CHAPTER 5

The Relationship between Human Sperm Apoptosis, Morphology and the Sperm Deformity Index

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ABSTRACT

Background: This study aimed to assess the relationship between apoptosis in human ejaculated spermatozoa, the sperm morphology and the novel sperm deformity index (SDI).

Method: Semen specimens from 50 healthy donors were prepared by density gradient centrifugation followed by incubating the prepared sperm with paramagnetic annexin V-conjugated microbeads and subjecting this to magnetic cell sorting (MACS). The procedure delivers 2 sperm fractions: annexin-negative (non-apoptotic) and annexin-positive (apoptotic). Activated caspase-3 levels and the integrity of the sperm mitochondrial membrane potential (MMP) were assessed as markers of apoptosis in the annexin-negative and positive aliquots following MACS. Sperm morphology and the SDI scores were assessed using the strict criteria.

Results: Compared to the apoptotic sperm subpopulations the non-apoptotic sperm subpopulations had an improved sperm morphology profile as demonstrated by significantly higher proportions of sperm with normal morphology and significantly lower SDI scores and percentages of sperm with acrosomal defects, midpiece defects, cytoplasmic droplet and tail defects. There was a significant correlation between sperm morphology attributes studied and the expressed apoptotic markers - caspase-3 activation and MMP integrity.

Conclusion: Non-apoptotic sperm subpopulation has morphologically superior quality sperm compared to apoptotic sperm as reflected by significantly lower SDI scores. The study results may support abortive apoptosis.

Keywords: Apoptosis, human, morphology, sperm, sperm deformity index

INTRODUCTION

Apoptosis is a mode of programmed cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations, leading the cell to suicide without eliciting an inflammatory response. Mature sperm cells have been reported to express distinct markers of apoptosis-related cell damage (Sun *et al*., 1997; Sakkas *et al*., 1999 b; Barroso *et al*., 2000; Gandini *et al*., 2000; Muratori *et al*., 2000; Oosterhuis *et al*., 2000; Shen *et al*., 2002), although they lack transcriptional activity and have a very small amount of cytoplasm (Weil *et al*., 1998, Grunewald *et al*., 2005a). Externalization of phosphatidylserine (PS) to the sperm outer membrane leaflet is considered to mark terminal apoptosis. Activated caspase-3, loss of the integrity of the mitochondrial membrane potential (MMP) and DNA fragmentation are other markers of terminal apoptosis expressed by a varying proportions of ejaculated sperm (Evenson *et al*., 2002; Paasch *et al*., 2004a,b).

There is an established consensus on the implication of apoptosis in male infertility (Oosterhuis *et al*., 2000; Oehninger *et al*., 2003; Sakkas *et al*, 2003; Taylor *et al*., 2004), however, the exact mechanisms of its involvement remains to be elucidated (Agarwal & Said 2005). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with different degrees of testicular insufficiency (Lin *et al*., 1997, Jurisicova *et al*., 1999). The proportions of apoptotic sperm is reported to be higher in ejaculated semen samples from infertile men compared to healthy men (Taylor *et al*., 2004). Moreover, sperm caspases become more activated in patients with infertility than in healthy donors during cryopreservation (Grunewald *et al*., 2005b). Nevertheless, it is not clear whether the apoptotic markers detected in spermatozoa are residues of an abortive apoptotic process started before ejaculation or whether they result from apoptosis initiated in the post-ejaculation period (Sakkas *et al*., 1999a,b; Tesarik *et al*., 2002; Lachaud *et al*., 2004).

Semen analysis including the assessment of sperm concentration, motility and percentage normal forms remains the standard procedure for evaluating the fertility potential of semen samples. Several studies have explored the relationship between these parameters and apoptosis in ejaculated semen. A significant negative correlation between the proportion of apoptotic cells and sperm viability and motility in ejaculated semen has been reported (Marchetti *et al*., 2002; Shen *et al*., 2002; Weng *et al*., 2002; Pena *et al*., 2003; Liu *et al*., 2004; Taylor *et al*., 2004; Said *et al.,* 2005b). On the other hand, the relationship between sperm morphology and apoptosis has not been well characterized. A significant negative relationship between the proportions of apoptotic sperm and the proportions of sperm with normal morphology in semen applying the Tygerberg's strict criteria (Kruger *et al*., 1988) has been reported (Siddighi *et al*., 2004; Chen *et al*., 2006). However, it is also known that the percentage of sperm with normal morphology in sperm preparations by WHO criteria (WHO, 1999) showed

no significant correlation with caspase-3 activation, intact MMP or PS externalization (Said *et al*., 2005b).

The sperm deformity index (SDI) score is a novel expression of the quality of sperm morphology which has been shown to be a more powerful predictor of male fertility and of in vitro fertilization outcome compared to the assessment of the proportion of sperm with normal morphology (Aziz *et al*., 1996). The objective of our study was to assess prospectively the relationship between apoptosis in human ejaculated spermatozoa, the sperm morphology and the SDI scores to chart the shift in sperm morphology profile in non-apoptotic and apoptotic sperm fractions. As a secondary outcome the sperm morphology assessment was utilized to evaluate any potential effect of magnetic forces used to isolate non- apoptotic and apoptotic sperm fractions on sperm structure as seen under the light microscopy.

MATERIALS AND METHODS

Experimental design

This study was approved by the Institution Review Board of the Cleveland Clinic Foundation. Semen samples were obtained from healthy donors following a period of 3 – 5 days of sexual abstinence. Semen analysis was performed according to the World Health Organization guidelines (WHO, 1999). Samples with $\geq$ 20 X 10^6 spermatozoa/mL and at least 50 % progressive sperm motility were selected for the study.

The study design (Figure 1) included preparing semen samples by double density gradient centrifugation (PureCeption®, SAGE BioPharma, Bedminster, NJ). Samples were loaded onto a 40 % and 80 % discontinuous gradient and centrifuged at 300 *g* for 20 minutes at room temperature (25 °C.). The resulting 80 % pellet was washed by centrifugation for additional 7 minutes and re-suspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA).

One aliquot of the sperm suspension served as control, while the other aliquot was subjected to MACS. Activated caspase-3 levels and integrity of the MMP were assessed as markers of apoptosis in the annexin-negative and positive aliquots following MACS as well as in the control aliquot.

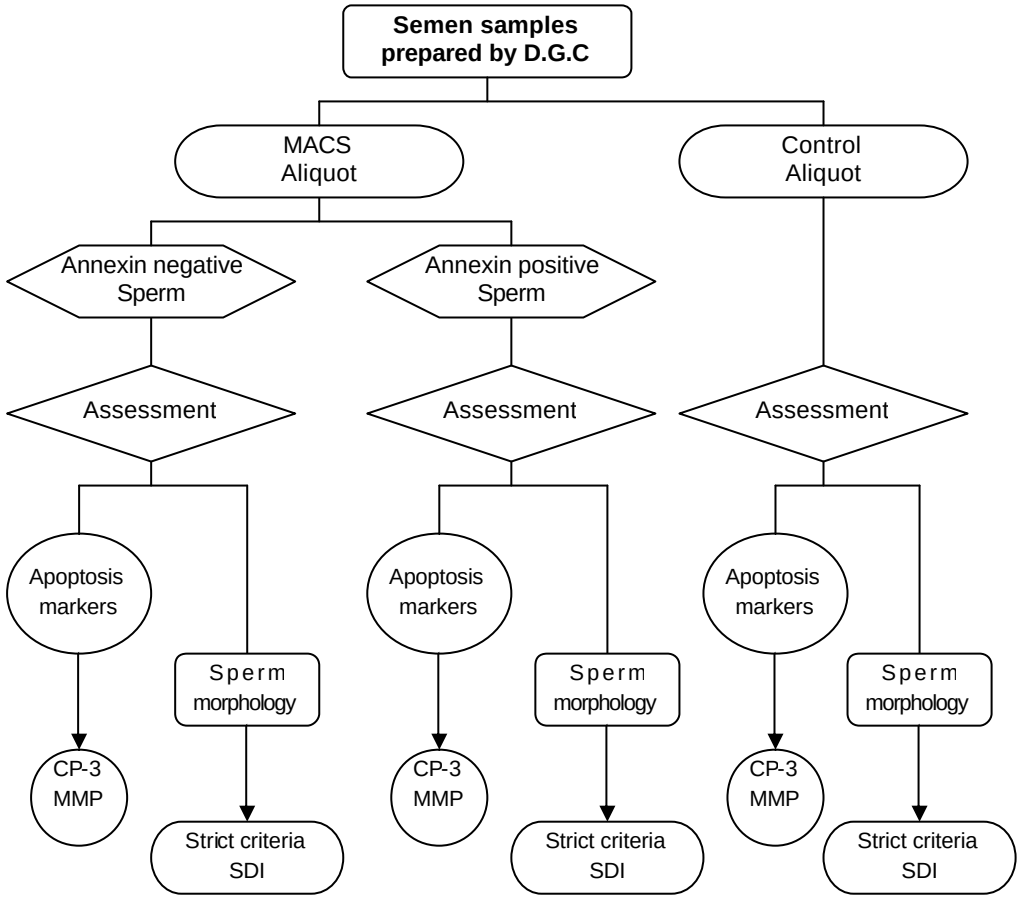

Figure1. *Flow chart depicting the study design. DGC=density gradient centrifugation; MACS=magnetic cell separation; CP-3 = active caspase-3; MMP = mitochondrial membrane potential; SDI = sperm deformity index.*

Isolation of spermatozoa with deteriorated membranes by MACS

Spermatozoa were incubated with annexin-conjugated microbeads (Miltenyi Biotec, Auburn, CA) for 15 minutes at room temperature. One hundred μL of microbeads was used for each 10 million separated cells. The sperm/microbeads suspension was loaded in a separation column containing coated cell-friendly matrix containing iron balls, which was fitted in a magnet (MiniMACS; Miltenyi Biotec, Auburn, CA). The fraction composed of apoptotic spermatozoa was retained in the separation column and labeled as annexin-positive, whereas the fraction with intact membranes that was eluted through the column was labeled as annexin-negative. The power of the magnetic field was measured as 0.5 tesla between the poles of the magnet and up

to 1.5 tesla within the iron globes of the column. After the column was removed from the magnetic field, the retained fraction was eluted using annexin-binding buffer (Miltenyi Biotec, Auburn, CA).

Detection of activated caspase-3

Levels of activated caspase-3 were detected in spermatozoa using fluorescein labeled inhibitor of caspase (FLICA), which is cell permeable, non-cytotoxic and binds covalently to active caspase-3 (Ekert *et al*., 1999). The inhibitor was used with the appropriate controls according to the kit instructions provided by the manufacturer (Carboxyfluorescein FLICA, Immunochemistry Technologies, Bloomington, MN). A 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide and further diluted in phosphate buffered saline (PBS) to yield a 30-fold working solution. All test aliquots and controls (with 100 µL PBS) were incubated at 37° C for 1 hour with 10 µL of the working solution and subsequently washed twice with the rinse buffer.

Evaluation of mitochondrial membrane potential

A lipophilic cationic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine chloride) was used to detect intact MMP in spermatozoa (ApoAlert Mitosensor Kit^{TM}, Clontech, CA). Spermatozoa with intact mitochondria excite an intense red fluorescence due to the formation of the dye aggregates whereas the monomer dye fluoresces green in the presence of sperm with a disrupted mitochondrial membrane. The kit was used according to the instructions of the manufacturer. Briefly, all aliquots were incubated at 37 °C for 20 minutes in 1 µg of the lipophilic cation diluted in 1 mL PBS. Negative controls were processed identically for each fraction except that the stain was replaced with 10 µL PBS.

Flow cytometry analysis

The extent of activated caspase-3 and intact MMP were evaluated by flow cytometric analyses. All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (Becton Dickinson, San Jose, CA). A minimum of 10,000 spermatozoa was examined for each assay at a flow rate of <100 cells/sec. The sperm population was gated using 90-degree and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FL-1 channel and red fluorescence (580–630 nm) in the FL-2 channel. The percentage of positive cells and the mean fluorescence was calculated on a 1023-channel scale using the flow cytometer software Expo32 ADC (Coulter, Krefeld, Germany).

Assessment of sperm morphology

Thin smears of the well-mixed semen were prepared in duplicate by placing 2 - 5 µL drops (depending on the sperm concentration) on clean poly-L-lysine coated slides. Thin semen smears facilitated sperm morphology assessment by avoiding sperm cell overlap and ensuring that the sperm were scattered at the same focal depth. After the slides were air-dried, they were stained using Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology.

Slides of seminal smears for morphological examination were shipped to LWH. One observer (N.A.) at LWH scored these slides utilizing a previously described technique (Aziz *et al*., 1996). Briefly, a total of 100 spermatozoa were scored per slide using bright field illumination and an oil immersion objective with a total magnification of X2000**.** At least 10 high-power fields were selected at random from different areas of the slide and examined. A calibrated micrometer on the eyepiece of the light microscope was used to measure sperm dimensions when there was doubt over sperm classification. All slides were assessed using a morphological classification based on a modification of the method of Eliasson (Eliasson, 1971) and the strict criteria for normal sperm morphology (Kruger *et al*., 1988). A multiple entry scoring technique was adopted in which an abnormal sperm was classified more than once if more than one deformity was observed. The SDI was calculated by dividing the total number of deformities observed by the number of sperm that were randomly selected and evaluated irrespective of their morphological normality. Borderline forms that were considered abnormal included 1) spermatozoa with slightly elongated head with loss of its oval shape, 2) those with rounded heads and intact acrosome, and 3) those with normal heads and a thickened midpiece. Strict quality control was maintained as each slide was in duplicate and coded. The scorer was blinded to the category that each slide had been assigned. The code was broken once the results were mailed back to CCF. Quality control assessment of sperm morphology slides revealed no significant difference in repeated estimation of different sperm morphological forms.

Statistical analysis

Data were analyzed by using inbuilt functions within the Statistical Package for Social Science Version 11 (SPSS UK Ltd., Chertsey, Surrey, UK). Study variables were not normally distributed. Summary statistics are presented as median (25^{th}, 75^{th} centile). Univariate comparison of continuous variables in sperm subgroups was performed with Wilcoxon's signed ranks test. Spearman's rank correlation was utilized to test the relationship between sperm apoptotic markers and sperm morphological subtypes and the SDI scores. All hypothesis testing were two-tailed; $P < 0.05$ was considered statistically significant.

RESULTS

Fifty healthy donors were included in this study. Summary statistics of parameters studied are presented as medians (25^{th}, 75^{th} centile) in Table 1. The non-apoptotic sperm fractions had significantly lower median percentage of sperm with activated caspase-3 compared to control and apoptotic sperm fractions. Also, the non-apoptotic sperm fractions had significantly higher median percentage of sperm with intact MMP compared to control and apoptotic sperm fractions.

The non-apoptotic sperm fractions had significantly lower median SDI score compared to the apoptotic sperm fractions ($P < 0.0001$) and to DGC preparations ($P < 0.0001$). The median percentages of sperm with normal morphology were similar in the non-apoptotic sperm fractions and the DGC preparations, but significantly higher in the non-apoptotic sperm fractions compared to the apoptotic sperm fractions (Table 1). The non-apoptotic sperm fractions had significantly lower median percentage of sperm with amorphous heads, acrosomal defects, midpiece defect and cytoplasmic droplets compared to the apoptotic sperm fractions ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, respectively) and to DGC preparations ($P = 0.026$, $P < 0.0001$, $P = 0.03$, $P = 0.0007$, respectively). While the non-apoptotic sperm fractions and the DGC preparations had similar median percentages of sperm with tail defects, the percentage was significantly higher in the apoptotic sperm fractions. Median percentages of sperm with borderline morphology and sperm with amorphous heads in DGC preparations, non-apoptotic and apoptotic sperm fractions were similar. Non-apoptotic sperm subpopulation had significantly higher percentage of motility compared with DGC preparation and the apoptotic sperm subpopulation.

Criteria	DDG preparation (control)	Non-apoptotic fraction	Apoptotic fraction	Non-apoptotic vs. DDG preparation 95% CI (P)	Non-apoptotic vs. apoptotic fraction 95% CI (P)
Activated Caspase-3 (%)	14 (7, 21)	6 (3, 12)	68 (43, 82)	8.2 to 8.5 (P < 0.0001)	-63.5 to -48.9 (P < 0.0001)
Intact MMP (%)	82 (65, 91)	89 (85, 93)	30 (18, 43)	-11.7 to -5.2 (P < 0.0001)	50.2 to 60.3 (P < 0.0001)
Motility (%)	76.5 (70, 82)	81 (74, 87)	9 (4, 19)	2 to 6 (P = 0.0002)	65 to 71 (P < 0.0001)
Normal morphology (%)	18 (12, 24)	18 (12, 26)	12 (9, 16)	-3 to 2 (P = 0.61)	3 to 8.5 (P < 0.0001)
SDI score	1.42 (1.34, 1.5)	1.34 (1.26, 1.42)	1.72 (1.6, 1.86)	-0.13 to -0.07 (P < 0.0001)	-0.48 to -0.34 (P < 0.0001)
Borderline morphology (%)	18 (12 to 20)	16 (11 to 20)	12 (9 to 19)	-1.5 to 2.5 (P = 0.71)	0 to 5.5 (P = 0.06)
Amorphous heads (%)	44 (35 to 53)	40 (32 to 36)	45 (38 to 61)	-8.5 to -0.5 (P = 0.026)	-12 to -4.5 (P < 0.0001)
Pyriform heads (%)	9 (5 to 60)	9 (6 to 19)	10 (5 to 17)	-2 to 2 (P = 0.96)	-2 to 2 (P = 0.89)
Acrosomal damage (%)	24 (18, 30)	17 (14, 24)	30 (23, 40)	-3 to -8.5 (P < 0.0001)	-15 to -7 (P < 0.0001)
Midpiece defects (%)	8 (4, 14)	6 (4, 11)	15 (10, 25)	4 to 0 (P = 0.03)	-10 to -4 (P < 0.0001)
Cytoplasmic droplet (%)	4 (2, 7)	1 (0.5, 3)	7 (3, 12)	-0.5 to -3.5 (P = 0.0007)	-7.5 to -3.5 (P < 0.0001)
Tail defects (%)	4 (2, 8)	4 (2, 7)	15 (10, 22)	-1.5 to 0.5 (P = 0.3)	-12.5 to -6.5 (P < 0.0001)

Table 1: *Descriptive statistics of study variables given as median (25th, 75th centiles). Sperm variables in the double density preparations (control) and the subsequent two sperm fractions resulting from MACS separation were compared utilizing Wilcoxon's signed ranks test. All hypothesis testing were two-tailed; P < 0.05 was considered statistically significant. The 95 % confidence intervals (CI) are given in each case.*

When the non-apoptotic and apoptotic fractions were considered together, there was significant negative correlation between the percentage of sperm with intact MMP and percentage of sperm expressing caspase-3 activation (r = -0.8, 95% *CI*: -0.86 to -0.72, P < 0.0001). The proportion of sperm with activated caspase-3 showed significant negative correlation with the proportions of sperm with normal morphology and significant positive correlation with the SDI scores and percentages of sperm with acrosomal defects, cytoplasmic droplets, midpiece defects and tail defects (Table 2). On the other hand, the proportion of sperm with intact MMP showed significantly positive correlation with the proportions of sperm with normal morphology and significant negative correlation with the SDI scores and percentages of sperm with acrosomal defects, cytoplasmic droplets, midpiece defects and tail defects (Table 2). Percentage sperm motility had significant positive correlation with percentage of sperm with intact MMP and significant negative correlation with percentage of sperm cells with activated caspase-3 (Table 2) and the sperm deformity scores (r = -0.81, confidence interval -0.86 to -0.73, P < 0.0001).

Criteria	Percentage of sperm with activated Caspase-3 Spearman's r (95% CI, P)	Percentage of sperm with intact MMP Spearman's r (95% CI, P)
Motility (%)	-0.77 (-0.84 to -0.67, P < 0.0001)	0.86 (0.81 to 0.91, P < 0.0001)
Sperm with normal morphology (%)	-0.37 (-0.53 to -0.18, P = 0.0002)	0.43 (0.25 to 0.58, P < 0.0001)
SDI score	0.75 (0.65 to 0.83, P < 0.0001)	-0.78 (-0.85 to -0.69, P < 0.0001)
Acrosomal damage (%)	0.5 (0.33 to 0.63, P < 0.0001)	-0.44 (-0.59 to -0.26, P < 0.0001)
Midpiece defects (%)	0.61 (0.47 to 0.72, P < 0.0001)	-0.56 (0.68 to -0.41, P < 0.0001)
Cytoplasmic droplet (%)	0.56 (0.4 to 0.68, P < 0.0001)	-0.58 (-0.69 to -0.43, P < 0.0001)
Tail defects (%)	0.49 (0.32 to 0.63, P < 0.0001)	-0.68 (-0.77 to -0.55, P < 0.0001)

Table 2: *Spearman's rank correlation was utilized to test the relationship between sperm apoptotic markers and sperm morphological subtypes and the SDI scores. The r statistics and the 95% confidence intervals (CI) are given in each case. Two tailed P < 0.05 was considered statistically significant.*

DISCUSSION

This is the first detailed study to the best of our knowledge that charted the shift in sperm morphological profile in sperm preparations before and after the isolation of the non-apoptotic and apoptotic sperm on the basis of PS externalization using annexin V labeling and MACS technique. Compared to the apoptotic sperm subpopulations the non-apoptotic sperm subpopulation had an improved sperm morphology profile as demonstrated by significantly higher proportions of sperm with normal morphology and significantly lower SDI scores and percentages of sperm with acrosomal defects, midpiece defects, cytoplasmic droplet and tail defects. A similar favorable shift was noted in the non-apoptotic sperm morphology when compared to the DGC control preparations, with the exception of the proportions of sperm with normal morphology and percentages of sperm with tail defect which remained similar in these two sperm subpopulations. The study results demonstrated a significant correlation between sperm morphology attributes studied and the expressed apoptotic markers - caspase-3 activation and MMP integrity. Judging by *r* statistics the strongest interdependency was between SDI scores and caspase-3 activation and MMP. Sperm motility also correlated significantly with apoptotic markers and the SDI scores.

The relationship between apoptotic markers in the ejaculated sperm and sperm morphology was previously studied in neat semen (Sakkas *et al*., 1999a; Gandini *et al*., 2000; Sakkas *et al*., 2002; Shen *et al*., 2000; Ricci *et al*., 2002; Siddighi *et al*., 2004; Chen *et al*., 2006). The design of these studies might not have facilitated the accurate distinction between moribund or necrotic sperm and motile sperm expressing apoptotic markers. Other studies correlated sperm morphology in neat semen with apoptosis in selected motile sperm subpopulation obtained after swim-up or double gradient centrifugation techniques (Weng *et al*., 2002; Benchaib *et al*., 2003; Muratori *et al*., 2003; Almeida *et al*., 2005). All these studies applied different criteria for the assessment of sperm morphology including WHO 1992 standards (Muratori *et al*., 2003), WHO 1999 standards (Gandini *et al*., 2000; Shen *et al*., 2000; Benchaib *et al*., 2003; Ricci *et al*., 2002; Almeida *et al*., 2005) and the Tygerberg's strict criteria (Sakkas *et al*., 2002; Weng *et al*., 2002; Siddighi *et al*., 2004; Barroso *et al*., 2006; Chen *et al*, 2006). The design of our study involved the selection of highly motile sperm population through DGC technique followed by MACS technique to facilitate the direct correlation of sperm morphology and the expressed apoptotic markers in the same sperm subpopulation avoiding interference by moribund or necrotic sperm found in semen. The correlation between normal sperm morphology and apoptotic markers observed in our study is in agreement with other reports that used apoptotic makers such as the percentage of sperm with PS externalization (Shen *et al*., 2000; Ricci *et al*., 2002), caspase-3 activation (Weng *et al*., 2002; Paasch *et al*., 2003; Almeida *et al*., 2005; Said *et al*., 2005b; Barroso *et al*., 2006), MMP integrity (Said *et al*., 2005b; Barroso *et al*., 2006) chromatin

fragmentation (Sakkas *et al*., 2002; Benchaib *et al*., 2003; Siddighi *et al*., 2004; Chen *et al*., 2006), membrane bound death receptor Fas and p53 (Sakkas *et al*., 1999a; Sakkas *et al*., 2002). Unlike our study some reports found a correlation between normal morphology and the studied apoptotic markers detectable under certain conditions and not others. For instance normal morphology in semen was found to correlate negatively with caspase-3 activation measured in swim-up preparation but not in semen (Almeida *et al*., 2005). In another study normal sperm morphology assessed in the swim-up preparations correlated negatively with chromatin fragmentation only in teratozoospermic semen samples but not in normospermic ones as determined by the WHO 1992 criteria (Muratori *et al*., 2003). Finally, normal morphology applying the strict criteria in sperm preparations was reported to correlate inversely with caspase-3 activation, but had no relationship with MMP integrity (Said *et al*., 2005b). The same study found no relationship between expressed apoptotic markers and sperm morphology applying WHO criteria (Said *et al*., 2005b).

The significant relationship between midpiece defects and caspase-3 activation observed in our study are in agreement with result of another study that utilized chromatin fragmentation as an apoptotic marker (Weng *et al*., 2002,). In this study the correlation was found only when the low motility fractions of sperm preparations in infertile men were considered. This relationship was absent in the high motility fractions of the same patients or in the high and low motility fractions of donors' sperm. Unlike our study results, two other studies failed to find a relationship between midpiece defects and apoptotic markers (Ricci *et al*., 2002; Chen *et al*., 2006). However, it has been demonstrated that in caspase-3 activation in ejaculated sperm was confined to the post acrosomal part in mature sperm (Paasch *et al*., 2004c) and to the midpiece where mitochondria and residual cytoplasm reside (Weng *et al*., 2002). Structurally, this is in agreement with our study results of significant interdependence between the percentages of sperm with cytoplasmic droplet and midpiece defects on one hand and caspase activation and MMP integrity on the other. Our group has previously shown that sperm cytoplasmic droplet deformity is associated with excessive reactive oxygen species (ROS) production (Aziz *et al*., 2004; Said *et al*., 2005a). In view of their positive correlation with apoptosis and excessive ROS production, these immature spermatozoa with excessive cytoplasmic remnants could be responsible for male subfertility when present in abundance.

It was noted that the significant increase in the proportion of sperm with tail defects in the apoptotic sub-population was not matched by a decrease in this deformity in the non-apoptotic subpopulations compared to control. In other words sperm selection may not offer an explanation to the observed significant increase in tail defects in the apoptotic subpopulation. This may suggest that subjecting apoptotic sperm labeled with paramagnetic microbeads to magnetic forces of 1.5 Tesla may have contributed to the observed increase in tail defects. This

may also suggest that the apoptotic fraction is more susceptible to the mechanical and magnetic forces within the column. However, the positive correlation between tail defects and nuclear fragmentation as an apoptotic marker was reported previously in a study where MACS technique was not utilized (Chen *et al*., 2006). This apoptotic sperm subpopulation would not be used under any circumstances for any assisted reproductive technique should MACS technique becomes incorporated in sperm preparations for therapeutic purposes.

The apoptotic markers in our study were found to correlate with sperm morphological features that are not subject to change after release from the seminiferous tubules. Unlike other animals, sperm epididymal passage in human is not associated with any morphological remodeling detectable under the light microscope (Bedford, 1994). Even the cytoplasmic extrusion is completed before the commencement of epididymal transport (Huszar *et al*., 1998). As a result persistent active apoptotic signals in ejaculated sperm are likely to have developed during spermatogenesis in defective spermatozoa to mark them for removal well before entering the epididymis. The presence of these sperm cells in ejaculate becomes a reflection of abortive apoptosis. This argument is further supported by the evidence that spermatozoa that are healthy after ejaculation are incapable of becoming apoptotic spontaneously (Lachaud *et al*., 2004; Oosterhuis *et al*., 2004) and their demise occur by necrosis rather than apoptosis (Lachaud *et al*., 2004). This was elegantly demonstrated by showing that after selection of healthy spermatozoa by swim-up, density gradient centrifugation and washing, no spermatozoa become apoptotic over time under standard incubation conditions.

In conclusion the non-apoptotic sperm subpopulation has morphologically superior quality sperm compared to apoptotic sperm as reflected by significantly lower SDI scores. The poor sperm morphology profiles seen in the apoptotic sperm fractions may be partly due to the inclusion of sperm with acrosomal damage, midpiece defects and cytoplasmic droplet. It was observed that there was excessive sperm tail damage in the annexin-positive (apoptotic) subpopulation. This may suggest that the apoptotic fraction is more susceptible to the mechanical and magnetic forces within the column. This study demonstrated how the multiple entry technique and SDI scoring system are more informative research tools compared to the routine assessment of the percentage of normal morphology. The study results may support abortive apoptosis where the apoptotic mechanism of spermatozoa is already triggered before ejaculation.

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