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Apoptosis Pathways:

Presence and Significance in Ejaculated Human Spermatozoa

by

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ABSTRACT

Apoptosis Pathways:
Presence and Significance in Ejaculated Human Spermatozoa

Steven Lewis Taylor
Old Dominion University, 2002
Co-Director: Dr. Sergio C. Oehninger
Co-Director: Dr. Mahmood S. Morshedi
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Ejaculated sperm display markers that are indicative of apoptosis in somatic cells. The question remains as to whether sperm have operative apoptosis mechanisms. The aim of this research was to test the hypothesis that apoptosis markers in sperm and somatic cells are different.

Ejaculated human sperm from patients and donors were separated into high and low motility fractions using Percoll™ gradients. Contaminating cells were removed using anti-CD45 conjugated paramagnetic beads. Fractions were divided into groups: staurosporine, anti-Fas antibody, and hydrogen peroxide treated and control. Direct enzymatic measurement of caspase activity, flow cytometric evaluation of phosphatidylserine translocation, immunoblots, and immunocytochemistry were used in this investigation.

Of the pro-apoptotic treatments, only staurosporine resulted in elevated caspase activity in patients and donors. Differences in caspase activity were found between donor and patient sperm. No elevations low motility fraction caspase activities were detected.

In Annexin-V-FITC labeled high motility fractions, staurosporine, anti-Fas, and 25 micromolar hydrogen peroxide failed to stimulate phosphatidylserine

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translocation. However, 200 micromolar hydrogen peroxide resulted in translocation increases. No treatment stimulated translocation in low motility fractions.

In immunoblots, the presence of procaspase-3 was detected in high and low motility fractions from donors and patients. Active caspase-3 was detected in high and low motility fractions from donors and patients. Procaspase-7, but not active, was also detected. Procaspase-9 was detected in high and low motility fractions from donors and patients. Active caspase-9 was only detected in a low motility patient fraction. Neither intact Poly-ADP-ribose Polymerase nor its breakdown product was detected. Apoptosis Inducing Factor was present in immunoblots from all high and low motility fractions. BID was not detected in either high or low motility fractions.

Active caspase-3 was detected using immunofluorescent microscopy in severe oligoasthenoteratozoospermic patients. Less than one percent of the sperm evaluated exhibited active caspase-3.

Sperm possess apoptosis markers. However, pro-apoptotic treatments indicated that apoptosis was not fully inducible in sperm. Sperm lack major signals associated with caspase dependent apoptosis in somatic cells. Since mitochondria are the only active organelle present in sperm, further work must be done to determine whether mitochondria dependent pathways are intact.

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To my parents Lewis H. Taylor, Jr. and Mary J. Taylor, I am eternally grateful for interminable support throughout my college education, long though it has been.

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CHAPTER I

INTRODUCTION

DEFINING APOPTOSIS

The term apoptosis derives from Greek terminology that is used to describe the shedding of leaves. Apoptosis was first coined by Kerr *et al.*, (1972) in a landmark paper describing a process of cell death distinct from necrosis in that it was the result of a normal physiological process in ontogeny. It was also known at this time that this newly described process could be induced through treatment with noxious stimuli.

Kerr's determination of apoptosis was based on morphological criteria. Cells classified as apoptotic had already separated themselves from other cells if part of a tissue. Apoptotic cells demonstrated pronounced condensation of the cell nucleus. Another noteworthy characteristic of apoptosis was membrane protuberances or "blebbing" and subsequent formation of apoptotic bodies. Apoptotic bodies were observed to contain cell organelles that appeared to be intact structurally as well as functionally. Other cells soon phagocytized the by-products of apoptosis. A distinguishing characteristic in apoptosis compared with necrosis was the lack of inflammation in apoptosis. Kerr also suggested that apoptosis plays a vital role in maintenance of normal tissue homeostasis and during embryonic development.

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PS translocation can occur under several mechanisms. During apoptosis, APT is inhibited and phospholipid scramblases are activated, resulting in the exposure of PS on the outer leaflet of the plasma membrane. This change stimulates the removal of apoptotic cells by phagocytes (Fadok *et al.*, 1992; Fadok *et al.*, 2001; Hoffmann *et al.*, 2001).

There is some evidence that PS oxidation occurs during apoptosis and that this modification can adversely affect recognition of PS by APT and scramblase (Kagan *et al.*, 2000)]. Oxidation of cysteine residues by reactive oxygen species (ROS) on APT inactivates the enzyme (de Jong *et al.*, 1997; Hermann and Devaux, 1990; Morrot 1989 *et al.*, 1989). Hence, failure of APT to catalyze internalization of PS may be due to inability of the enzyme to recognize oxidized PS or may be due to inactivation of APT by ROS. Oxidation of PS, as well as other phospholipids, can also increase their tendency to spontaneously translocate or "flip" to the outer leaflet of the plasma membrane (Hampton *et al.*, 1996). Although it could not be discounted, caspase inactivation of APT is not thought to be an exclusive mechanism (Zhaung *et al.*, 1998).

A caspase-3 role in PS translocation was supported by a recent study (Mandal *et al.*, 2002). In this study, erythrocytes were stimulated through oxidative stress to undergo PS translocation, which was shown to be inhibited, but not totally abolished, by the caspase-3 inhibitor Z-DEVD-FMK. Erythrocytes are known to have functional caspases-3 and -9 (Wolf *et al.*,

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1999). Further, these cells were determined to have reduced aminophospholipid translocase (APT) activity.

Caspases are a family of aspartic acid directed cysteine proteases that are involved in apoptosis. The appearance of active caspases is a well-known biochemical marker for apoptosis. Caspases are synthesized in an inactive, proenzyme form. The proenzyme consists of three domains: an NH_2-terminal sequence, a large subunit, and a small subunit. Proteolytic cleavage of the proenzyme results in separation of the three domains and association, in the form of heterodimers, of large and small subunits to form the active enzyme. The presence of caspase concensus sequences at cleavage sites of the domains in the proenzyme makes these proteins targets for other activated caspases (Stennicke *et al.*, 1998). Alternatively, procaspases may also activate autocatalytically (Muzio *et al.*, 1998).

The first caspase identified was caspase-1, then known in mammalian cells as interleukin-1β converting enzyme (ICE) (Thornberry and Lazebnik 1998). This enzyme, termed CED-3 (cell death abnormal-3) when first discovered in nematodes was implicated in cell death in *Caenorhabditis elegans* (Ellis and Horvitz 1986) and was found to have sequence homology to mammalian ICE, also known as caspase-1 (Yuan *et al.*, 1993). To date, 14 caspases have been discovered. Among them, caspases-3, -9, and –8 are the best characterized major players in apoptosis.

Caspases are classified as initiator or effector caspases. Initiator caspases are activated early in apoptosis and often require a co-factor. An

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example of cofactors involved in apoptosis are apoptosis protease activating factor-1 (Apaf-1) and cytochrome c. Effector or executioner caspases are downstream of initiator caspases and are activated by initiator caspases. Caspases -3, -6, and -7 are examples of an effector caspase, downstream of initiator caspases such as caspase-8 or -9 (Thornberry and Lazebnik 1998). However, caspase-3 is a common point of convergence in both caspase-8 and -9 pathways. Caspase-3 acts to amplify the apoptotic signal by activating other caspases (-3, -6, and -7) as well as inactivating essential cellular proteins involved in homeostasis.

During apoptosis, the release of cytochrome c from mitochondria may be a key event in oxidative modification of PS. Release of cytochrome c from mitochondria occurs concurrently with a dramatic intracellular increase in reactive oxygen species (ROS) production and release into the cytosol (Cai and Jones 1998). These ROS may be directly responsible for oxidation of PS. Kagan (2000) suggested that cytochrome c might oxidize PS on the cytosolic face of the plasma membrane and cause an increase in oxidized PS translocation.

An early event in apoptosis is a reduction in mitochondrial membrane potential ($\Delta\Psi_m$). This occurs prior to completion of fragmentation of cellular DNA into oligonucleosomal fragments. $\Delta\Psi_m$ decrease is thought to occur by formation of pores on the inner mitochondrial membrane during permeability transition. Permeability transition pores destabilize $\Delta\Psi_m$ by facilitating equilibration of ion gradients between the cytoplasm and mitochondrial matrix.

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Alternatively, $\Delta\Psi_m$ can be disrupted through a Fas activated pathway. In this pathway, caspase-8 cleaves the full-length 22 kDa Bid, a cytoplasmic protein involved in the Fas type II apoptosis pathway (Nagata 1999; Li *et al.*, 1998). The shortened 15 kDa Bid protein then proceeds to promote disruption of $\Delta\Psi_m$ by promoting pore formation and facilitating the escape of cytochrome c (Lou *et al.*, 1998), which then promotes caspase cascades and results in apoptosis (Gross *et al.*, 1999). Cleavage of Bid by other caspases may result in lower molecular weight Bid species (11 and 13 kDa). After reduction of $\Delta\Psi_m$, mitochondrial apoptosis promoters escape to the cytosol. Among them are cytochrome c and Apoptosis Inducing Factor (AIF).

AIF is released from the mitochondrial intermembrane space and is translocated to the nucleus where it promotes DNA fragmentation (Daugas *et al.*, 2000). Caspase inhibition has been demonstrated not to affect the release of AIF and cytochrome c (Daugas *et al.*, 2000; Susin *et al.*, 1999b). However, Bcl-2 overexpression inhibits AIF translocation, as BCL-2 counteracts apoptotic signals to mitochondria (Susin *et al.*, 1999b). AIF causes large scale DNA fragmentation (50-200 kb) rather than the oligonucleosomal fragments seen during later stages of apoptosis. AIF has also been shown to adversely affect permeability of isolated mitochondria, an event that could lead to a positive feedback loop intensifying the apoptotic signal (Susin *et al.*, 1999b). The apoptotic effects of staurosporine (STS) can be counteracted, to a degree by overexpression of Bcl-2. Bcl-2 hinders AIF and cytochrome c release from mitochondria (Yang *et al.*, 1997; Kluck *et al.*,

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1997). Staurosporine has been shown to induce AIF release from the mitochondria concurrent with the release cytochrome c (Daugas *et al.*, 2000; Susin *et al.*, 1999b). In addition, it has been found that AIF transport to the nucleus is energy dependent.

Procaspase-3 has also been found in mitochondria and is likely liberated from the intermembrane space in its active form during loss of $\Delta\Psi_m$ (Mancini *et al.*, 1998). In this study, apoptosis was induced in human keratinocytes by either irradiation with UVB, C2-Ceramide treatment, or staurosporine treatment. Mitochondrial membrane potential was monitored during treatment and in control replicates. With staurosporine treatment, cells lacked both the mitochondrial label indicating intact mitochondrial membrane potential and caspase-3 staining. Additionally, procaspases-2 and -9 have also been localized to the intermembrane space in similar studies (Susin *et al.*, 1999a).

Poly (ADP-ribose) polymerase-1 (PARP-1) is an approximately 116 kDa eukaryotic protein that functions to help repair DNA damage. PARP-1 activity increases with increasing DNA fragmentation (Halldorsson *et al.*, 1978). Currently, four PARP enzymes have been discovered. PARP-1 functions to bind to and catalyze the repair of DNA strand breaks. During apoptosis, PARP-1 is inactivated by one or more of caspases-3, -7, and/or -9. The inactive PARP-1, like caspase activation, is a telltale marker of active caspase-dependent apoptosis pathways.

DNA fragmentation in apoptosis is the culmination of the combined influences of a number of pathways. In apoptotic cells, caspase cascades

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result in the activation of Caspase Activated DNase (CAD), which acts on DNA to produce oligonucleosomal fragmentation. DNA is initially digested into segments ranging from 50-200 kb (Susin *et al.*, 1999b; Oberhammer *et al.*, 1993; Lagarkova *et al.*, 1995). Later, DNA is cleaved into smaller, nucleosomal units. Apoptosis also requires new mRNA and protein synthesis in early stages (Kidd 1998).

Caspase-3 acts to amplify the cell death signal and eventually activates CAD. CAD is initially associated with ICAD (inhibitor of CAD). Caspases target ICAD and cause disassociation of ICAD and CAD, activating the CAD enzyme (Nagata 2000). CAD is responsible for nuclear DNA fragmentation in apoptotic cells. Caspase-3 also inactivates PARP-1, a protein involved in the repair of DNA damage. The result is a fail-safe system in which caspase-3 activation ultimately leads to DNA fragmentation while simultaneously inactivating DNA repair mechanisms.

APOPTOSIS INDUCTION

Apoptosis is, in many cases, initiated by the binding of the Fas ligand (FasL) to extracellular receptors (CD95). FasL is a type II 40 kDa membrane protein in the tumor necrosis factor (TNF) protein family. *In vivo*, a common apoptosis inducer is the FasL. FasL is expressed by immune cells of T-cell lineage and natural killer cells (Suda *et al.*, 1995; Oshimi *et al.*, 1996). FasL binds to a Fas/APO-1/CD95 receptor that has been found in cells targeted for apoptosis. Fas stimulated apoptosis results from trimerization of Fas receptors. Experimentally, Fas receptors can be cross-linked using

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recombinant FasL and certain stimulatory antibodies (Trauth *et al.*, 1989; Rokhlin *et al.*, 1997). Fas receptors are a type I membrane protein.

Two major apoptotic signaling schemes are seen in cells undergoing CD95 stimulated apoptosis. These two pathways have come to be known as Type I and Type II pathways. Typically, one pathway will predominate (cell type dependent) and the other will play a supportive or reinforcement role. The determination of whether a particular cell type reacts to apoptotic stimuli in a type I or type II manner depends upon the amount of active caspase-8 generated by the trimerization of CD95 receptors. Generation of large amounts of caspase-8 result in the assumption of the type I pathway, while a much more modest caspase-8 recruitment results in the cell taking the type II route (Scaffidi *et al.*, 1998).

In Type I cells, apoptosis FasL binding causes trimerization of Fas receptors (APO-1 or CD95). Fas receptors have associated with them an adaptor molecule, the Fas Associated Death Domain (FADD). FADD binds to Fas through carboxy-terminal death domain interactions. An N-terminal area of FADD is called a death effector domain (DED) that binds to N-terminal DED domain located on procaspase-8. The association of these molecules is termed the death inducing signaling complex (DISC). Procaspase-8 has some protease activity and, under these conditions, the enzyme activates through autocatalysis during oligomerization. Hence, trimerization of Fas receptors and their associated FADD causes processing of procaspase-8 into its active form (Nagata 1997). A hallmark of type I Fas mediated apoptosis is

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the recruitment of large amounts of caspase-8. Within seconds of Fas trimerization, caspase-8 is activated, which then activates caspases-3, -6, and -7.

In Type II cells, apoptosis is also initiated by the binding of FasL or through chemical stimuli and primarily involves mitochondrial factors. Recruitment of DISC is drastically reduced compared with type I cells, although adequate component proteins are present. Apoptosis inducing conditions cause reduction of $\Delta\Psi_m$ and the escape of major apoptotic mitochondrial associated proteins such as cytochrome c and AIF. In Fas mediated pathways, activation of caspase-8 results in the cleavage of Bid. The truncated form of Bid is a pro-apoptotic factor that, in turn, triggers the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$).

Stimulation of the release of cytochrome c from mitochondria initiates an apoptotic cascade in type II cells. Cytochrome c and the cytoplasmic protein Apaf-1 contribute to the activation of caspase-9. Apaf-1 is a 130 kDa protein with an N-terminal caspase recruitment domain (CARD) that binds to procaspase-9 proteins. Apaf-1 first binds to and hydrolyzes dATP in the presence of cytochrome c, forming a multimeric Apaf-1/cytochrome c complex. This complex then associates with procaspase-9 proteins, resulting in autocatalytic activation of caspase-9. The Apaf-1/cytochrome c/procaspase-9 complex is termed the apoptosome (Li *et al.,* 1997).

Caspase-9 serves to activate effector caspases-3, -6, and -7. AIF, released into the cytosol along with cytochrome c, is translocated to the

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nucleus where it promotes DNA fragmentation through an unknown mechanism. Type II responses can be countered by overexpression of two anti-apoptotic proteins, BCL-2 and/or BCL-$_{XL}$.

Timeline differences in the activation of major apoptotic enzymes are also markedly different between type I and type II cells (Scaffidi *et al.*, 1998). In type I cells, recruitment of large amounts of caspase-8 is immediate and caspase-3 recruitment can be recognized within 30 minutes. Additionally, type I cells are not susceptible to the anti-apoptotic effects of overexpression of BCL-2 and BCL-$_{XL}$. In type II cells, recruitment of large amounts of caspase-8 does not occur until as late as an hour after CD95 stimulation. In type II cells, activation of the caspase cascade can be inhibited altogether by BCL-2 mediated inhibition of the release of mitochondrial pro-apoptotic factors.

Alternative designations of caspase-dependent and caspase-independent have also been used to classify cellular pathways. Caspase-dependent pathways involve activating caspases during initiation of apoptosis. Caspase-independent pathways of apoptosis generally involve mitochondrial factors. In many cell types, cross-activation and reinforcement between caspase dependent and independent pathways occurs.

Apoptosis can be experimentally induced in somatic cells using any number of stimuli. Early studies induced apoptosis through exposure to ionizing radiation. Irradiation was found to induce PS translocation (Vermes *et al.*, 1995).

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marker for incomplete spermatogenesis or as an indicator for immature sperm. Cytoplasmic droplets are composed of unnecessary or discarded cellular material unused during spermatogenesis and are normally phagocytized by sertoli cells.

Since the use of morphological criteria to demonstrate apoptosis in ejaculated spermatozoa is precluded by the unique specializations of this cell type, attention must be turned towards biochemical means of evaluating the question of whether apoptosis occurs in sperm. Few biochemical markers of apoptosis have been demonstrated in sperm.

DNA fragmentation, an important end result of apoptosis, has been demonstrated in sperm. Aitken, et al. (1998), demonstrated that DNA damage in sperm was associated with high levels of reactive oxygen species in sperm fractions.

The presence of caspase-3 in sperm has been reported in one previous study (Weng *et al.*, 2002). However, active caspase-3 was only detectable by immunofluorescent microscopy in a small percentage of the sperm evaluated. Enzymatic measurements of caspase activity were similarly low in untreated patient and donor sperm fractions.

A single previous report of PARP-1 presence in sperm fractions has been published. Blanc-Layrac, et. al. (2000) reported that both the intact 116 kDa and the caspase modified 85 kDa forms of PARP-1 were present in fractions from semen samples. Three fractions were derived from each sample: whole semen, washed semen, and motile sperm separated by density gradient

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centrifugation. Both 116 kDa and 85 kDa forms of PARP-1 were observed in the whole semen and washed semen fractions. The 85 kDa form was specifically mentioned as not being present in sperm fractions isolated by density gradient centrifugation. Curiously, no mention was made of whether intact PARP-1 was observed in this fraction. No other studies have been published investigating the presence of PARP-1 in sperm fractions.

AIF is a mitochondrial protein that is released and is translocated to the nuclei of somatic cells undergoing apoptosis. AIF alone is sufficient stimulus to induce apoptotic changes to isolated nuclei (Susin *et al.*, 1999b). AIF is associated in somatic cells with Type II or mitochondrial dependent mechanisms of apoptosis. AIF has not been previously demonstrated in ejaculated spermatozoa.

It has been suggested that apoptosis markers may be used as semen quality indicators (Jurjen *et al.*, 2000). Assays were proposed to detect two common apoptosis markers, PS translocation and DNA fragmentation. The assay used for the detection of PS translocation was flow cytometric evaluation of Annexin-V-FITC binding to PS in sperm plasma membranes, using propidium iodide (PI) as a counterstain for viability. PI binds to DNA and is membrane impermeable, hence serving as a reliable indicator of intact plasma membranes. Inverse correlations were found between Annexin-V-FITC binding and concentration and motility.

The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay was used to detect DNA fragmentation. TUNEL results

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indicated an inverse correlation between concentration and DNA fragmentation, but no correlation between TUNEL positive (DNA fragmentation) and Annexin-V binding (PS translocation) could be detected. Although the suggestion that sperm undergo apoptosis is intriguing, no evidence has yet been presented to indicate that sperm do, in fact, undergo the process. Recent studies have indicated that PS translocation occurs during acrosome reaction (Gadella and Harrison 2000 and 2002). Annexin-V binding in ejaculated spermatozoa, then, may merely be indicative of the early stages of acrosome reaction.

SPECIFIC AIMS

1. To identify changes in ejaculated sperm normally associated with apoptosis in somatic cells.

   The working hypothesis for this specific aim is that ejaculated human spermatozoa undergo membrane associated changes similar to those seen in apoptotic somatic cells.

2. To determine the presence of caspases in ejaculated sperm and whether they can be initiated by known pro-apoptotic agents.

   The working hypothesis for this aim is that ejaculated human sperm express procaspases that are capable of becoming activated by treatments known to promote apoptosis in somatic cells.

3. To identify key apoptotic proteins in ejaculated sperm.

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CHAPTER II

MATERIALS AND METHODS

INSTITUTIONAL REVIEW BOARD (IRB) APPROVAL

These studies were approved by the Eastern Virginia Medical School Institutional Review Board (IRB).

SUBJECTS

Subjects were drawn from pools of two main groups. Donor semen samples were obtained through cooperation with the Jones Institute Cryo Laboratory sperm bank. Patient semen samples were drawn from two populations: All patients were consulting for primary infertility at the Jones Institute for Reproductive Medicine. For caspase activity assays, samples were obtained from patients with otherwise normal semen samples who were consulting for primary infertility. A second group of patients exhibited oligozoospermia (sperm concentrations below 20 x 10^6/milliliter (mL) and motility below 40%). Semen samples were collected by masturbation following a period of sexual abstinence of a minimum of two days and a maximum of five days. Patients with a history of antisperm antibodies were excluded from the studies or with round cell concentrations in excess of 1x10^6/mL.

SAMPLE PREPARATION

SEMEN ANALYSIS

Donor and patient semen samples were allowed to liquefy for at least 30 minutes prior to semen analysis. Samples were evaluated for sperm

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concentration, sperm motility, sperm morphology, and round cell concentration.

Five µL of raw semen was placed on a Makler™ chamber and evaluated using a Hamilton-Thorne™ computer assisted semen analyzer (CASA) IVOS version 10.8 (Hamilton Thorne Research, Beverly, MA). Measurements were performed in duplicate, with concentration and motility manually verified upon completion of CASA measurements. Viability was performed as previously published (Eliasson 1971) as part of the standard semen analysis, before the start of incubations, and immediately after completion of incubation periods. A 0.5% (w/v) solution of Eosin Y (C.I. 45380, Sigma, St. Louis, MO) in saline was mixed with the sample in a 1:3 ratio. Twenty µL of the suspension was placed on a microscope slide, coverslipped, and evaluated. Live sperm, with intact membranes, excluded the dye and appeared white. Sperm with compromised membranes were stained red.

Sperm morphology smears were prepared by placing 5 µL droplets of semen on a microscope slide and smearing droplets with a clean second slide. Slides were allowed to dry at 37^0 C. Dried smears were stained using Diff-Quik™ stain (Dade Behring AG, Düdingen, Switzerland). Morphology was assessed using Strict Criteria (Menkveld *et al.*, 1990). A minimum of 200 sperm were evaluated on each slide.

Semen samples not meeting or exceeding World Health Organization (World Health Oorganization, 1999) requirements for normal semen with respect to concentration, motility, and round cell concentration were rejected

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for all assays except for caspase immunofluorescent microscopy. Round cells were identified using a peroxidase staining method for neutrophils. Sperm morphology, although evaluated for statistical purposes, was not an exclusion factor when abnormal. Upon completion of diagnostic testing, suitable semen samples were identified based upon preliminary results of the semen analyses.

PERCOLL™ SEPARATION

With the exception of semen specimens used for Caspase-3 immunofluorescence microscopy, all semen samples were separated using Percoll™ (Sigma, St. Louis, Missouri) density gradient centrifugation into high (90% isotonic Percoll™) and low (45% isotonic Percoll™) motility fractions. Isotonic Percoll™ was prepared by mixing Percoll™ with 10X concentrated synthetic Human Tubal Fluid (HTF, Irvine Scientific, Santa Ana, California) culture medium in a 1:10 ratio. The resulting mixture was used as 100% isotonic Percoll™. Isotonic 90% Percoll™ was prepared by adding HTF medium supplemented with 0.2% human serum albumin (HSA, Irvine Scientific) in a 1:10 ratio. Forty-five percent isotonic Percoll™ was then prepared by 1:2 dilution of 90% Percoll™ with HTF medium, supplemented with 0.2% (volume/volume) HSA supplemented HTF medium.

Separation gradients were prepared by overlaying 1 mL of 90% Percoll™ with 1 mL of 45% Percoll™ in 15 mL conical centrifuge tubes (Fisher Scientific, Pittsburgh, PA). Great care was taken during overlaying to prevent mixture of the two layers and to preserve a stable interface between the two layers. A

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maximum of 2 mL of raw semen was layered onto the 45% Percoll™ layer. Samples with volumes greater than two 2 were split equally among several conical centrifuge tubes.

Centrifugation was carried out at 546 xg for 20 minutes. Pellets containing motile sperm and approximately 0.75 mL of the 90% Percoll™ layers were removed using fine bore transfer pipets (Fisher Scientific, Pittsburgh, PA). A fresh transfer pipet was used to remove each layer. The 45%/90% Percoll™ interface, containing mainly immotile sperm, was removed together with approximately 0.5-0.75 mL of the 45% Percoll layer™. Aspirates were deposited into appropriately labeled fresh 15 mL conical centrifuge tubes.

Aspirates of each layer there then diluted with two-fold volumes of fresh HTF-0.2% HSA supplemented medium for a wash phase. Apportioned samples were centrifuged for 10 minutes at 425 xg to pellet the sperm in the aspirates. Supernates were removed and discarded. Pellets were resuspended in 1 mL of fresh HTF with 0.2% HSA.

REMOVAL OF CONTAMINATING NON-SPERMATOGENIC CELLS

Prior to use in assays, cell types of non-spermatogenic lineage that could complicate, even invalidate, assay results were separated from sperm fractions. Apoptosis pathways in polymorphonuclear lymphocytes (PMNs) are well characterized. A primary concern is caspase activity due to contamination with PMNs. Relatively abundant proapoptotic factors from these cells could falsely elevate activity measurements from sperm fractions. Major contaminants of semen include cell types of hematopoietic lineage that

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express CD45 immunologic markers. A reliable method of removing CD45-positive cells is the use of anti-CD45 conjugated paramagnetic beads. Previous studies have validated this method (Krausz *et al.*, 1992).

Percoll™ separated samples were incubated with anti-CD45 conjugated Dynabeads (Dynal, Lake Success, NY) for 30 minutes. Anti-CD45 Dynabeads are paramagnetic beads conjugated with Anti-CD45 antibodies. When mixed with cellular suspensions, anti-CD45 Dynabeads function to bind, with high specificity, cells expressing CD45 on their outer membrane. The cell suspension is then subjected to a strong magnetic field from a Magnetic Particle Concentrator (MPC-L, Dynal) through a glass centrifuge tube (Fisher Scientific, Pittsburgh, PA), allowing the removal of the remaining cell suspension. Dynabeads were well mixed by vortexing prior to distribution into centrifuge tubes. Twenty-five μL of Dynabeads were distributed to centrifuge tubes using an appropriate pipet and subjected to a magnetic field for two minutes to remove the antibody coated paramagnetic beads. The remaining fluid was removed using a 25 μL pipet. The tubes were removed from the magnetic field and resuspended in excess washing buffer (phosphate buffered saline [PBS] supplemented with 0.1% [weight/volume] Bovine Serum Albumin [BSA]). The resuspended beads were subjected to the magnetic field for another two minutes and excess binding buffer was removed. Beads were then resuspended to their original volume and allowed to stand until just before use.

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The beads were subjected to the magnetic field once again for two minutes and the excess fluid removed. This time, however, the beads were resuspended with a maximum of 1 mL of either motile (90% Percoll™) or immotile (45% Percoll™) sperm suspension. The cell suspension was shaken gently for thirty minutes. Upon completion of the timed incubation, the suspension was again subjected to a magnetic field to discard the beads together with their bound CD45-positive cells. The immunodepleted cell suspension was then used for caspase activity assays, protein immunoblots, and flow cytometry assays.

After cellular depletion of CD45-positive cells, sperm fractions were evaluated for the presence of PMNs using a Makler™ chamber to determine the concentration of round cells followed by peroxidase staining to determine the percentage of round cells that were PMNs. Samples containing more than 1000 PMNs/mL were subjected to an additional Dynabead separation step prior to use in assays. Sperm fractions with PMN contamination greater than 1000 cells/mL were discarded.

CASPASE ACTIVITY ASSAYS

Three separate conditions designed to stimulate an apoptotic response were tested with sperm fractions. After sperm fractions were purified through Percoll™ isolation and anti-CD45 antibody conjugated paramagnetic bead depletion of contaminating leukocytes, fractions were divided equally between four treatments. Staurosporine (S-5921, Sigma, St. Louis, MO), a well-known protein kinase inhibitor and inducer of apoptosis, was incubated in one sperm

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fraction for four hours. Staurosporine was dissolved and diluted in DMSO to a concentration of 10 mM. Final concentration in test samples was 10 μM.

A second treatment utilized two equivalent anti-FAS antibodies (Clones CH-11 and IPO-4, Kamiya Biomedical Company, Seattle, WA) demonstrated to induce apoptotic responses in somatic cells. Both antibodies were demonstrated to induce apoptosis in human neutrophils in caspase activity assays before use in experiments on sperm. Final antibody concentrations in sperm fractions were 1 μg/mL. As with the staurosporine treatment, incubations spanned four hours.

Finally, hydrogen peroxide treatment was included in an attempt to induce apoptosis as a result of oxidation stress or damage. Peroxide concentrations were 200 μM with a four-hour incubation.

A fourth aliquot was a control aliquot consisting of a sperm suspension only, also incubated for a period of four hours. Upon completion of the incubation, each aliquot was evaluated for sperm concentration, motility, using the Hamilton-Thorne CASA and viability.

Sperm fractions (20-50 x 10^6 total spermatozoa) were centrifuged to a pellet for 10 minutes at 325 xg and disrupted in a lysis buffer containing 10 mM potassium phosphate, 5 mM K_2HPO_4, 5 mM KH_2PO_4, 1 mM EDTA, 10 mM dithiothreitol (DTT), and 0.5% Triton with pH adjusted to 6.9. Additionally, the lysis buffer solution contained the general protease inhibitors phenylmethanesulfonyl fluoride (PMSF), leupeptin, and pepstatin (Parvanthenani *et al.*, 1998). To aid in protein extraction, the sperm

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suspension was sonicated. Sonication periods were kept brief and the sample was frequently returned to an ice bath to prevent conditions that could lead to denaturation of enzymes. Following sonication, suspensions were centrifuged at 16000 xg at 4° C for 10 minutes to remove insoluble particulate matter. Supernates were removed as close to the pellet as possible while taking care not to disturb the pellet. Aliquots were stored frozen for no more than 7 days at -70° C prior use in caspase activity assays.

Upon thawing, an aliquot of sperm lysate was diluted with a solution containing ICE buffer (50 mM HEPES, 10% sucrose, 0.1% CHAPS, pH 7.5) and the fluorogenic substrate Ac-DEVD-afc (N-acetyl-aspartate-glutamate-valine-aspartate-AFC, 7-amino-4-trifluoromethyl coumarin) (Parvathenani, *et al.*, 1998). Fluorescent emission (excitation 400 nm and emission 505 nm) was measured after incubation for 45 minutes at 37º C. Fluorescence without sperm was evaluated to determine background status. Samples containing 7.813, 15.625, 31.25, 62.5, 125, 250, and 500 pmol AFC (7-amino-4-trifluoromethyl coumarin) were prepared as standards to determine the amount of fluorochrome released. These standards were used to generate standard curves from which enzymatic activity was derived. Sperm lysates were evaluated in duplicate. Each sample contained 195 µL ICE buffer, 25 µL of DTT solution (15mg/mL), 10 µL of 2.5 mM Ac-DEVD-AFC, and 20 µL sperm lysate. Reaction mixtures were incubated in the dark for 45 minutes in a 37° C incubator with a 5% CO_2 in 100% humidified room air atmosphere.

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Fluorescence was measured at λ^{max}= 505 nm using a SpectraMax Gemini XS (Molecular Devices, Sunnyvale, CA). Caspase activity was expressed as femtomole (fmol)/min/10^6 cells and picomole (pmol)/min/mg protein. Human neutrophils treated with 1 mM cycloheximide (to induce apoptosis) were used as positive controls. The level of sensitivity of this assay was determined to be 250,000 cells using standard curves of caspase activity in known numbers of neutrophils, Jurkat cells and HL-60 cells.

PROTEIN ASSAYS

Protein assessment for caspase assays was performed using the Bio-Rad Protein Assay (Bio-Rad). This colorimetric assay is a microplate modification of the Bradford method (Bradford 1976). Bovine serum albumin (BSA) was used to prepare standards. BSA standards were serial dilutions of 1 mg/mL H_2O stock solutions. Protein standards were: 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL. Protein dye was diluted 1:5 with deionized H_2O prior to use. Protein concentrations were performed in 96-well micro-titer plates (Corning). For each sample, 200 µL of the diluted protein dye was added to each well. Cell lysates with unknown protein concentration were diluted at least 1:3 prior to mixing with the protein dye. To the protein dye mixture was added 5 µL of each sample. Protein concentrations were measured on a Tecan Spectra Micro-Titer Plate Reader with 595 nm filters.

PROTEIN IMMUNOBLOTS

Protein immunoblot analysis was performed on dedicated aliquots of both high motility and low motility sperm fractions. Sperm fractions were

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centrifuged at 325 xg for 10 minutes, the supernate discarded and the pellet resuspended in PBS. This procedure was repeated twice. After the second centrifugation step, 5x electrophoresis sample buffer was added directly to the pelleted sperm. The pellet was resuspended by pipetting thoroughly through an Eppendorf pipet tip. It was necessary, in certain instances, to force highly viscous mixtures through a 23-gauge (hypodermic) needle to reduce the viscosity. Reconstituted samples were then boiled for 10 minutes at 95^{o} C. Samples were either immediately used for gel electrophoresis or frozen at -20^{o} C until use in SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Discontinuous polyacrylamide gel electrophoresis was used in all experiments. Stacking gels in each experiment were 4% acrylamide. Resolving gels ranged from 6.3% to 14% acrylamide, with the latter being most useful for most applications. In cases where protein digestion products of interest were close in molecular weight to undigested proteins, concentrations of acrylamide were adjusted to allow for resolution of distinct breakdown products.

Gels were cast using a Bio-Rad Mini-Protean 3 Electrophoresis System (Bio-Rad Laboratories, Hercules, CA) casting apparatus (catalog 165-3301. Prior to casting glass plates were thoroughly cleaned, rinsed with deionized water, and dried. Glass plates with integral 0.75 mm spacers and 10 well combs were used. During polymerization, the resolving gel was overlayered with a 0.5% SDS in ultrapure water. When polymerization of the resolving gel

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was complete, the SDS overlay was carefully removed and the stacking gel was added. A 10 well comb was used when casting the stacking gel.

When polymerization was complete, the gels were mounted in a Bio-Rad Mini-Protean III electrophoresis tank. Electrophoresis was performed at 200V until the dye front from the samples was running uniform, then the voltage was reduced to 150V for the remainder of the electophoresis. Electrophoresis was stopped when the dye front reached a point within 0.5 cm of the bottom of the gel.

Gels were removed from the electrophoresis tank and glass support plates. Stacking gels were separated and resolving gels were soaked in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol) for 30 minutes. Ten minutes prior to transfer PVP electrophoresis membranes and blotting paper were cut to slightly larger dimensions than the gel size and soaked in transfer buffer. PVP membranes required a short rinse with methanol followed by transfer buffer prior to use.

A Bio-Rad SD Semi-Dry Transfer Cell (Bio-Rad) was used for transfers. Blots were assembled in order with blot paper, membrane, gel, and a second blot paper. Care was taken to prevent or reduce the incidence of air bubbles that could retard efficient transmission of proteins from the gel. Protein transfer was carried out for approximately 20-30 minutes to ensure complete transmission. The apparatus was disassembled and remaining protein binding sites on the membrane were blocked for one hour at room

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temperature under constant agitation. The blocking agent was 5% non-fat dry milk in wash buffer (10 mM Tris base, 100 mM NaCl, and 0.1% Tween-20).

PRIMARY ANTIBODIES

Primary antibodies were chosen for major apoptotic markers in somatic cells. All antibodies were obtained from B-D Pharmingen Antibodies were: polyclonal anti-caspase-3, anti-caspase-7, anti-caspase-9, anti-poly-ADP-ribose polymerase (PARP), and anti-BID. BD Pharmingen mouse anti-human caspase-7 monoclonal antibody 551237 was used to assay for the presence of caspase-7 in sperm. The antibody detects the unmodified procaspase-7 at 35 kDa, the intermediate form at 32 kDa, and the 20 kDa subunit of the active enzyme. Purified rabbit anti-caspase-9 polyclonal antibody 550437, also from BD Pharmingen was used to assay for the presence and activation of caspase-9. The procaspase-9 enzyme is processed from a 48 kDa inactive form into 37 kDa large and 10 kDa small subunits. BD Pharmingen mouse anti-PARP [Poly (ADP-ribose) Polymerase] monoclonal antibody 65196E was used to assay for the presence and inactivation of this chromatin-associated enzyme. The 116 kDa intact enzyme is processed by caspases-3 and -9 into 85 kDa and 25 kDa inactive fragments. Anti-Apoptosis Inducing Factor (AIF) rabbit polyclonal antibody (NT) X1109P was obtained from Exalpha Biologicals, Incorporated (Boston, MA) and detects the 67 kDa AIF protein. Becton-Dickinson Pharmingen rabbit anti-BID polyclonal antibody 550365 was used to detect the presence of this 24 kDa Bcl-2 family member. The antibody recognizes full-length BID as well as the active 15 kDa cleaved form.

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Primary antibody incubation began with addition of fresh blocking buffer and the addition of the primary antibody so that the adjusted concentration of antibody was 1:1000 dilution. Primary antibody incubation was continued with gentle agitation overnight.

Upon completion of incubation with primary antibody, blots were washed for three consecutive ten-minute periods with wash buffer prior to addition of the secondary antibody. An appropriate secondary, peroxidase conjugated secondary antibody was used for visualization of the labeled protein bands. The blots were incubated with the secondary antibody for two hours at room temperature with gentle agitation. As a final step prior to filming, blots were washed three additional times at ten-minute intervals in fresh wash buffer.

ECL western blotting detection kits (Amersham Pharmacia Biotech, Piscataway, NJ) were used to visualize the protein bands. The two part developing solution was mixed in equal aliquots just prior to filming. The resulting chemiluminescence bands were recorded on Hyperfilm ECL films (Amersham Pharmacia, Piscataway, NJ) and developed. The position of labeled protein bands relative to molecular weight markers allowed identification of proteins and their condition (e.g., either active or inactive in the case of enzymes).

CASPASE-3 IMMUNOFLUORESCENCE

Semen samples selected for caspase-3 immunofluorescence were washed twice with PBS to remove seminal fluid. Sperm concentrations were adjusted to 1 million/mL. For these samples, no effort was made to deplete

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non-spermatogenic round cells from the processed samples. The presence of PMNs in the suspension was actually desirable since these cells provided positive controls when apoptotic. Twenty-five μL of the adjusted sperm suspension was placed on a poly-L-lysine coated microscope slide. The samples were immediately diluted with 25 μL of 4% paraformaldehyde (Sigma, St. Louis , MO) in PBS for fixation. Cells were fixed for a minimum of 10 minutes. During this time, sperm settled to contact the slide. After fixation the aqueous phase was removed by touching one edge of the drop with a Kim-Wipe™ (Fisher Scientific, Pittsburgh, PA). The remaining fluid was allowed to dry and the slide was stored at -70^{O} C until evaluation.

Just prior to evaluation, slides were removed and washed three times with 25 μL PBS. Non-specific binding of primary antibody was prevented by a single 1 hour incubation in 25 μL of blocking buffer containing 10% normal goat serum (NGS) in PBS. The primary antibody, anti-active caspase-3 (BD Pharmingen 559565), was mixed with incubation buffer at 10 μg/mL. Spots containing sperm were incubated for one hour at room temperature with 25 μL of primary antibody solution. Spots were washed three times with wash buffer. The secondary antibody, a fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit polyclonal antibody (Pharmingen 554020), was diluted at a 1:200 ration with incubation buffer. After one hour, the spots were once again washed three times with wash buffer. After the final wash, the spots were drained and 3 μL of an anti-bleaching agent anti-quench was added. Slides were immediately coverslipped and evaluated using a Nikon

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Eclipse E600 stereo microscope with a Y-FL epifluorescence attachment (Nikon, Melville, NY). At least 200 cells were evaluated per sample. Images were recorded using a Spot RT Slider digital camera (Spot Diagnostic Instruments, Inc, Sterling Heights, MI) using Spot RT version 3.2 software attached to a Gateway Pentium 4 computer.

FLOW CYTOMETRY

Recombinant FITC conjugated Annexin-V (human) catalog 209-250-T300 was obtained from Alexis Biochemicals (Alexis Corporation, San Diego, CA). High and low motility sperm fractions were separated from whole semen using Percoll™, immunodepleted using anti-CD45 conjugated paramagnetic beads, and separated into control and treatment fractions. Each sperm fraction was incubated in their respective treatment group for four hours prior to flow cytometry. Treatment included 10 μM STS, anti-Fas antibody, and 200 μM H_2O_2. Fraction concentrations were adjusted for a total 1 x 10^6 sperm.

Upon completion of incubation, sperm fractions were centrifuged to a pellet in a microcentrifuge (Eppendorf), the supernate discarded and the pellet resuspended. Each sample was washed twice in PBS. After the final wash, pelleted sperm were resuspended in 195 μL sterile-filtered Annexin-V binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$,). A dual labeling method was used to distinguish cells in which PS translocation had occurred from necrotic cells (Lagarkova *et al.*, 1995). FITC labeled Annexin-V was used to detect PS on the outer leaflet of the plasma membrane. Necrotic cells were labeled using ethidium homodimer-1. To the cell suspension was

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added 5 µL of Annexin-V-FITC. As a viability counter-stain, a 1 µM solution of ethidium homodimer-1 (Molecular Probes, Eugene, OR) was used. Suspensions were well mixed and allowed to stand in the dark for 10 minutes at room temperature. After incubation with Annexin, an additional 190 µL of binding buffer was added and 10 µL of 1mM ethidium homodimer-1 in DMSO was added to the each suspension. Sperm suspensions were evaluated on a Becton-Dickinson FACalibur bench-top flow cytometer (Becton-Dickinson, San Jose, CA) through excitation with a 15 milliwatt 488 nm air-cooled argon-ion laser.

For each suspension, 15,000 sperm were analyzed for Annexin-V binding and/or ethidium homodimer uptake. Emission data were collected and analyzed using CellQuest (Becton-Dickinson, San Jose, CA). Three different types of binding could be observed: Annexin-V only (indicates cells with PS translocation to the outer membrane leaflet), ethidium homodimer only binding (cells with compromised membranes), and cells labeled with both Annexin-V and ethidium homodimer. Cells displaying both Annexin-V and ethidium homodimer labeling were considered necrotic due to the possibility that Annexin binding could be occurring on the inner membrane leaflet.

STATISTICAL ANALYSIS

Viability data were transformed and analyzed using analysis of variance (ANOVA). Results of caspase activity assays were compared using Generalized Estimating Equations analysis. Flow cytometry data was analyzed by rank transformation percentages of cells in each of three groups

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(Annexin-V bound live cells, unlabeled live cells, and necrotic cells) from contour plots of the data. The transformed data was then analyzed using a single factor analysis of variance (ANOVA) with post-hoc multiple comparisons tests.

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CHAPTER III

RESULTS

CASPASE ACTIVITY ASSAYS

Sperm motion parameters were analyzed to evaluate associations between motility and caspase activity. Motion parameters analyzed were VAP, VSL, VCL, ALH, BCF, STR, LIN, RAPID, MEDIUM, SLOW, and STATIC (Table 1). Motion parameters in semen, high motility, and low motility were not significantly different between patients and donors. In high motility fractions control and Fas antibody treated samples were not significantly different in any motion parameter after four hours of incubation. Treatment with H_2O_2 resulted in complete loss of motility. Staurosporine treatment also resulted in significant decrease (to nearly zero) in every motility parameter examined. In staurosporine-treated fractions, sperm motility typically was limited to twitching, non-progressive types of movement with occasional sluggish, forward progression in very few sperm.

Table I. Motility Parameters

Abbreviation	Parameter
VAP	Average path velocity (μm/s)
VCL	Path velocity (μm/s)
VSL	Straight line velocity (μm/s)
STR	VSL/VAP x 100
LIN	VSL/VCL x 100
BCF	Cross beat frequency (Hz)
Rapid	VAP greater than 25 μm/s
Medium	5 μM/s < VAP < 25 μm/s
Slow	Less than 5 μm/s
Static	Non-moving

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Caspase activity was significantly and negatively correlated to VAP, VSL, and BCF in Fas antibody treated samples. Caspase activity decreased 1.91 pmol/min/mg protein for every percent increase in VAP, 2.5 pmol/min/mg protein for every percent increase in VSL, and 8.44 pmol/min/mg protein for every percent increase in BCF.

In staurosporine-treated fractions, caspase activity expressed in pmol/min/mg protein was significantly and positively correlated to medium and slow parameters. Caspase activity increased 43.68 pmol/min/mg protein for every percent increase in medium and 20.52 pmol/min/mg protein for every percent increase in slow motion parameters.

When analyzing caspase activity expressed as fmol/min/10^6 cells, activity was significantly and positively related to ALH in staurosporine-treated fractions. Caspase activity increased 37.52 fmol/min/10^6 cells for every unit increase in ALH. Additionally, two associations of marginal significance were noted. For Fas antibody treated fractions, caspase activity decreased 25.82 fmol/min/10^6 cells ($p=0.059$). In staurosporine-treated fractions, caspase activity increased 5.21 fmol/min/10^6 cells for every unit increase in linearity ($p=0.056$). Table 2 details correlations and significance between motility parameters and caspase activity.

Morphology results were evaluated to determine association with caspase activity. No correlation between caspase activity and morphology percent normal sperm could be made. In patient low motility fractions regardless of activity units, caspase activity increased with increasing cytoplasmic droplets,

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but decreased in high motility fractions (p=0.031 for pmol/min/mg protein; p=0.01 for fmol/min/10^6 cells). In donors, no significant change in caspase activity was detected with the presence of cytoplasmic droplets, although trends followed the same pattern seen in patients. H_2O_2 treated fractions had significantly lower viability than any other treatment group.

Table II. Correlations Between Motility and Caspase Activity

Parameter	Treatment	Correlation with Caspase Activity	
		pmol/min/mg protein	fmol/min/10^6 sperm
VAP	Control	-0.103 (0.615)	-0.271 (0.181)
	Fas	-0.521 (0.038)*	-0.387 (0.125)
	STS	0.154 (0.516)	0.229 (0.332)
VSL	Control	-0.078 (0.705)	-0.261 (0.198)
	Fas	-0.517 (0.040)*	-0.348 (0.171)
	STS	0.184 (0.437)	0.341 (0.141)
VCL	Control	-0.099 (0.629)	-0.220 (0.280)
	Fas	-0.350 (0.183)	-0.443 (0.075)
	STS	0.187 (0.429)	0.150 (0.529)
ALH	Control	-0.005 (0.982)	-0.033 (0.871)
	Fas	-0.115 (0.672)	-0.467 (0.059)
	STS	0.201 (0.396)	0.503 (0.024)*
BCF	Control	-0.128 (0.396)	-0.023 (0.913)
	Fas	-0.687 (0.003)*	-0.065 (0.806)
	STS	0.154 (0.517)	0.088 (0.712)
Linearity	Control	0.018 (0.932)	-0.165 (0.419)
	Fas	-0.187 (0.488)	0.182 (0.485)
	STS	0.065 (0.784)	0.434 (0.056)-
Rapid	Control	-0.039 (0.849)	-0.340 (0.089)
	Fas	0.232 (0.388)	-0.358 (0.158)
	STS	0.405 (0.077)	0.106 (0.657)
Medium	Control	0.091 (0.660)	-0.170 (0.406)
	Fas	0.280 (0.294)	-0.063 (0.811)
	STS	0.637 (0.003)*	0.381 (0.097)
Slow	Control	0.012 (0.952)	-0.021 (0.919)
	Fas	0.286 (0.283)	-0.054 (0.837)
	STS	0.508 (0.022)*	0.374 (0.104)
Static	Control	0.029 (0.887)	0.323 (0.107)
	Fas	-0.258 (0.335)	0.337 (0.186)
	STS	0.004 (0.988)	0.383 (0.096)

*Significant correlations

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Viability evaluation was performed at the completion of each four-hour treatment cycle. Percent viable sperm in anti-Fas antibody staurosporine-treated fractions did not differ significantly from control fractions in either high or low motility fractions (Table 3). This indicated a negligible toxicity effect of these two treatments upon sperm viability. The H_2O_2 treatment resulted in profound toxicity, significantly lower than corresponding control values. In comparisons of patients and donors responses, patient high motility H_2O_2-treated fractions had significantly higher ($p<0.001$) viability than matched donor fractions.

Table III. Viability in High and Low Motility Fractions

Fraction	Patient	n	Donor	n
High Motility				
Control	78.4 ± 3.7	21	84.3 ± 2.6	8
Fas	69.7 ± 7.0	10	81.0 ± 4.9	8
H_2O_2	16.0 ± 3.0	10[1]	3.8 ± 0.9[3]	8
Staurosporine	77.0 ± 3.5	19	79.0 ± 4.9	8
Low Motility				
Control	40.9 ± 3.8	16	58.3 ± 19.3	8
Fas	43.5 ± 4.1	11	51.7 ± 9.2	6
H_2O_2	10.5 ± 4.5	4[2]	5.4 ± 2.1[4]	5
Staurosporine	41.9 ± 3.2	12	76.3 ± 9.3	4

[1] $P=0.001$ Patient High Motility Control vs. H_2O_2. [2] $P=0.037$ Patient Low Motility Control vs. H_2O_2. [3] $P<0.001$ Donor High Motility Control vs. H_2O_2. [4] $P<0.001$ Donor Low Motility Control vs. H_2O_2.

Caspase activity was expressed in pmol/min/mg protein and fmol/min/10^6 cells. Early experimental results demonstrated variability in protein

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concentrations of sperm fractions containing identical number of cells. The possibility of interference from components of the lysis buffer was explored. The lysis buffer contained Triton X-100 to facilitate solubilization of cellular proteins. Bradford (1976) demonstrated that Triton X-100 and other strong alkaline buffering agents (sodium diodecyl sulfate, SDS, and commercial detergents) interfere with the protein dye-binding assay that he championed and lead to significant error in protein determination. The error effect of trace amounts of these compounds could be corrected by appropriate controls. However, in higher concentrations, the interference from these chemicals is insurmountable. In order to determine the level at which the lysis buffer interfered with the dye binding, lysis buffer was serially diluted with ICE buffer in ratios 1:5, 1:4, 1:3, 1:2, and undiluted (Graph 1). Interference from Triton X-100 began to be detectable in dilutions less than 1:3 and was clearly pronounced at 1:2 dilutions. Undiluted lysis buffer caused protein measurements to be inflated far above the actual concentration. Due to the small amount of protein in most sperm fractions, it was necessary for dilutions to be a maximum of 1:3. A 1:3 dilution, for most fractions, would bring protein concentration into the lowest reliably detectable range based on standard curves. Dilutions of 1:2 would have solidly placed protein concentrations on the curve. To avoid the pitfall of lysis buffer interference in protein determinations, an alternative expression of caspase activity was found in expressing activity in units/10^6 sperm. It is believed that the fmol/min/10^6 sperm is a more reliable figure than pmol/min/mg protein.

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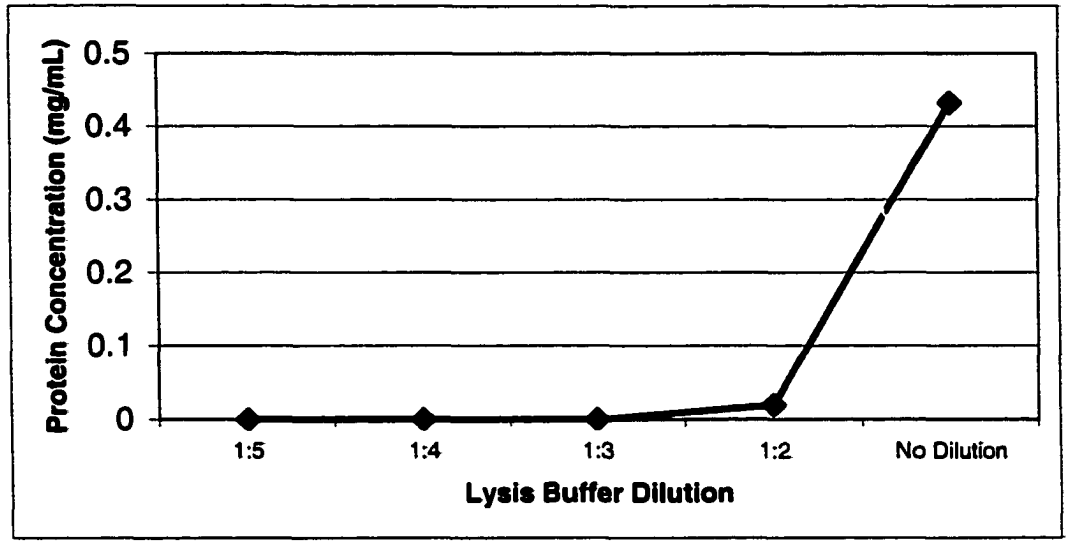

Graph 1. Lysis Buffer Interference With Protein Assays.

Control and three treatment fractions (Fas antibody, staurosporine, and H_2O_2) were compared for caspase activity. For patient high motility fractions expressed as pmol/min/mg protein, there were 25 control, nine Fas antibody, twelve H_2O_2, and twenty-one staurosporine-treated fractions. When caspase activities were expressed as fmol/min/10^6 cells, the number of replicates was the same with the exception of Fas antibody treated fractions that increased to ten replicates. For donor fractions, nine control, eight Fas antibody, eight H_2O_2, and nine staurosporine fractions were analyzed for both pmol/min/mg protein and fmol/min/10^6 cells.

In order to determine whether caspase activity could be induced, caspase activities were compared first between control and treatment groups. Comparisons were then made between both control and treated donor and patient fractions. Other analyses involved comparing high and low motility fractions for both control and treatment groups.

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When results were expressed in pmol/min/mg protein, no significant difference was detected in patient high motility fractions between the control (n=25) and any of the three treatment groups (Table 4 and Graph 2). In low motility fractions, caspase activity for H_2O_2 fractions were significantly lower (p=0.021) than corresponding control fractions (Graph 3). Staurosporine-treated fraction had significantly higher (p=0.039) caspase activity than corresponding Fas antibody treated fractions. However, there were no significant differences between control and Fas antibody treated fractions.

Table IV. Patient Caspase Activities (pmol/min/mg protein)

Fraction	Caspase Activity[1] pmol/min/mg protein	n
High Motility		
Control	78.8 ± 22.9	25
Fas Antibody	101.0 ± 50.3	9
H_2O_2	84.4 ± 23.3	12
Staurosporine	119.7 ± 34.7	21
Low Motility		
Control	72.8 ± 21.8	18
Fas Antibody	59.1 ± 27.0[2]	11
H_2O_2	54.1 ± 16.2	4
Staurosporine	72.4 ± 30.7	14

[1]Values are means ± SEM. [2]*P*=0.039 compared with Fas antibody.

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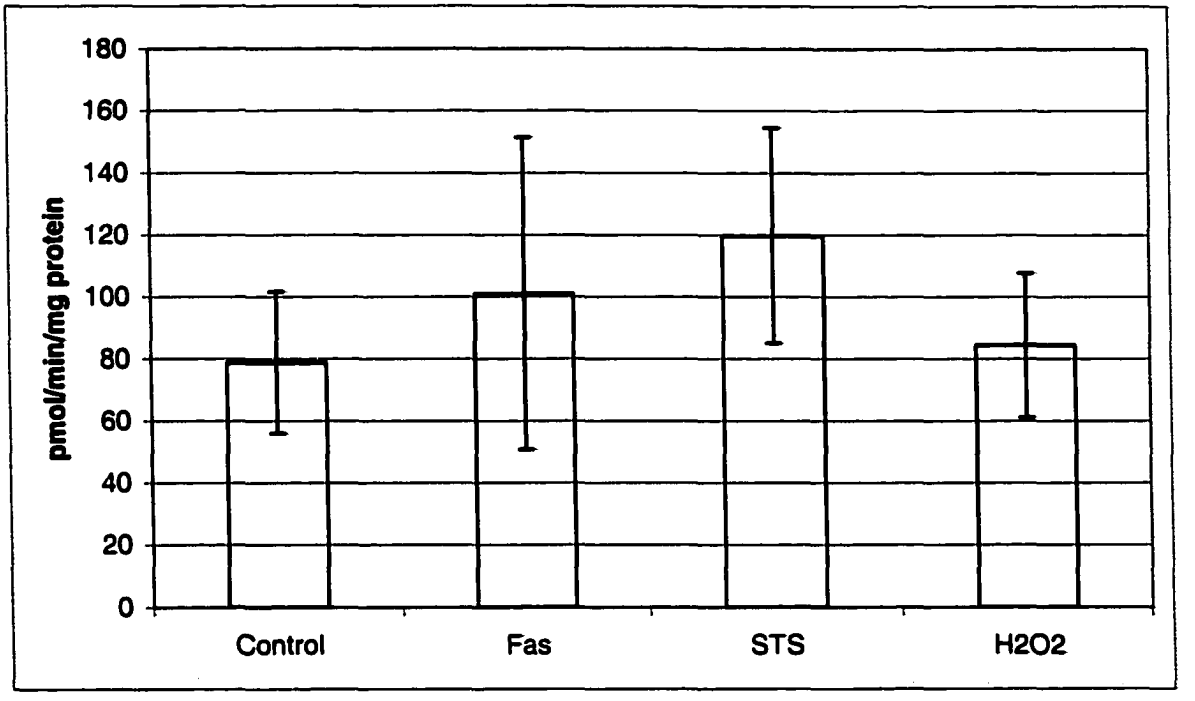
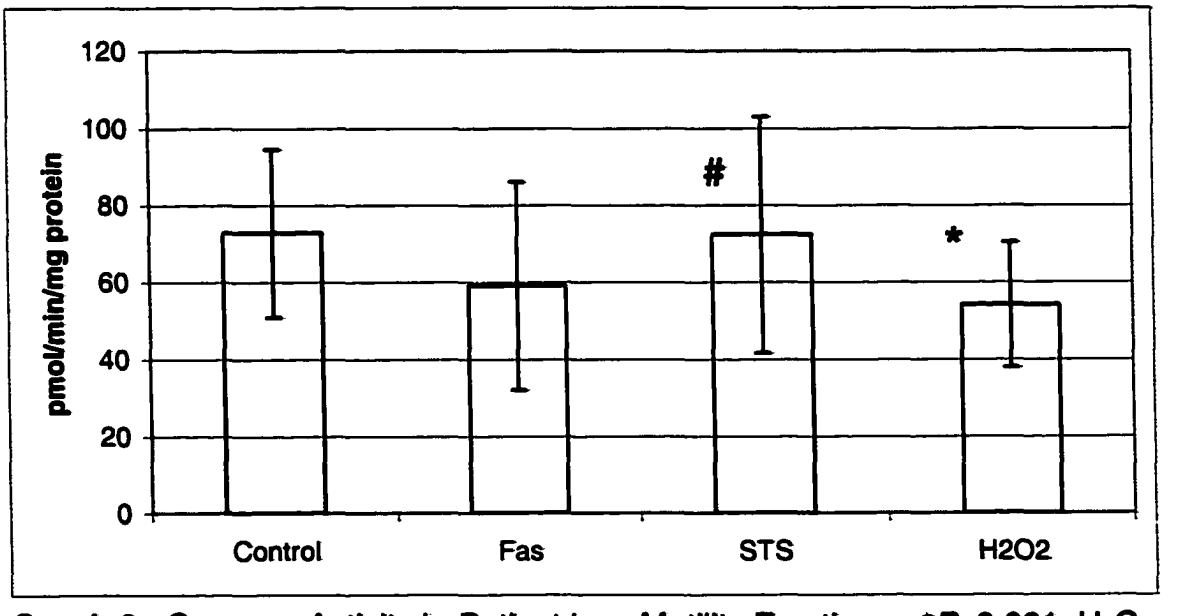


Graph 2. Caspase Activity In Patient High Motility Fractions. No significant differences. Values are means ± SEM.



Graph 3. Caspase Activity In Patient Low Motility Fractions. *P=0.021, H_2O_2 caspase activity was lower compared with control. #P=0.039, staurosporine was higher compared with Fas antibody treated fractions. Values are means ± SEM.

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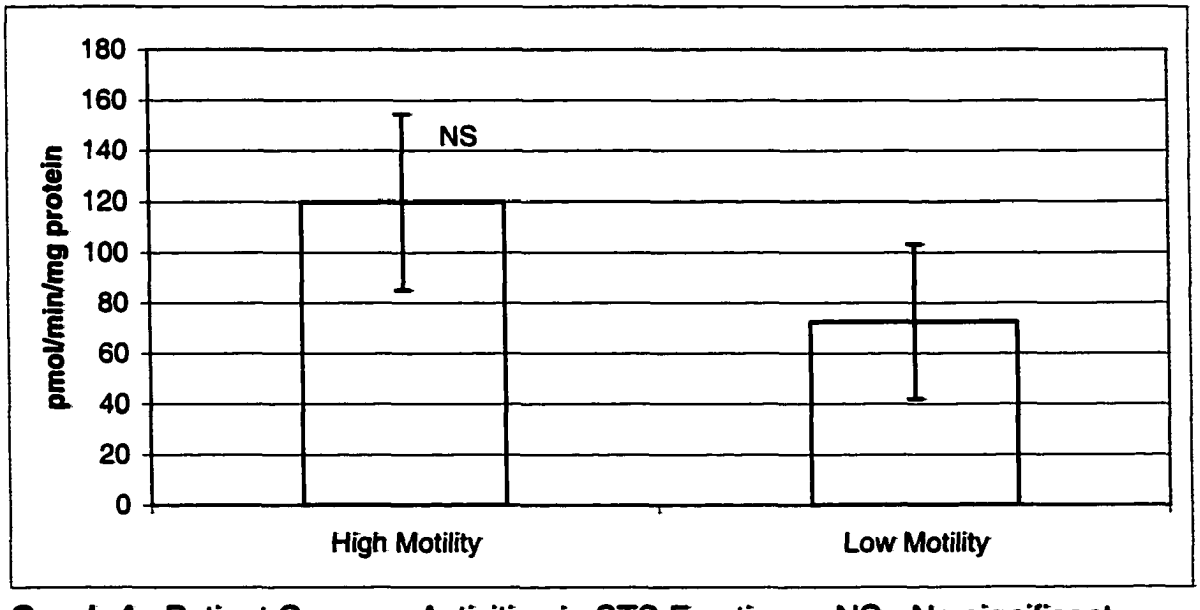
Comparisons between high and low motility fractions were also done. When comparing controls high and low motility, caspase activities were comparable. Of the remaining two treatment groups, Fas antibody and staurosporine treated fractions demonstrated no significant differences between high and low motility fractions (Graph 4). H_2O_2 treated high motility fractions had significantly higher ($p=0.009$) caspase activity than low motility fractions (Table 5).

Table V. Patient Baseline Caspase Activities (pmol/min/mg protein)

Fraction	Caspase Activity[1] pmol/min/mg protein	n
Low Motility	72.8 ± 21.8	18
High Motility	78.8 ± 22.9	25

[1]Values are means ± SEM.



Graph 4. Patient Caspase Activities in STS Fractions. NS: No significant differences. Values are means ± SEM.

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Sperm fractions from donors were compared in a similar manner. With high motility fractions, two (Fas antibody and H_2O_2) of the three treatment groups did not significantly differ from control fractions. Staurosporine-treated fractions, however, had significantly higher ($p<0.001$) (Graph 5) caspase activities than control fractions and was also significantly higher than either Fas antibody ($p=0.005$) or H_2O_2 ($p=0.043$) treated fractions (Table 6).

In low motility fractions and with the exception of the H_2O_2 treated fractions, none of the treatments were significantly different from the control fractions (Graph 6). The H_2O_2 treated fractions had significantly lower caspase activity than corresponding control fractions.

Table VI. Donor Caspase Activities (pmol/min/mg protein)

Fraction	Caspase Activity[1] pmol/min/mg protein	n
High Motility		
Control	18.8 ± 7.5	9
Fas Antibody	19.9 ± 6.0	8
H_2O_2	24.4 ± 8.6	8
Staurosporine	41.6 ± 9.7[2]	9
Low Motility		
Control	32.0 ± 8.8	9
Fas Antibody	33.1 ± 7.6	6
H_2O_2	9.7 ± 5.8[2]	5
Staurosporine	51.1 ± 17.3	5

[1]Values are means ± SEM.
[2]$P<0.001$ lower caspase activities compared with Control fractions.

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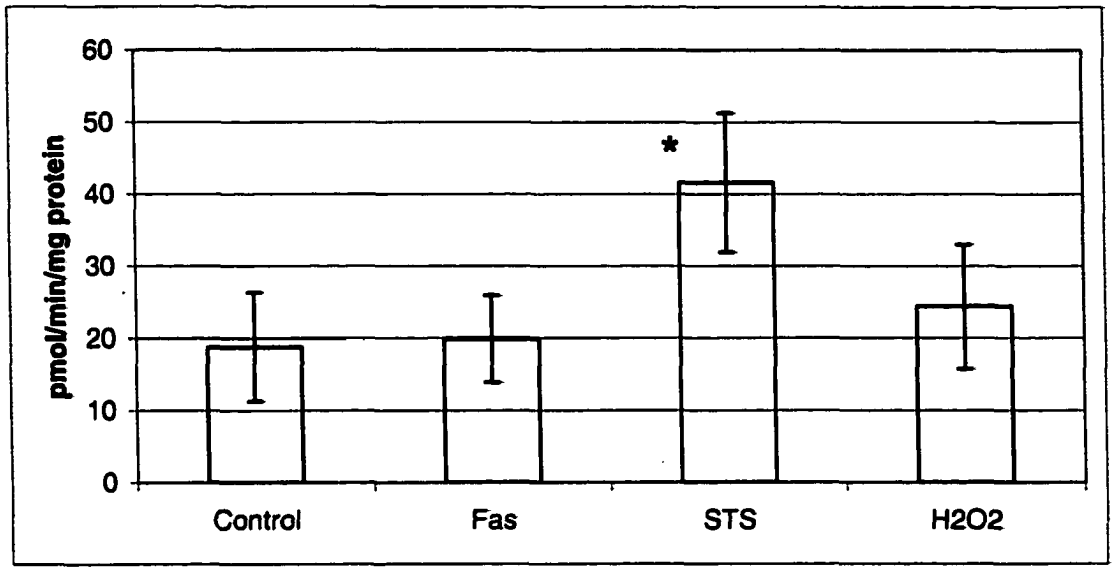
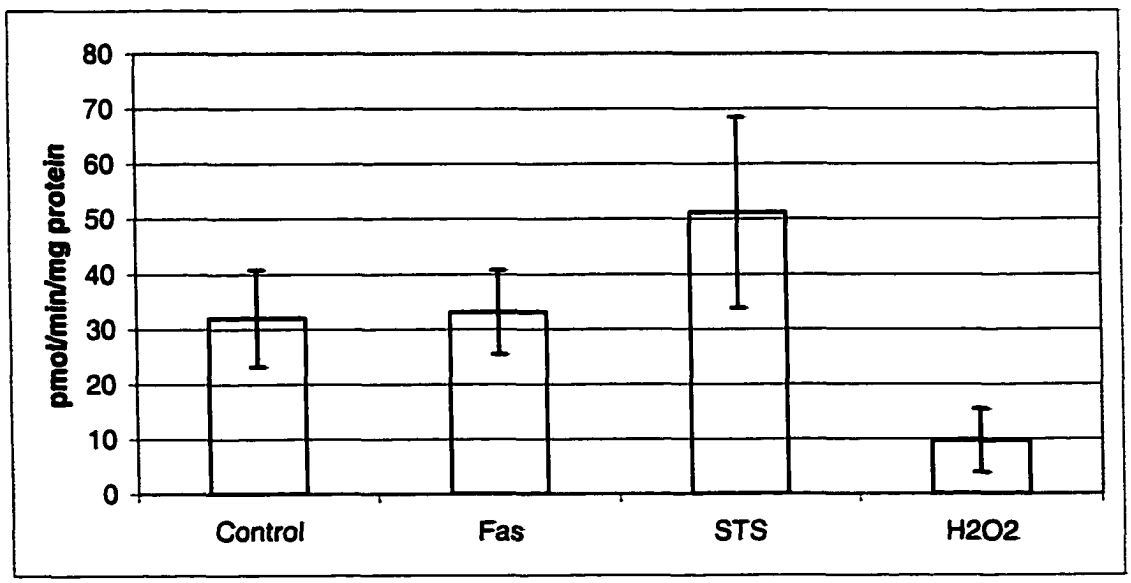


Graph 5. Donor High Motility Fractions (pmol/min/mg). *P<0.001 compared with matched control fractions. Values are means ± SEM.



Graph 6. Donor Low Motility Fractions (pmol/min/mg). No significant differences. Values are means ± SEM.

Comparisons between high and low motility fractions detected significantly higher (p=0.049) caspase activities in low motility control fractions compared with high motility fractions (Table 7).

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Table VII. Donor High and Low Motility Fraction Caspase Activities

Fraction	Caspase Activity[1] pmol/min/mg protein	n
Low Motility	32.0 ± 8.8	9
High Motility	18.8 ± 7.5[2]	9

[1]Values are means ± SEM.
[2]*P*=0.049.

Donor and patient samples were compared (Tables 8 and 9) to distinguish whether caspase activity in donors were different than patients. Comparisons were first made with caspase activities expressed in pmol/min/mg protein between donors and patient high and low motility control, Fas antibody-, staurosporine-, and H_2O_2-treated fractions. High motility fractions from patients had significantly (p=0.012) higher caspase activities than control (Graph 7), staurosporine (p=0.029, Graph 8), and H_2O_2-treated fractions (p=0.030). No other significant differences were detected between patient and donor sperm fractions.

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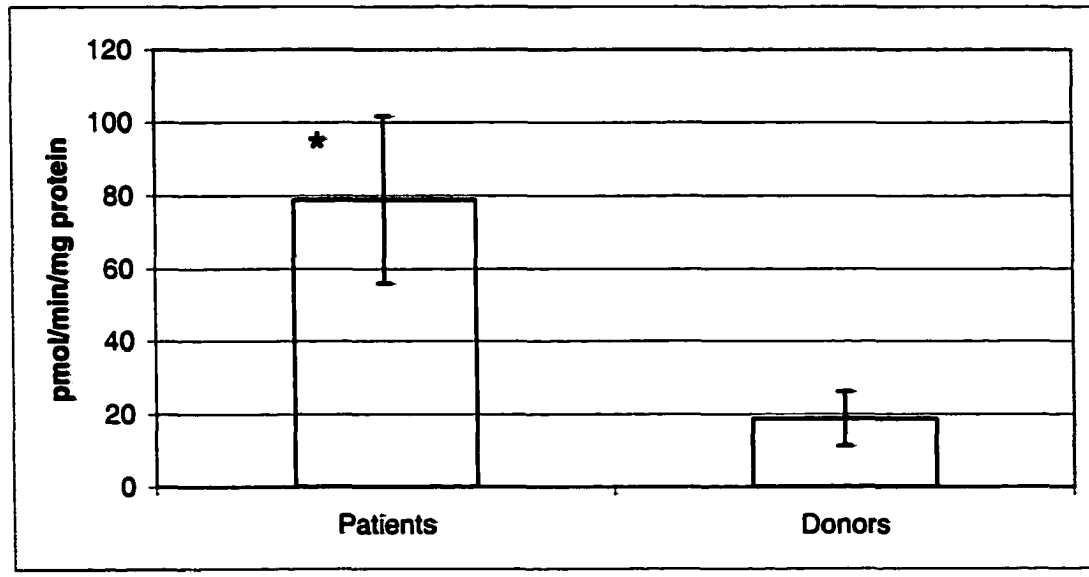
Table VIII. Donor and Patient High Motility Caspase Activities

Fraction	Caspase Activity[1] pmol/min/mg protein	*P*-value
Control		
Patients	78.8 ± 22.8	0.012
Donors	18.8 ± 7.0	
Fas Antibody-Treated		
Patients	89.2 ± 58.7	0.218
Donors	16.6 ± 5.4	
Staurosporine-Treated		
Patients	122.9 ± 36.2	0.029
Donors	41.6 ± 9.2	
H_2O_2-Treated		
Patients	77.5 ± 23.8	0.030
Donors	22.8 ± 8.2	

[1]Values are means ± SEM.



Graph 7. Patient and Donor High Motility Sperm (pmol/min/mg). *P=0.012. Values are means ± SEM.

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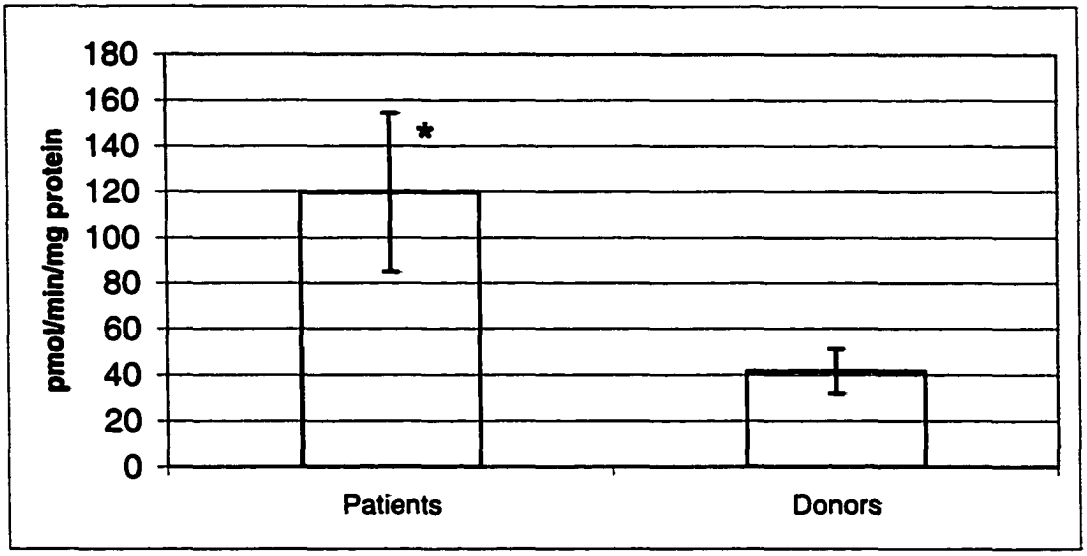

Graph 8. Patient and Donor High Motility STS (pmol/min/mg). *P=0.029. Values are means ± SEM.

Table IX. Donor and Patient Low Motility Caspase Activities

Fraction	Caspase Activity[1] pmol/min/mg protein	p-value
Control		
Patients	72.8 ± 21.6	0.078
Donors	32.0 ± 8.3	
Fas Antibody-Treated		
Patients	58.6 ± 27.4	0.309
Donors	29.3 ± 6.8	
Staurosporine-Treated		
Patients	78.2 ± 31.6	0.448
Donors	50.7 ± 17.5	
H_2O_2-Treated		
Patients	11.6 ± 5.7	0.490
Donors	6.5 ± 4.8	

[1]Values are means ± SEM.

Because of concerns about protein measurement, caspase activity was also measured in fmol/min/10^6 cells. An advantage of this method of caspase

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measurement is that it eliminates the necessity of performing protein measurements on samples and error that could be introduced as a result of using these measurements.

In high motility fractions from patients, only the staurosporine treatment group had significantly higher caspase activities (p=0.003) than the control fractions (Table 10 and Graph 9). The staurosporine fractions also differed significantly from Fas antibody- (p=0.003) and H_2O_2-treated fractions (p=0.039). No differences were detected among low motility fractions (Graph 10).

Table X. Patient High Motility Fraction Caspase Activities

Fraction	Caspase Activity[1] fmol/min/10^6 cells	n
Control	151.5 ± 28.4	25
Fas Antibody	109.1 ± 28.8	10
H_2O_2	324.5 ± 110.9	12
Staurosporine	378.4 ± 85.3[2]	21

[1]Values are means ± SEM.
[2]Staurosporine fractions were significantly (P=0.003) higher than Control fractions, Fas antibody treated (p=0.003), and H_2O_2 treated (P=0.039) fractions.

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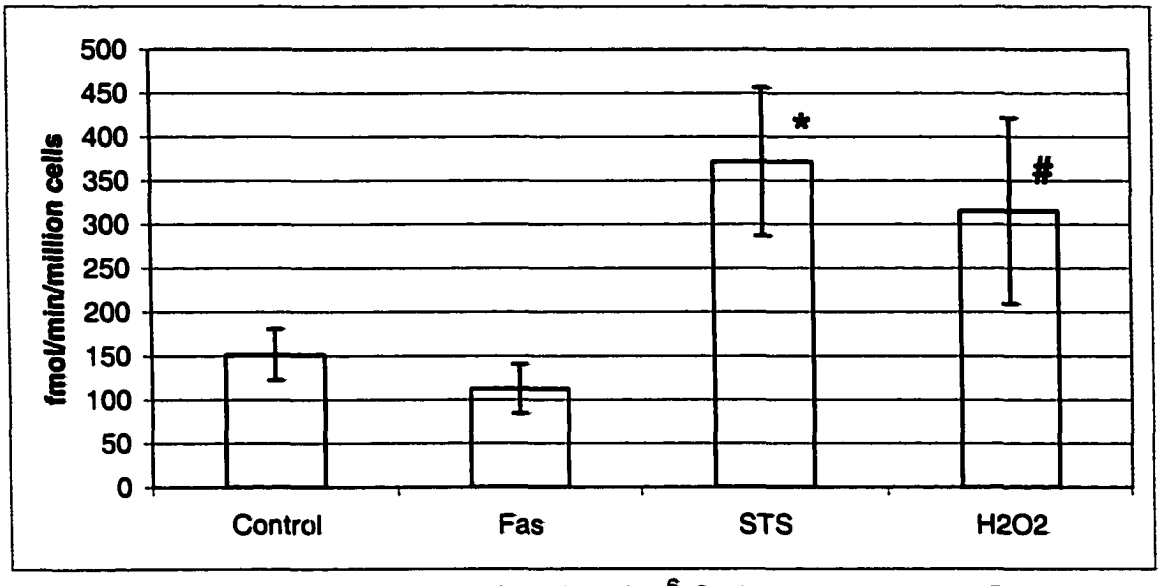
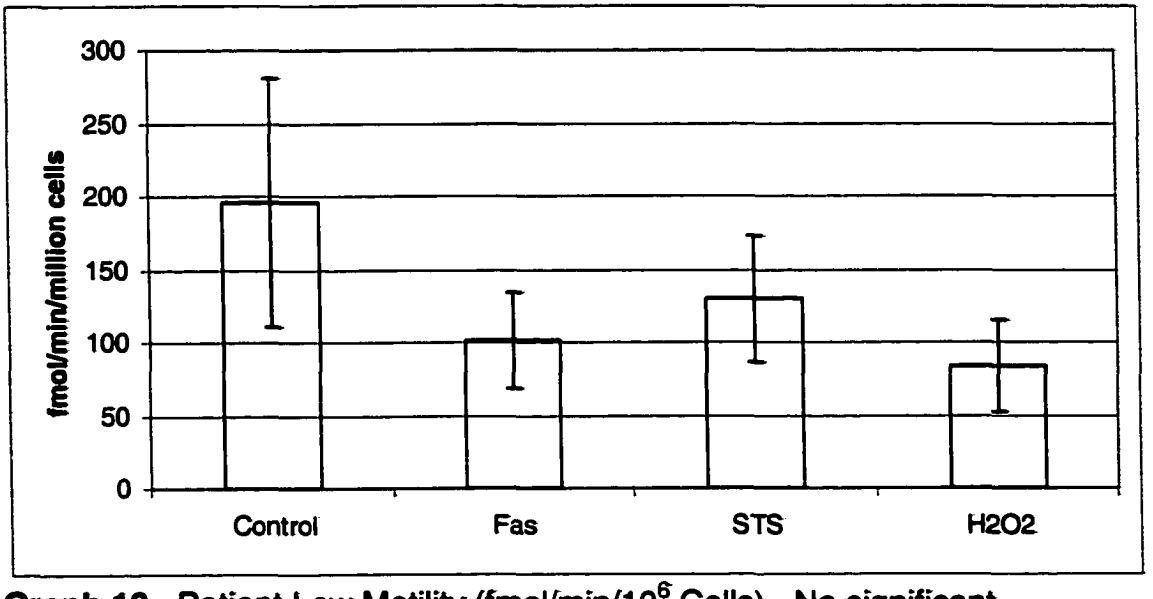


Graph 9. Patient High Motility (fmol/min/10^6 Cells) *P=0.003. #P=0.039. Values are means ± SEM.



Graph 10. Patient Low Motility (fmol/min/10^6 Cells). No significant differences. Values are means ± SEM

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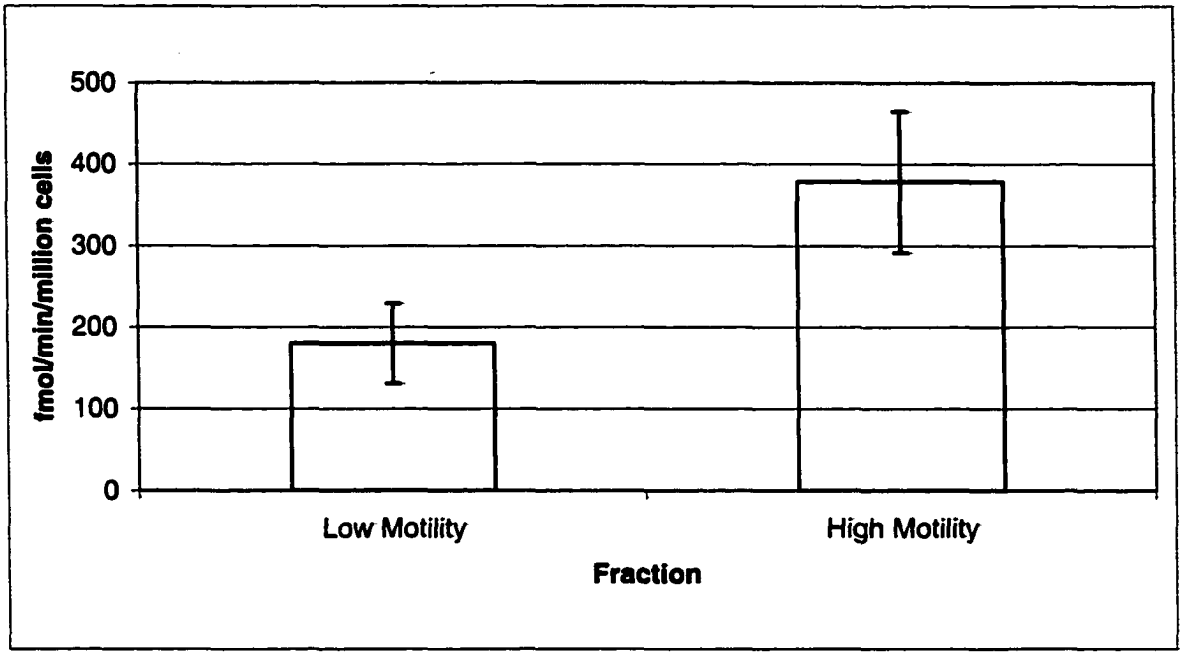

Graph 11. Patient STS Treated (fmol/min/10^6 Cells). No significant differences. Values are means ± SEM.

Table XI. Patient Low Motility Caspase Activities

Fraction	Caspase Activity[1] fmol/min/10^6 cells	n
Control	195.4 ± 83.3	18
Fas Antibody	101.3 ± 33.3	11
H_2O_2	98.9 ± 37.3	4
Staurosporine	68.6 ± 48.0	14

[1]Values are means ± SEM. No significant comparisons.

For patients, no differences were detected between high and low motility control fractions (Table 12) and high and low motility Fas antibody-treated fractions. High motility H_2O_2 treated fractions had significantly higher (p=0.016) caspase activities compared with low motility fractions. High motility

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staurosporine-treated fractions were significantly higher (p=0.004) than low motility fractions (Graph 11).

Table XII. Patient High and Low Motility Baseline Caspase Activities

Fraction	Caspase Activity[1] fmol/min/10^6 cells	n
Low Motility	195.4 ± 83.3	18
High Motility	151.5 ± 28.4	25

[1]Values are means ± SEM. No significant differences.

In donor sperm fractions, no significant differences were detected between high motility control fractions and either Fas antibody or H_2O_2 treatments (Table 13). Staurosporine-treated fractions were significantly higher than control (p<0.001) and Fas antibody (p=0.016) treated fractions (Graph 12). No comparison between low motility control, Fas antibody, H_2O_2, and staurosporine fractions were significantly different (Graph 13).

Donor high and low motility control fractions were not significantly different in caspase activities (Table 14). Comparisons between high and low motility Fas antibody treated fractions revealed significantly higher (p=0.023) caspase activities in the low motility fraction. Both H_2O_2- and staurosporine-treated high and low motility fractions had comparable caspase activities. Caspase activities were lower, but not significantly so, in low motility H_2O_2 fractions compared with high motility fractions in the same treatment group. Caspase activities in staurosporine-treated high and low motility fractions were nearly identical.

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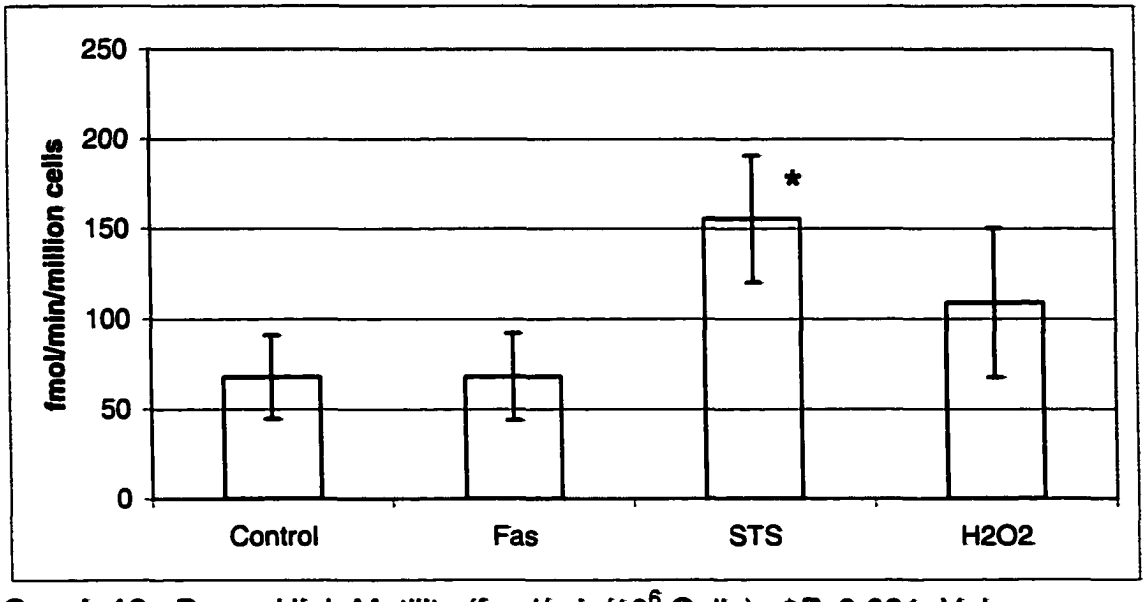
Table XIII. Donor High Motility Fraction Caspase Activities

Fraction	Caspase Activity[1] fmol/min/10^6 cells	n
High Motility		
Control	67.8 ± 22.5	9
Fas Antibody-Treated	66.9 ± 23.8	8
H_2O_2-Treated	108.4 ± 40.4	8
Staurosporine-Treated	155.4 ± 34.3[2]	9
Low Motility		
Control	133.3 ± 32.0	9
Fas Antibody-Treated	150.2 ± 27.9	6
H_2O_2-Treated	53.9 ± 48.6	5
Staurosporine-Treated	198.1 ± 42.3	5

[1]Values are means ± SEM. [2]Staurosporine-treated fractions were significantly ($p<0.001$) different compared with Control fractions and Fas antibody treated ($p=0.016$).



Graph 12. Donor High Motility (fmol/min/10^6 Cells). *P=0.001. Values are means ± SEM.

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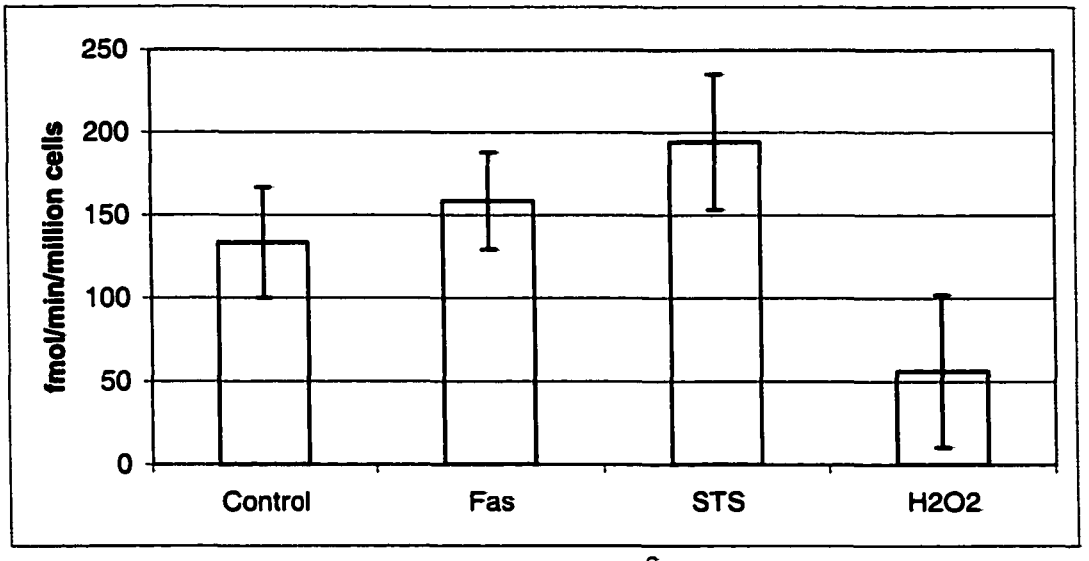

Graph 13. Low Motility Donor (fmol/min/10^6 Cells). No significant differences. Values are means ± SEM.

Table XIV. Donor High and Low Motility Baseline Caspase Activities

Fraction	Caspase Activity[1] fmol/min/10^6 cells	n
Low Motility	133.3 ± 32.0	9
High Motility	67.8 ± 22.5	9

[1]Values are means ± SEM.

When comparing patient and donor high motility control fractions (Graph 14), patient fractions had significantly higher (p=0.02) caspase activity than donor fractions (Table 15). No significant difference was detected between low motility patient and donor fractions. Patient staurosporine-treated fractions had significantly higher (p=0.016) caspase activities that were nearly two and one-half times higher than donor fractions (Graph 15). Conversely, no significant difference was detected between low motility patient and donor staurosporine-treated fractions (Table 16). No statistically significant

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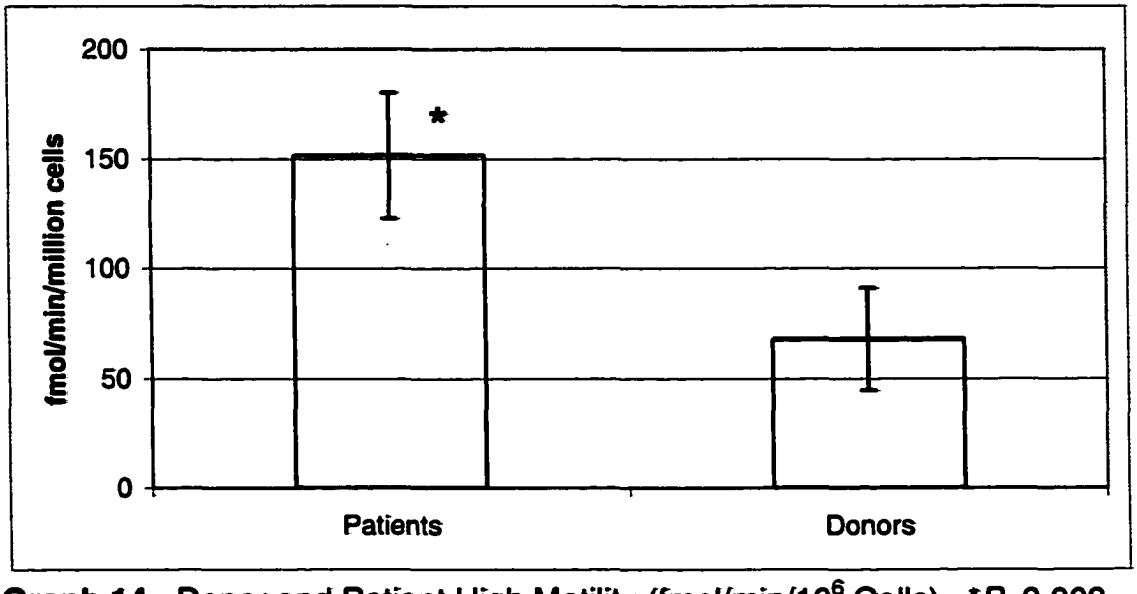
difference was detected in comparisons between either high or low motility fractions in Fas antibody or H_2O_2 treatment groups.

Table XV. Donor and Patient High Motility Fractions Caspase Activities

Fraction	Caspase Activity[1] fmol/min/10^6 cells	p-value
Control		
Patients	151.5 ± 28.8	0.02
Donors	67.8 ± 21.8	
Fas Antibody		
Patients	109.1 ± 29.0	0.258
Donors	66.9 ± 23.6	
Staurosporine		
Patients	378.5 ± 86.2	0.016
Donors	155.4 ± 33.4	
H_2O_2		
Patients	324.5 ± 111.9	0.069
Donors	108.4 ± 27.1	

[1]Values are means ± SEM.



Graph 14. Donor and Patient High Motility (fmol/min/10^6 Cells). *P=0.002. Values are means ± SEM.

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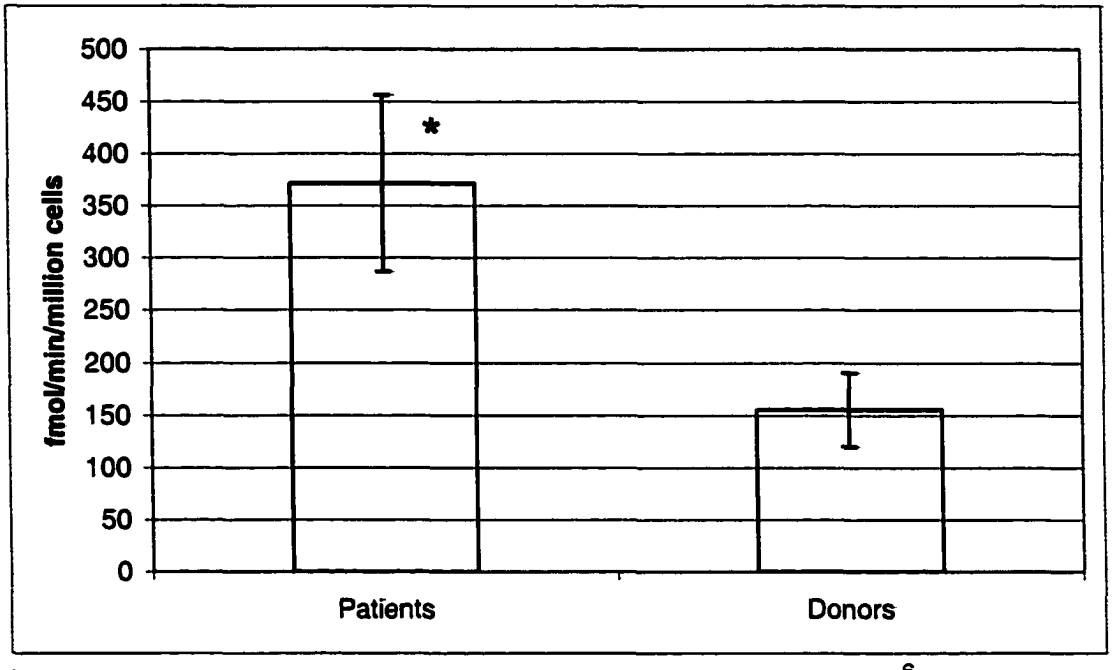

Graph 15. Donor and Patient High Motility STS (fmol/min/10^6 Cells). *P=0.016. Values are means ± SEM.

Table XVI. Donor and Patient Low Motility Fractions Caspase Activities

Fraction	Caspase Activity[1] fmol/min/10^6 cells	p-value
Control		
Patients	195.4 ± 84.1	0.489
Donors	133.3 ± 31.3	
Fas Antibody-Treated		
Patients	109.1 ± 29.0	0.258
Donors	66.9 ± 23.6	
Staurosporine-Treated		
Patients	151.6 ± 48.9	0.461
Donors	198.1 ± 40.0	
H_2O_2-Treated		
Patients	68.6 ± 36.6	0.802
Donors	53.2 ± 49.3	

[1]Values are means ± SEM. No significant differences.

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PROTEIN IMMUNOBLOTS

CASPASE-3

Caspase-3 is one of three caspases that can give positive reading using the caspase activity assay. Fractions from 9 donors and 6 patients were evaluated. Between 20 million and 50 million sperm were necessary to visualize the inactive, procaspase-3 protein. Thus, this assay was limited to patients with adequate sperm concentrations. Procaspase-3 (32 kDa) was demonstrated, to a variable degree, in both high and low motility sperm fractions from both donors and patients. Active capase-3, however, was either absent or below the detection limits of the assay. Some very light 17-20 kDa bands were demonstrated in a small minority of samples. Figure 1 is a representative immunoblot showing only procaspase-3.

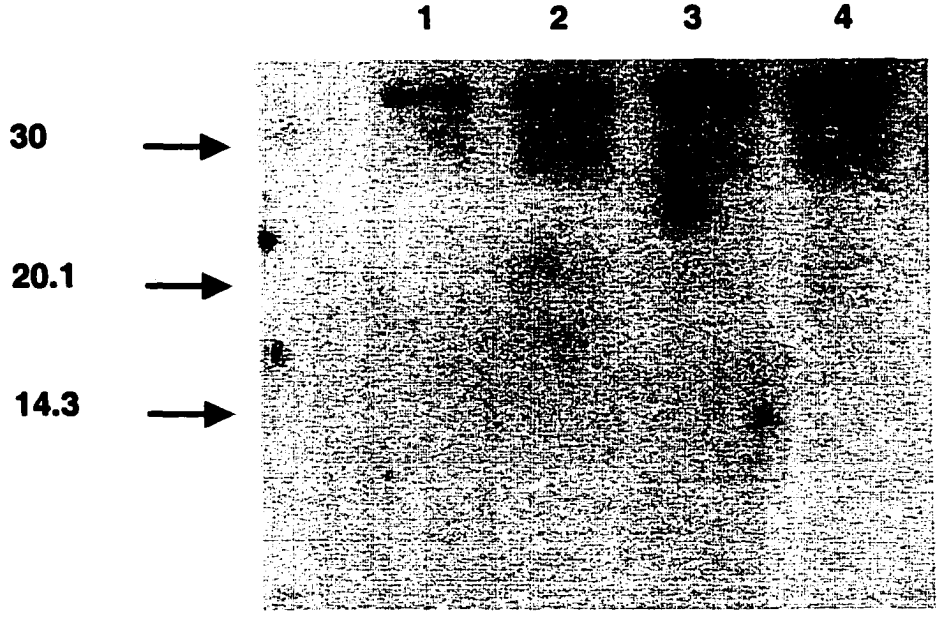

Figure 1. Caspase-3 Immunoblot. All fractions are donor high motility. Lane 1: Untreated. Lane 2: Fas Antibody-treated. Lane 3: STS-treated. Lane 4 Peroxide-treated.

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CASPASE-7

Capase-7 is a second caspase that is capable of cleaving the AC-DEVD-AFC substrate used for caspase activity assays. The inactive procaspase-7 typically migrates to approximately 35 kDa during gel electrophoresis. An intermediate caspase-7 protein migrating to 32 kDa is formed upon cleavage of the 35 kDa proenzyme. Finally, the active enzyme is composed of 20 and 11 kDa subunits. With fractions from two patients and four donors, the 35 kDa band was readily visible. In low motility fractions from both patients, the intermediate procaspase-7 band was also observed. Sperm concentrations greater than 20 $x10^6$/mL were necessary, as with caspase-3 immunoblots. In some sperm fractions, the intermediate 32 kDa band was also visible. In no case was either of the component subunits of active enzyme detected. A representative caspase-7 immunoblot is shown in Figures 2 and 3.

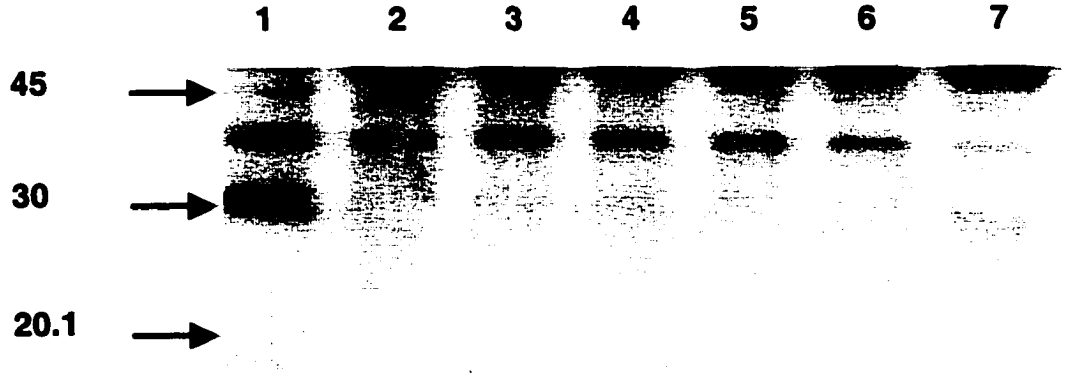

Figure 2. Caspase-7 Immunoblot. Lane 1: PMNs, untreated. Lanes 2-7, fractions. Lane 2: High motility STS-treated. Lane 3: High motility untreated. Lane 4: High motility STS-treated. Lane 5: Low motility untreated. Lane 6: Low motility STS-treated. Lane 7: High motility untreated.

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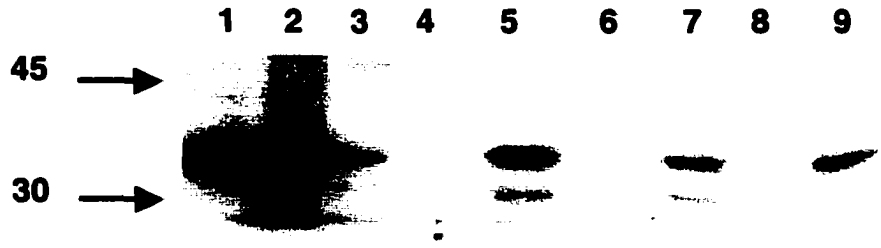

Figure 3. Caspase-7 Immunoblot, 7.5% Acrylamide Gel. Lane 1: Jurkat cells, untreated. Lane 2: Jurkat cells, STS-treated. Lanes 3-9: untreated sperm fractions. Lane 3: Donor high motility. Lane 4: Patient high motility. Lane 5: Patient low motility. Lane 6: Patient high motility. Lane 7: Patient low motility. Lane 8: Patient high motility fraction. Lane 9: Patient low motility.

CASPASE-9

Caspase-9 is an enzyme involved in apoptosis upstream of caspase-3 in somatic cells. The inactive, procaspase-9 protein migrates to 46-48 kDa during SDS-PAGE. Procaspase-9 is processed into a 37 kDa large subunit and a 10 kDa small subunit in cells undergoing apoptosis. Representative immunoblots are depicted in Figure 4. Sperm fractions from three patients and one donor were used for this experiment. As was common with donors throughout this research, Percoll™ separation resulted in a low motility fraction with too few sperm for immunoblotting. Two Jurkat cell extracts (4 million cells) were used as controls. One extract was composed of untreated cells (lane 1) while in the second (lane 2), Jurkat cells were treated with staurosporine for four hours. All sperm fractions were untreated Active caspase-9 was confined to a single patient low motility fraction. No other fractions exhibited active caspase-9. Some caspase-9 activation was seen in

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the untreated Jurkat cell lysates, although the intensity of the bands were clearly less than that seen in STS-treated Jurkat cells.

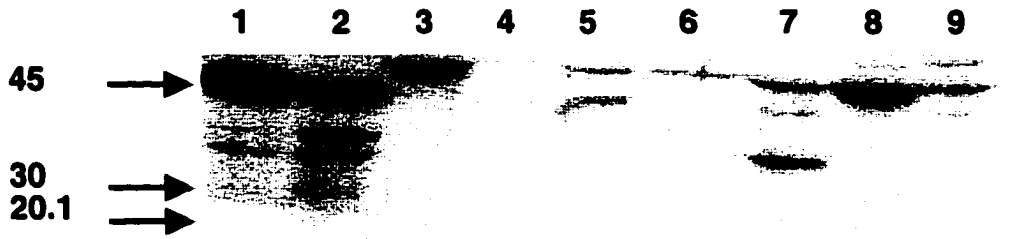

Figure 4. Caspase 9 Immunoblot. Lane 1: Jurkat cells, untreated. Lane 2 Jurkat cells, STS treated. Lanes 3-9: untreated sperm fractions. Lane 3: donor high motility. Lanes 4, 6, 8: patient high motility. Lane 5, 7, 9: patient low motility.

Poly(ADP-ribose) Polymerase-1 (PARP-1)

Poly(ADP-ribose) Polymerase-1 (PARP-1) typically migrates to 116 kDa during SDS-Page. In apoptotic cells, PARP-1 is a prime target of caspase-3, which processes the enzyme to 85 kDa and 25 kDa fragments. No intact PARP-1 was detected in either sperm or PMNs . The antibody did, however, react with a 66 kDa protein in every sperm fraction examined. This 66 kDa band is seen in somatic whole cells extracts and is of cytoplasmic origin. Immunoblots from 9 donors and 8 patients all exhibited the 66 kDa band and no other. Since PMNs do not have the PARP-1 enzyme, Jurkat cells were used as controls. As expected, untreated Jurkat cells displayed a band at slightly above the 97 kDa band in the expected position of 116-120 kDa (Figure 5, lane 1). Jurkat cells were stimulated to undergo apoptosis by four hour incubation with two different anti-Fas antibodies (CH-11 and IPO-4, both

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from Kaimya Biomedical Company, Seattle, WA) and STS. As expected, a band of approximately 86 kDa was seen (Figure 5, lanes 2, 3, and 4). On the same blot, two sperm fractions (from the same donor, but one as a control and the other treated with STS) and one PMN extract were evaluated. The 116 kDa PARP-1 band was not detected in any of the three fractions. However, very faint 66 kDa bands were observed in each fraction.

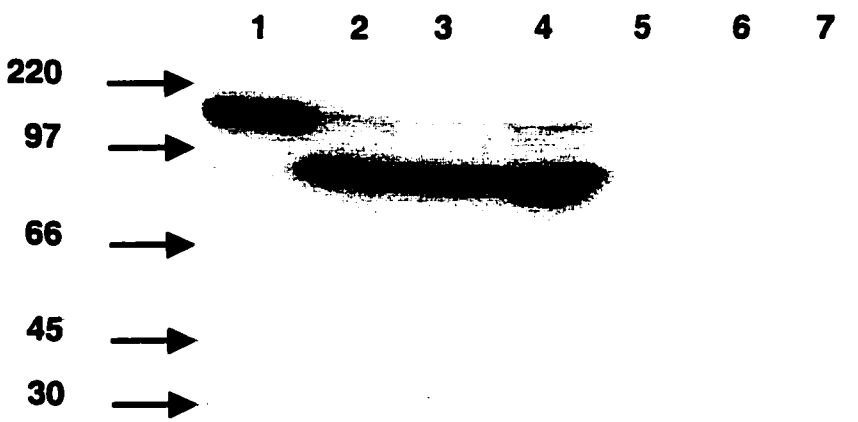

Figure 5. PARP-1 Untreated, Staurosporine, and Fas Antibody. Lane 1: untreated Jurkat cells. Lane 2: CH-11 Fas antibody-treated Jurkat cells. Lane 3: IPO-4 Fas antibody-treated Jurkat cells. Lane 4: STS-treated Jurkat cells. Lane 5: untreated donor high motility sperm fraction. Lane 6: STS-treated donor high motility sperm fraction. Lane 7: STS-treated PMNs.

Figures 6 and 7 are representative of sperm fractions, probed with the anti-PARP-1 antibody. In both blots, each sperm fraction exhibited the 66 kDa band and the complete absence of both the 116 kDa and 85 kDa bands. Two PMN extracts (one control and one STS-treated fraction) were also evaluated. Curiously, the untreated PMN extract failed to exhibit even the 66 kDa band seen in sperm and with STS-treated extracts.

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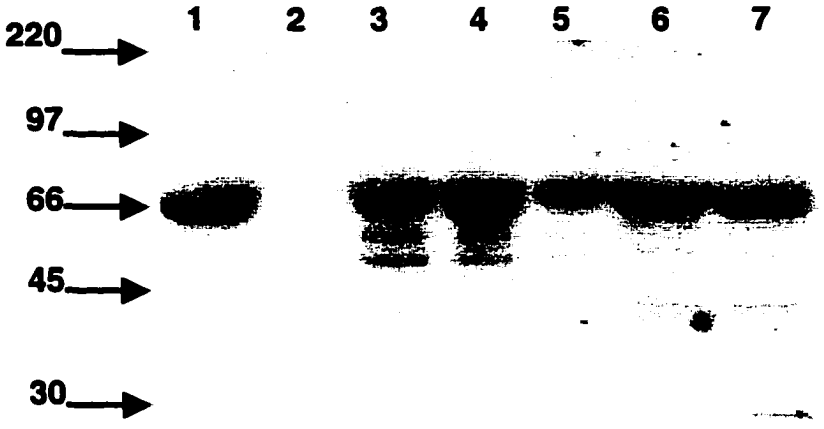

Figure 6. PARP-1 Donor Sperm Untreated and Staurosporine. Lane 1: STS-treated PMNs. Lane 2: untreated PMNs. Lanes 3-7: sperm fractions. Lane 3, 6: untreated donor high motility. Lanes 4, 7: untreated low motility. Lane 5: STS-treated donor low motility.

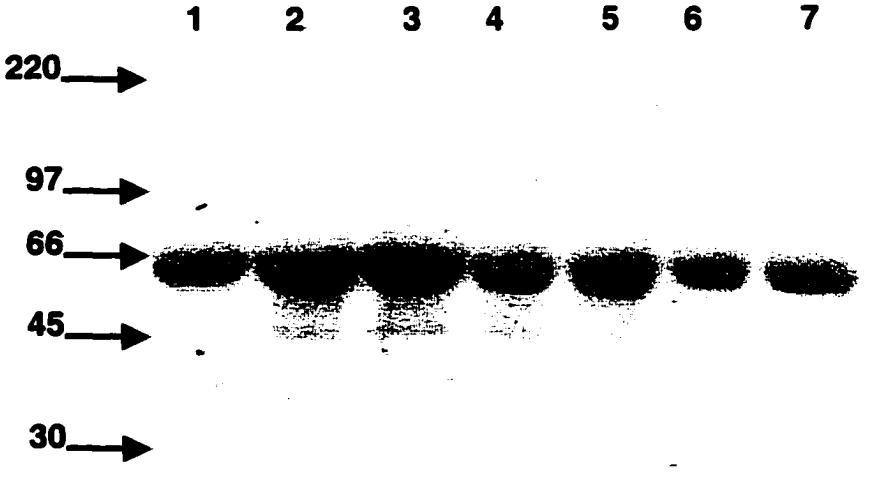

Figure 7. PARP-1 Patient Sperm Untreated and Staurosporine. Lane 1: STS-treated PMNs. Lanes 2-9: patient sperm fractions. Lanes 2, 6: high motility. Lane 3: STS-treated high motility. Lanes 4, 7: untreated low motility. Lane 5: STS-treated low motility.

AIF

The presence of Apoptosis Inducing Factor (AIF), a 67 kDa protein released by mitochondria in response to apoptotic stimuli was evaluated.

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Unlike many of the other apoptotic proteins, AIF does not undergo any processing and is produced in its active form. For this experiment, 3 patients and 1 donor were used. In Figure 9, AIF was detected in Jurkat cell controls as well as sperm extracts from the donor and three infertility patients.

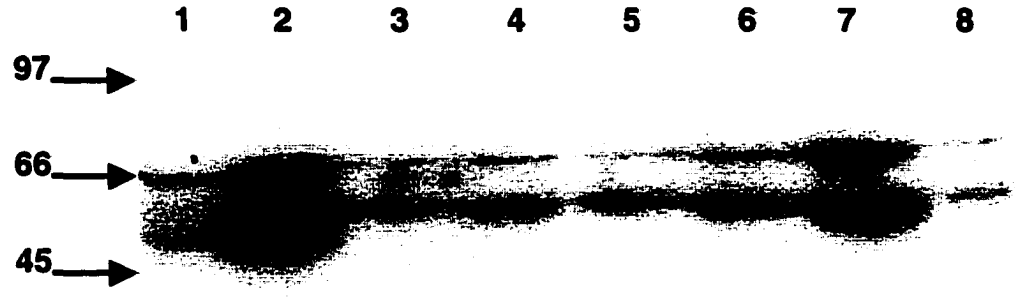

Figure 8. AIF Immunoblot with Patient Sperm Fractions. Lane 1 Jurkat cells, STS-treated. Lanes 2-8: untreated sperm fractions. Lane 2: donor high motility. Lanes 3, 5, 7: patient high motility. Lane 4, 6, 8: patient low motility.

BID

Like AIF, BID is another important factor in mitochondria regulated apoptosis. Full-size BID, the inactive form, may migrate to three molecular weights during SDS-PAGE, 22 kDa, 24 kDa, or 26 kDa. During activation, BID is primarily processed to a 15 kDa. Minor products have also been seen at 13 kDa and 11 kDa. In Figure 10, Bid was detected in Jurkat cells, but not in any of the donor or patient sperm fractions. One donor high motility and three patient high and low motility fractions were evaluated.

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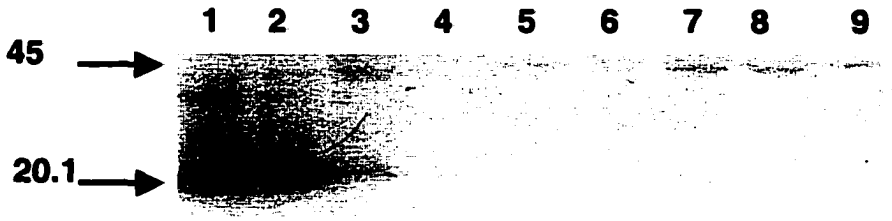

Figure 9. Bid Immunoblot. Lane 1: Jurkat cells, untreated. Lane 2 Jurkat cells, STS treated. Lanes 3-9: untreated sperm fractions. Lane 3: donor high motility. Lanes 4, 6, 8: patient high motility. Lane 5, 7, 9: patient low motility.

The effect of four hour H_2O_2 treatment was studied on sperm fractions was studied with four H_2O_2 concentrations, 200 μM, 100 μM, 50 μM, and 25 μM (Table 17, Graphs 16 and 17). As with data collected during the caspase activity assays, sperm from a donor semen sample was separated into high and low motility fractions. Contaminating cells of non-spermatogenic origin were immunodepleted using the aforementioned paramagnetic method. High motility fractions incubated with 200 μM had virtually no viable sperm. Motility was also non-existent for these fractions. The 100 μM fractions displayed increased viability, although sperm motility was still non-existent. At 50 μM, viability had reached control values, however, motility was still at or near zero. At a H_2O_2 concentration of 25 μM, viability remained at control levels and progressive motility was noted in some sperm. Findings with low motility fractions were similar to the high motility fractions (Graphs 18 and 19).

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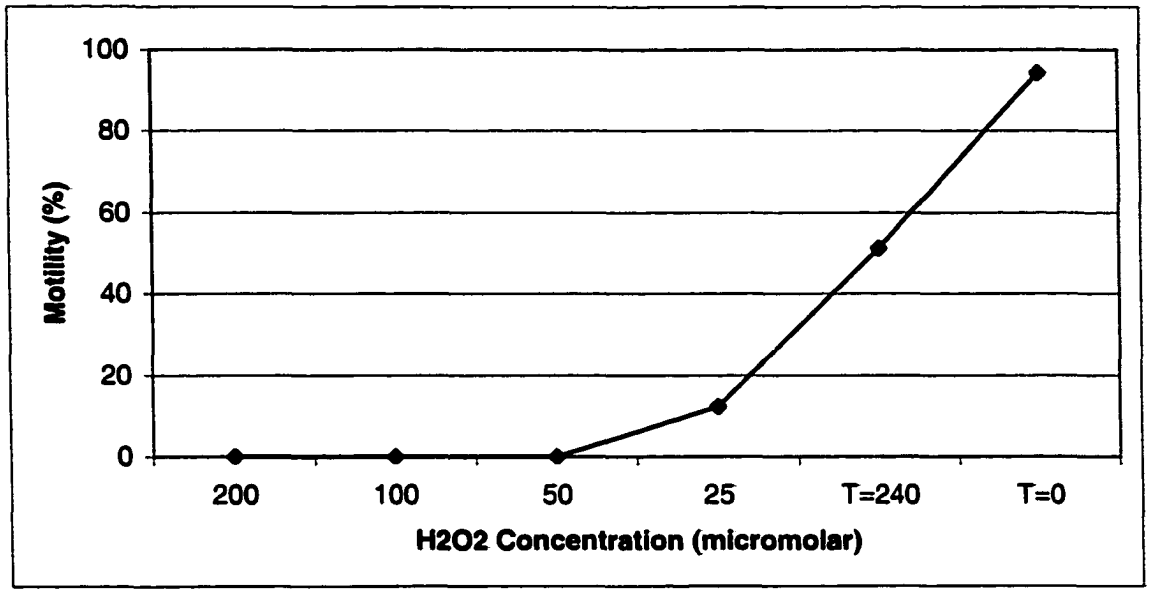
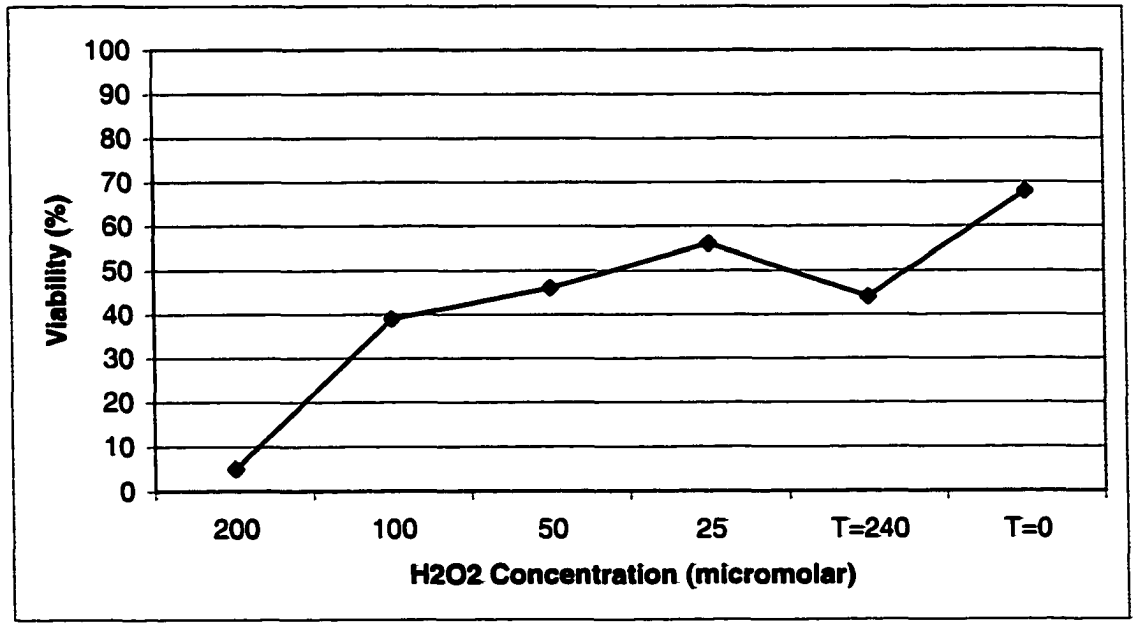


Graph 17. High Motility H_2O_2 Motility Dose Response Fractions.



Graph 18. Low Motility H_2O_2 Viability Dose Response.

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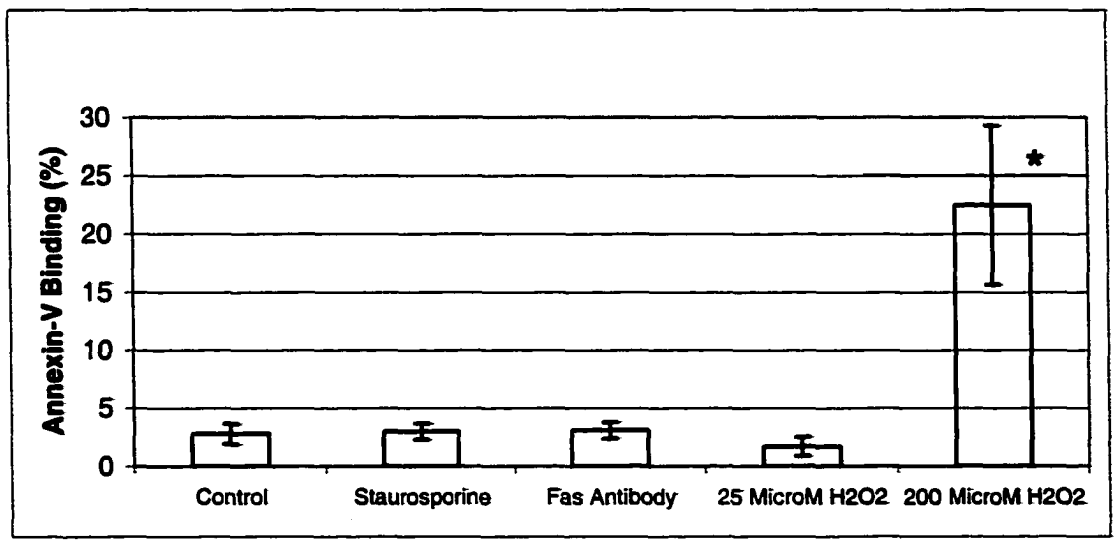
Table XVIII. Annexin-V Binding in Ethidium Negative Sperm

Fraction	Treatment	n	Annexin-V Binding Average ± SEM
High Motility	Control	10	2.78 ± 0.87
	STS	10	2.98 ± 0.69
	Fas Antibody	9	3.08 ± 0.71
	25 μM H_2O_2	4	1.69 ± 0.82
	200 μM H_2O_2	4	22.47 ± 6.81[1]
Low Motility	Control	8	3.96 ± 0.69
	STS	8	3.78 ± 0.84
	Fas Antibody	8	4.13 ± 1.01
	25 μM H_2O_2	4	4.06 ± 1.06
	200 μM H_2O_2	4	17.00 ± 8.49

[1] *P*=0.012 compared with Control fractions. *P*=0.033 compared with STS treated fractions. *P*=0.045 compared with Fas antibody treated fractions. *P*=0.012 compared with 25 μM H_2O_2 treated fractions.



Graph 20. High Motility Cells With Only Annexin-V Binding. *P=0.012 compared with control fractions.

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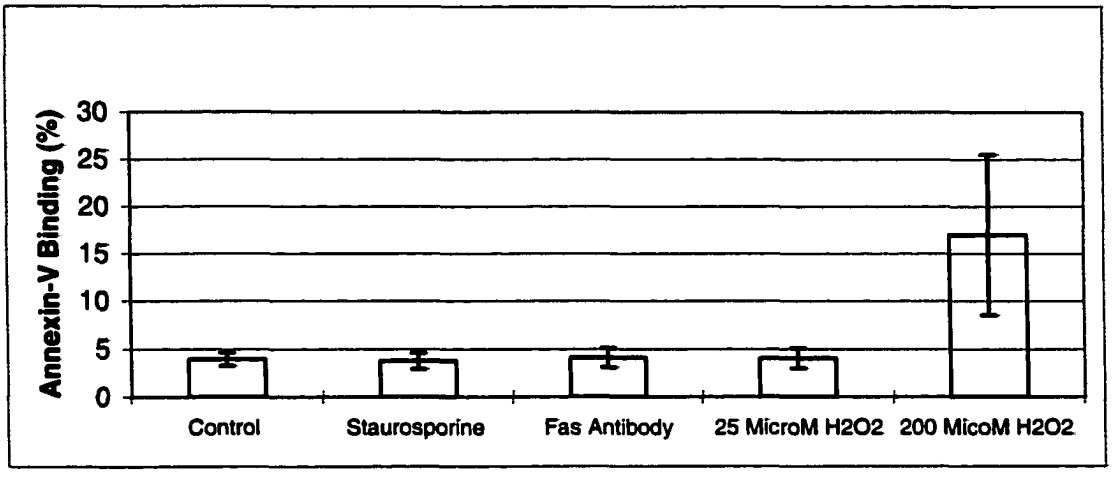

Graph 21. Annexin-V Binding In Low Motility Live Cell Populations. No significant differences. Values are means ± SEM.

Percentages of live and dead (necrotic) cells were not significantly different from control fractions. Figures 11 is a series of representative contour plots of flow cytometry data for high and low motility fractions, respectively.

CASPASE-3 IMMUNOFLUORESCENT MICROSCOPY

Nine patients exhibiting severe oligoasthenoteratozoospermia (S-OAT) were used for caspase immunofluorescence experiments. Of the nine patients, none had more than 1.2% of the sperm binding. Two patient samples had no binding. An average of 0.5% of the sperm evaluated demonstrated active caspase-3.

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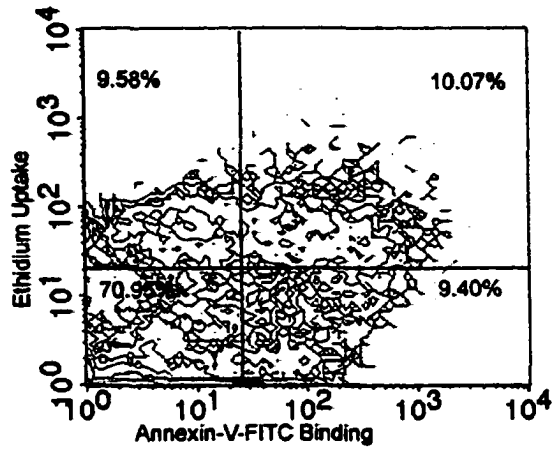
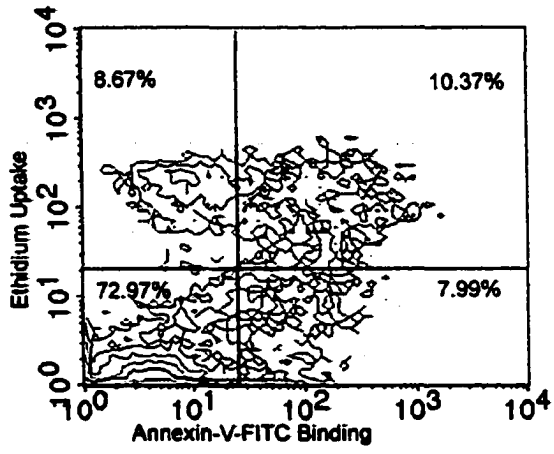
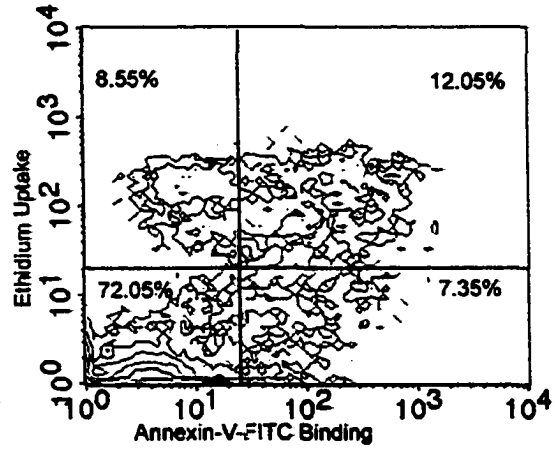


(a) High Motility Control



(b) High Motility 1 μg/mL Fas Antibody 2 hour incubation.



(c) High Motility 1 μg/mL Fas Antibody 4 hour incubation.

Figure 10. High Motility Flow Cytometry Contour Plots. Shown are representative log density contour plots from Annexin-V flow cytometry studies. The innermost contour was represented by yellow (50% of the peak height), followed by dark blue (25%), orange (12%), light blue (6%), pink (3%), and green (1%). Annexin-V binding, a maker for PS translocation, was on the x-axis and Ethidium homodimer uptake, a marker for compromised membrane integrity, was on the y-axis. The percentages in each quadrant were the percentage of the total population of cells exhibiting a labeling pattern. Cells in the lower left quadrant were normal, live cells with no Annexin-V binding, the lower right quadrant were cells to which only Annexin-V-FITC was bound (considered apoptotic in somatic cells), the upper right and upper left quadrants were necrotic cells as evidenced by ethidium uptake. (a) untreated control fraction. (b, c) Fas antibody treated 2 and 4 hour incubations. No significant differences in Annexin-V-FITC binding were observed with incubation with a Fas antibody.

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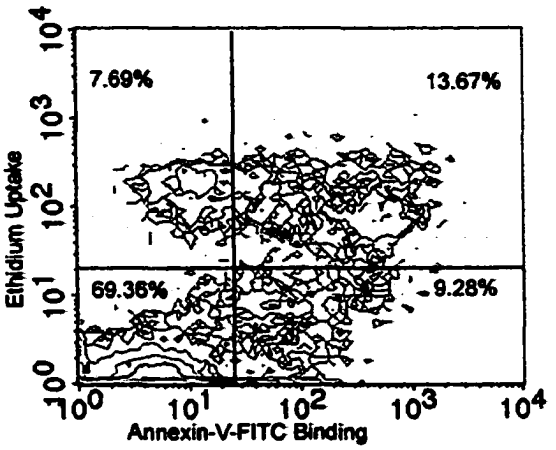
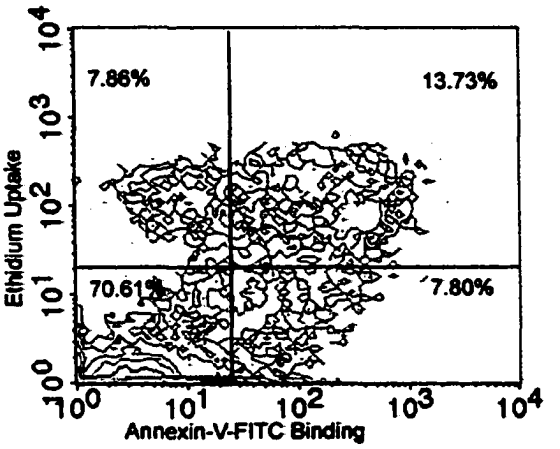
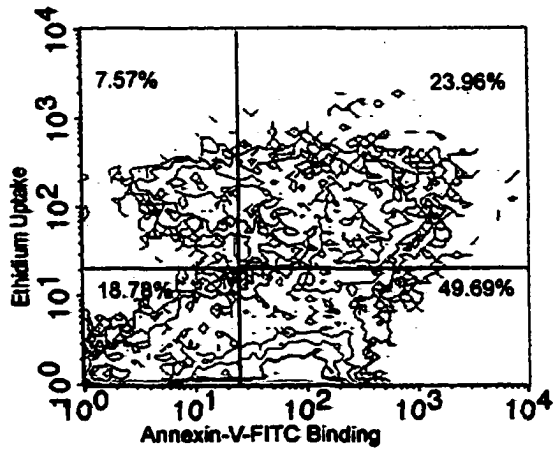


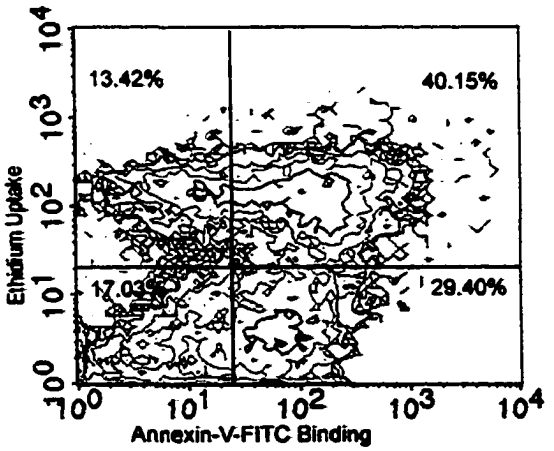
(d) High Motility 10 μM STS 2 hour incubation.



(e) High Motility 10 μM STS 4 hour incubation.



(f) High Motility 200 μM H_2O_2 2 hour Incubation.



(g) High Motility 200 μM H_2O_2 4 hour Incubation.

Figure 10 (Continued). High Motility Flow Cytometry Contour Plots. (d, e) STS treated after 2 and 4 hour of incubation. No significant increases were detected with STS treatment. (f, g) 200 μM H_2O_2 after 2 and 4 hours incubation. Significant increases (P=0.012) in Annexin-V-FITC were detected. Percentages of cells binding Annexin-V-FITC increased from 9.4% in untreated cells to 49.69% at the 2 hour incubation with 200 μM H_2O_2. At 4 hours, fewer cells were binding Annexin-V-FITC than at 2 hours, however, increases were still significant over untreated cells. Gradual progression in ethidium uptake was detected as incubation progressed, indicating increasing numbers of cells experiencing loss of membrane integrity.

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Blanc-Layrac study did not use a method of separating non-spermatogenic cells contaminating semen samples. Since the present study used a method of separating these cells from sperm, it seems possible the failure to remove these cells in the Blanc-Layrac study may have contributed to their detection of both active and inactive PARP-1 proteins.

The 66 kDa band does not match the expected molecular weights of either the active or inactive form of PARP-1. However, a PARP reactive cytoplasmic protein of approximately 66 kDa has been noted in whole cell extracts from somatic cells. The only other human cell that lacks PARP-1 is neutrophils (Bhatia *et al.*, 1995; Sanghavi *et al.*, 1998). Bhatia (1995) demonstrated that intact PARP-1 is missing from non-apoptotic mature human neutrophils and explored the point during differentiation that immunoreactive PARP-1 is lost. In the Sanghavi study, and confirmed in the present study (see figure 6, lane 2), both intact PARP-1 was absent from non-apoptotic neutrophils. Given the highly condensed and therefore inaccessible nature of normal sperm DNA, it would seem logical that PARP-1 be absent from human sperm.

Selection of the 200 μM H_2O_2 concentration for use in caspase activity studies was drawn from a previously published study performed at the Jones Institute (Duru *et al.*, 2000). In that study, the H_2O_2 concentration significantly associated with DNA fragmentation was 200 μM. In somatic cells, caspase activation, particularly caspase-3 activation, induces a cascade of enzymatic activity that ultimately culminates in the fragmentation of cellular DNA. The 200 μM H_2O_2 concentration was tested in the caspase activity assays based

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on the possibility that DNA fragmentation observed at this concentration could be the result of caspase-dependent apoptotic events in sperm. The alternative possibility remains that the DNA fragmentation could be the result of simple, direct oxidative damage to the DNA by H_2O_2.

It appears from the significantly lower caspase activity seen in H_2O_2 treated sperm fractions could be explained by the detrimental effect of the H_2O_2 concentration on sperm plasma membrane integrity. In sperm with compromised membranes, the potential exists for caspases present to diffuse from the intracellular compartment to the surrounding medium where they were removed with the supernatant during the concentration process prior to sonication. H_2O_2 fractions were the only treatment group with significantly decreased viability after incubation. This explanation could account for the dramatically decreased caspase activity in H_2O_2 treated sperm fractions. Additionally, H_2O_2 has been recently shown to inhibit caspase activity (Borutaite and Brown 2001). Absent the escape of caspases from compromised sperm membranes, the finding that H_2O_2 inactivates caspases could explain the significantly lower caspase activity in fractions treated in this manner. Significantly, the inhibition of caspase activity was shown to be reversed by the addition of dithiothreitol (DTT) to the suspension. Given that the lysis buffer used in preparation of the sperm extracts contained DTT, makes the explanation of caspase inactivation by H_2O_2 unlikely. Therefore, it appears most likely that, in H_2O_2 treated fractions, any caspases that may

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have been activated through mechanisms secondary to oxidative stress were lost due to compromised integrity of the sperm plasma membrane.

Two stimulatory anti-Fas antibody clones (CH-11 and IPO-4) were used in an attempt to induce caspase activation in sperm fractions through Fas mediated pathways. Neither antibody was able to induce increases in caspase activity in sperm, though both were able to induce a dramatic caspase activation in human blood neutrophils. Both Neutrophils and sperm were treated identical regimens. At the end of incubation, neutrophils had high levels of caspase activity, but sperm caspase activity remained at levels comparable to control fractions.

The presence of Fas on the sperm plasma membrane has been conclusively demonstrated using flow cytometry (Sakkas *et al.,* 1999). The Sakkas study demonstrated a distinct and significant difference between fertile donors and oligozoospermic men in Fas expression on ejaculated human sperm. Therefore, if an intact Fas mediated apoptotic system is present in human sperm, treatment with an activating anti-Fas antibody could be expected to induce apoptotic events in at least a significant subpopulation of sperm. Sakkas postulated that ejaculated sperm lacked the full complement of apoptotic machinery present in somatic cells and that, such machinery, so much as present, were simply disconnected remnants of incomplete apoptosis. The failure of either of the two anti-Fas antibody clones used in the present study to induce caspase activation in sperm would tend to

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support Sakkas incomplete apoptosis theory, at least so far as Fas induced apoptotic caspase dependent pathways are concerned.

Flow cytometry results supported the theory that PS translocation is not directly caspase dependent. Sperm has been shown to have Fas receptors on the plasma membrane (Sakkas *et al.*, 1999). Treatment with an apoptosis stimulating anti-Fas antibody resulted in no significant increase in PS translocation and therefore would seem to rule out the type I apoptotic pathways. Likewise, STS treatment also failed to stimulate PS translocation, leaving Annexin-V binding levels equivalent to both control and Fas antibody levels. The lack of stimulation of PS translocation in STS treated samples suggests that if, mitochondria dependent or type II pathways are present, they are not linked to PS translocation. The significant increase in Annexin-V binding in fractions treated with cytotoxic (200 μM) levels of H_2O_2 may merely be a secondary due to oxidative stress. Direct action of H_2O_2 on PS and/or APT may cause the appearance of PS on the outer membrane leaflet. While it was possible to stimulate Annexin-V binding in sperm, the stimulus for PS translocation was different from that necessary in caspase assays. In flow cytometry experiments, STS treatment, the only effective treatment for stimulating caspase activity, resulted in no measurable increase in Annexin-V-FITC binding.

Low motility fractions, representing (theoretically) lower quality live sperm and sperm already dead, did not have any significant differences in Annexin-V binding between any of the treatment fractions. As with caspase activity

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assays, sperm in low motility fractions appeared impervious to any of the treatments. Annexin-V binding in the 200 μM fractions was somewhat higher, but not significantly so, compared with other fractions.

The extremely low number of sperm in a semen sample exhibiting active caspase-3 via immunofluorescent microscopy explains the difficulty in detecting this enzyme in sperm fractions. Further, active caspase-3 was always localized to the mid-piece region and, in some cases, a cytoplasmic droplet. In somatic cells, procaspase-3 has been shown to have a mitochondrial distribution as well as cytoplasmic (Mancini *et al.*, 1998). In Mancini's research, procaspase-3 was activated and released from mitochondria during induction of apoptosis. This same process may very well be occurring in sperm, however, diffusion out of the immediate vicinity of the mitochondria may be limited by compartmentalization characteristic of sperm structure.

Sperm have been shown to have, at least, procaspases -3, -7, and -9, as well as the mitochondria dependent, caspase independent factor AIF. In some cases, active caspases -3 and -9 were detected. Bid, an important protein in the induction of mitochondrial apoptotic pathways, was not detected in any sperm fraction.

PARP-1, another marker of apoptosis was detected in neither of its common forms. This feature makes sperm only the second, after polymorphonuclear leukocytes, cell type that lacks this important protein. Given the role of PARP-1 in somatic cell DNA repair, the fact that PARP-1 is

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absent in sperm is not surprising. Mature spermatozoa, with their highly compacted and virtually inaccessible DNA, would not have the need for PARP-1. Even if PARP-1 proteins were present in sperm, it would be necessary to unwind the sperm DNA in order to repair damaged sequences.

Do sperm undergo apoptosis? Sperm certainly do have some of the major components necessary to undergo the process as it is understood today, however, it is unclear how these components work together, if at all. It was not possible to induce apoptosis using known stimuli of caspase dependent pathways (anti-Fas antibodies), even though sperm have been shown to express Fas receptors (Sakkas *et al.*, 1999; Sakkas *et al.*, 2002). Additional evidence of the lack of involvement of caspases in sperm lies in flow cytometry data. PS translocation during apoptosis has been viewed as a caspase dependent phenomenon, yet, as with the caspase activity assays, anti-Fas antibody treatment failed to promote this event. A possibility is that some critical link in the caspase dependent pathway is absent, effectively short-circuiting a cascade of apoptotic events.

The increase in caspase activity with STS treatment may indicate mitochondrial pathways are active in sperm. However, BID was not detected in protein immunoblots of sperm fractions. It is possible that BID is present in sperm, except that the protein level in these experiments falls below the threshold for immunoblots. AIF is present in sperm, however, it is not clear whether any communication exists between the mid-piece and the nucleus.

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AIF exerts all of its influence in the nucleus where it causes DNA fragmentation.

Sperm have many of the essential components for apoptosis, however, what seems to be lacking is a pathway for bringing together the components for induction of apoptosis. It is clear that some apoptosis inducers used for somatic cells fail to have the comparable effects on sperm. Perhaps other inducers of apoptosis will be successfully in stimulating these events in sperm. Or, as Sakkas suggests, maybe these proteins are merely leftovers from defective or incomplete apoptosis during spermatogenesis.

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CHAPTER V

CONCLUSIONS

Specific aim one was to identify changes in ejaculated sperm normally associated with apoptosis in somatic cells. It was expected that ejaculated human sperm would have changes similar to those in somatic cells undergoing apoptosis. Many of the hallmarks of apoptosis are indemonstrable in ejaculated sperm. A common early apoptosis marker in somatic cells is loss of membrane asymmetry due PS translocation to the outer membrane leaflet. This marker was identified to investigate specific aim 1. While sperm do exhibit PS translocation, pro-apoptotic stimuli involving a Fas antibody and staurosporine, two potent inducers of apoptosis, failed to elicit a detectable increase in PS translocation. Oxidative damage from H_2O_2 also induces apoptosis in somatic cells, however, a selected low concentration (25 μM) of H_2O_2 was unsuccessful for inducing PS translocation. At 200 μM H_2O_2, a significant increase in PS translocation was observed. This significant translocation may have been due to either simple oxidative damage to PS and associated monitoring systems or may have been due to an induced apoptosis-like event in sperm.

Specific aim two was to determine the presence of caspases in ejaculated sperm and whether they can be initiated by known pro-apoptotic agents. If sperm were capable of apoptosis, the expected outcome of these studies would have been that pro-apoptotic treatments would result in an increase in caspase activity in sperm. With approximately 1000x less caspase activity per

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cell than stimulated PMN-positive controls, the extremely low caspase activity present in human sperm necessitated at least 20 x 10^6 sperm be used for each treatment. Fas antibody and H_2O_2 treatments resulted in no significant induction of caspase activity. However, STS did result in significant induction of caspase activity in both patient and donor high motility samples. The STS induction is indicative of a mitochondrial dependent, type II-like, pathway in sperm.

Specific aim three was to identify key apoptotic proteins in ejaculated sperm, with the expectation that sperm would express similar apoptotic proteins compared with somatic cells. Sperm were found to have procaspases-3, -7, and -9 by protein immunoblot. Active caspase-3 was present in sperm. Procaspase-7 and an intermediate form procaspase-7 were also detected in sperm fractions, however, no active caspase-7 (neither 20 kDa subunit nor 11 kDa subunit) was observed. Procaspase-9 was found in high and low motility fractions from both patients and donors. However, active caspase-9 was only detected in a single patient low motility fraction. PARP-1, a caspase-3 target, was not found in any sperm fraction, making sperm and PMNs the only two cell types in which PARP-1 has not been detected (Sanghavi, *et al.,* 1998). AIF, an important protein in caspase independent DNA fragmentation, was found in all sperm fractions. Since mitochondrial are present in sperm, the presence of AIF was expected. Bid, a cytoplasmic pro-apoptotic protein, was not detected in any sperm fraction. Bid may be totally absent in sperm of simply below the threshold of detection for protein

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immunoblotting. Active-caspase-3 immunofluorescent microscopy with severe oligoasthenoteratozoospermic patients was found to be comparable to Weng's (2002) observations for normozoospermic patients. Less than 1% of the sperm evaluated exhibited positive immunofluorescence for active caspase-3.

Specific aim four was to define differences between fertile donors and infertility patients with regard to apoptotic markers. This specific aim was accomplished by comparing caspase activity assays between donors and patients. Patients had significantly higher basal levels of caspase activity compared with fertile donors. STS treated patient high motility fractions also were approximately twice the donor values.

Further studies will involve immunoblots of stimulated patient and donor sperm fractions for caspases-7, -9, and -6. Caspase-8 has yet to be evaluated using immunoblots in sperm fractions. PARP activity is present in other cells in up to three other related proteins. Additional immunoblots are necessary to evaluate whether these proteins are present in human sperm. Additionally, it will be necessary to localize AIF in STS stimulated sperm to determine whether this protein is released from sperm mitochondria and whether it is capable of migration to the nucleus.

In summary, the following has been demonstrated in ejaculated spermatozoa:

1. That an apoptotic membrane bio-marker PS is present in sperm and is inducible through H_2O_2 treatment.

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2. Caspase activity in ejaculated sperm and that this activity is inducible.
3. The presence of procaspases-3, -7, and -9, as well as active caspases-3 and -9.
4. That PARP-1 is absent from human ejaculated spermatozoa.
5. That Bid, a pro-apoptotic protein when processed by caspase-8, is absent in human spermatozoa.

The potential significance of these studies lies in the benefits towards treating infertility patients. Identification of apoptosis bio-markers in otherwise normal semen samples from infertility patients may serve to identify a subpopulation of male factor infertility patients. The possibility exists that inappropriate apoptosis in sperm may be prevented or arrested through inhibiting these process.

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