 1.5.3. The alteration of phospholipids distribution is assessed using the Annexin-V assay. This assay is used to detect the number of apoptotic sperm characterised by the translocation of PS on the outer leaflet of the plasma membrane (Van Engeland *et al.*, 1998). In the early stages of apoptosis, PS is translocated from the inner leaflet of the plasma membrane to the outer leaflet. Annexin-V

is a calcium dependant phospholipid that has a high affinity for PS to detect apoptotic cells. The simultaneous application of propidium iodide (PI), allows the discrimination of necrotic cells from the Annexin-V positively stained populations. The procedure for this assay has been described in more detail in Chapter 2. The Annexin-V assay has been applied by numerous authors in an attempt to identify this early apoptotic event (Glander and Schaller, 1999; Barroso *et al.*, 2000; Blanc-Layrac *et al.*, 2000; Oosterhuis *et al.*, 2000; Anzar *et al.*, 2002; Ricci *et al.*, 2002; Shen *et al.*, 2002; Henkel *et al.*, 2004; Lachaud *et al.*, 2004; Moustafa *et al.*, 2004; Taylor *et al.*, 2004). The translocation of phosphatidylserine during capacitation in human spermatozoa is found on the head region as reported by Kotwicka *et al.* (2002) but shows no relation to the apoptotic process (Gadella and Harrison, 2002; Harrison and Gadella, 2005).

1.8.3 Assays that measure caspases and apoptosis–related proteins

The Fas (CD95/APO-1) molecule is a cell surface receptor that mediates apoptotic cell death. The presence of a membrane receptor, Fas has been investigated with anti-Fas antibodies by flow cytometry (Sakkas *et al.*, 1999b; Sakkas *et al.*, 2002; Castro *et al.*, 2004) and immunofluorescence (Castro *et al.*, 2004; Henkel *et al.*, 2004; McVicar *et al.*, 2004). A number of other key apoptotic markers in human ejaculated sperm have been investigated using immunofluorescence staining. These include Bcl and p53 (Sakkas *et al.*, 2002).

The apoptotic cascade is regulated by caspases as described under the heading 1.4.2.1. and one of the major caspases identified is caspase-3. This enzyme is one of the key enzymes responsible for the induction of apoptosis resulting in many of the morphological changes observed in apoptotic cells. Various assays have been used to identify several key markers for apoptosis. The presence of caspase-3, -6, -7, -8 and -10 activities was identified in sperm samples by Taylor *et al.* (2004). They reported that caspase activity was

higher in the ejaculates of infertile patients than donors. Marchetti *et al.* (2004) used flow cytometry to analyze cells stained with a fluorescein conjugate that binds to the active centre of most caspases. These authors also detected caspase-3 using western blot analysis. Wang *et al.* (2003) evaluated the presence of caspase-3 and -9 in ejaculated spermatozoa in patients with male factor infertility by means of western blot analysis. They reported higher levels of these caspases in patients than donors. Active caspase-8; -9; -1 and -3 were detected in human ejaculated spermatozoa using caspase inhibitors and evaluated using flow cytometry (Paasch *et al.*, 2004). The immunodetection of caspase-3 was investigated using fluorescence microscopy by Weil *et al.* (1998).

These are just some of the apoptotic events that have been identified in sperm cells using microscopy based methods or the rapid, quantification flow cytometric method. Flow cytometric methods should be confirmed by the inspection of the cells using the light or electron microscope.

1.9 Aims of this study

It was not an aim of this study to continue with the debate as to whether apoptosis is initiated in the testes and the products visible in the ejaculate samples. This study reviewed the presence of selected apoptotic markers in an attempt to identify which apoptotic events are representative of the apoptotic process and which are relevant in infertility studies. The objectives of this study are outlined below.

- This study was undertaken to test the application of a range of apoptotic marker assays using flow cytometry on sperm from patients with abnormal semen parameters.
- To establish whether any associations existed between the number of apoptotic sperm and the standard semen analysis results.

- The identification of possible apoptotic-like sperm cells by transmission electron microscopy was undertaken to ascertain whether the ultra-structural changes indicate apoptotic sperm in the ejaculate sample or whether these ultra-structural features are indicative of the poor sperm morphology based on the semen analysis results.
- To identify a possible single apoptotic assay to be performed in conjunction with the standard semen analysis which defines the possible fertility status of a male.
- To ascertain the value of apoptosis as a determinant of male infertility which may be valuable to confirm the outcome of assisted reproductive technological procedures.

This study did not correlate the expression of the selected apoptotic markers with the clinical outcome of fertilisation success as it was a scientific-based study only.

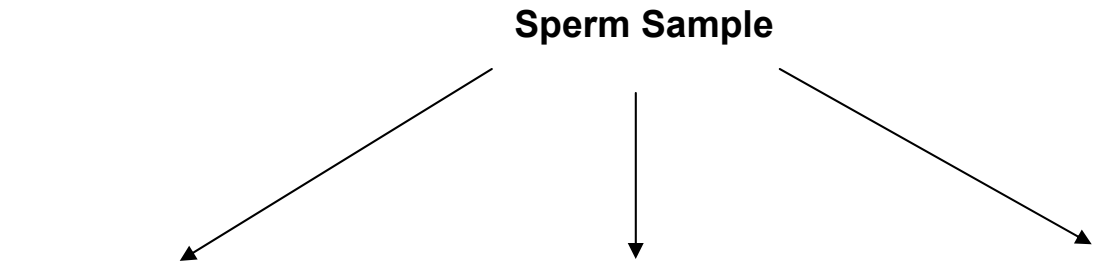
CHAPTER 2

Study design and Methodology

The presence of apoptotic markers and nuclear damage in ejaculated sperm samples was investigated using semen samples from patients who attended the Andrology Laboratory of the Reproductive Unit, Groote Schuur Hospital and Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa. Semen samples were obtained from 162 patients attending the Andrology Laboratory of the Reproductive Unit during the study period. The semen samples were from men classified as infertile with one or more abnormal semen parameters. All subjects were the partners of women who had failed to conceive after at least 12 months of unprotected intercourse. Informed consent was obtained from all the patients. Patient information remained confidential and within the institution. The protocol was approved by the Ethics Committee of the institution.

2.1 Standard semen analysis

Ejaculate semen samples were collected by masturbation from patients after an abstinence of 2-4 days from sexual intercourse. Ejaculate samples were allowed to liquefy at 37°C for 30 minutes. The semen samples were assessed for various qualitative parameters according to the World Health Organization [WHO] (1999) including liquefaction, viscosity, coagulation, colour and pH after 30 minutes for liquefaction. Assessment of the quantitative parameters was performed according to WHO criteria (1999) and these included sperm concentration, motility, morphology and mixed antiglobulin reaction only. The sperm vitality by dye exclusion test and the peroxidase test for leukocytes was not performed as part of the semen analysis. Figure 2.1 depicts a flow diagram of the procedure for the standard semen analysis.



Standard Semen Analysis Parameters assessed:

- **Volume**
- **Liquefaction**
- **Viscosity**
- **Coagulation**
- **Colour**
- **pH**
- **Concentration** (Haemocytometer Method)
- **Quantitative Motility** (% Motile sperm)
- **Morphology** (% Normal forms according to Tygerberg strict Criteria)
- **Mixed Antiglobulin Reaction** (MAR)

Flow Cytometry Apoptotic Marker Assays:

- **Annexin-V** Translocation of phosphatidylserine from inner to outer leaflet of cell membrane
- **APO-Direct** Quantification of DNA fragmentation of sperm
- **Caspase-3** Quantification of the expression of caspase-3 on sperm cells
- **Fas (CD95)** Quantification of the presence of the Fas surface receptors on sperm cells

Electron Microscopy Protocol

- **Fixation** (2.5% glutaraldehyde)
- **Post-fixation** (1.5% Osmium tetroxide)
- **Dehydration** (Graded series of ethanol changes)
- **Impregnation** in epoxy resin
- **Polymerisation** overnight (60°C)
- **Sectioning** Ultramicrotomy
- **Specimen viewing** Transmission Electron Microscope

Figure 2.1. Flow diagram illustrating the procedure for the standard semen analysis, apoptotic marker assays and transmission electron microscopy.

A 20 μl drop of semen was placed onto a clean glass slide and then covered with a coverslip. The wet preparation was examined using a phase-contrast microscope [Olympus CK2, Tokyo, Japan] at a magnification of 400X. Qualitative motility was assessed by evaluation of the percentage of forward progression of sperm by determination of the movements of a hundred sperm and categorized according to a scale of a to d (WHO, 1999). Category a, rapid progressive motility; b, slow or sluggish progressive motility; c, nonprogressive motility and a, immotility. The quantitative motility of sperm involved the determination of the percentage of motile sperm by enumeration of the motile and immotile sperm in several randomly selected fields at a magnification of 400X until one hundred sperm was assessed.

Sperm concentration was measured with the use of the haemocytometer method. A 1:10 dilution with a mixture (sodium bicarbonate, formalin, gentian violet and distilled water) was prepared for each well-mixed semen sample (WHO, 1999). The diluted sample was transferred to a Neubauer haematocytometer covered with a coverslip and after the sperm cells had settled, they were counted at a magnification of 400X using bright field microscopy [Olympus CX40, Tokyo, Japan].

The presence of sperm antibody IgG (human-IgG antiserum from rabbit) was evaluated using the mixed antiglobulin reaction [MAR] test. Briefly, this test involved mixing sensitised red blood cells (Western Province Blood Transfusion Services, South Africa) coated with IgG to anti-human-IgG antiserum (Behring Diagnostics, Marburg, Germany) and a drop of semen was added to this mixture. The formation of mixed agglutinates between particles and sperm indicates the presence of IgG antibodies on the sperm. When 50% or more of the motile spermatozoa have adherent particles, the diagnosis of immunological infertility has to be considered.

The evaluation of sperm morphology was done by the assessment of the Papanicolaou stained slides (Mortimer, 1994; WHO, 1999). The procedure for preparation of slides for the Papanicolaou staining technique involved placing a 10 μl drop of semen onto a clean glass slide and a smear was made which was air-dried and fixed in 95% ethanol for 15 minutes. The staining procedure is briefly outlined. The smears were sequenced through a series of ethanol solutions before staining with Mayer's haematoxylin (3 minutes). This was followed by rinsing of the slides with water followed by sequencing through ethanol solutions before the eosin staining for 5 minutes. The smears were sequenced through another series of ethanol solutions followed by mounting with DPX as outlined by Mortimer (1994) and WHO (1999). At least 100 sperm were counted from each smear at a magnification of 1000X and classification of sperm morphology was based on the Tygerberg strict criteria (Menkveld *et al.*, 1990). The results were expressed as percentage of morphologically normal spermatozoa.

An ejaculate sample was considered to have normal semen parameters if the following requirements were fulfilled: a sperm count of ≥20 x 10^6 sperm/ml of semen; sperm motility of more than 50% (grades a + b) or 25% or more with progressive motility (grade a), MAR ≤ 50% and ≥ 14% morphologically normal sperm. The ejaculate samples were classified into groups based on the criteria for normal concentration, motility and morphology according to the nomenclature by the WHO (1999). Semen samples were classified as teratozoospermic [G-pattern] (n=26), teratozoospermic [P-pattern] (n=98) and oligoteratozoospermic [P-pattern] (n=36). Refer to Chapter 1 for explanations of these classifications. The G-pattern teratozoospermic group has a "good" prognosis for fertilisation. Ideally, a normozoospermic group should have been included in the study; however, due to a number of obstacles it was abandoned and therefore not included. The reference values of semen parameters has been detailed in Chapter 1 and illustrated in Table 1.1.

Further experimentation was performed with these semen samples, which included various assays for studying cell death in these sperm cell populations (Figure 2.1). Quantification of cell death, specifically apoptosis was identified for each of the sperm samples of the three groups. At the nuclear level, labelled DNA strand breaks to detect possible apoptotic sperm were measured by flow cytometry. At the plasma membrane level, the percentage of apoptotic cells based on the translocation of phosphatidylserine during the early stages of apoptosis was measured by flow cytometry and morphologically the sperm cells were studied by transmission electron microscopy. A cytoplasmic event, caspase-3 expression during apoptosis was quantified by flow cytometry and the presence of Fas (CD95) was studied by flow cytometry. These flow cytometric assays allow the automated measurement of apoptosis at a single cell level in ejaculated spermatozoa with enormous precision.

2.2 Annexin-V Assay

Annexin-V is a Ca^{2+} dependant phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Apoptotic cells become Annexin-V positive after nuclear condensation has started, but before the cell becomes permeable to PI indicative of the loss of plasma membrane integrity. The Annexin-V assay allows the quantification of cells at an early stage of apoptosis (Cat. No. 1 828 681, Roche Diagnostics, Mannheim, Germany). The simultaneous staining of cells with FITC-Annexin-V and propidium iodide (PI) (Sigma-Aldrich Co., UK) allows the discrimination between apoptotic, necrotic and live cell populations. Sperm suspensions were adjusted to a concentration of 5 x 10^6 sperm/ml in Dulbecco's phosphate buffered saline (PBS) as formulated as detailed in the WHO laboratory manual (1999) and centrifuged at 200 x g for 5 minutes. The reagents used for the PBS preparation were from Sigma-Aldrich Co. (USA). These cells were washed twice in PBS and the pellets were resuspended in 100 μl of labelling solution [20 μl Annexin-V-Fluos labelling reagent in 1000 μl Hepes (Sigma, USA) buffer

and 20 μl PI] for 15 minutes at room temperature. The preparation of the components of the labelling solution was followed according to the manufacturer's instructions [Roche Diagnostics, Mannheim, Germany].

Prior to the analysis of these samples by flow cytometry, 0.5 ml of Hepes buffer was added to each sample. For each sample, 10 000 sperm were analysed in a flow cytometer (FACSCalibur, Becton-Dickinson, San Josa, California). The excitation wavelength was 488 nm and a 515 nm bandpass filter for fluorescein isothiocyanate (FITC)-labelled Annexin-V (green fluorescence), simultaneously with dye exclusion of PI (negative for red fluorescence). Based on the ability of cells to exclude PI, sperm cells are classified into three possible groups: viable/intact cells (Annexin-V negative and PI negative), necrotic sperm (Annexin-V positive and PI positive) and cells with impaired plasma membranes/apoptotic cells (Annexin-V positive and PI negative). The cytograms were formulated with Cell Quest Pro (Becton Dickson, San Josa, California) and calculations of the percentage apoptosis populations were determined. WinMDI 2.8 (Scripps Research Institute, La Jolla, California) was used to construct the cytograms and these were imported into MS word and saved as a document file; these cytograms are shown in the figures illustrated in Chapter 3.

2.3 DNA Integrity

A characteristic feature of apoptosis is DNA fragmentation, one of the later steps of apoptosis, which results from the activation of endonucleases during the apoptotic process (Gandini *et al.*, 2000; Nagata, 2000). These endonucleases degrade double-stranded DNA at internucleosomal sites into fragments of oligonucleosomal size 180-200 bp. The detection of fragmented DNA, a later step in the apoptotic process, has been quantified using the APO-DIRECT method and performed using the manufacturer's protocol (Cat. No. 556381, BD Biosciences, Pharmingen, USA). This procedure includes the

fixation of 2 x 10^6 cells/ml sperm cells in 1% paraformaldehyde (Sigma-Aldrich Co., UK) in phosphate buffered saline (PBS) solution followed by storage on ice for 30-60 minutes. The cells were washed twice in PBS and centrifugation between the washing steps was carried out for 5 minutes at 300 x g. The sperm cells were resuspended in 70% ice-cold ethanol and stored on ice for 30 minutes. For the staining procedure according to the manufacturer's protocol, sperm cells were incubated in the staining solution (Reaction buffer, TdT Enzyme, FITC-dUTP suspended in distilled water) for 60 minutes at 37°C. Following the staining procedure, the sperm cells were rinsed twice in rinsing buffer with centrifugation (300 x g for 5 minutes) between the washing steps. The cell pellets were re-suspended in a propidium iodide/RNase (PI/RNase) staining buffer and incubated in the dark for 30 minutes at room temperature. The sperm cells were analysed within 3 hours of staining by flow cytometry for the detection of labelled DNA strand breaks to detect apoptotic sperm cells in the ejaculate sperm samples. Positive and negative control cells (purchased component of the APO-Direct Kit; BD Biosciences, Pharmingen, USA) were stained in the same manner according to the manufacturer's protocol and 10 000 cells per sample was analysed by flow cytometry (FACSCalibur, Becton-Dickson, San Josa, California).

2.4 Caspase-3 Assay

Antibodies against caspase-3 in cells, which have undergone apoptosis serves to differentiate and quantify cell populations into apoptotic and non-apoptotic cells as measured by flow cytometry (Weil *et al.*, 1998). Spermatozoa at a concentration of 5x10^6 cells/ml were fixed in a 4% paraformaldehyde solution at 37°C for 30 minutes. The pellet was obtained following centrifugation at 200 x g and then a double wash was performed with PBS solution at room temperature and centrifugation at 200 x g. The final pellet was resuspended in 1 ml of blocking buffer [10% fetal Calf serum (Gibco, UK), 0.4% Triton-X-100 (Sigma Chemicals, USA) in PBS] at room temperature for 1 hour. Following

centrifugation at 200 x g, the pellet was suspended in an incubation buffer [2% fetal calf serum, 0.4% Triton-X-100 in PBS] with 20 μl of FITC-conjugated polyclonal rabbit active anti-caspase-3 antibodies (Cat. No. 559341, Pharmingen, USA) for 1 hour. These spermatozoa were washed three times with 0.1% Tween-20 (Merck, Schuchardt, Germany) in PBS, resuspended in PBS and filtered through a nylon filter to reduce any clumping of cells, thus ensuring a single cell suspension. In all the analyses, spermatozoa unlabelled with FITC caspase-3 antibody served as the negative control. The samples were examined by flow cytometry and 10 000 cells were counted for each sample analysed with the FACSCalibur (Becton-Dickson, San Josa, California).

2.5 Fas (CD 95) Assay

The presence of the cell surface receptor, Fas, in ejaculate samples has been investigated following the protocol according to Sakkas *et al.* (1999a). Spermatozoa adjusted to a concentration of $5x10^6$ cells/ml was fixed in 4% paraformaldehyde solution for 30 minutes at 37°C followed by washing spermatozoa twice with 1% Bovine Serum Albumin (BSA, Roche Diagnostics, Mannheim, Germany) in PBS. Spermatozoa were incubated with 20μl of FITC-conjugated mouse anti-human monoclonal antibody (CD95), non-death inducing (Cat. No. 33454X, BD Biosciences, Pharmingen) at 37°C for 2 hours. The spermatozoa was washed twice in 1% BSA in PBS and the pellet was resuspended in a solution of 1% BSA and 100 μl of PBS and filtered through a nylon filter to minimize cell clumping. Analysis was performed using a FACSCalibur (Becton-Dickson, San Josa, California) and 10 000 cells were counted for each sample. In all the analyses, spermatozoa unlabelled with FITC Anti-human antibody (CD95) served as negative control.

2.6 Electron Microscopy

Transmission electron microscopy allows the identification of ultrastructural nuclear and cytoplasmic features in ejaculated spermatozoa (Baccetti *et al.*, 1996; Gandini *et al.*, 2000; Muratori *et al.*, 2000; Larson *et al.*, 2001). The procedure for the processing of sperm for transmission electron microscopy was followed according to the method outlined by Mortimer (1994). Only a limited number of sperm samples (n=30), ten from each of the three groups [P-pattern teratozoospermic, G-pattern teratozoospermic and P-pattern oligoteratozoospermic] were processed for electron microscopy in an attempt to verify the presence of possible structurally apoptotic-like features associated with the sperm cells. The electron microscopy procedure is summarised in Figure 2.1 (See page 38). The ejaculated human sperm samples of the patients were fixed in 2.5% glutaraldehyde (Agar Scientific, Essex, UK) in 0.1M-phosphate buffer (pH 7.4) overnight at 4°C. Sperm were rinsed in phosphate buffer and post-fixed in 1.5% osmium tetroxide (Spi-Chem, USA) for 1 hour followed by rinsing twice with distilled water. After exposure of sperm to 2% uranyl acetate (Merck, Darmstadt, Germany) in 70% alcohol solution, it was dehydrated in alcohol (70% for 5 minutes followed by immersion in 96% alcohol for 5 minutes). The specimens were then transferred to 2% uranyl nitrate (Merck, Darmstadt, Germany) in 96% alcohol solution for 10 minutes. The sperm were transferred to 100% alcohol before it was added to a 1:1 mixture of resin (TAAB Spurr Resin, UK) and alcohol for 90 minutes. After impregnation through two sequences of resin for 1 hour each, the sperm was embedded in resin in BEEM capsules and polymerisation was at 60°C overnight. Ultra-thin sections were prepared with an ultratome control unit (LKB Type 8802A, Sweden) and placed onto 200 mesh copper grids (Wirsam Scientific, Rondebosch, South Africa). These sections were viewed with a Transmission Electron microscope [Hitachi-800, 200kV].

2.7 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Results were analysed using the statistical software SYSTAT (SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis non-parametric analog of the one-way Analysis of variance (ANOVA) was used to identify statistically significant differences for the variables between the three groups. Differences were considered significant if the probability of their occurrence was $p<0.05$. The Spearman Correlation matrix was employed to evaluate the correlation between the flow cytometry assay results and the semen parameters. Box-and-whisker plots were used to represent data where significant differences between groups were evident. These plots were obtained using the programme, Medcalc (Belgium) as described by Schoonjans *et al.* (1995) and Schoonjans (1996). The following descriptions of the box-and-whisker plots apply to all plots. The central box represents 50% of data points and the solid middle line in this box represents the median. The line that extends upward from the central box represents values from the upper quartile while the line extending downward from the central box represents values from the lower quartile range. Small T-bars inside the central box indicate 95% confidence interval and the outliers are shown as separate points (small squares).

While the Kruskal-Wallis test (ANOVA) is useful to show if two or more groups differ significantly, it is not useful to predict and attain cut-off points with an increased level of sensitivity and specificity. Receiver operating characteristic curves (ROC) was used to construct a graphic representation between the sensitivity and specificity for each of the assays by comparing two groups using the programme Medcalc (Belgium) as outlined by Schoonjans *et al.* (1995) and Schoonjans (1996). The areas under the ROC curve were also

determined. Clinically, the ROC curve analysis allows the discrimination between diseased and normal cases within a population. ROC curves were only presented for the assays which displayed significant differences ($p<0.05$) between groups.

CHAPTER 3

Results

3.1 Standard semen analysis

The results were based on 162 subjects and were represented in the three sample groups based on sperm morphology and classified as teratozoospermic [G-pattern] (n=26), teratozoospermic [P-pattern] (n=98) and oligoteratozoospermic [P-pattern] [P-pattern] (n=36) according to the Tygerberg strict criteria for assessment of sperm morphology. The three variables of the standard semen analysis: sperm concentration, the percentage sperm motility and the percentage of normal sperm morphology were compared between the three groups as illustrated in Table 3.1.

Table 3.1 presents the summary statistics (means ± SEM) for the sperm concentration, sperm motility and sperm morphology for the three groups. In this study, the mean (±SEM) sperm concentration for the teratozoospermic [G-pattern] group was 122.58 ± 16.20, which was considered significantly different ($P<0.05$) from the oligoteratozoospermic [P-pattern] group with a sperm concentration of 9.51 ± 0.88. A significant difference ($P<0.05$) was also evident between the oligoteratozoospermic [P-pattern] group and the teratozoospermic [P-pattern] group, which had a mean sperm concentration of 101.30 ± 8.74. Comparison of the sperm concentration values between the teratozoospermic [G-pattern] group and the teratozoospermic [P-pattern] group revealed no significant difference ($P>0.05$).

Comparison of the differences in sperm motility between the three groups revealed that the mean sperm motility of the oligoteratozoospermic [P-pattern] group (49.44 ± 1.60) differed significantly ($P<0.05$) from the teratozoospermic [G-pattern] group (58.85 ± 1.70) and the

teratozoospermic [P-pattern] group (57.35 ± 0.73). There was no significant difference (P>0.05) observed for sperm motility between the teratozoospermic [G-pattern] and teratozoospermic [P-pattern] groups.

Table 3.1. Comparison of semen parameters (sperm concentration, motility and morphology) between the study groups.

Variable	**Teratozoospermic [G-Pattern]**	**Teratozoospermic [P-Pattern]**	**Oligoteratozoospermic [P-Pattern]**
Sperm concentration ($x10^6$ per ml)	122.58 ± 16.20 (22 – 331)	101.30 ± 8.74† (20 – 420)	9.51 ± 0.88‡ (1 – 19)
% Motile sperm	58.85 ± 1.70 (40 - 70)	57.35 ±0.73† (40 - 80)	49.44 ± 1.60‡ (10 - 65)
% Normal sperm morphology	5.54 ± 0.15 (5 – 7)	2.47 ± 0.10* (1 – 4)	1.86 ± 0.19‡ (0 – 5)

Note: Values are represented as means ± SEM. Minimum and maximum values are represented in parentheses. Kruskal-Wallis test was used for the statistical analysis. ‡P<0.05 was considered statistically significant from teratozoospermic [G-pattern] and teratozoospermic [P-pattern] groups.

†P>0.05 was not considered statistically significant from the teratozoospermic [G-pattern] group.

*P<0.0.5 was considered statistically significant from the teratozoospermic [G-pattern] group.

Additionally, the percentage normal sperm morphology was compared between the three groups. The normal sperm morphology for the teratozoospermic [G-pattern] group was 5.54 ± 0.15, the teratozoospermic [P-pattern] group was 2.47 ± 0.10 and the oligoteratozoospermic [P-pattern] group was 1.86 ± 0.19. Statistically significant differences (P<0.05) were revealed between all three groups when compared for normal sperm morphology.

3.2 Apoptotic Marker Assays

In the present study, four apoptotic marker assays were applied to examine the apoptotic alterations in ejaculated sperm: Annexin-V staining for the determination of PS exposure, APO-Direct to identify DNA fragmentation, caspase-3 to detect expression of this protease during early apoptosis and Fas expression of ejaculate sperm. Table 3.2 presents the means ± SEM for the four cell death marker assays for the three groups.

Table 3.2. Descriptive statistics for the Annexin-V assay, APO-Direct Assay, Caspase-3 activity and Fas expression for the three groups as assessed by flow cytometry. The results are shown as the percentage of apoptotic spermatozoa for each of the assays.

Apoptotic assay	Teratozoospermic [G-pattern]	Teratozoospermic [P-pattern]	Oligoteratozoospermic [P-pattern]
Annexin-V binding	11.52 ± 1.29 (2.8 - 31.30)	15.30 ± 1.21† (2.1 - 75.10)	20.50 ± 1.91‡ (8.7 - 69.50)
APO-Direct	9.34 ± 1.43 (0.30 - 31.70)	10.22 ± 0.71 (0.75 - 39.20)	12.13 ± 1.55 (2.3 - 36.90)
Caspase-3 expression	10.78 ± 1.75 (2.0 - 37.90)	11.44 ± 0.82 † (1.10 - 39.50)	18.73 ± 1.98‡ (2.70 - 45.80)
Fas expression	8.06 ± 1.33 (2.10 - 26.00)	10.00 ±0.82† (1.10 - 41.50)	11.75 ± 1.57† (0.30 - 37.20)

Note: Values are represented as means ± SEM. Minimum and maximum values are represented in parentheses. Kruskal-Wallis test was used for the statistical analysis. ‡P<0.05 was considered statistically significant from teratozoospermic [G-pattern] and teratozoospermic [P-pattern] groups.

†P>0.05 was not considered statistically significant from the teratozoospermic [G-pattern] group.

The results shown in Table 3.2 will be described under each of the specific apoptotic assay headings below. Annexin-V staining, DNA fragmentation, caspase-3 activity and Fas expression of sperm in relation to the semen parameters will be addressed under the heading, “correlation analysis”.

3.2.1 Annexin-V assay

The externalisation of phosphatidylserine (PS) in cells that undergo apoptosis is expressed as the percentage of Annexin-V positive sperm and propidium iodide (PI) negative as indicated in Table 3.2. The oligoteratozoospermic [P-pattern] group of patients showed a significantly higher level of Annexin-V expression (20.50 ± 1.91) when compared to the G-pattern teratozoospermic, (11.52 ± 1.29) and the P-pattern, teratozoospermic (15.30 ± 1.21) groups. No statistically significant difference ($P>0.05$) was evident between the G-pattern teratozoospermic and P-pattern teratozoospermic groups for Annexin-V staining. Figure 3.1 depicts the above trends graphically. Box-and-whisker plots as well as the subsequent ROC curves used the above-mentioned groupings as classification criteria according to WHO criteria.

An analysis using the Receiver operating characteristic curve (ROC) showed that the cut off value for the Annexin-V assay results between the teratozoospermic [G-pattern] group and the teratozoospermic [P-pattern] group was 12.8% This threshold value of 12.8% yielded a sensitivity of 46.5% (95% confidence interval 36.4% to 56.8%) and a specificity of 73.1% (52.2% to 88.4%). The area under the curve was 0.59 (95% confidence interval 0.5 to 0.67). The ROC curve is shown in Figure 3.2A.

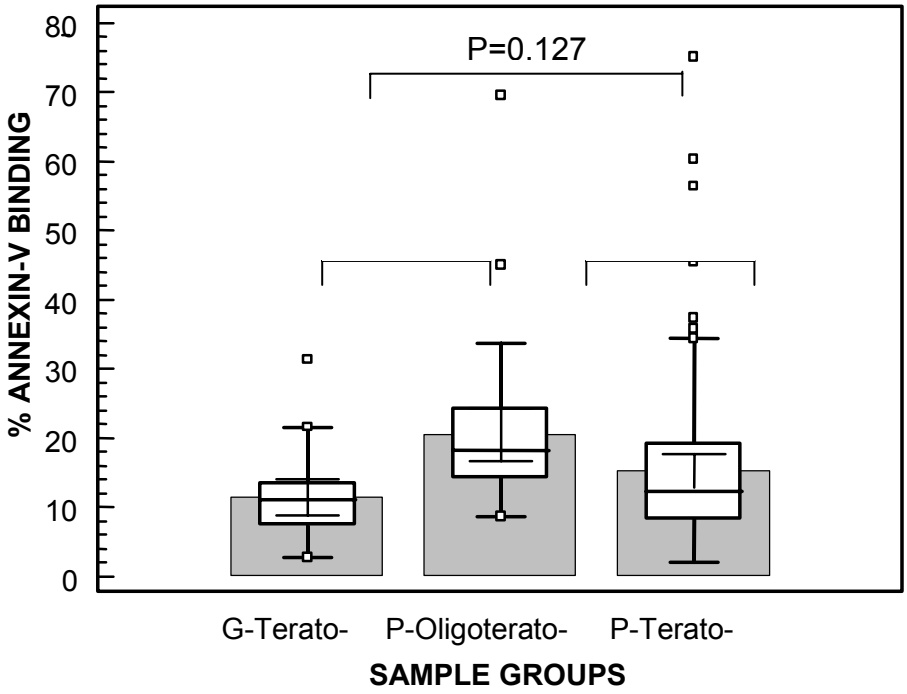


Figure 3.1. *Box and whisker plots of the Annexin-V assay between the three groups indicating the percentage of Annexin-V binding. P values are shown to indicate significant differences between groups. [G-Terato- represents G-pattern teratozoospermic, P-Oligoterato- represents oligoteratozoospermic [P-pattern] and P-Terato- represents P-pattern teratozoospermic].

The cut off value for the Annexin-V assay results between the teratozoospermic [G-pattern] group and the oligoteratozoospermic [P-pattern] group was 13.8%. At this threshold value, a sensitivity of 61.6% (51.3% to 71.2%) and a specificity of 78.4% (61.8% to 90.1%) was obtained. The area under the curve was 0.69 (0.61 to 0.77). The ROC curve is shown in Figure 3.2B.

*Explanation of box-and-whisker plots: Central box represents 50% of data points; solid line in middle box represents the median; line that extends upward and downward from central box represents values from upper quartile and lower quartiles respectively. Small T-bars inside central box represents 95% confidence intervals and outliers are shown as small squares.

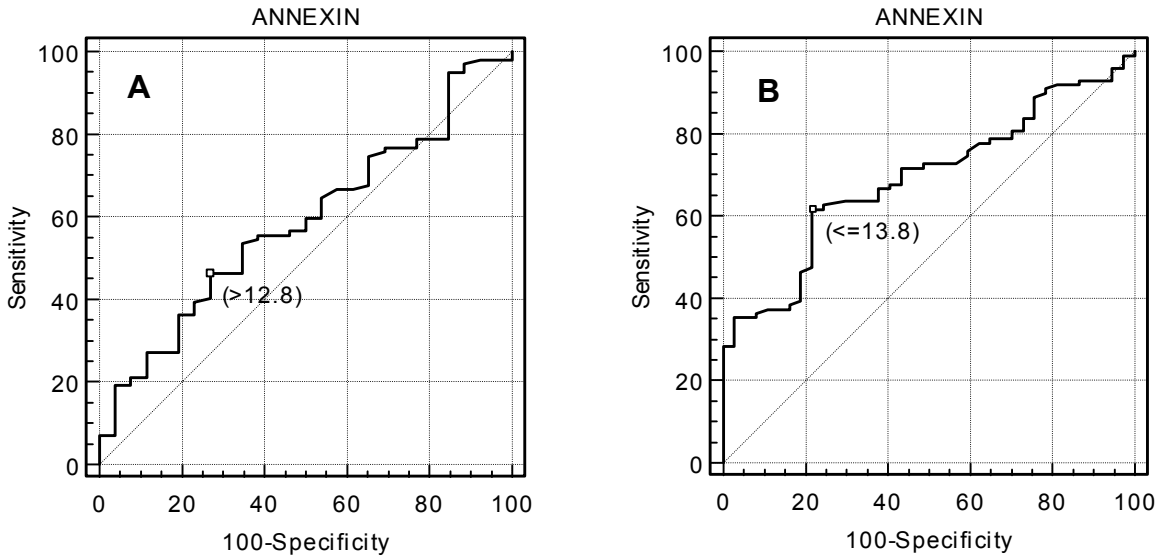


Figure 3.2. Receiver operating characteristic (ROC) curves of the Annexin-V assay between **(A)** teratozoospermic [G-pattern] and teratozoospermic [P-pattern] groups, and **(B)** teratozoospermic [G-pattern] and oligoteratozoospermic [P-pattern] groups as an apoptotic marker assay for apoptosis. For each ROC curve analysis, the classification criteria refer to G-pattern teratozoospermia, P-pattern teratozoospermia and P-pattern oligoteratozoospermia.

Figure 3.3 A, B and C illustrates cytograms representing the bivariate Annexin V/PI analysis for one sperm sample stained simultaneously with Annexin-V and propidium iodide (PI) for each of the three groups. The lower left quadrant of the cytogram shows the viable cells, which exclude PI and are negative for FITC-Annexin-V binding. The upper right quadrant represents the non-viable, necrotic cells, positive for FITC-Annexin-V binding and showing PI uptake. The lower right quadrant represents the apoptotic cells, FITC-Annexin-V positive and PI negative, demonstrating Annexin- V binding and cytoplasmic membrane integrity. The cytograms showing the percentage of apoptotic cells for each of the groups are illustrated in Figure 3.3.

The cytogram shown in Figure 3.3A indicates that this patient from the teratozoospermic [G-pattern] group has 7.22% of apoptotic sperm. The cytogram of the one patient from the teratozoospermic [P-pattern] group indicates 25.85% of apoptotic sperm (Figure 3.3B) as compared to the

oligoteratozoospermic [P-pattern] group where 69.47% of apoptotic sperm were present (Figure 3.3C).

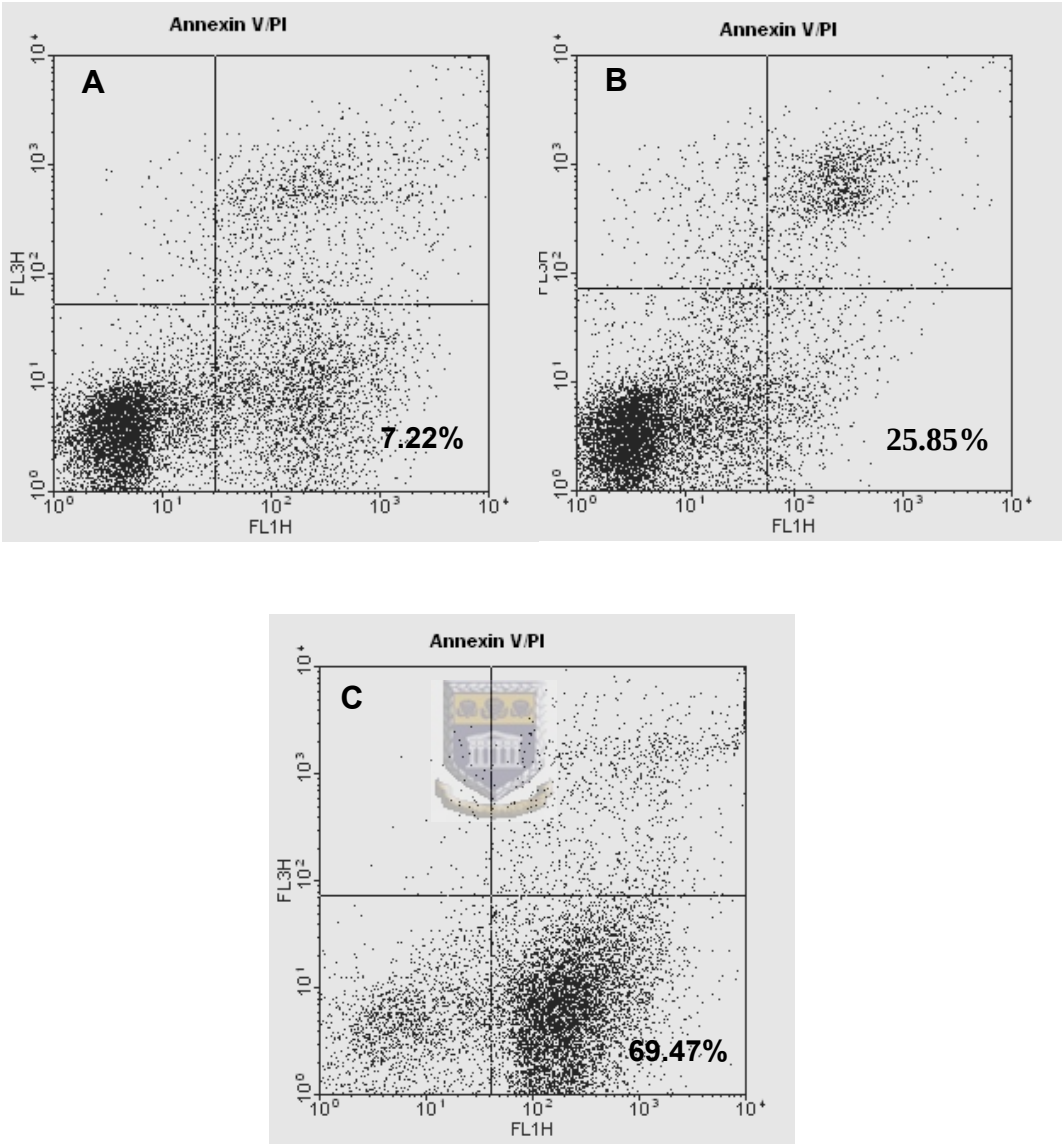


Figure 3.3. Bivariate Annexin-V/PI analysis used to identify the three distinctive cell populations. The lower left quadrant of each graph contains the viable, non-apoptotic cells, (Annexin-V and PI negative). The lower right quadrant represents the apoptotic cell population (Annexin-V positive and PI negative) and the upper right quadrant represents the necrotic cell population (Annexin-V and PI positive). Cytogram of one subject from the teratozoospermic [G-pattern] group **(A),** the teratozoospermic [P-pattern] group **(B)** and a cytogram of one subject from the oligoteratozoospermic [P-pattern] group **(C)** with a high incidence of apoptosis.

3.2.2 APO-Direct Assay

In this study, apoptotic alterations in sperm were further examined using the APO-Direct assay in an attempt to quantify the fragmented DNA by means of flow cytometry as presented in Table 3.2. The determination of the percentage fragmented DNA; indicative of apoptosis was compared between the three study groups. The mean (± SEM) percentage of fragmented DNA for the teratozoospermic [G-pattern] group was 9.34 ± 1.43, the teratozoospermic [P-pattern] group had 10.22 ± 0.71 and the oligoteratozoospermic [P-pattern] group had 12.13 ± 1.55. No significant differences ($P>0.05$) were found between the percentages of fragmented DNA for any of the study groups and therefore no box-and-whisker plot has been included.

Receiver operating characteristic curves were used for determination of the cut off values in the APO-Direct Assay. A cut off value of 3.5% was obtained for the assay results between the teratozoospermic [G-pattern] group and the teratozoospermic [P-pattern] group. This threshold value of 3.5% yielded a sensitivity of 84.8% (76.2% to 91.3%) and a specificity of 30.8% (14.4% to 51.8%). Figure 3.4A shows this ROC curve illustrating the sensitivity and specificity for the percentage of fragmented DNA. The area under the ROC curve was 0.55 (0.56 to 0.64).

An analysis using ROC showed that the cut off value for using the APO-Direct assay as a test for apoptosis between the teratozoospermic [G-pattern] and oligoteratozoospermic [P-pattern] groups was 23.3%. At this cut off value, the sensitivity yielded was 97% (91.4 to 99.3%) but the specificity was only 18.9% (8.0 % to 35.2%). While there might have been an increase in the sensitivity, the specificity was indeed very low. This resulted in the area under the ROC curve being only 0.54 (0.45 to 0.62). Figure 3.4B illustrates the sensitivity and specificity of the percentage of fragmented DNA as an apoptotic marker assay.

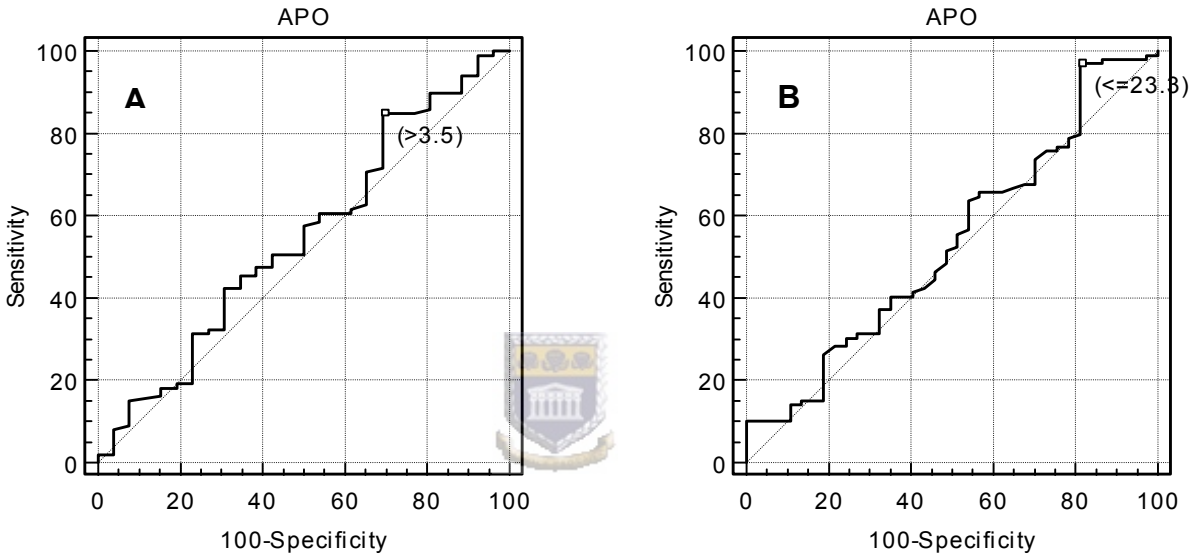


Figure 3.4. Receiver operating characteristic (ROC) curves of the percentage DNA fragmentation between **(A)** teratozoospermic [G-pattern] and teratozoospermic [P-pattern] groups, and **(B)** teratozoospermic [G-pattern] and oligoteratozoospermic [P-pattern] groups as an apoptotic marker assay for apoptosis.

Figure 3.5 shows typical frequency histograms obtained using flow cytometry of a negative control with 1.77% fragmented DNA, a positive control with 29.03% fragmented DNA and one patient with 31.70% fragmented DNA. However, no significant differences and relatively poor cut-off values were obtained for this parameter.

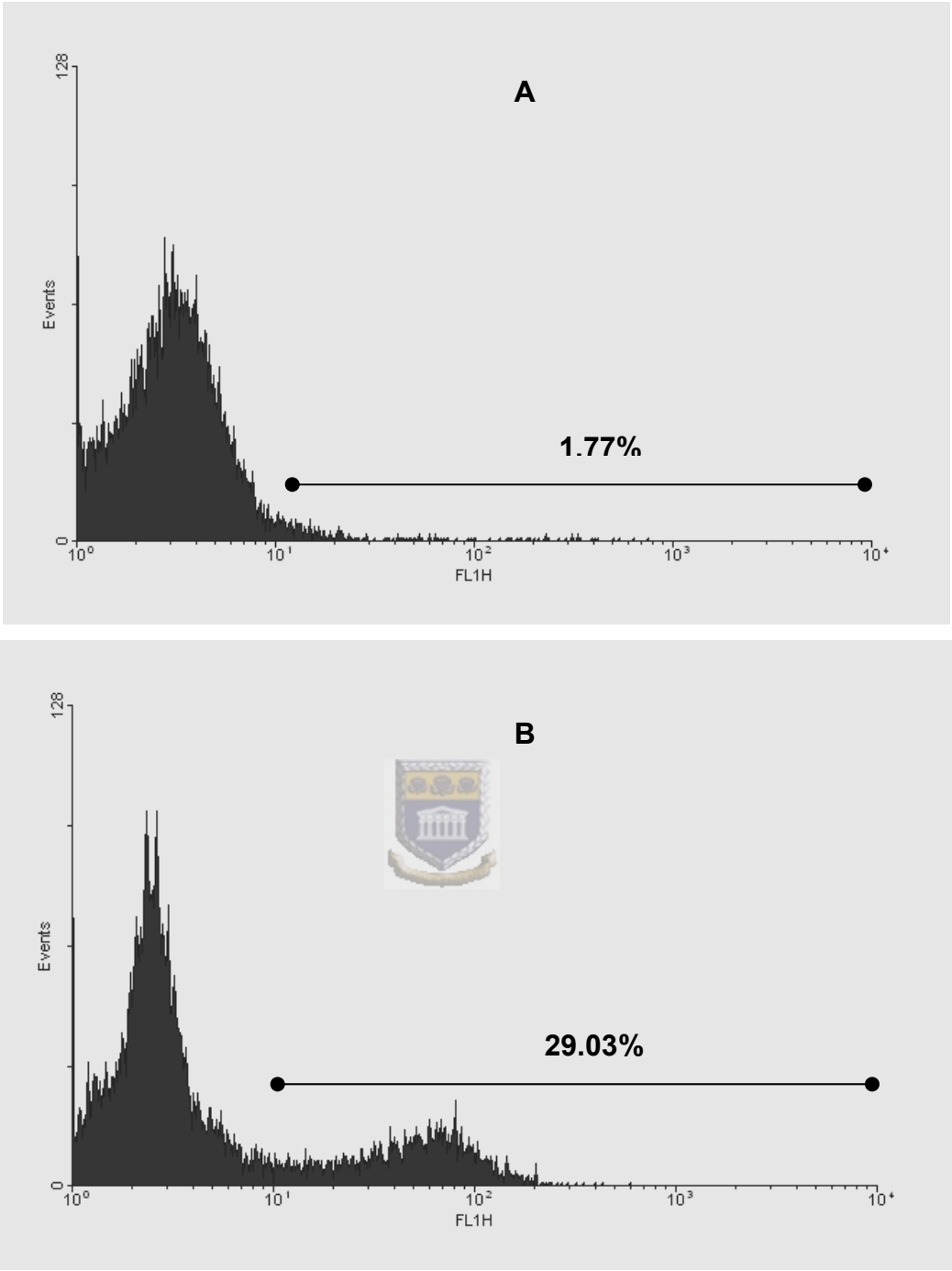


Figure 3.5. An illustration of the typical frequency histogram obtained for the negative control **(A)** and a positive control **(B)**.

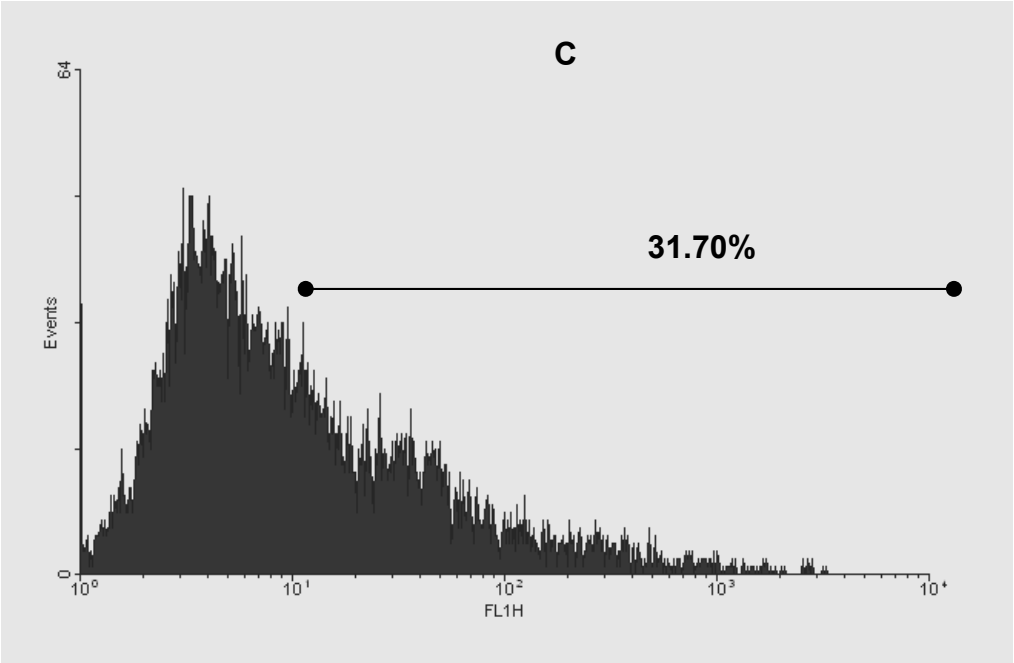


Figure 3.5. Continued. An illustration of the typical frequency histogram obtained for one patient with 31.70% fragmented DNA **(C)**.

3.2.3 Caspase-3 expression

Caspase-3 is considered to be a major executioner protease in the apoptotic pathway cascade. The level of caspase-3 expression was significantly higher in the oligoteratozoospermic [P-pattern] group of patients (18.73 ± 1.98) when compared to the teratozoospermic [G-pattern] group (10.78 ± 1.75) and the teratozoospermic [P-pattern] group (11.44 ± 0.82). There were significant differences ($P<0.05$) between the caspase-3 expression on sperm in all the groups (Table 3.2). These trends are depicted graphically in Figure 3.6.

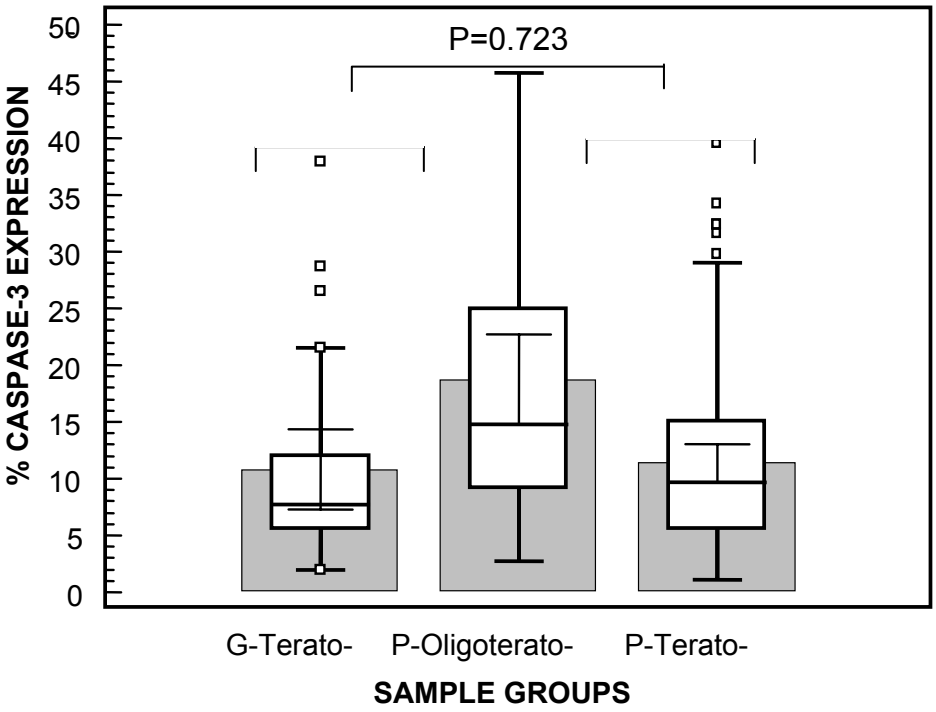


Figure 3.6: *Box and whisker plots of the caspase-3 expression assay between the three groups. P values are shown to indicate significant differences between groups. [G-Terato- represents G-pattern teratozoospermic, P-Oligoterato- represents oligoteratozoospermic [P-pattern] and P-Terato- represents P-pattern teratozoospermic].

There was more than 10% of caspase-3 expression in 26 of the 36 (72.2%) oligoteratozoospermic [P-pattern] samples analysed. In contrast, 9 of the 26 (34.6%) teratozoospermic [G-pattern] samples contained more than 10% of caspase-3 expression as compared to 47 of the 98 (48%) teratozoospermic [P-pattern] samples, which had more than 10% of caspase-3 expression on ejaculated sperm.

*Explanation of box-and-whisker plots: Central box represents 50% of data points; solid line in middle box represents the median; line that extends upward and downward from central box represents values from upper quartile and lower quartiles respectively. Small T-bars inside central box represents 95% confidence intervals and outliers are shown as small squares.

An analysis using ROC curves showed a cut off value of 10.7% for the caspase-3 assay results between the teratozoospermic [G-pattern] group and the teratozoospermic [P-pattern] groups. This threshold value of 10.7% yielded a sensitivity of 45.5% (35.4% to 55.8%) and a specificity of 73.1% (52.2% to 88.4%). Figure 3.7A shows this ROC curve illustrating the sensitivity and specificity for caspase-3 expression. The area under the ROC curve was 0.54 (0.45 to 0.63).

The cut off value for the caspase-3 assay results between the teratozoospermic [G-pattern] group and the oligoteratozoospermic [P-pattern] group was 18.5%. In this instance, the sensitivity was very high 88.9% (81.0% to 94.3%) and the specificity was low at 43.2% (27.1% to 60.5%). However, the area under the ROC curve was 0.69 (0.61 to 0.77). The ROC curve is shown in Figure 3.7B.

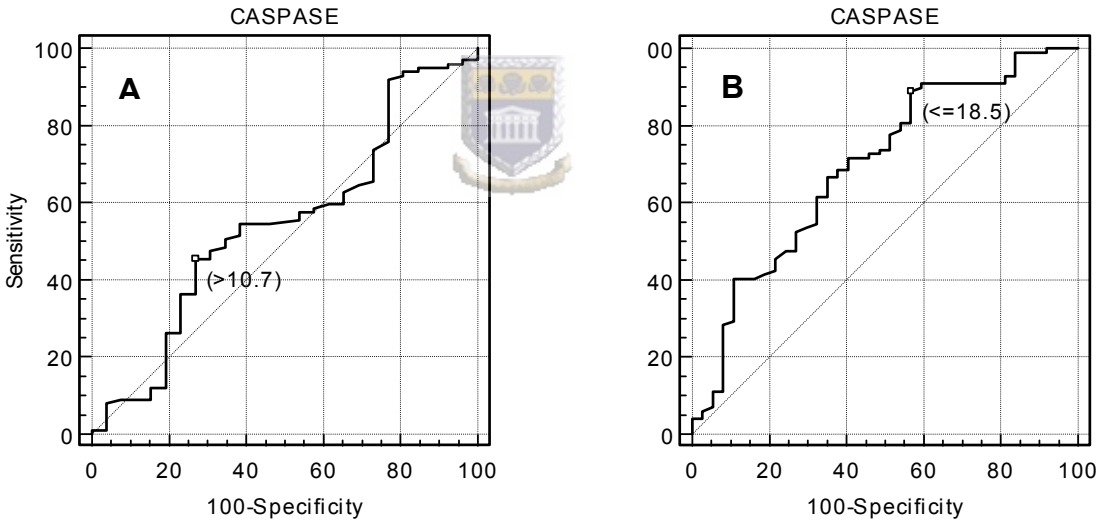


Figure 3.7. Receiver operating characteristic (ROC) curves of the percentage of caspase-3 expression between **(A)** teratozoospermic [G-pattern] and teratozoospermic [P-pattern] groups, and **(B)** teratozoospermic [G-pattern] and oligoteratozoospermic [P-pattern] groups as an apoptotic marker assay for apoptosis.

A typical flow cytometric histogram of a negative control (A) displaying 2.78% of caspase-3 positivity on the sperm cell and a histogram of one patient (B) from the oligoteratozoospermic [P-pattern] group showing 23.94% caspase-3 expression on the sperm is presented in Figure 3.8.

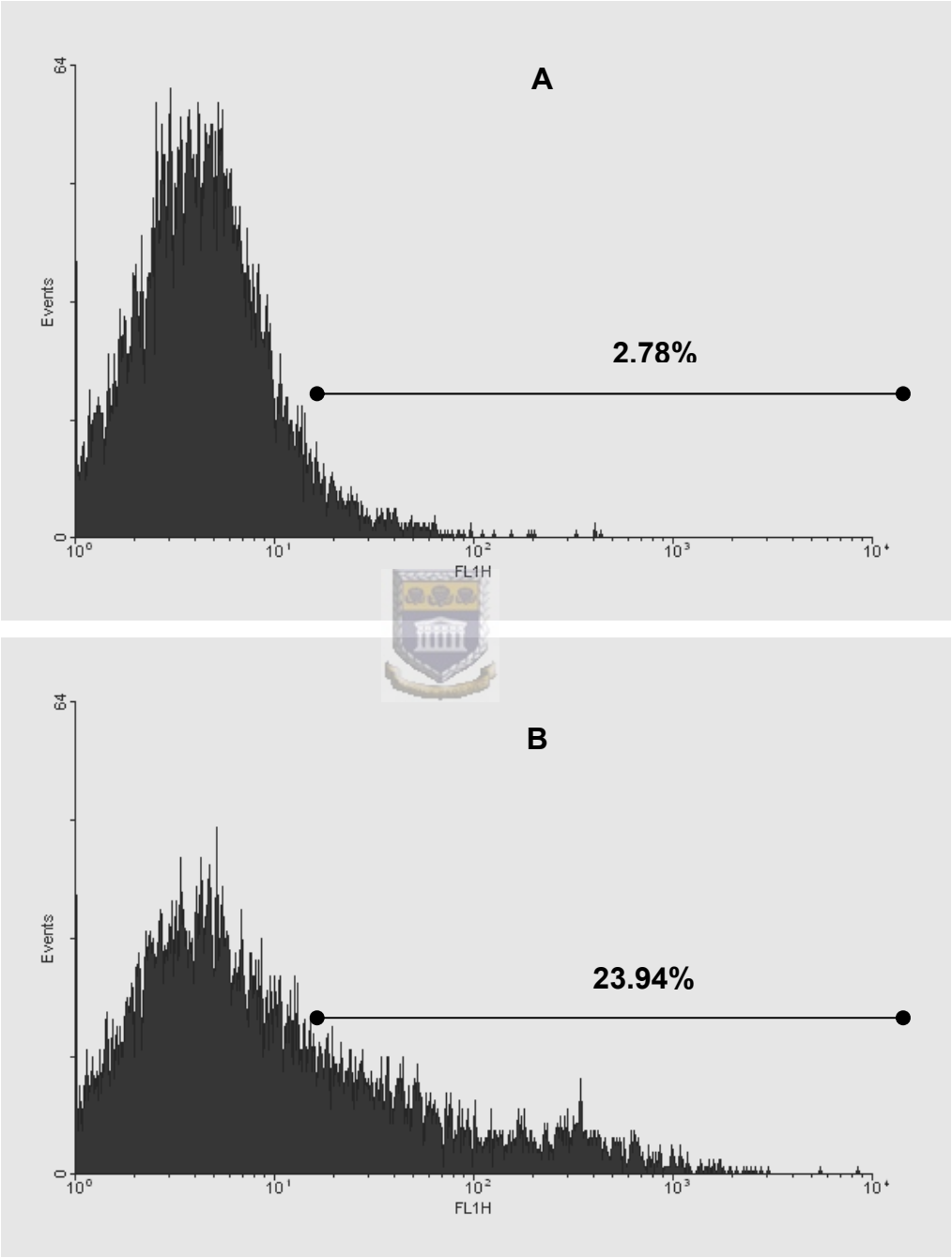


Figure 3.8. A typical flow cytometric histogram of caspase-3 activity of a negative control **(A)** and a patient from the oligoteratozoospermic [P-pattern] group with 23.94% caspase-3 labelled cells **(B)**.

3.2.4 Fas expression

The apoptotic marker Fas was studied to investigate the presence of Fas in ejaculated sperm. The Fas expression for the oligoteratozoospermic [P-pattern] group was 11.75 ± 1.57 and 10.00 ± 0.82 for the teratozoospermic [P-pattern] which was not significantly different from the teratozoospermic [G-pattern] group who displayed 8.06 ± 1.33 of Fas positivity as presented in Table 3.2. Figure 3.9 further depicts these trends graphically.

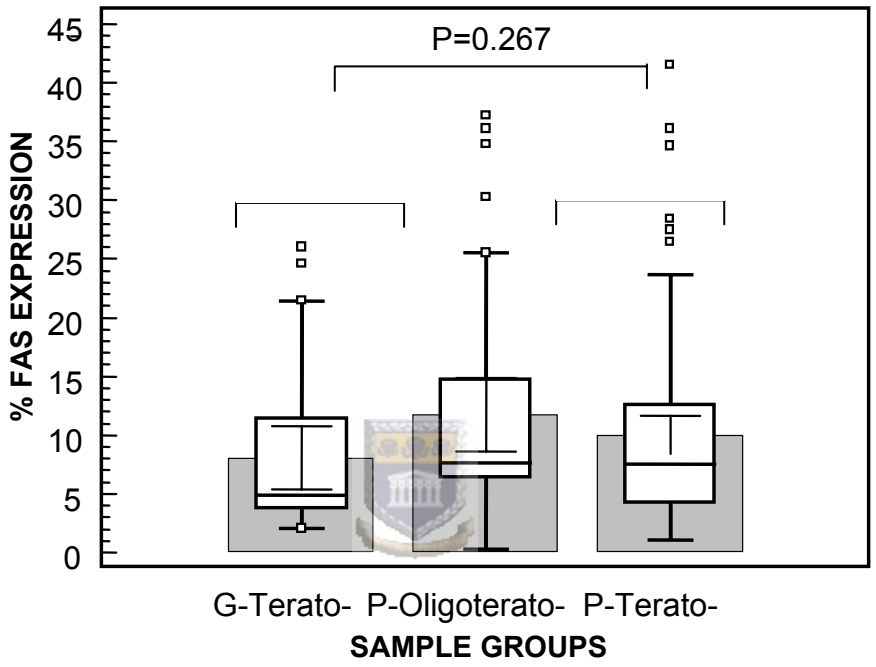


Figure 3.9: *Box and whisker plots of the percentage of Fas expression on sperm for the three groups. P values are shown to indicate significant differences between groups. [G-Terato- represents G-pattern teratozoospermic, P-Oligoterato- represents oligoteratozoospermic [P-pattern] and P-Terato- represents P-pattern teratozoospermic].

*Explanation of box-and-whisker plots: Central box represents 50% of data points; solid line in middle box represents the median; line that extends upward and downward from central box represents values from upper quartile and lower quartiles respectively. Small T-bars inside central box represents 95% confidence intervals and outliers are shown as small squares.

There was more than 10% of Fas positive sperm in 14 of the 36 (38.9%) oligoteratozoospermic [P-pattern] samples analysed. In contrast, 7 of the 26 (26.9%) teratozoospermic [G-pattern] samples contained more than 10% of Fas positive sperm as compared to 39 of the 98 (39.8%) teratozoospermic [P-pattern] samples, which had more than 10% of Fas positive sperm. These results indicate that the expression of Fas protein on the surface of the sperm cell was evident in samples from each of the three study groups, however, the Fas positivity percentage varied between samples. Fas expression in relation to the semen parameters will be assessed under the next heading where a correlation analysis will be performed to identify any correlations.

The ROC curve was used for the determination of the cut off value for the Fas assay between the teratozoospermic [G-pattern] and the teratozoospermic [P-pattern] groups. A cut off value of 4.8% yielded a sensitivity of 71.7% (61.8% to 80.3%) and a specificity of 50.0% (29.9% to 70.1%). Based on this cut off value, any patient with a value above 4.8% would fall into the teratozoospermic [P-pattern] group. Figure 3.10A shows this ROC curve illustrating the sensitivity and specificity for percentage Fas expression. The area under the ROC curve was 0.58 (0.49 to 0.67).

The cut off value for the Fas expression between the teratozoospermic [G-pattern] group and the oligoteratozoospermic [P-pattern] group was 5.5%. At this threshold value, a sensitivity of 39.4% (29.7% to 49.7%) and a specificity of 81.1% (64.8% to 92.0%) were obtained. The area under the curve was 0.56 (0.47 to 0.64). The ROC curve is shown in Figure 3.10B.

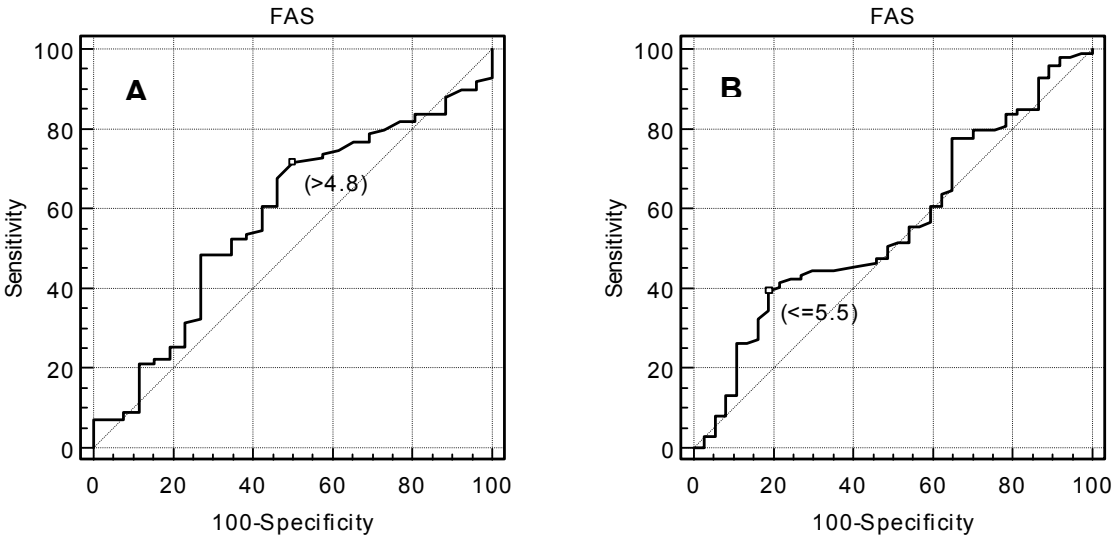


Figure 3.10. Receiver operating characteristic (ROC) curves of the percentage Fas expression between **(A)** teratozoospermic [G-pattern] and teratozoospermic [P-pattern] groups, and **(B)** teratozoospermic [G-pattern] and oligoteratozoospermic [P-pattern] groups as an apoptotic marker assay for apoptosis.

A typical flow cytometric histogram of a negative control displaying 1.91% of Fas expression on the sperm cells is shown in Figure 3.11A and a histogram of one patient from the oligoteratozoospermic [P-pattern] group showing 13.12% Fas expression on sperm is presented in Figure 3.11B.

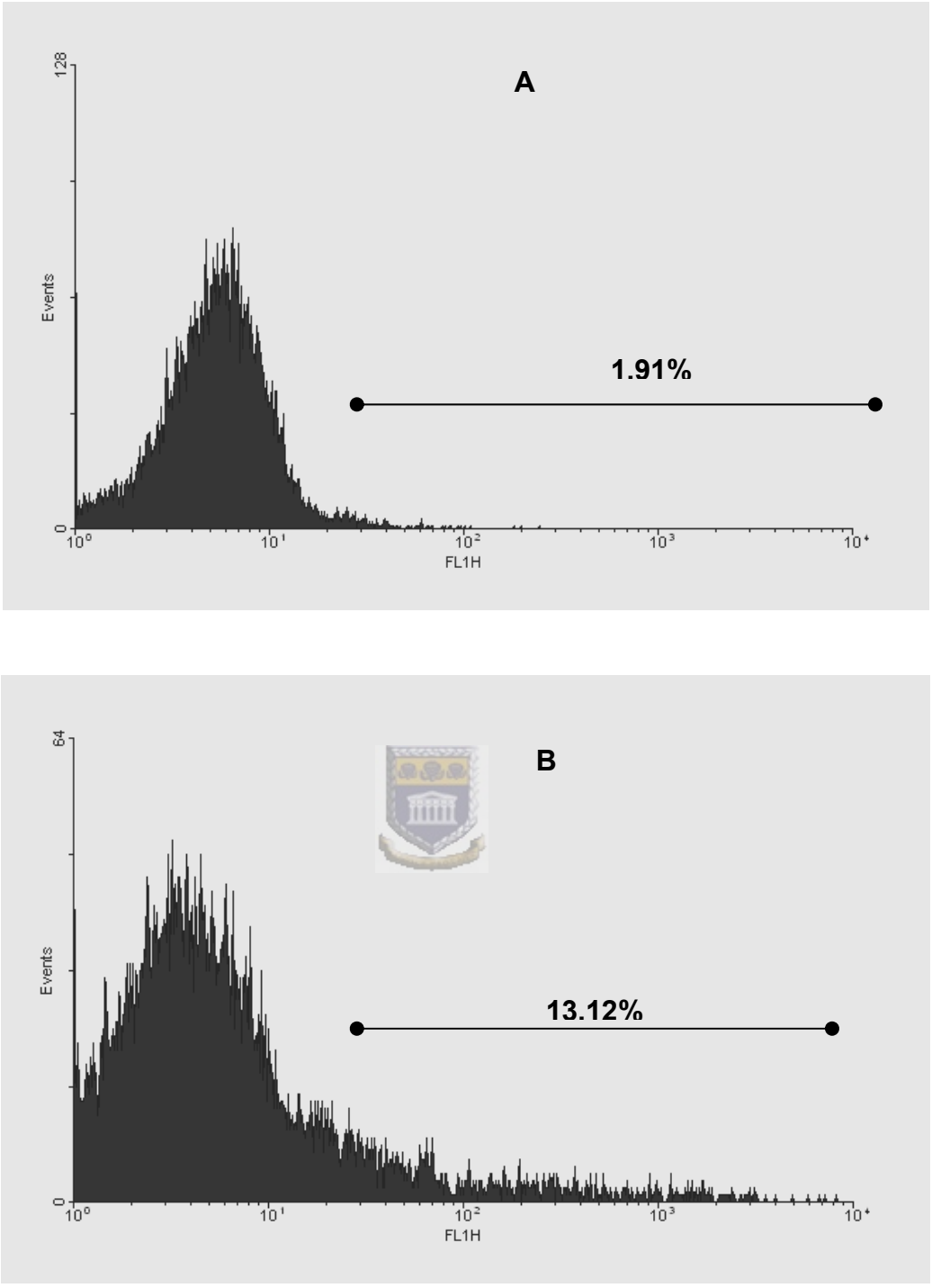


Figure 3.11. A typical flow cytometric histogram of a negative control displaying 1.91% of Fas labelled cells **(A)** and of a patient from the oligoteratozoospermic [P-pattern] group with 13.12% of Fas labelled cells **(B).**

3.2.5 Correlation analysis

In order to assess the possible association of sperm apoptosis and sperm quality, further analysis of the correlations between the apoptotic marker assays and the various semen parameters were performed. Associations between the four apoptotic marker assays and sperm concentration, motility and morphology parameters for each group was then assessed using the Spearman Correlation matrix (r_s) as indicated in Table 3.3. All strong positive correlations were shown to be significant ($P<0.05$) and these were indicated in Table 3.3.

In the teratozoospermic [G pattern] group, the only strong positive correlation was observed between the caspase-3 expression and Fas activity in sperm cells. Strong correlations were evident between the percentage DNA fragmentation and the caspase-3 expression; percentage of DNA fragmentation and the Fas activity as well as caspase-3 and Fas expression in sperm from the teratozoospermic [P-pattern] group. The only strong positive correlation in the oligoteratozoospermic [P-pattern] group was shown to exist between the expression of caspase-3 and the percentage of fragmented DNA obtained using the APO-Direct Assay.

Table 3.3. Correlation of the apoptotic markers of Annexin-V, DNA fragmentation, Caspase-3 and Fas assays with semen variables for the three groups.

Correlation-efficient (r_s)	Variables						
	Concentration	**Motility**	**Morphology**	**Annexin**	**Apodirect**	**Caspase-3**	Fas
Teratozoospermic [G-pattern]							
Concentration		0.147					
Motility			-0.325				
Morphology	0.144						
Annexin-V	-0.394	0.045	0.252		0.094	0.172	0.139
APO-Direct	-0.047	0.134	0.016			0.236	0.113
Caspase-3	-0.061	0.129	-0.310				0.680* p=0.003
Fas	0.091	0.415	-0.432				
Teratozoospermic [P-pattern]							
Concentration		0.031					
Motility			0.207				
Morphology	0.207						
Annexin-V	-0.260	0.003	-0.158		-0.117	0.170	-0.014
APO-Direct	-0.054	-0.112	-0.115			0.377* p=0.003	0.331* p=0.018
Caspase-3	-0.057	-0.083	-0.115				0.312* p=0.037
Fas	-0.038	-0.054	-0.045				
Oligoteratozoospermic [P-pattern]							
Concentration		-0.075					
Motility			0.161				
Morphology	-0.241						
Annexin-V	-0.148	0.136	0.183		-0.026	-0.011	-0.015
APO-Direct	0.118	0.326	-0.138			0.536	0.290
Caspase-3	0.142	0.157	-0.004		0.536* p=0.023		0.432
Fas	-0.210	0.088	0.067				

The Spearman Correlation matrix was used for the correlation analysis. P-values are indicated only for significant correlations.

r_s value between -0.5 and 0 indicates weak negative correlation between assays.

r_s value between 0 and 0.5 indicates weak positive correlation between assays.

* r_s value between 0.5 and 1.0 indicates strong positive correlation between two assays ($P<0.05$).

3.3 Electron Microscopy

Electron microscopy has been applied in this study in an attempt to identify any morphological characteristics suggestive of apoptosis. This valuable tool assists in the identification not only of the presence of sperm defects, but the nature of the defect as well. To verify whether possible sperm apoptosis identified by flow cytometry was associated with the presence of apoptotic-like features of sperm cells, thirty sperm samples (10 from each group: G-pattern teratozoospermic, P-pattern teratozoospermic and P-pattern oligoteratozoospermic) were examined by electron microscopy in each of the three groups. Identification of the presence of any apoptotic like characteristics of sperm was noted in the ten specimens from each of the three groups. Only a qualitative assessment of the apoptosis-like characteristics of sperm was performed.

The ultra-structural features indicative of apoptosis observed will be described in the characteristic sequence as described by Wyllie *et al.* (1980). Chromatin alterations were evident in some sperm cells from all the groups (Figures 3.12-3.15). One of the earliest morphological events suggestive of apoptosis is the condensation of the nuclear chromatin where most of the chromatin has aggregated into large compact granular masses (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Instead, of nuclear chromatin condensation, the chromatin of some sperm had a disorganized pattern forming a loose fibrillar-microgranular chromatin network (Figures 3.12A-F and Figure 3.14F). The nuclear membrane was intact in most of the samples; however a few sperm cells had partially disrupted nuclear membranes (Figures 3.12A, B and E). The chromatin of a few sperm samples showed a partial disruption by the presence of one or more nuclear vacuoles in the nuclear chromatin. The number and diameter of the vacuoles within the nuclear chromatin showed variation between samples (Figures 3.12-3.15). A feature, rarely observed was the presence of multilayered membranous bodies (myelin figures) within the vacuoles of the nuclear chromatin (Figures 3.12F, 3.13E and 3.14A).

The presence of apoptotic spermatids has been identified in some of the ejaculate samples illustrated in Figures 3.13F, 3.15B, C and D. Figure 3.13F shows a spermatid with a typically marginal nucleus and chromatin is condensed along the periphery due to the presence of a large intranuclear vacuole within the cytoplasmic residue. Also present in this spermatid are numerous vacuoles. Figure 3.15B depicts a spermatid with nucleus and various vacuoles while Figure 3.15C depicts a binucleated spermatid. Figure 3.15D illustrates a spematid with a peripherally placed, incompletely condensed nucleus and various vacuoles.

The plasma membrane was intact and generally normal in most of the sperm cells observed by electron microscopy (Figures 3.12-3.15). In a few cases, plasma membrane protuberances were present (Figures 3.12F, 3.13A, 3.13C, 3.14B, 3.14C, 3.14E and 3.15F). The acrosomes appeared to be intact in most samples and retained their shape. Cytoplasmic residues were evident containing vacuoles and these are indicated in Figures 3.12E, 3.13C, 3.14C, 3.14F and 3.15F.

Large spheroid bodies of variable diameters were identified within some ejaculate samples (Figures 3.12B, 3.13B, 3.13D, 3.13E, 3.13F, 3.14D, 3.15A to C and 3.15E). The very large apoptotic bodies as shown in Figures 3.14D and 3.15C may be due to the aggregation of smaller apoptotic bodies.

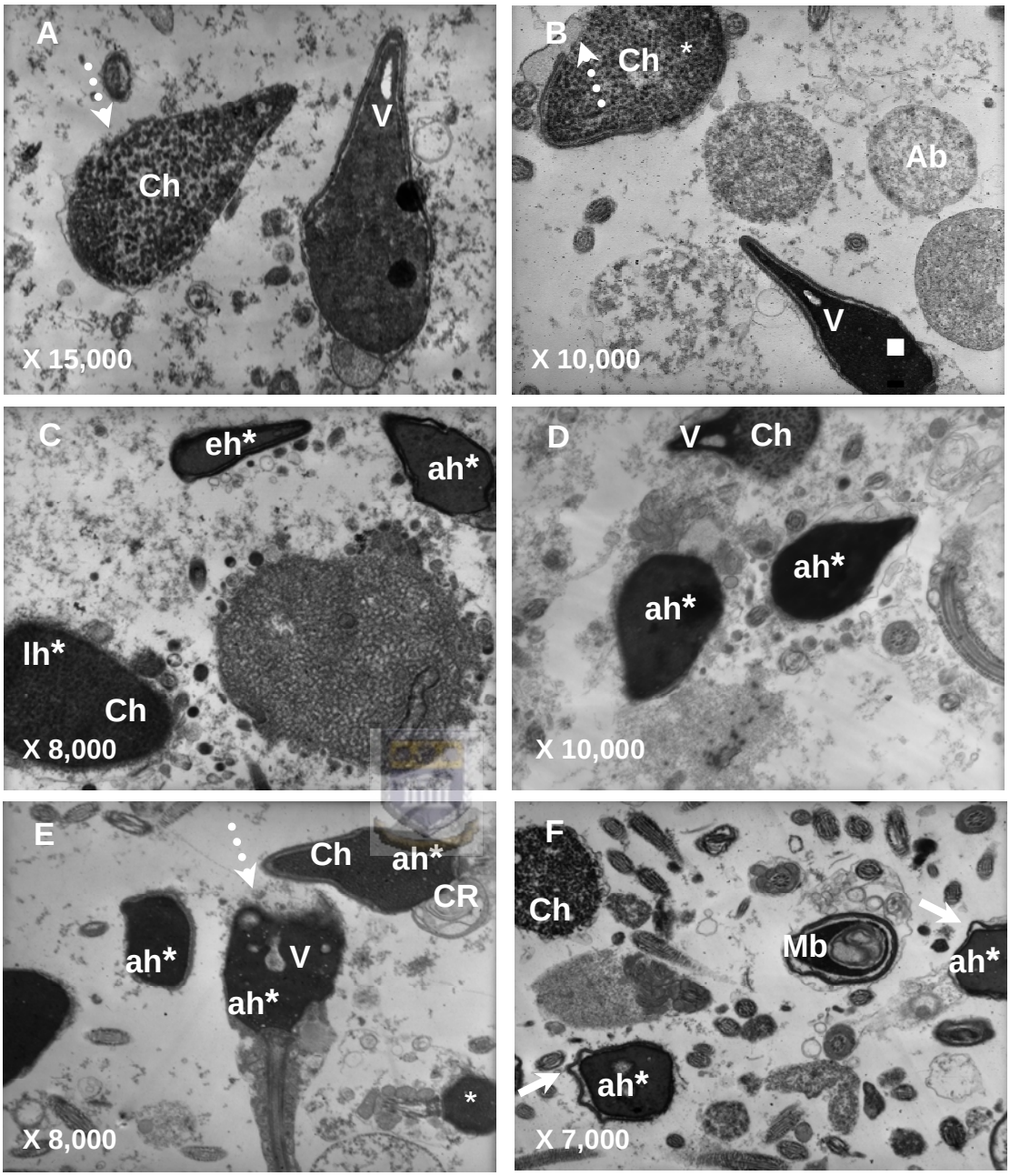


Figure 3.12: Electron micrographs of human sperm (A-F). Loose fibrillar-microgranular nuclear chromatin (Ch) with partially disrupted nuclear membrane (broken arrow) and nuclear membrane protuberances (solid arrow). Vacuoles within the nuclear chromatin (V), a membranous body within the vacuole (Mb) and apoptotic bodies (Ab) are indicated. The cytoplasmic residue (CR) is shown in E. Morphologically normal sperm (■) and some defective sperm are indicated above (*) and these are explained in the text.

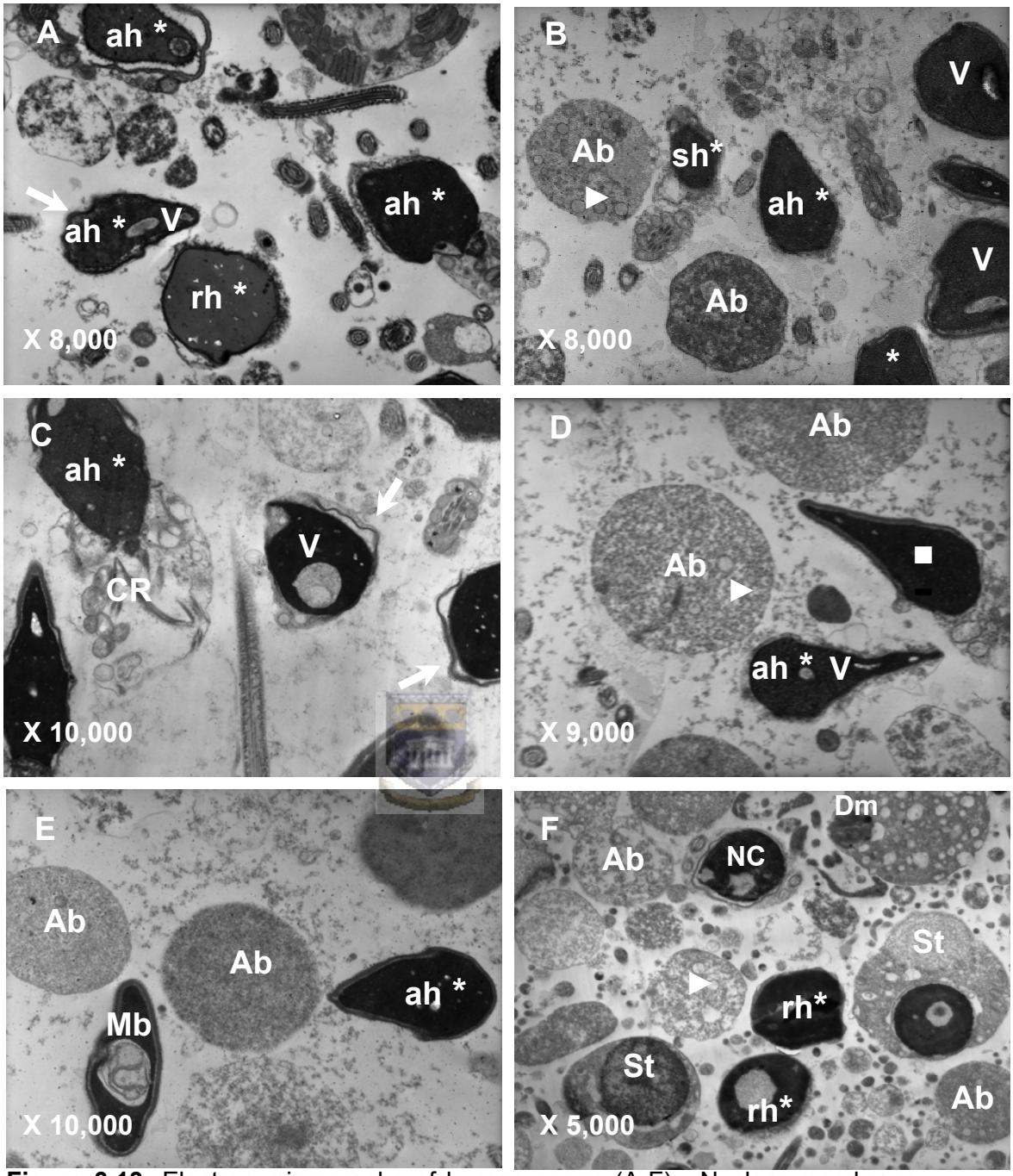


Figure 3.13: Electron micrographs of human sperm (A-F). Nuclear membrane protuberances (solid arrow) are indicated. Note the presence of vacuoles within the nuclear chromatin (V) and a membranous body (myelin figure) within the vacuole (Mb). Apoptotic bodies (Ab), cytoplasmic residues (CR) and spermatids (St) are indicated. Morphologically normal sperm (■) and defective sperm are indicated above (*). See text for explanations. Cytoplasmic vacuoles within the apoptotic bodies (►), dense mass (Dm) and necrotic features (NC) is indicated.

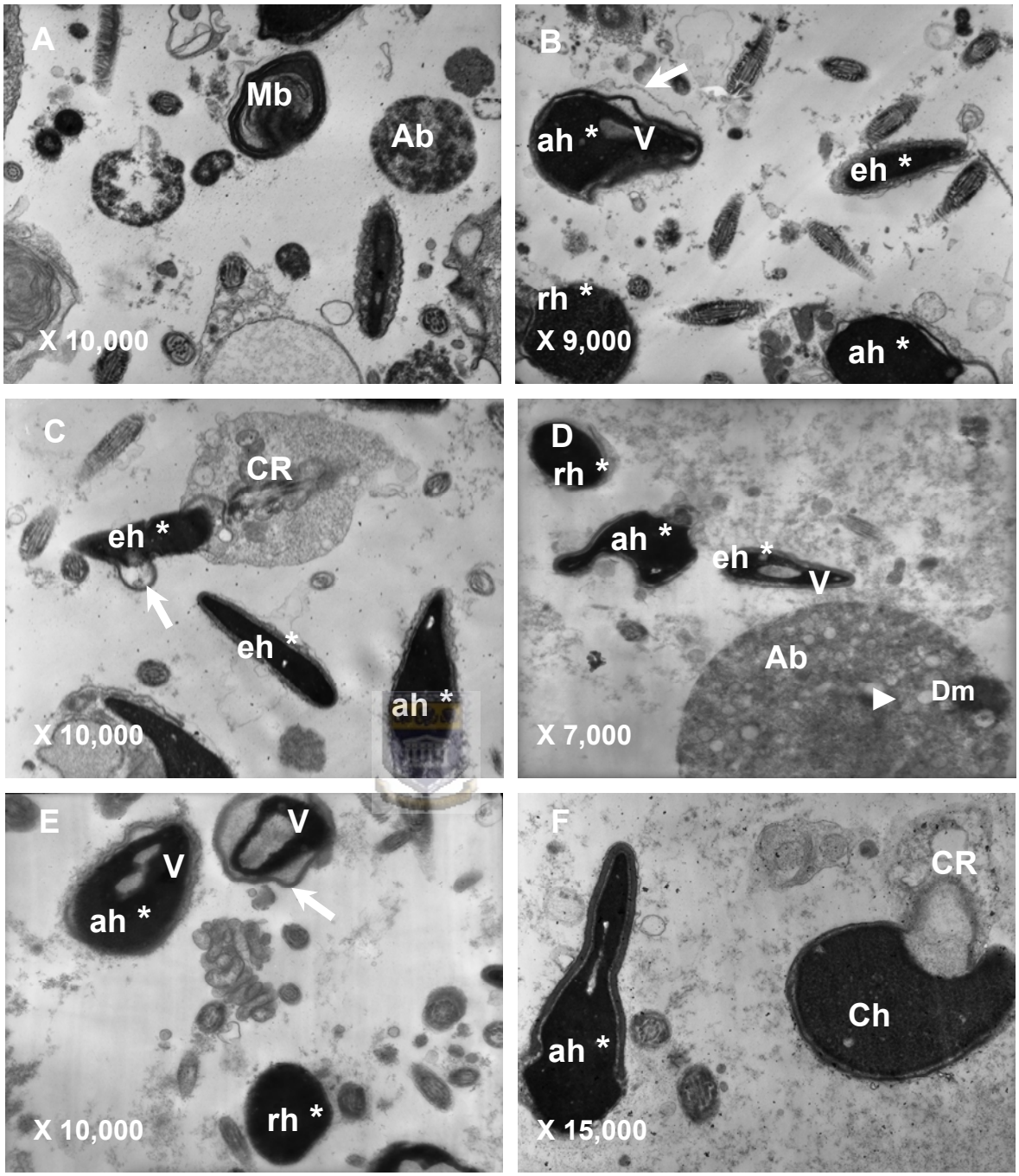


Figure 3.14: Electron micrographs of human sperm (A-F). Loose fibrillar-microgranular nuclear chromatin (Ch) with nuclear membrane protuberances (solid arrow). Note the presence of vacuoles within the nuclear chromatin (V) and a membranous body (myelin figure) within the vacuole (Mb). Also, apoptotic bodies are indicated (Ab) and some defective sperm are indicated above (*), which are explained in the text. Cytoplasmic vacuoles within the apoptotic bodies (►) and dense masses (Dm) is indicated.

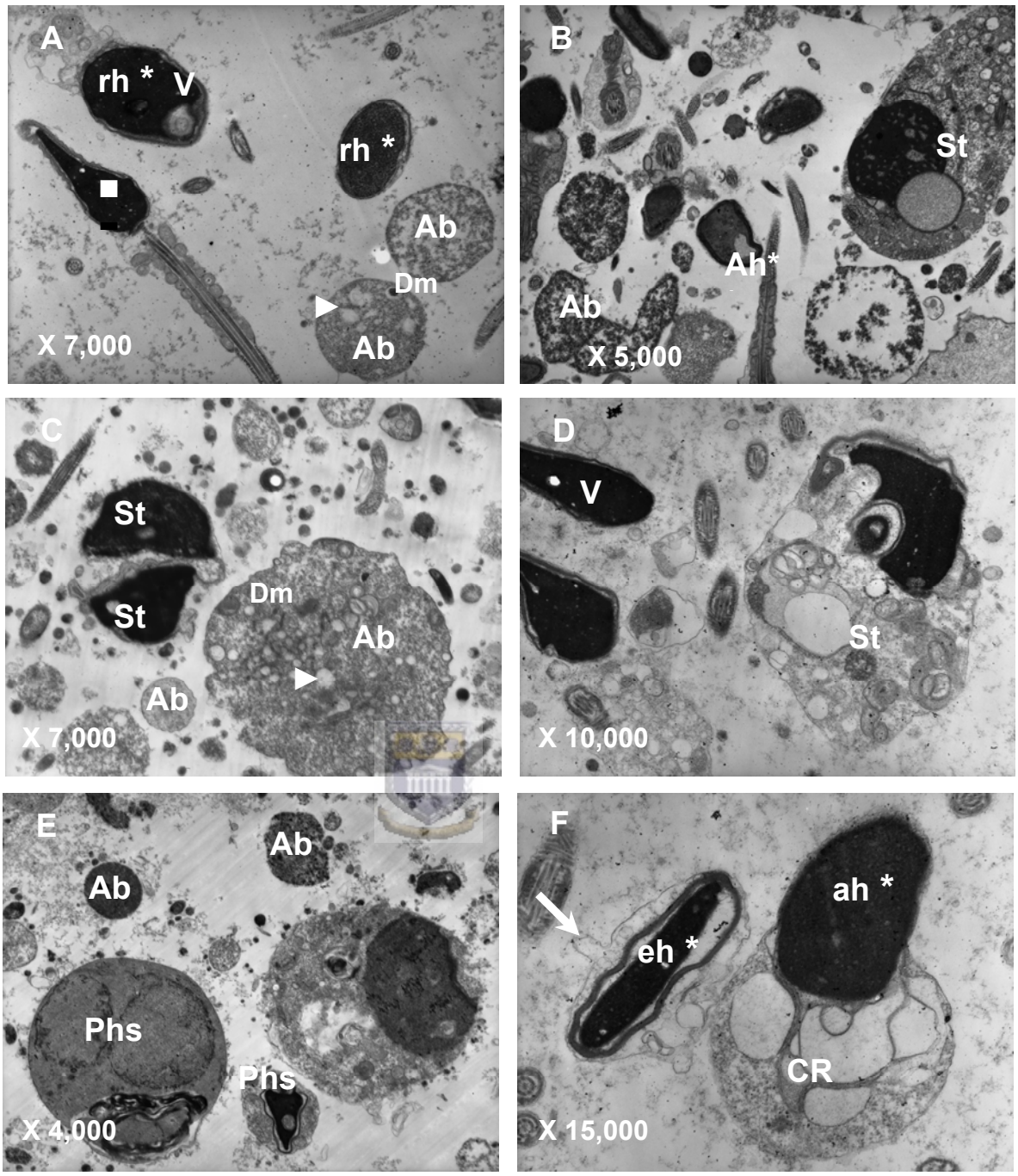


Figure 3.15: Electron micrographs of human sperm (A-F). Nuclear membrane protuberances (solid arrow) are indicated. Note the presence of vacuoles within the nuclear chromatin (V). Apoptotic bodies (Ab), cytoplasmic residues (CR), phagosomes (Phs) and spermatids (St) are indicated. Morphologically normal sperm (■) and defective sperm (explanations in text) are indicated above (*). Cytoplasmic vacuoles within the apoptotic bodies (►) and dense masses (Dm) is indicated.

Cytoplasmic vacuoles are seen in some of these apoptotic bodies (Figures 3.13B, D and F; Figure 3.14 D; Figures 3.15 A, and C) and dense masses of nuclear material (Figures 3.13F, 3.14D, 3.15A and C. These apoptotic bodies are possibly phagocytosed by the macrophages.

In addition to the apoptosis like features observed in the sperm samples, many sperm head abnormalities were evident and these are illustrated in Figures 3.12-3.15. These head abnormalities include; large heads, small heads, round heads, elongated heads and amorphous heads (Figures 3.12-3.15). Sperm with fairly morphologically normal sperm heads are indicated in Figures 3.12 B, 3.13D and 3.15A. An example of a sperm with a large head (lh) is indicated in Figure 3.12C and sperm with small head (sh) is shown in Figure 3.13B. Amorphous sperm heads (ah) are indicated in Figures 3.12C-F; 3.13A-E; 3.14B-E and Figures 3.15 B and F. Sperm with elongated heads (eh) are shown in Figures 3.12C, 3.14B-D and 3.15F. Round sperm heads (rh) are shown in Figures 3.13A and F, 3.14B, D and E and 3.15 A.

3.3.1 Phagocytosis in the ejaculate

The subsequent description under 3.3.1 of apoptosis and necrosis yielded cellular packages and debris material randomly removed by phagocytosis. The phagocytes in ejaculate seem to play an important role here (Figures 3.16A to D). From the morphological evidence of the transmission electron micrographs, it is evident that phagocytes (macrophages) were present in some of the ejaculate samples. The macrophages found in ejaculate sperm samples are shown in Figures 3.16 A to D.

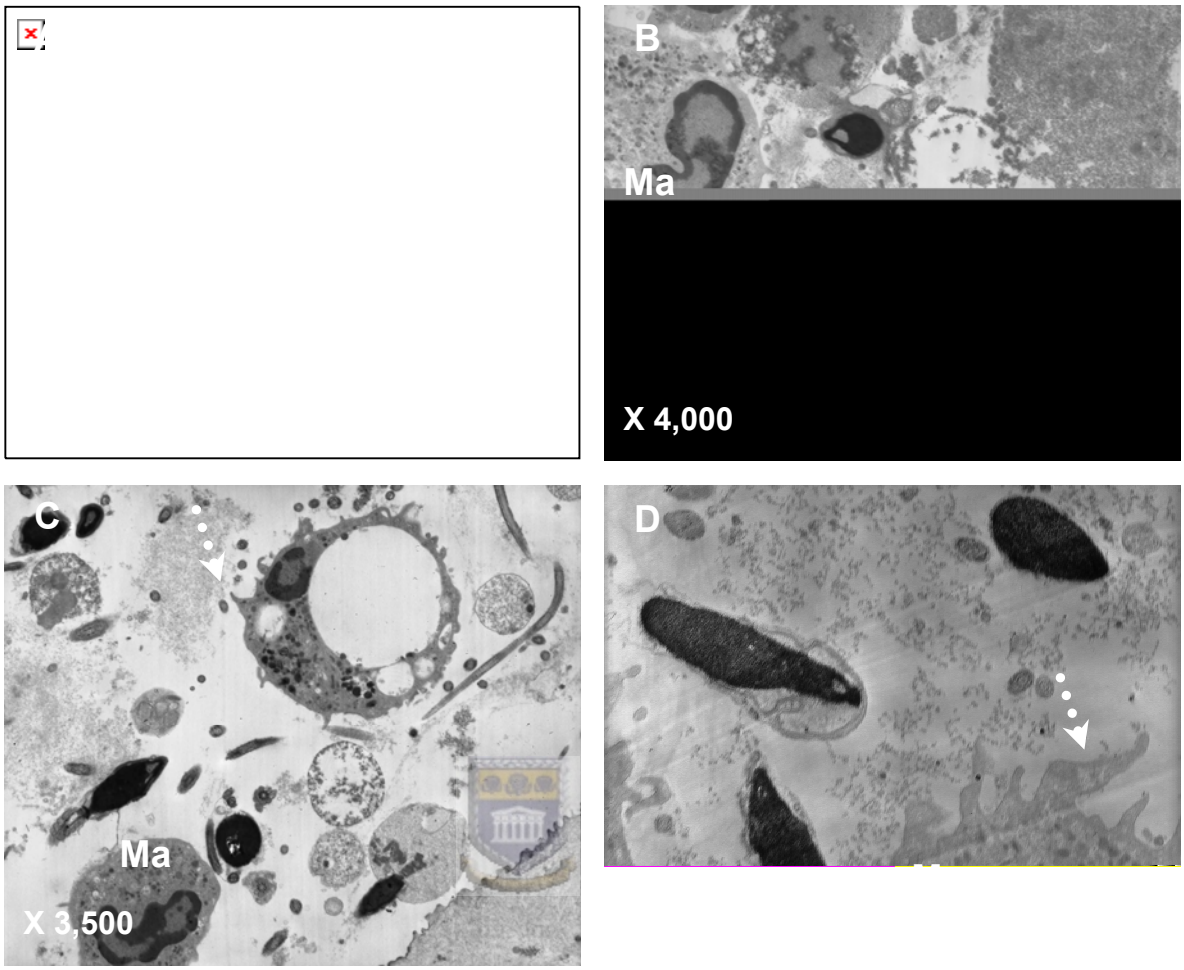


Figure 3.16. Electron micrographs of monocyte/macrophages (Ma) cells found in some of the ejaculate samples (A-D). Note the pseudopodia like extensions indicated by the broken arrow and the dense granules (*).

These macrophages are cells with a characteristic kidney-shaped nucleus as illustrated in Figure 3.16B, C and D. Figure 3.16A indicates a macrophage with its large nucleus and the presence of membrane folds (extensions). The macrophage illustrated in Figure 3.16C shows evidence of the presence of digestive vacuoles and cytoplasmic debris within this macrophage. Figure 3.16D indicates a macrophage with large extensions and dense granules within the cytoplasm. The presence of this type of seminal leukocyte namely monocytes/macrophages has not been quantified as the leukocyte profile was

not determined in this study. The identification of these structures in the ejaculate samples seem to suggest that active sperm phagocytosis is a process required to remove several types of foreign matter as a result of apoptosis or necrosis.

3.3.2 Morphological evidence of apoptosis and necrosis in sperm

Refer to Figure 1.1 in Chapter 1 (page 12) as described previously which compares the apoptotic and necrotic cell death pathways in somatic cells. These two processes have been shown to exist in ejaculate sperm in this study as determined by the Annexin-V/PI assay which determines the percentage of apoptotic as well as necrotic spermatozoa (Figure 3.3 on page 55). From a morphological point of view, using transmission electron microscopy, any structural features indicative of apoptosis and necrosis have been identified.

In summary, the sequential steps of the apoptotic pathway are outlined below. The cell shrinks and becomes denser and the chromatin becomes aggregated in masses while the nuclear outline is abnormally convoluted. Figure 3.12A, B, D and F indicates the microgranular appearance of the chromatin with the partially disrupted nuclear membrane of the sperm cells. Budding of the plasma membrane is evident and these are indicated in Figures 3.12C and F; 3.13A and C, Figures 3.14B, C and E and Figure 3.15F. Cytoplasmic condensation results and organelles usually retain their integrity, while cytoplasmic vacuoles are evident. The formation of the apoptotic bodies occur due to the breaking off of nuclear fragments and these are phagocytosed by macrophages or remains free. These apoptotic bodies are indicated in Figures 3.12 to 3.15 and the macrophages are shown in Figure 3.16 A to D. The phagosomes contain digestive enzymes for the degradation process of the contents (Figure 3.15E and 3.16C).

In contrast, necrosis involves chromatin aggregation with disruption of the nuclear membrane and characterised by swelling of the organelles (Wyllie *et al.*, 1980; Kerr and Harmon, 1991; Majno and Joris, 1995). There is an increase in membrane permeability leading to the acceleration of disintegration. A later stage involves the disappearance of the chromatin and is usually accompanied by inflammation. Figure 3.13F indicates a possible apoptotic sperm cell showing necrotic features as the compacted chromatin is clumped with ill-defined edges. This study has not emphasized the identification of the morphological features of necrosis using transmission electron microscopy.

In summary, spermatozoa in the ejaculate samples display some of the classical apoptotic features of somatic cells as initially described by Kerr *et al.* (1972) and Wyllie *et al.* (1980). The electron microscopy of this study clearly demonstrates many morphological features that depict apoptosis as demonstrated in somatic cells. The difference between these samples is the varying amounts of each of the above-mentioned apoptotic-like characteristics observed by transmission electron microscopy (no quantification performed) and the apoptotic assay results confirmed by flow cytometry.

CHAPTER 4

Discussion

It has previously been demonstrated that the underlying mechanism of germ cell death during spermatogenesis is apoptosis and it is an important mechanism regulating spermatogenesis in mammals (Sinha Hikim and Swerdloff, 1999; Print and Loveland, 2000; Ricci *et al.*, 2002). The study of apoptosis in spermatogenic cells has been reported by many authors (Billig *et al.,* 1996; Blanco-Rodrĩguez, 1998; Fujisawa *et al.*, 1999; Sinha Hikim and Swerdloff, 1999; Kierszenbaum, 2001; Martinčič *et al.*, 2001; Sinha Hikim *et al.*, 2003), however, there have been conflicting reports regarding apoptosis in ejaculated sperm (Gandini, *et al.*, 2000; Oosterhuis *et al.*, 2000; Ricci *et al.*, 2002; Shen *et al.*, 2002; Lachaud *et al.*, 2004). What remains unclear is whether the presence of these apoptotic markers on ejaculated sperm is residues of the "abortive" apoptotic process started at the testicular or post-testicular level or whether they are the result of apoptosis initiated during the post-ejaculation phase. It was not the purpose of this study to address or debate the above-mentioned conflicting hypotheses.

Firstly, a discussion of the standard semen analysis results followed by the apoptotic marker assay results and their correlations to the semen analysis results and finally the morphological evidence of features suggestive of apoptosis will be discussed.

4.1 Standard semen analysis

Standard semen analysis is fundamental to the andrological management of infertility as it forms part of the initial investigation of the couple. However, standard semen analysis assays do not provide any information regarding the presence of DNA fragmentation of sperm. As only a single sperm is used for intracytoplasmic sperm injection (ICSI), selecting a sperm with intact DNA would be crucial for fertilisation. The concern exists as spermatozoa with damaged DNA may lead to the transmission of defective genetic material into the female egg, and consequently, to possible abnormal embryo development (Kruger and Coetzee, 1999; Oehninger, 2000; Giwercman *et al.*, 2003; Larson-Cook *et al.*, 2003).

In this study, it was found that a significant decrease in sperm concentration for the oligoteratozoospermic [P-pattern] group when compared to the teratozoospermic [G-pattern] group is evident (Table 3.1). According to the WHO guidelines (1999), a sperm concentration below 20 X 10^6/ml is classified as abnormal. Sperm motility is an important factor of fertilisation, as poor sperm motility affects the sperm's fertilising ability (Giwercman *et al*., 2003). The decrease in sperm motility and sperm morphology in the oligoteratozoospermic [P-pattern] group could be attributed to the possible reactive oxygen species (ROS) production as found by Henkel *et al*. (2005a). These authors reported that a motility of less than 10% may represent a possible problem in assisted reproductive technologies. More details on ROS production by sperm and its effects are discussed under the heading "DNA Fragmentation" below.

Clearly, the mean value for normal sperm morphology among the subjects in the three study groups all fell below the Tygerberg strict criteria cut-off value of 14%. According to many studies, when the normal sperm morphology falls between 5 and 14% thresholds, a positive prediction for assisted reproduction

was obtained which can be used to identify a patient with subfertility in the clinical practice (Grow and Oehninger, 1995; Vawda *et al*., 1996; Coetzee *et al*., 1998; Gunalp *et al*., 2001; Van Waart *et al*., 2001; Van der Merwe *et al*., 2005). A study conducted by Menkveld *et al.* (2001) reported the cut-off value range may be between 3 to 4% morphologically normal sperm with the strict criteria. The sperm morphology according to the Tygerberg strict criteria appears to be a more sensitive and objective method as compared with the evaluation of sperm morphology according to the WHO (Menkveld *et al*., 1990; Vawda *et al*., 1996). However, sperm morphology evaluated according to WHO criteria was found to be comparable with those according to strict criteria (Menkveld *et al*., 2001). The Tygerberg strict criteria for evaluation of sperm morphology are now accepted by the WHO (1999).

The percentage of morphologically normal spermatozoa was decreased for all three groups; however, both the teratozoospermic [P-pattern] and the oligoteratozoospermic [P-pattern] groups had percentage normal sperm morphology below 4%. These two groups of patients would, according to the Tygerberg strict criteria, be at risk of poor fertilisation rates. The mean percentage of morphological normal sperm morphology of the teratozoospermic [G-pattern] group was 5.54%. Morphological evaluation by the strict criteria therefore assists to predict the male fertility potential for the outcome of fertilisation *in vitro* and assisted reproduction (Grow and Oehninger, 1995; Coetzee *et al.*, 1998; Kruger and Coetzee, 1999; Gunalp *et al.*, 2001; Van Waart *et al.,* 2001; Henkel *et al.*, 2005b; Van der Merwe *et al.*, 2005). However, sperm morphology merely provides a sensitive measure for fertilising potential, but cannot conclusively evaluate all the many prerequisites for fertilisation. This parameter has been shown to have the highest prediction for the outcome of assisted reproductive technologies (Coetzee *et al*., 1998; Van Waart *et al*., 2001; Van der Merwe *et al*., 2005).

The original contribution of this study was to identify key markers of the apoptotic process in sperm and these included Annexin-V expression, DNA damage, caspase-3 expression and Fas activity. The results of each of these assays are discussed below. Annexin-V and the Fas assay are early events of the apoptosis cascade and thus form part of the initiation phase of apoptosis. The caspase-3 and APO-Direct assays are later apoptotic events and are involved as the executioner molecules during the final phase of apoptosis; the proteases (caspases) are responsible for the DNA degradation.

4.2 Annexin-V Assay

The simultaneous staining of sperm cells with fluorescein isothiocyanate (FITC)-labelled Annexin-V and propidium iodide (PI) enables the discrimination of intact cells (live), apoptotic cells and necrotic cells (Vermes *et al.*, 1995; Overbeeke *et al.*, 1998; Van Engeland *et al.*, 1998; Oosterhuis *et al.*, 2000; Shen *et al.*, 2002). The expression of phosphatidylserine (PS), an early stage in the apoptotic process was assessed using bivariate Annexin-V/PI analysis. This assay clearly distinguishes between the intact (live) cell population (Annexin-V negative and PI negative), the apoptotic cell population (Annexin-V positive and PI negative) and the necrotic cells (Annexin-V and PI positive). PS exposure at the outer membrane surface of a cell is a universal process occurring during early apoptosis. The externalization of PS is a downstream event of caspase activation possibly preceding nuclear condensation and a phenomenon of the execution phase of apoptosis (Vermes *et al.*, 1995; Van Engeland *et al*., 1998). Using the Annexin-V assay, the number of apoptotic cells in suspension can be determined in a fast, simple and sensitive way.

In this study, it was found that the Annexin-V finding of the oligoteratozoospermic [P-pattern] group was higher and statistically significant when compared to the two other groups (teratozoospermic [G- and P-pattern] groups). These results indicate that men with poorer semen analysis results

exhibit an increase in Annexin-V expression which could be attributed to possible apoptosis of sperm and could be an indication of the outcome of fertilisation.

The results of a study by Shen *et al.* (2002) demonstrated that an average of 20% of spermatozoa was labelled with Annexin-V in sperm from subfertile subjects. These percentage apoptotic values were in the same range as those reported by Oosterhuis *et al.* (2000) where 20% of ejaculated spermatozoa were shown to be apoptotic by the Annexin-V assay. Similar findings were reported by Barroso *et al.* (2000) where the translocation of PS in fractions with low and high sperm motility was identified. They reported a similar proportion of cells exhibiting the translocation of PS in the fractions with fractions of high and low sperm motility. A study analysing the cell death in human ejaculate sperm reported that Annexin-V decreased significantly after 24 hours for the control and the centrifugation alone groups as compared to the increase in Annexin-V staining observed for the density gradient centrifugation group (Blanc-Layrac *et al.*, 2000).

The externalisation of PS has been related to other sperm conditions, such as capacitation rather than possible apoptosis (Gadella and Harrison, 2002; Kotwicka *et al.*, 2002; Harrison and Gadella, 2005). These authors demonstrated the presence of Annexin-V binding sites in the midpiece and the posterior portion of the head prior to capacitation. Following capacitation, these binding sites were found on the acrosomal region and the posterior part of the head. However, in this study we have worked entirely on ejaculate sperm and have not evaluated capacitated sperm, therefore the findings by Gadella and Harrison (2002) are not applicable to this study.

Our results have demonstrated a weak positive correlation between Annexin-V expression on sperm and percentage motility (Table 3.3). These findings are

supported by the results of Glander and Schaller (1999) who found that the early loss of sperm membrane asymmetry is combined with an increase in forward sperm motility. A study undertaken by Ricci *et al.* (2002) reported that a decrease in Annexin-V binding could be an indication of a high degree of membrane disorganization or an indication that cells may be in a later stage of apoptosis. Our findings indicate that Annexin-V expression of ejaculated sperm correlates negatively with sperm concentration. This result is supported by the findings of Oosterhuis *et al.* (2000). In addition, we found a weak positive correlation between Annexin-V and sperm motility, a result which is in contrast to the findings of Oosterhuis *et al.* (2000). In this study, no significant correlation was obtained between Annexin-V and sperm morphology.

The lack of correlation between the Annexin-V assay and the other apoptotic marker assays indicate that these assays are independent assays indicating the levels of apoptosis present, however the number of apoptotic sperm varied between the assays. Oosterhuis *et al.* (2000) showed no correlation between the sperm that express PS and the number that show DNA strand breaks. The authors concluded that these two phenomena occur during different phases of the apoptotic process. The Annexin-V assay is a fast, sensitive, reliable and easy to perform test for the identification of apoptotic cells to detect loss of plasma membrane asymmetry during apoptosis. While Annexin-V expression is evident in ejaculate sperm it may not necessarily just represent apoptosis. Annexin-V is a sensitive and relatively accurate indicator in somatic cells, but has variable and inconclusive results in ejaculate sperm. It therefore appears that Annexin-V may not be a suitable indicator for predicting the fertility potential of sperm (Henkel *et al.*, 2004).

4.3 DNA Fragmentation

The findings of this study have indicated that the oligoteratozoospermic [P-pattern] group has the highest percentage of fragmented DNA, however, not

significantly different from the other two groups. Although there were no significant differences for the percentage of DNA fragmentation between groups, the individual high incidences of DNA fragmentation reflects a possible problem which may affect fertilisation rates. Semen samples with low sperm concentration, poor motility and morphology show higher levels of DNA damage. These results indicate that men from the oligoteratozoospermia [P-pattern] and teratozoospermia [P-pattern] groups possess poorer sperm DNA integrity than the teratozoospermic [G-pattern] only group. These results are supported by the findings of Moustafa *et al.* (2004) where semen samples with low sperm concentration and poor sperm morphology demonstrated higher levels of TUNEL positivity. However, they reported that not all apoptotic sperm possessed DNA damage. On average, 15% of sperm were identified as TUNEL positive in a study conducted on a group of infertile patients (Shen *et al.*, 2002).

A study conducted by Sakkas *et al.* (2002) investigated the presence of DNA damage by TUNEL positivity in men with abnormal semen parameters. They proposed that the DNA damage is not linked directly to an apoptotic process occurring in spermatozoa. A study employing the COMET assay for DNA damage of sperm in fertile and infertile men reported that the DNA from the infertile group was more susceptible to damage (Hughes *et al*., 1996). The proportion of spermatozoa with DNA fragmentation was found to be significantly higher (mean level of 11%) in the fractions with low sperm motility when compared to the samples with higher motility (mean level of 1%) (Barroso *et al.*, 2000). Findings reported by McVicar *et al.* (2004) using the COMET assay suggested that fertile men have fewer fragmented DNA in their sperm when compared to infertile men and concluded that not all DNA damage is attributed to apoptosis. Their findings confirmed that DNA fragmentation is a useful indicator of sperm quality.

The results of this study demonstrate that there were no significant correlations between DNA fragmentation and Annexin-V for any of the groups. Also, no significant correlation existed between DNA damage and caspase-3 expression for the teratozoospermic [G-pattern] group. There was a positive significant correlation between DNA damage and caspase-3 expression for the teratozoospermic and oligoteratozoospermic [P-pattern] groups. This result indicates that caspase-3 activation occurs and this may lead to DNA damage of sperm in samples with poor semen parameters. Based on the results, significant positive correlations were evident only for the teratozoospermic [G-pattern and P-pattern] groups between Fas expression and DNA fragmentation indicating that Fas expression is associated with the execution of the possible cell death process. These results furthermore indicate that apoptotic markers do not exist simultaneously and support the findings by other investigators (Sakkas *et al.*, 2002; Mc Vicar *et al.*, 2004). The DNA fragmentation assay may give an indication of the DNA damage of sperm, however, it is a suitable indicator to predict the outcome of assisted reproductive technologies as its clinical relevance remains to be investigated further.

The amount of DNA damage reflected by the APO Direct assay could be attributed to possible apoptosis and possibly also due to oxidative stress, which was not examined in the present study. Henkel *et al*. (2005a) reported that an increase in DNA damage in sperm is possibly due to an increase in ROS production, produced by the sperm cells themselves or by the leukocytes. Henkel *et al.* (2003) found that spermatozoa are only a minor source of ROS production in the ejaculate. However, the impact of these cells and the production of ROS on male fertility potential are still poorly understood. The presence of DNA strand breaks in ejaculated spermatozoa could be due to incomplete sperm chromatin packaging due to faulty protamine deposition during spermiogenesis (Sakkas *et al.*, 1999; Sakkas *et al.*, 2002; Shen *et al.*, 2002; Oehninger *et al.*, 2003; Sakkas *et al.,* 2003). Another theory proposed

was that the presence of endogenous nicks in ejaculated human spermatozoa is due to apoptosis, a mechanism of eliminating defective germ cells from the reproductive pool (Gorczyca *et al.*, 1993; Sakkas *et al.*, 1999; Sakkas *et.al.*, 2002; Moustafa *et al.*, 2004). This study supports the findings of Wang *et al.* (2003) where an increase in sperm damage was obtained in patients with male factor infertility. Other authors have supported the findings that sperm DNA fragmentation was shown to be induced by ROS production which has a significant impact on sperm function and possibly contributing to male infertility (Lopes *et al*., 1998; Ochsendorf, 1999; Barrosa *et al.*, 2000; Gandini *et al.*, 2000; Irvine *et al*., 2000; Moustafa *et al*., 2004; Henkel *et al.*, 2005a). Sperm with an increase in DNA damage, possibly due to an increase in ROS production could indicate that these samples possess a decrease in antioxidant enzymes. Ricci *et al.* (2002) investigated the relationship between apoptosis and seminal leukocytes and indicated that seminal leukocytes probably provide the removal of apoptotic sperm constituting a control system of abnormal spermatozoa.

No significant positive or negative correlations were found to exist between DNA damage and standard semen analysis results (sperm concentration, sperm motility and sperm morphology). Our results indicate that these semen parameters do not appear to be indicative of the quality of sperm DNA. It has been shown that a negative correlation exists between the percentage of sperm with fragmented DNA and abnormal semen parameters (Sun *et al.*, 1997; Sakkas *et al*., 1999a; Gandini *et al*., 2000). The findings by Lopes *et al.* (1998) and Gandini *et al.* (2000) confirmed that DNA fragmentation of sperm is in line with the decrease in sperm concentration, sperm motility and also sperm morphology. Muratori *et al.* (2000) indicated that sperm showing DNA breakage are cells that have failed to complete maturation, and failed to complete the packaging of chromatin during spermatogenesis.

Poor-quality sperm chromatin structure was demonstrated to be highly indicative of male subfertility, regardless of the number, motility and the morphology of spermatozoa and seen as a predictor of the probability to conceive (Spanò *et al.*, 2000). Gandini *et al*. (2000) confirmed that a high level of DNA fragmentation is one of the characteristics of spermatogenetic failure. A study by Muratori *et al*. (2000) confirmed that no significant relationship exists between DNA breakage and the characteristics suggestive of apoptosis. The results of a study conducted by Giwercman *et al.* (2003) demonstrated that sperm motility and chromatin integrity are significantly associated. They concluded that motile and otherwise normal spermatozoa with damaged DNA has an impact on the fertility status and provides additional criteria for male factor infertility evaluations.

Larson-Cook *et al*. (2003) investigated the diagnostic and prognostic role of sperm nuclear DNA fragmentation and its effect on fertilisation in the assisted reproductive program. These findings indicate that fertilisation success was associated with sperm DNA fragmentation as men with decreased fertility potential display an increase in sperm DNA fragmentation (Host *et al.*, 1999; Zini *et al*., 2001; Marchetti *et al.*, 2002; Henkel *et al.*, 2003; Larson-Cook *et al*., 2003; Henkel *et al.*, 2004). A case study by Alvarez *et al*. (2004) demonstrated that sperm samples with >30% DNA fragmentation are at a much higher risk for not achieving a term pregnancy in assisted reproduction. These authors have proposed that morphologically normal sperm may harbour DNA fragmentation and therefore necessitated the importance of the sperm chromatin structure assay to provide accurate information for patients. Benchaib *et al*. (2003) indicated that sperm DNA fragmentation >10% influences the fertilisation rate and is therefore a potentially useful indicator of the outcome of ICSI. A study conducted by Payne *et al.* (2005) evaluated the relationship between sperm deoxyribonucleic acid damage by the sperm chromatin structure assay (SCSA) and the outcome of assisted reproductive

techniques. These authors reported that patients with a low DNA fragmentation index (≤9%) were least likely to achieve a pregnancy. They suggested that the SCSA should be used as part of the infertility investigation with great caution until further investigation has been conducted.

Sakkas *et al.* (1999a and 1999b) proposed two possible hypotheses to describe the origin of DNA damage in ejaculated human spermatozoa. His first hypothesis is based on the manner in which the sperm chromatin is packaged and could indicate incomplete endogenous nuclease activity during spermiogenesis. The second hypothesis attributes nuclear DNA damage of spermatozoa to apoptosis. The presence of DNA damage on ejaculate spermatozoa indicates that these spermatozoa have failed to be eliminated due to the "abortive apoptotic" mechanism; however, the reason human ejaculated sperm show these abnormalities remains unclear. This apoptotic process could be a pre-programmed event in the testes and only triggered by the appropriate stimulus. While the apoptotic events in sperm are part of the abortive process in germ cells, they are retained in sperm as remnants, thus the presence of these apoptotic markers. In conclusion, all sperm samples have displayed some degree of fragmented DNA.

4.4 Caspase-3 Expression

The results of this study demonstrate that spermatozoa from the oligoteratozoospermic [P-pattern] group display a higher degree of caspase-3 expression, statistically significant when compared to the teratozoospermic [G- and P-pattern] groups. These findings are in accordance with the findings of several authors (Wang *et al*., 2003; Marchetti *et al*., 2004). The results of a study by Weil *et al*. (1998) were in contrast to our findings and the above-mentioned findings, as these authors confirmed that caspases were lacking in epididymal mouse spermatozoa. Their findings raised the possibility that mouse sperm has a cell death independent of caspases; however, the mammalian species differs.

Taylor *et al.* (2004) demonstrated that caspase enzyme activity was higher in the ejaculates of infertile patients than fertile donors and caspase activity was higher in low motility fractions. A study undertaken by Marchetti *et al.* (2004) demonstrated the presence of caspase-3 activation on ejaculate sperm from infertile patients in preparation for IVF. The level of caspase activity found by these authors was higher than our results and may be due to the antibody specificity for other caspases as well. They also found that the frequency of caspase-3 expression was higher than the DNA fragmentation which indicates that some sperm cells are caspase-3 positive but without DNA damage. The results of our study support these findings as we found a higher level of caspase-3 expression compared to DNA fragmentation.

A positive correlation was noted between active caspase-3 expression and DNA fragmentation in all three groups, however, not significant for the oligoteratozoospermic [P-pattern] group (Table 3.3). This result indicates that in ejaculated sperm, caspase-3 is expressed and it possibly increases DNA fragmentation. Our findings supported the results of Wang *et al*. (2003). These authors also evaluated ROS production and its correlation with caspase-3 and -9 and cytochrome c. Their findings may suggest that caspases may function to increase PS translocation and DNA fragmentation. No relationship existed between caspase-3 expression and any of the semen parameters for any of the groups in our study.

A study by Paasch *et al.* (2004) confirmed that apoptosis might act via the CD95-death-inducing signalling complex, caspase-8 pathway in human sperm based on the caspases detected on sperm. They demonstrated the presence of active caspase-3 predominantly on the post acrosomal region of sperm. Said *et al.* (2004) confirmed that caspases play a major role in the pathogenesis of a multiplicity of andrological disorders, such as impaired

spermatogenesis, decreased sperm motility and an increased level of sperm DNA fragmentation. Our findings indicate the presence of caspase-3 activity on human ejaculate sperm and demonstrate the existence of a caspase-dependant apoptosis pathway in human sperm. However, whether caspase-3 activity was initiated in the testis, post-testicular level or post ejaculatory level could have an impact on the function and quality of sperm. The presence of caspase-3 activity on sperm may be an indicator of sperm dysfunction and possible infertility as it had the best correlation with the semen analyses results.

4.5 Fas Assay

From these apoptotic assay results, the teratozoospermic [G-pattern] group of patients displayed the lowest percentage of Fas expression as compared to the teratozoospermic [P-pattern] and oligoteratozoospermic [P-pattern] groups. An increase in Fas positivity in ejaculate spermatozoa from the teratozoospermic and oligoteratozoospermic [P-pattern] groups may indicate that there may have been errors in the activation of apoptosis and therefore the presence of these markers on ejaculate sperm. Ejaculate sperm with apoptotic markers appear to have escaped programmed cell death and reflect the failure of the sperm to complete apoptosis confirming the “abortive apoptosis” theory proposed by Sakkas *et al*. (1999a and 1999b). The results of this study confirm that an increase in Fas expression in the infertile male population was evident (Donnelly *et al.*, 2000; Sakkas *et al.*, 2002). This increase in Fas expression could be attributed to the poor functional activity of sperm and our results have confirmed that men with the poorest sperm parameters have the highest expression of Fas positive sperm. These results could also indicate that apoptosis via CD95 could be the mechanism by which possible “sperm apoptosis” occurs.

Sakkas *et al.* (1999a and 1999b) also indicated that men with reduced sperm parameters display an increase in Fas-positive spermatozoa while men with reduced semen parameters displayed as high as 50% Fas positive spermatozoa. Moustafa *et al.* (2004) reported an increase in Fas expression in semen samples exhibiting poor parameters. The presence of Fas positive sperm is an indicator that the elimination of spermatozoa via apoptosis is not occurring and an "abortive apoptosis" has taken place (Sakkas *et al.*, 1999a and 1999b). Thus, the Fas-mediated pathway appears to play a key role in controlling sperm populations during spermatogenesis.

In this study, no significant correlations were found between Fas expression and any of the semen parameters as illustrated in Table 3.3. A study undertaken by McVicar *et al.* (2004) found that fertile men displayed no Fas positivity, whereas 70% of infertile samples were Fas positive. Of the infertile samples, 96% of the couples displayed male infertility only. Thus they suggested a relationship between Fas signalling of sperm and male infertility. These authors also found a relationship between Fas expression and morphology and motility suggesting that Fas expression is a mechanism of eliminating defective sperm. Henkel *et al.* (2004) did not find any correlation of fertilisation and pregnancy to early markers of apoptosis (Annexin V binding and Fas expression).

A study investigating the expression of Fas on ejaculate sperm of normozoopermic and nonnormozoospermic men found that Fas was absent from these samples (Castro *et al.*, 2004). Their findings did not support the "abortive apoptosis" theory proposed by Sakkas (1999a and 1999b). The Sertoli cells activate the Fas-mediated system and initiate the germ cell death process as a means to eliminate inadequately supported germ cells that express Fas (Lee *et al.*, 1997; Blanco-Rodrĩguez, 1998; Lee *et al.*, 1999; Pentikäinen *et al.*, 1999; Sinha Hikim and Swerdloff, 1999; Print and Loveland,

2000; Kierszenbaum, 2001; McVicar *et al.*, 2004). The presence of apoptosis in the germ cells of the testes of men has been investigated by several authors; however, it was not performed in this study (Blanco-Rodrĩguez, 1998; Fujisawa *et al.*, 1999; Print and Loveland, 2000; Kierszenbaum, 2001; Martinčič *et al.*, 2001; Sinha Hikim *et al.*, 2003). The exact role of Fas in apoptosis of ejaculated sperm requires further investigation.

4.6 Apoptotic Marker Assays

The percentage apoptotic sperm as shown by the apoptotic markers are relatively low (Table 3.2), however, the current results have confirmed that ejaculate sperm exhibit DNA damage as well as the presence of various apoptotic markers previously described. Sakkas *et al.* (2002) confirmed, however, that the DNA damage and the presence of apoptotic markers do not always exist in unison. However, the presence of the apoptotic markers on ejaculate sperm indicate that these sperm have escaped programmed cell death (Sakkas *et al.*, 2003). They concluded that spermatogonia earmarked for apoptosis, escapes the process and therefore leads to combinations of anomalies such as histone-protamine exchange, DNA remodelling and cytoplasmic and membrane abnormalities of spermatozoa. A study by Silva and Gadella (2005) reported that sperm deterioration at the DNA level may not affect fertilisation rates as long as sperm membranes and organelles are functionally intact. However, Henkel *et al.* (2004) have demonstrated that DNA fragmentation, determined using the TUNEL assay is predictive for pregnancy in IVF. Our findings suggest that a possible relationship exists between the presence of apoptosis markers and the presence of poor sperm parameters, however it warrants further research.

The predictive power of each of the assays was examined using the statistical tool of ROC curve analysis. The values for the areas under the curve obtained by the ROC curves for most of the assays were all close to 0.5 in this study.

This implies that the variables under study cannot clearly distinguish between two groups and therefore these tests do not provide sufficient evidence. Also, the cut-off values obtained for each of the assays between the various groups did not correlate with our flow cytometric data for each of the samples and was not an accurate indicator. For caspase-3, the sensitivities were increased, specificities were relatively good and the area below the ROC curve was 0.69 and this provided a better predictor compared to the other assay results. With the addition of further work, the value of this analysis could be pertinent in the clinical setting. ROC curve analysis has been used in this study to determine cut-off values for each of the apoptotic marker assays comparing two groups at a time and has been useful in this context.

4.7 Electron Microscopy

In the present study, morphological aspects indicating possible apoptotic features of sperm cells were examined by means of electron microscopy. Any morphologically abnormal defects of sperm were also identified. The ultra-structural features suggestive of apoptosis have been originally described by Kerr *et al.* (1972). Our findings demonstrate the disruption of nuclear chromatin exhibiting a disorganized pattern of nuclear chromatin in some samples as described by Baccetti *et al.* (1996), Blanc-Layrac *et al.* (2000), Muratori *et al*. (2000) and Baccetti *et al.* (2002). We report the presence of vacuoles within the nuclear chromatin and the occurrence of membranous bodies (myelin figures) within some of these vacuoles as previously described (Baccetti *et al.*, 1996; Blanc-Layrac *et al.,* 2000; Muratori *et al.*, 2000; Kubo-Irie *et al.*, 2004). The occurrence of spheroidal bodies was identified in most of the ejaculate samples as reported by Baccetti *et al.* (1996). Smith *et al.* (1989) also described the presence of anucleate cytoplasmic masses in the ejaculate of fertile men.

The apoptotic bodies contain nuclear fragments and condensed cytoplasm, disrupted organelles and cytoplasmic material and considered the final phase of the apoptotic process as described by Kerr *et al.* (1972), Wyllie *et al.* (1980), Smith *et al.* (1989) and Baccetti *et al.* (1996). These apoptotic bodies contain nuclear fragments which bud off and form dense, rounded masses. The presence of macrophages within the ejaculate is common in the final phase of the apoptotic process as reported by Smith *et al.* (1989), Wolf *et al.* (1990), Blanco *et al.* (1992), Tomlinson *et al.* (1992), Aitken *et al.* (1994), Baccetti *et al.* (1996), Rossi and Aitken (1997) and Gil *et al.* (1998). The presence of macrophages in the ejaculate has been demonstrated in this study and their possible role includes the clearing of foreign matter or debris and degenerate or abnormal spermatozoa. Macrophage ingestion of the apoptotic bodies occurs by phagocytosis and these phagosomes contain lysosomes to rapidly degrade most of their contents. Most apoptotic bodies undergo rapid phagocytosis, while some may escape ingestion by macrophages. These apoptotic bodies eventually undergo spontaneous degeneration and swelling with membrane rupture (Wyllie *et al*., 1980). Apoptotic sperm cells that are not phagocytosed exhibit necrotic features, except for the inflammation process (Van Cruchten and Van den Broeck, 2002). The possible mechanisms involved in the phagocytic recognition of apoptotic cells by macrophages have been described (Hale *et al*., 1996; Rossi and Aitken, 1997; Afford and Randhawa, 2000; Ravichandran, 2003). The functional significance of these phagocytic cells such as the monocytes/macrophages within the ejaculate remains to be further elucidated.

A study undertaken by Smith *et al*. (1989) identified the presence of non-sperm cells in the ejaculates of fertile men using transmission electron microscopy. These authors reported that normal ejaculate samples host active phagocytosis and possibly macrophage activation. Tomlinson *et al.* (1992) found that ejaculates with >50% morphologically normal forms according to the

WHO criteria of spermatozoa contain significantly larger leukocyte populations. These authors suggested that the quality of sperm morphology is directly correlated with the size of the seminal leukocyte population and leukocytes might play a role in shaping the semen profile by phagocytosing morphologically abnormal or degenerate spermatozoa. A study conducted by Hughes *et al.* (1981) identified three types of leukocytes capable of phagocytosing spermatozoa: polymorphonuclear cells, small monocytes/macrophages and larger macrophages. Our study has confirmed the presence of macrophages within some ejaculate sperm samples; however, no quantification of leukocytes was performed. The upper limit of normality for leukocyte numbers in semen is 1 X 10^6 leukocytes/ml and values above this has been classified as leukocytospermia by the WHO (1999). The presence of macrophages (above the stipulated value) within the ejaculate samples is indicative of a possible infection and poor sperm quality. Punab *et al.* (2003) suggested that a cut-off value of 1 X 10^6 leukocytes/ml indicative of leukocytospermia might be too high. Henkel *et al.* (2005a) found that leukocyte counts <1 X 10^6/ml caused a significant decrease of motility and DNA integrity and in this context; the number of leukocytes in semen is controversial. Thus, the WHO cut-off value for leukocytospermia has seriously been questioned.

Transmission electron microscopy has been applied in a study to detect sperm abnormalities in samples from a group of infertile men (Baccetti *et al.*, 1999; Baccetti *et al.*, 2002; Kubo-Irie *et al.*, 2004). Carbone *et al.* (1998) reported a number of benefits of electron microscopy prior to assisted reproductive technologies. These authors indicated that patients with severely abnormal sperm motility and morphology may benefit from electron microscopy study of sperm as none of the patients in the study with abnormal sperm ultra-structure achieved success with assisted reproductive technologies. Electron microscopy may assist to identify patients who might pass on inheritable

genetic disorders through assisted reproductive technologies, specifically ICSI (Carbone *et al.*, 1998).

The findings of this study have indicated the presence of features suggestive of possible apoptosis of ejaculate human spermatozoa as previously reported. Transmission electron microscopy clearly showed variation in the incidence of possible sperm apoptosis between ejaculate sperm samples in this semi-quantitative study. Electron microscopy is a valuable tool to evaluate ultra-structural morphological sperm defects and possible apoptosis. It adds another dimension to the evaluation of sperm of both fertile and infertile individuals for assisted reproductive technologies.

4.8 Conclusion

It remains unclear whether apoptosis in ejaculated sperm occurs in a manner similar to somatic cells or whether sperm undergo an "abortive apoptosis" as proposed by Sakkas *et al.* (1999b). Some researchers propose that sperm do not undergo apoptosis; however, these findings are conflicting. The questions still remain as to whether apoptosis occurs during the process of spermatogenesis or at the post-testicular level and whether abnormal sperm are removed by apoptosis and necrosis. It was not the purpose of this study to identify exactly where sperm apoptosis is initiated, but rather to identify selected apoptotic markers on ejaculate sperm samples. Our results have confirmed the presence of selected apoptotic markers on ejaculate sperm and appear to support the "abortive apoptosis" mechanism.

This study has conclusively compared four apoptotic assays for each of the ejaculate sperm samples and their correlation with the standard semen analysis parameters. No other study has demonstrated these different apoptotic markers [DNA fragmentation, Annexin-V, Caspase-3 and Fas expression] on human ejaculate sperm and correlated these apoptotic markers

with each other and with the semen parameters in the same study. Annexin-V seems to be problematic to consider due to other variables including capacitation which may indicate the loss of plasma membrane symmetry in apoptotic cells. This study has shown that the Fas assay was not a good indicator of apoptosis in this context as it has shown no correlation with any other assay or standard semen analysis results. This study also convincingly shows that caspase-3 is potentially valuable for future work particularly as it relates to DNA damage and correlated with electron microscopy. This study has clearly confirmed that the poorer the semen sample, the higher the levels of the apoptotic markers. The possibility for not having major differences within the groups is that some sperm may be arrested before caspase-3 expression and therefore this marker was not detected on sperm. Our observations have indicated that the expression of these apoptotic markers on human ejaculate sperm suggest that apoptosis has possibly taken place. The exact molecular mechanisms for the possible apoptotic process, however, remain to be elucidated. Questions still remain as to which markers are representative of apoptosis and which ones relate to fertility. In this study, caspase-3 was related to DNA damage, which was confirmed by the morphological features identified by electron microscopy.

In addition, we have attempted to identify ultrastructural features of apoptosis by means of electron microscopy. While there have been suggestions of phagocytotic activity in semen samples, this work supports the evidence of the presence of macrophages in human ejaculate samples and future studies could investigate this aspect in further detail. The transmission electron microscopy observations have further supported the apoptotic marker assay findings. The characteristics suggestive of apoptosis observed by electron microscopy could be an indication of the poor morphology results obtained for the standard semen analysis.

The findings of this study suggest that the Fas mediated system is involved as a regulator of the activation of the apoptotic process. These apoptotic marker assays provide additional information about sperm deterioration and may prove to be adequate tools to further study the quality of sperm. Future studies should aim to determine the exact molecular mechanism involved in possible sperm apoptosis and the possibility of these apoptotic markers as a diagnostic and accurate indicator of sperm dysfunction and male infertility.

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