

# Association of sperm apoptosis and DNA ploidy with sperm chromatin quality in human spermatozoa

Reda Z. Mahfouz, M.D.,<sup>a</sup> Rakesh K. Sharma, Ph.D.,<sup>a</sup> Tamer M. Said, M.D.,<sup>b</sup> Juris Erenpreiss, M.D.,<sup>c</sup> and Ashok Agarwal, Ph.D., H.C.L.D.<sup>a</sup>

<sup>a</sup> Reproductive Research Center, Glickman Urological and Kidney Institute and Department of Obstetrics and Gynecology, Cleveland Clinic, Cleveland, Ohio; <sup>b</sup> Toronto Institute for Reproductive Medicine, Toronto, Ontario, Canada; and <sup>c</sup> Andrology Laboratory, Riga Stradins University, Riga, Latvia

**Objective:** To examine the relationship among sperm apoptosis, sperm chromatin status, and DNA ploidy in different sperm fractions.

**Design:** Prospective study.

**Setting:** Reproductive research center in a tertiary care hospital.

**Intervention(s):** Sperm prepared by density gradient were evaluated for sperm count, motility, apoptosis, and sperm chromatin assessment.

**Main Outcome Measure(s):** Sperm count, sperm motility, toluidine blue (TB) results, DNA fragmentation index (%DFI), high DNA stainability, DNA cytometry, and early and late apoptosis.

**Result(s):** Sperm motility was related to late apoptotic and subhaploid apoptotic sperm ( $r = -0.56$  and  $-0.53$ , respectively). The sperm %DFI showed significant correlation with late apoptotic and subhaploid sperm ( $r = 0.62$  and  $0.68$ ). TB-stained sperm were significantly correlated with late apoptotic sperm ( $r = 0.51$ ). Significantly higher proportions of haploid sperm and light blue TB-stained sperm were seen in mature compared with immature fractions.

**Conclusion(s):** Even in semen samples with low %DFI, semen processing results in a lower incidence of nuclear immaturity and subhaploidy, but the incidence of late apoptotic sperm remains unchanged. Therefore, simultaneous evaluation of apoptosis and sperm chromatin status is important for processing sperm in assisted reproductive procedures. (Fertil Steril® 2009;91:1110–8. ©2009 by American Society for Reproductive Medicine.)

**Key Words:** Sperm DNA integrity, sperm apoptosis, sperm fractions, sperm DNA cytometry

Sperm preparation techniques are a vital component of assisted reproductive technologies (ART). Density-gradient centrifugation is commonly used for sperm preparation, which leads to an increased conception rate (1). This technique separates superior motile spermatozoa with normal morphology from the total sperm population, leaving behind immature, morphologically abnormal, and senescent spermatozoa with damaged DNA (2–6).

In mature spermatozoa, the compact chromatin results from replacement of histones by arginine- and cysteine-rich protamines during spermatogenesis. Additional conformational changes in sperm chromatin structure occur during maturation by formation of disulfide bridges between cysteine residues providing highly compacted chromatin. Sperm DNA integrity is critical for accurate transmission of paternal genetic information (7, 8). Although fertilization can occur

with damaged DNA spermatozoa, abnormal sperm chromatin structure has been negatively correlated with the fertility potential of the spermatozoa and subsequent embryo development (9).

Sperm chromatin status can be evaluated by several methods; the more widely reported are the sperm chromatin structure assay (SCSA) and the terminal transferase dUTP nick-end labeling (TUNEL) assay. Numerous reports indicate that SCSA correlates with clinical outcomes (9–12). In this assay, the DNA fragmentation index (%DFI) represents the proportion of sperm with abnormal DNA integrity, while high DNA stainability (%HDS) is suggested to indicate sperm chromatin immaturity (11). The toluidine blue (TB) image cytometry test can also be used as a relatively inexpensive, feasible, alternative test for sperm chromatin conformation assessment. In this assay, spermatozoa with intact and mature chromatin when stained with TB will stain light blue (%LB), while sperm with damaged and/or immature chromatin will stain dark violet (%DV) with two intermediate reaction colors, light violet (%LV) and blue (%B), according to the degree of sperm chromatin maturity and integrity (12).

Although the mature spermatozoa are transcriptionally inactive and the mature apoptosis characteristics seen in somatic cells are absent, recent studies have demonstrated

Received November 14, 2007; revised and accepted January 11, 2008; published online May 7, 2008.

R.Z.M. has nothing to disclose. R.K.S. has nothing to disclose. T.M.S. has nothing to disclose. J.E. has nothing to disclose. A.A. has nothing to disclose.

Reprint requests: Ashok Agarwal, Ph.D., H.C.L.D., Professor and Director, Reproductive Research Center, 9500 Euclid Avenue, Desk A19.1, Cleveland Clinic, Cleveland OH 44195 (FAX: 216-445-6049; E-mail: Agarwaa@ccf.org).

apoptosis-like conditions (or abortive apoptosis), including single-stranded DNA damage, presence of activated caspases, and externalization of the phosphatidyl serine on the sperm plasma membrane in ejaculated human spermatozoa (13–21). In addition, we have reported the separation of non-apoptotic spermatozoa from apoptotic spermatozoa using annexin V–labeled microbeads by the magnetic-activated cell-sorting technique (17, 22, 23). All these studies indicate that ejaculated spermatozoa may exhibit some form of apoptotic features.

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated to the outer leaflet of the plasma membrane, exposing the PS to the cellular environment (24). Changes in plasma membrane composition and function can be detected by the appearance of PS on the plasma membrane, which reacts with annexin V–fluorochrome conjugates. When annexin V is combined with propidium iodide (PI) staining, this method can distinguish among the viable, necrotic, early, and late apoptotic cells (25–27). During capacitation and acrosome reaction of spermatozoa, PS does not become exposed on the outer surface of the viable cells. Only in a subpopulation of PI-positive sperm cells does PS become accessible upon capacitation (28). Caspase activation also has been reported only in an annexin V–positive sperm fraction after cryopreservation (29).

Decreased elimination and subsequent accumulation of the DNA-damaged spermatozoa results in poor-quality sperm. This is due largely to inefficient apoptotic machinery and poor DNA integrity and abnormal chromatin packaging (15, 30). In the embryonic genome, any modifications at the level of the DNA nucleotides and/or DNA strand breaks that originate from the paternal genome that are beyond the oocyte repair capacity after fertilization are not compatible with normal embryo and fetal development (31).

The association of the indices of sperm chromatin structure tests such as the SCSA and the TB test and the possible ramifications of apoptosis (annexin V–PI assay) in ejaculated spermatozoa are unclear. Characterization of this relationship may be helpful in evaluating many of the diagnostic and therapeutic intervention procedures, ranging from semen collection and preparation to analysis of sperm for use in any assisted reproduction program (9, 32).

Our aim was to examine the relationship of the sperm chromatin status with apoptosis markers in different sperm fractions after preparation of sperm by density-gradient separation and to analyze immature and mature sperm for chromatin packaging, distribution of subhaploid, haploid, and apoptotic sperm using TB staining, SCSA, and sperm DNA cytometry.

## MATERIALS AND METHODS

### Sample Collection and Preparation

This study was approved by the Cleveland Clinic Institutional Review Board. Semen samples were collected from 18 do-

nors according to World Health Organization guidelines (33). All samples were collected by masturbation after sexual abstinence for at least 48 hours. After complete liquefaction, routine semen analysis was done for all samples using MicroCell counting chambers (Conception Technologies, San Diego) under a phase-contrast microscope. Samples with low volume and high viscosity and those with leukocytospermia (white blood cell count  $>1 \times 10^6$  /mL) were excluded.

Each sample was divided into two aliquots; 300  $\mu$ L of unprocessed (neat) semen sample was removed, and the remaining was subjected to double density-gradient centrifugation (40%: 80%; PureCeption, SAGE BioPHARMA, Bedminster, NJ). After centrifugation at 300 *g* for 20 minutes, the resulting interfaces between the 40% and 80% layers comprising immature spermatozoa were aspirated and resuspended in human tubal fluid media (HTF; Irvine Scientific, Santa Ana, CA). The resulting pellet in the 80% phase consisting largely of highly motile, mature spermatozoa was resuspended in HTF media. Neat as well as mature and immature fractions were washed with phosphate-buffered saline (PBS). All fractions were evaluated for sperm chromatin status and apoptosis.

### Toluidine Blue (TB) Test

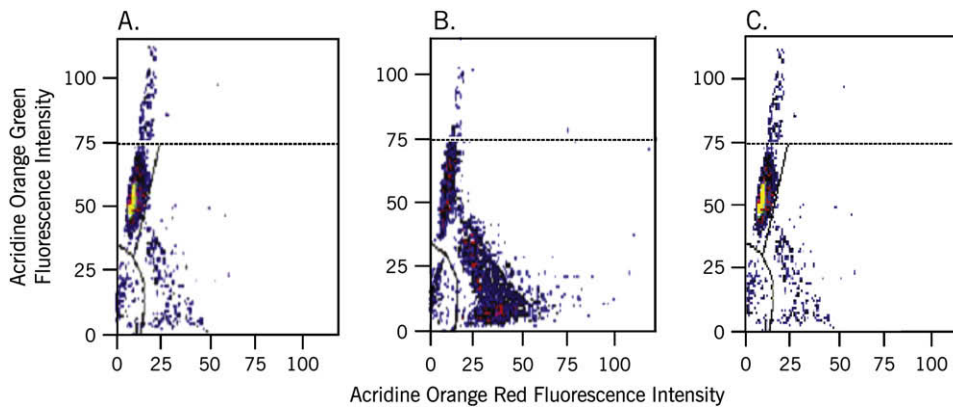
The TB test was performed as described elsewhere (12). Briefly, 200  $\mu$ L of sperm suspension from each fraction was washed with PBS at 250 *g* for 10 minutes and resuspended in 5% bovine serum albumin. Thin smears were prepared on precleaned slides and air-dried for 30–60 minutes. These were fixed with freshly prepared fixative (96% ethanol: acetone; 1:1) at 4°C and air-dried. Hydrolysis was performed in 0.1 M HCl at 4°C for 5 minutes, followed by three washes of distilled water, each wash for 2 minutes. Smears were stained in TB (0.05% in 50% McIlvain's citrate phosphate buffer at pH 3.5) for 5 minutes. Slides were rinsed briefly in distilled water and dehydrated in tertiary butanol at 37°C for 2  $\times$  3 minutes followed by xylene at room temperature (2  $\times$  3 minutes) and mounted with distrene plasticizer xylene. The stained slides were scored by light microscopy by counting at least 200 cells. The TB test was categorized on the basis of the chromatin staining as normal (%LB), abnormal (%DV), and two intermediate chromatin conformations (%B and %V). All slides were coded and scored in a blinded fashion by one experienced observer (JE) only. The assay is reproducible with acceptable intraobserver variability as demonstrated by our earlier studies (12, 34, 35).

### Sperm Chromatin Structure Assay

Aliquots of 250  $\mu$ L of neat, mature, and immature sperm fractions were treated with an acid detergent solution (pH 1.2) containing 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 mol/L HCl for 30 seconds and stained with 6  $\mu$ g/L purified acridine orange (Polysciences Inc., Warrington, PA) in a phosphate-citrate buffer, pH 6.0, to be subjected to analysis

## FIGURE 1

Flowcytograms showing examples of data generated by SCSA. Example of: (A) Normal DNA integrity (normal %DFI); (B) poor sperm DNA integrity (high %DFI); and (C) Normal sperm chromatin maturity (normal HDS).



Mahfouz. Sperm chromatin status and apoptosis. *Fertil Steril* 2009.

by flow cytometry (FCM). Sperm chromatin damage was quantified by the FCM measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red versus green fluorescence intensity cytogram patterns (Fig. 1). The %DFI is the ratio of the percentage of sperm showing an increased red fluorescence/total fluorescence intensity (red + green). In addition, we included the fraction of cells with high DNA stainable (%HDS) cells representing immature spermatozoa with incomplete chromatin condensation (11, 36).

### Annexin V–Propidium Iodide Assay

To perform this assay the Annexin-V FITC Apoptosis Detection Kit was used (Pharmingen, San Diego). Aliquots of 100  $\mu\text{L}$  of neat, mature, and immature sperm fractions were resuspended in 400  $\mu\text{L}$  cold reaction buffer (HEPES; N2-hydroxyethyl piperazine-N'2-ethanesulfonic acid) containing 2.5 mM  $\text{CaCl}_2$ . Sperm cells were labeled with 10  $\mu\text{L}$  each of annexin-V/fluorescein isothiocyanate solution and PI for detecting apoptotic and necrotic sperm. Samples were incubated for 15 minutes at room temperature in the dark. Cells were washed with 1 mL PBS, centrifuged, and resuspended in 300  $\mu\text{L}$  of reaction buffer. The FCM analysis was done to quantitatively determine the percentage of early and late apoptotic, necrotic, and viable cells (37–39).

Although the term “cell ploidy” is usually used in cytogenetics to refer to the chromosomal status/number, various investigators have used this term to indicate the DNA ploidy and cell cycle analysis by calculating the DNA index (ratio of normal, G0-G1; ratio of abnormal, G0-G1). Therefore, by using DNA cytometry, we can differentiate aneuploidy, triploidy, or tetraploidy from diploidy (40, 41) and even dif-

ferentiate various maturation stages of spermatozoa (42–45). Essentially, these are the haploid sperm with different levels of differentiation (representing the immature haploid and mature condensed spermatozoa) and loss of DNA content (representing the subhaploid sperm). DNA cytometry analysis was done on PI-stained sperm and reanalyzed for PI fluorescence intensity histograms. The PI-negative aliquot (control) did not contain any PI stain. This aliquot was run simultaneously along with each specimen. The PI-negative sperm population was gated on the basis of the PI-negative control. The PI-negative sperm population therefore did not show PI red (FL-2) fluorescence. The entire sperm population was classified based on the presence or absence of PI fluorescence into positive PI and negative PI populations. The PI-positive population was further subclassified based upon the PI fluorescence intensity into subhaploid, haploid, and immature sperm subpopulations. PI intercalates between bases in sperm DNA strands and displays red fluorescence. Apoptotic sperm will lose DNA, and thus PI will produce lower fluorescence intensity. FCM analysis of cells stained with PI can distinguish different sperm cell subpopulations according to their fluorescence. PI red fluorescence intensity (which reflects DNA content) recorded on a logarithmic scale allows easy differentiation among the intensities of tetraploid, diploid, and haploid round spermatid and spermatozoa containing condensed chromatin (42, 46). DNA fragmentation associated with apoptotic stages leads to subdiploid cells in somatic cells or subhaploid cells in germinal cells such as spermatozoa; these are called apoptotic bodies (42, 47, 48).

### FCM Analysis

All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (Becton Dickinson, San Jose, CA). About 10,000 spermatozoa were examined

for each assay at a flow rate of <100 cells/second. 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) was measured in the FL-2 channel. Gating was done to exclude debris and aggregates using 90° and forward-angle light scatter. Both percentage of positive cells and the mean fluorescence were calculated on a 1023-channel scale using the flow cytometer software FlowJo version 6.2.4 (FlowJo, LLC, Ashland, OR).

### Statistical Analysis

The data were analyzed for normality. One-way analysis of variance was used for multiple group comparison, and the Bonferroni test was used post hoc for comparison of two groups. Results are represented as mean ± SEM throughout the study. Bivariate Pearson's correlations were also tested.  $P \leq .05$  was considered statistically significant. All analyses were done using SPSS version 11 for windows (SPSS Inc., Chicago).

### RESULTS

The mean ejaculate volume (mL) was  $3.0 \pm 1.7$ , the mean sperm concentration ( $\times 10^6/\text{mL}$ ) was  $57.9 \pm 33.9$ , and the mean sperm motility (%) was  $59.5 \pm 18.5$ . Motility was significantly improved in all the samples after density-gradient separation. Descriptive data of neat, mature, and immature sperm for various sperm chromatin and sperm apoptosis parameters are shown in Table 1.

### Relationship of Sperm Ploidy and Apoptosis with Routine Semen Parameters and Sperm Chromatin Integrity

In Figure 2A, the sperm concentration was positively correlated with haploid sperm ( $r = 0.628$ ,  $P = .005$ ). In Figure 2B, a significant negative correlation was seen between sperm motility and late apoptotic sperm ( $r = -0.56$ ,  $P = 0.01$ ). Sperm motility was negatively related to percentage of subhaploid apoptotic sperm ( $r = -0.53$ ,  $P = .02$ ; Fig. 2E). The %DFI was positively correlated with the percentage of subhaploid apoptotic sperm ( $r = 0.717$ ;  $P < .001$ ; Fig. 2C). The %DFI was correlated with PI-positive (late apoptotic) sperm ( $r = 0.65$ ;  $P < .001$ ; Fig. 2D). Similarly, a significant positive correlation was seen between TB-stained sperm and late apoptotic sperm ( $r = 0.51$ ;  $P = .03$ ). The TB-DV stained cells showed a significant positive relationship with subhaploid sperm ( $r = 0.2$ ,  $P = .03$ ).

### Sperm Apoptosis and Chromatin Structure Characteristics

A significantly lower %HDS was seen in the mature sperm compared with the neat or immature fraction ( $P < .004$  and  $< .001$ , respectively). A higher but nonsignificant level of %DFI was seen in the immature sperm fraction compared with the neat and mature fractions (Fig. 1A–1C).

Using the TB staining test, a higher incidence of spermatozoa stained light blue (normal chromatin conformation) was seen in the mature fraction compared with the immature fraction samples ( $P = .01$ ). The mature sperm fraction sample showed a lower incidence of spermatozoa with dark violet

**TABLE 1**

#### Sperm chromatin structure and apoptosis stages in different sperm fractions.

Sperm chromatin/apoptosis assays: parameter	Neat sample (n = 18), mean ± SE	Mature fraction, (n = 18), mean ± SE	Immature fraction (n = 18), mean ± SE
TB (%):			
Light blue	58.6 ± 4.4	68.7 ± 3.7 <sup>a</sup>	51.4 ± 4.2
Blue	0.8 ± 0.25	0.7 ± 0.2	0.6 ± 0.2
Light violet	16.6 ± 1.4	15.7 ± 1.6	16.3 ± 1.4
Dark violet	24.4 ± 3.4	16.9 ± 2.7 <sup>a</sup>	31.9 ± 3.8
SCSA (%):			
DFI	17.6 ± 3.5	17.5 ± 4.3	18.7 ± 3.4
HDS	6.4 ± 0.6	3.1 ± 0.5 <sup>a,b</sup>	7.5 ± 1.0
Annexin V-PI (%):			
Early apoptotic sperm	15.4 ± 3.7	10.8 ± 3.5	15.9 ± 4.2
Necrotic sperm	4.9 ± 1.6	2.9 ± 1.7	4.6 ± 1.8
Late apoptotic sperm	32.0 ± 2.8	30.2 ± 3.2	25.5 ± 1.7
Sperm DNA cytometry (%):			
Haploid sperm	17.5 ± 2.0	18.9 ± 3.1 <sup>a</sup>	12.1 ± 1.9
Immature sperm	4.7 ± 0.7	4.6 ± 1.5	6.7 ± 2.2
Subhaploid sperm	15.9 ± 1.4	10.4 ± 0.9 <sup>b</sup>	13.4 ± 1.7

Note:  $P < .05$  was statistically significant between each pairs.

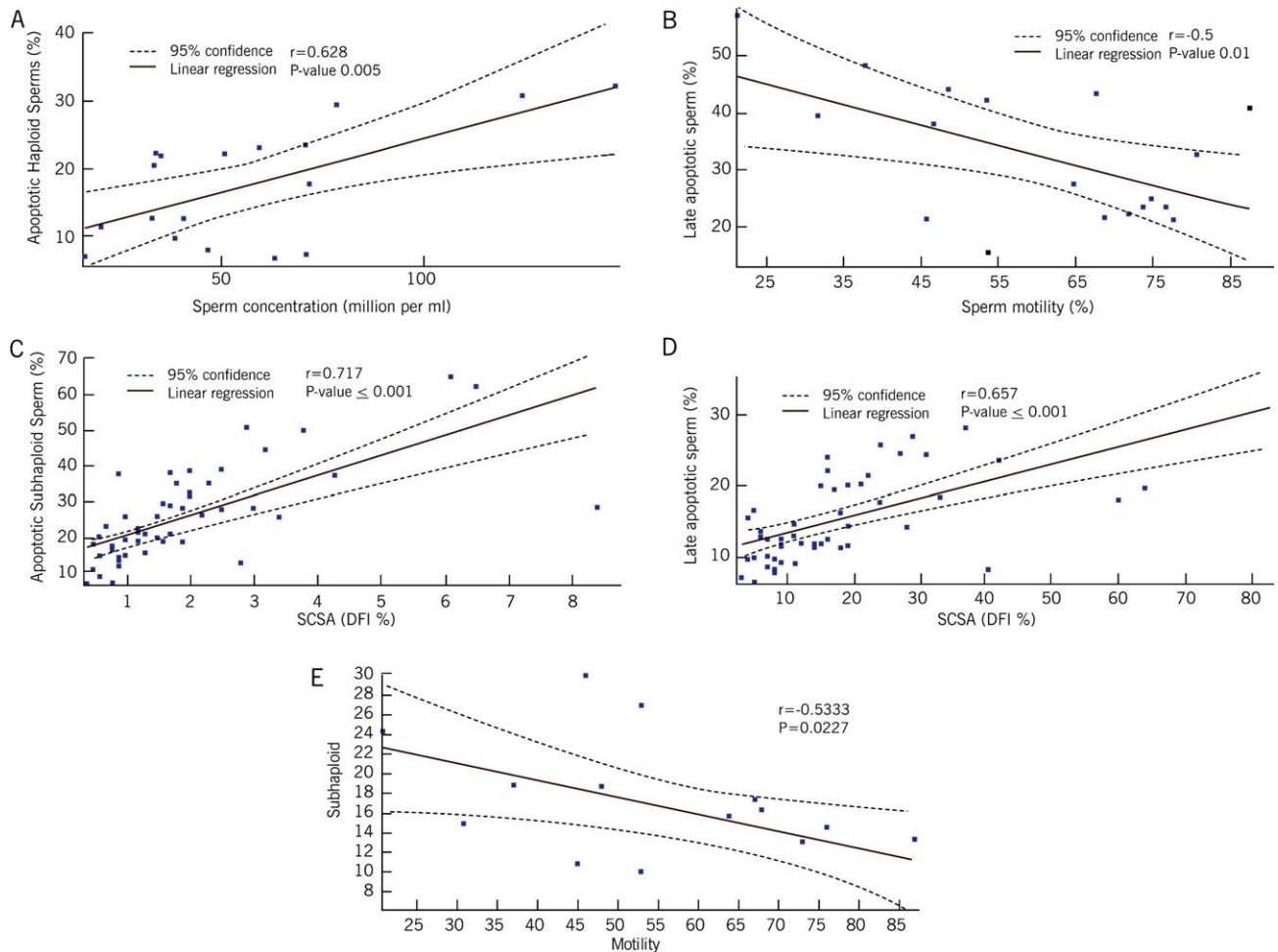
<sup>a</sup> Significant difference between mature and immature fractions.

<sup>b</sup> Significant difference between mature and neat sample.

Mahfouz. Sperm chromatin status and apoptosis. *Fertil Steril* 2009.

**FIGURE 2**

Sperm parameters and their correlations. (A) Sperm concentration and percentage of apoptotic haploid sperm%; (B) sperm motility and percentage of late apoptotic sperm%; (C) SCSA-%DFI and subhaploid % apoptotic sperm; (D) late apoptotic sperm and SCSA-%DFI; and (E) sperm motility and subhaploid %.



Mahfouz. Sperm chromatin status and apoptosis. *Fertil Steril* 2009.

stain (abnormal chromatin conformation) compared with the immature fraction sample ( $P=.002$ ).

In the mature fraction, low levels of early apoptotic and necrotic sperm were seen that were comparable with the neat and immature fractions. Low levels of late apoptotic sperm were comparable in the immature fraction with both the neat and mature fractions (Fig. 3). This figure shows pseudocolor FCM histograms of the neat, mature, and immature fractions with the percentage of the viable, early apoptotic, late apoptotic, and necrotic spermatozoa in each fraction.

A higher percentage of haploid sperm were seen in the mature fraction compared with the immature fraction ( $P=.04$ ). The percentage of subhaploid sperm in the mature fraction was significantly lower compared with the neat fraction ( $P=.007$ ; Fig. 4). This figure shows overlay marker FCM histograms to compare viable, apoptotic sperm types in different sperm fractions.

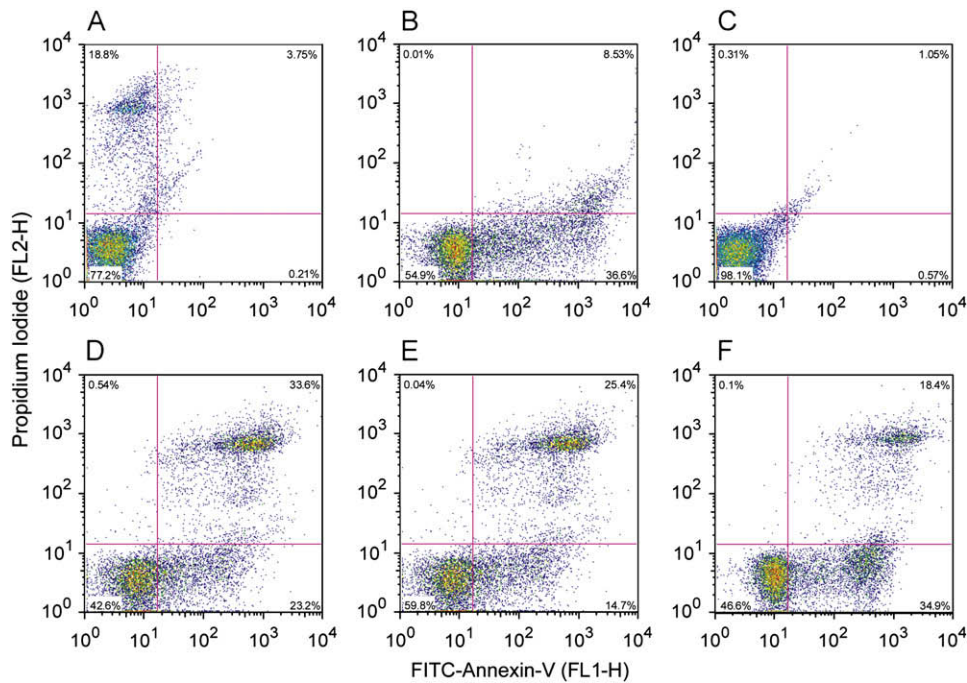
## DISCUSSION

Sperm chromatin status and sperm DNA integrity are critical factors that may affect individual fertility potential. Infertile men are reported to have a higher incidence of sperm with chromatin defects and DNA breaks than are found in fertile controls (49). Some investigators suggest that resolving the nature of sperm DNA lesions present in the spermatozoa of infertile men will be an important step toward uncovering the etiology of this damage and developing strategies for its clinical management (50). Others reports indicate that sperm damage in infertile men may be due to abortive apoptosis in the germ cells (43) and oxidative stress (51).

ART is essential for infertility management of either male or female factors or both. Sperm processing procedures used for ART are aimed at improving sperm quality by increasing the number of progressive motile and morphologically normal sperm cells (52). Sperm preparation by density-gradient

**FIGURE 3**

Dot plot histograms showing apoptotic and necrotic changes in (A) PI control, (B) annexin V control, (C) unstained control, (D) neat sperm, (E) mature sperm, and (F) immature sperm. The lower left quadrant shows viable sperm (%). The lower right quadrant shows early apoptotic sperm (%). The upper left quadrant shows necrotic sperm (%). The upper right quadrant shows late apoptotic sperm (%).



Mahfouz. Sperm chromatin status and apoptosis. *Fertil Steril* 2009.

centrifugation allows the selection of good-quality mature and actively motile spermatozoa, depending on their size, shape, and density (2, 5).

The mature sperm fraction prepared by density-gradient centrifugation has improved longevity and sperm DNA integrity (53), although a recent report recommended immediate semen preparation before use for ART to overcome the negative effect of extended (6–24 hour) incubation (54). The aim of this study was to evaluate the association of sperm chromatin integrity and maturity with sperm apoptotic stages and the apoptotic subpopulation in unprocessed as well as mature and immature sperm fractions processed by density-gradient separation. To achieve our objective, SCSA and TB staining were employed, and their relationship with the stages of sperm apoptosis was assessed by annexin V–PI assay.

Our results reveal a definite correlation between the %DFI measured by SCSA with late apoptotic and necrotic and the percent of subhaploid sperm. From the correlative results between %DFI in the SCSA and two direct tests for sperm DNA fragmentation (the TUNEL and COMET assays), it has been suggested that %DFI reflects sperm DNA fragmentation. However, SCSA is an indirect probe for sperm DNA integrity, and it is still not clear exactly what it tests. Therefore, our results also help in understanding the biological mechanisms of this assay.

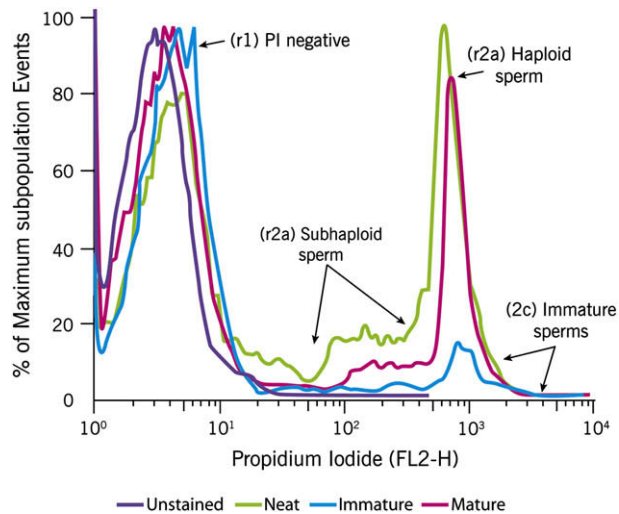
Our results (correlation between %DFI and apoptotic markers) are in agreement with the findings of Weng et al. (55), who measured sperm DNA integrity by the TUNEL assay. Our findings are also in agreement with Chen et al., who reported that percentage of sperm apoptosis was associated with several measures of semen quality, including sperm motility and morphology (56). In contrast, Stronati et al. reported no correlation between sperm DNA damage as measured by TUNEL assay and apoptotic (Fas) markers after exposure to organochlorine pollutants. However, the apoptotic markers measured by Stronati et al. were different from the apoptotic markers examined by us. They used immunofluorescence for detecting apoptotic (Fas) and anti-apoptotic (Bcl-xL) markers (57). In our study, we examined both early and late apoptosis and apoptotic sperm by DNA cytometry to identify early membrane and sperm DNA damage.

Our findings of a positive correlation of the percentage of subhaploid sperm with both %DFI and %DV sperm (abnormal chromatin conformation) with TB staining also agree with the findings of Kajstura et al. (58), who reported that apoptotic cells can be identified using DNA ploidy frequency histograms as “subnormal” cell subpopulations with low DNA content due to apoptotic DNA fragmentation.

Recent reports demonstrate the occurrence of aneuploidy during sperm maturation. This may lead to sperm DNA

**FIGURE 4**

Histograms showing fluorescent intensity of PI staining on the X-axis with cells count on the Y-axis. Region 1 (r1) shows PI-negative sperm (equal to the unstained negative control). Region 2 (r2) shows PI-positive sperm. The PI-positive sperm were further divided into subpopulations according to their fluorescent intensity. Region 2a (r2a) shows subhaploid fragmented sperm. Region 2b (r2b) shows haploid condensed sperm. Region 2c (r2c) shows haploid noncondensed spermatid.



Mahfouz. Sperm chromatin status and apoptosis. *Fertil Steril* 2009.

fragmentation as part of a genomic screening mechanism developed to genetically inactivate sperm with a defective genomic makeup. The investigators suggest that the burden of genomic damage in sperm cannot be inferred from semen quality and that a small fraction of men are at increased risk for transmitting multiple genetic and chromosomal defects (59–61). These reports confirm the disequilibria of sperm chromosome and the degree of DNA fragmentation that provide a clue for the sperm ploidy importance.

The biological significance of the two intermediate groups in relation to normal sperm chromatin structure by the TB test identified by sperm staining violet and blue is not clear (35). Interestingly, in our study, spermatozoa that stained blue, indicating chromatin structure disorganization, correlated with late apoptotic spermatozoa, confirming the earlier suggestion that these sperm may represent a population with deeply disorganized chromatin structure (12).

We report that mature fractions separated by density gradient demonstrate low but not significant levels of early apoptotic sperm and necrotic sperm compared with both neat and immature fractions. This may be due to the small sample size in our study. Other investigators have also demonstrated that density-gradient centrifugation is more efficient than

simple washing in recovery of actively motile, nonapoptotic spermatozoa (62). Furthermore the presence of apoptotic sperm cells in both fractions is in accordance with the reports by Weng et al. (55). However, Paasch et al. recommended selection of annexin V–negative spermatozoa from mature sperm fraction prepared after density gradient for clinical use. They found this selection to be effective in removing spermatozoa with activated caspase from the mature sperm fraction. These investigators reported caspase activation before and after artificial capacitation (29).

Our study findings show some interesting correlations and may help explain the biological meaning of one of the TB-stained intermediate cell groups: blue stained spermatozoa, suggesting disorganized sperm chromatin that may be related to late apoptosis. The %DFI was positively related to late apoptosis and subhaploid apoptotic spermatozoa. The fact that our results on the %DFI were similar in the neat semen fraction and in the fraction after sperm preparation by density gradient may be explained by the fact that we used semen samples from normal, healthy men. The mean %DFI in all these semen samples was well below (<18%) the cutoff established by the SCSA, that is, 27%–30%. The density gradient can separate only the spermatozoa with normal chromatin maturity and good motility; it cannot differentiate spermatozoa with DNA or membrane damage (annexin V–PI assay). This finding may not be critical in normal healthy men, but it is important in semen samples from infertile men. We recommend a large-scale study to include infertile patients with high spermatozoa damage.

We report in this study some unique observations. [1] We have shown for the first time the usefulness of the TB assay, SCSA, annexin V assay, and sperm DNA cytometry in identifying sperm chromatin status in sperm prepared after density gradient in men with normal sperm parameters. [2] These differences may be more relevant in sperm prepared from infertile men. [3] Both TB (sperm staining blue) and %DFI may be associated with late apoptotic sperm. [4] Sperm prepared by density gradient may still have some degree of sperm DNA fragmentation.

Correct interpretations of the sperm chromatin integrity and immaturity results are important and may be related to different stages of apoptosis. These associations may provide a better understanding of the various sperm chromatin assays that are available and may explain the repeated assisted reproduction failures despite the use of sperm with normal chromatin structure. Our results may be of value in the management of unexplained male factor infertility and may provide technical interpretation for the laboratory testing of spermatozoa.

In conclusion, our findings suggest that even in semen samples with a low %DFI, the incidence of late apoptotic sperm remains unchanged, although semen processing results in a lower incidence of nuclear immaturity and subhaploidy. Therefore, simultaneous evaluation of apoptosis and sperm chromatin status is important for processing sperm in assisted reproductive procedures.

## REFERENCES

- Allamaneni SS, Agarwal A, Rama S, Ranganathan P, Sharma RK. Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatozoa. *Asian J Androl* 2005;7:86–92.
- Morrell JM. Update on semen technologies for animal breeding. *Reprod Domest Anim* 2006;41:63–7.
- Canto CL, Segurado AC, Pannuti C, Cedenho A, Srougi M, Spaine D, et al. Detection of HIV and HCV RNA in semen from Brazilian coinfecting men using multiplex PCR before and after semen washing. *Rev Inst Med Trop Sao Paulo* 2006;48:201–6.
- Mousset-Simeon N, Rives N, Masse L, Chevallier F, Mace B. Comparison of six density gradient media for selection of cryopreserved donor spermatozoa. *J Androl* 2004;25:881–4.
- Henkel RR, Schill WB. Sperm preparation for ART. *Reprod Biol Endocrinol* 2003;1:108.
- Marchesi DE, Feng HL. Sperm DNA integrity from sperm to egg. *J Androl* 2007;28:481–9.
- Dias GM, Retamal CA, Tobella L, Arnholdt AC, Lopez ML. Nuclear status of immature and mature stallion spermatozoa. *Theriogenology* 2006;66:354–65.
- Perdichizzi A, Nicoletti F, La Vignera S, Barone N, D'Agata R, Vicari E, et al. Effects of tumour necrosis factor- $\alpha$  on human sperm motility and apoptosis. *J Clin Immunol* 2007;27:152–62.
- Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod* 2007;22:174–9.
- Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 2003;9:331–45.
- Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. *Reprod Biomed Online* 2006;12:466–72.
- Erenpreiss J, Jepson K, Giwercman A, Tsarev I, Erenpreisa J, Spano M. Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow cytometric sperm chromatin structure and TUNEL assays. *Hum Reprod* 2004;19:2277–82.
- Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod* 2002;66:1061–7.
- Sakkas D, Leppens-Luisier G, Lucas H, Chardonnens D, Campana A, Franken DR, et al. Localization of tyrosine phosphorylated proteins in human sperm and relation to capacitation and zona pellucida binding. *Biol Reprod* 2003;68:1463–9.
- Angelopoulou R, Plastira K, Msaouel P. Spermatozoal sensitive biomarkers to defective protaminosis and fragmented DNA. *Reprod Biol Endocrinol* 2007;5:36.
- Aziz N, Said T, Paasch U, Agarwal A. The relationship between human sperm apoptosis, morphology and the sperm deformity index. *Hum Reprod* 2007;22:1413–9.
- Grunewald S, Baumann T, Paasch U, Glander HJ. Capacitation and acrosome reaction in nonapoptotic human spermatozoa. *Ann NY Acad Sci* 2006;1090:138–46.
- Grunewald S, Paasch U, Said TM, Sharma RK, Glander HJ, Agarwal A. Caspase activation in human spermatozoa in response to physiological and pathological stimuli. *Fertil Steril* 2005;83(Suppl 1):1106–12.
- Grunewald S, Said TM, Paasch U, Glander HJ, Agarwal A. Relationship between sperm apoptosis signalling and oocyte penetration capacity. *Int J Androl*. Published online 15 June 2007 [Epub ahead of print].
- Paasch U, Agarwal A, Gupta AK, Sharma RK, Grunewald S, Thomas AJ Jr, et al. Apoptosis signal transduction and the maturity status of human spermatozoa. *Ann NY Acad Sci* 2003;1010:486–8.
- Paasch U, Grunewald S, Agarwal A, Glander HJ. Activation pattern of caspases in human spermatozoa. *Fertil Steril* 2004;81(Suppl 1):802–9.
- Said TM, Agarwal A, Grunewald S, Rasch M, Glander HJ, Paasch U. Evaluation of sperm recovery following annexin V magnetic-activated cell sorting separation. *Reprod Biomed Online* 2006;13:336–9.
- Paasch U, Grunewald S, Glander HJ. Sperm selection in assisted reproductive techniques. *Soc Reprod Fertil Supp* 2007;65:515–25.
- Grizard G, Ouchchane L, Roddier H, Artonne C, Sion B, Vasson MP, et al. In vitro alachlor effects on reactive oxygen species generation, motility patterns and apoptosis markers in human spermatozoa. *Reprod Toxicol* 2007;23:55–62.
- Oancea M, Mazumder S, Crosby ME, Almasan A. Apoptosis assays. *Methods Mol Med* 2006;129:279–90.
- Shen HM, Dai J, Chia SE, Lim A, Ong CN. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod* 2002;17:1266–73.
- Varum S, Bento C, Sousa AP, Gomes-Santos CS, Henriques P, Almeida-Santos T, et al. Characterization of human sperm populations using conventional parameters, surface ubiquitination, and apoptotic markers. *Fertil Steril* 2007;87:572–83.
- Kurz A, Viertel D, Herrmann A, Muller K. Localization of phosphatidylserine in boar sperm cell membranes during capacitation and acrosome reaction. *Reproduction* 2005;130:615–26.
- Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ Jr, et al. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod* 2004;71:1828–37.
- O'Neill DA, McVicar CM, McClure N, Maxwell P, Cooke I, Pogue KM, et al. Reduced sperm yield from testicular biopsies of vasectomized men is due to increased apoptosis. *Fertil Steril* 2007;87:834–41.
- Alvarez JG. DNA fragmentation in human spermatozoa: significance in the diagnosis and treatment of infertility. *Minerva Ginecologica* 2003;55:233–9.
- Fraser L, Strzezek J. Effect of different procedures of ejaculate collection, extenders and packages on DNA integrity of boar spermatozoa following freezing-thawing. *Anim Reprod Sci* 2007;99:317–29.
- World Health Organization WHO. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press, 1999.
- Beletti ME, Mello ML. Comparison between the toluidine blue stain and the Feulgen reaction for evaluation of rabbit sperm chromatin condensation and their relationship with sperm morphology. *Theriogenology* 2004;62:398–402.
- Erenpreisa J, Erenpreiss J, Freivalds T, Slaidina M, Krampe R, Butikova J, et al. Toluidine blue test for sperm DNA integrity and elaboration of image cytometry algorithm. *Cytometry A* 2003;52:19–27.
- Boe-Hansen GB, Fedder J, Ersboll AK, Christensen P. The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. *Hum Reprod* 2006;21:1576–82.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J Immunol Methods* 1995;184:39–51.
- Moreau MF, Guillet C, Massin P, Chevalier S, Gascan H, Basle MF, et al. Comparative effects of five bisphosphonates on apoptosis of macrophage cells in vitro. *Biochem Pharmacol* 2007;73:718–23.
- Chaveiro A, Santos P, da Silva F. Assessment of sperm apoptosis in cryopreserved bull semen after swim-up treatment: a flow cytometric study. *Reprod Domest Anim* 2007;42:17–21.
- Andre S, Pinto AE, Laranjeira C, Quaresma M, Soares J. Male and female breast cancer—differences in DNA ploidy, p21 and p53 expression reinforce the possibility of distinct pathways of oncogenesis. *Pathobiology* 2007;74:323–7.
- Graesslin O, Chantot-Bastaraud S, Lorenzato M, Birembaut P, Quereux C, Darai E. Fluorescence in situ hybridization and immunohistochemical analysis of p53 expression in endometrial cancer: prognostic value and relation to ploidy. *Ann Surg Oncol*. Published online 11 December 2007 [Epub ahead of print].
- Levek-Motola N, Soffer Y, Shochat L, Raziel A, Lewin LM, Golan R. Flow cytometry of human semen: a preliminary study of a non-invasive method for the detection of spermatogenetic defects. *Hum Reprod* 2005;20:3469–75.
- Marchiani S, Tamburrino L, Maoggi A, Vannelli GB, Forti G, Baldi E, et al. Characterization of M540 bodies in human semen: evidence that they are apoptotic bodies. *Mol Hum Reprod* 2007;13:621–31.

44. Srinivas M, Degaonkar M, Gupta DK, Raghunathan P, Das SN, Mitra DK. Evaluation of developing rat testis by phosphorus 31P magnetic resonance spectroscopy and DNA flow cytometry. *Pediatr Surg Int* 2001;17:316–20.
45. Oliveira H, Loureiro J, Filipe L, Santos C, Ramalho-Santos J, Sousa M, et al. Flow cytometry evaluation of lead and cadmium effects on mouse spermatogenesis. *Reprod Toxicol* 2006;22:529–35.
46. Sarkar S, Jones OW, Shioura N. Constancy in human sperm DNA content. *Proc Natl Acad Sci U S A* 1974;71:3512–6.
47. He Y, Cao Y, Xu YJ, Huang J, Sun ZX, Zhou ZM, et al. [Experimental study on the effects of Jujingwan on oligospermia]. *Zhonghua Nan Ke Xue* 2006;12:1135–8.
48. Cui FJ, Li Y, Xu YY, Liu ZQ, Huang DM, Zhang ZC, et al. Induction of apoptosis in SGC-7901 cells by polysaccharide-peptide GFPS1b from the cultured mycelia of *Grifola frondosa* GF9801. *Toxicol In Vitro* 2007;21:417–27.
49. Tarozzi N, Bizzaro D, Flamigni C, Borini A. Clinical relevance of sperm DNA damage in assisted reproduction. *Reprod Biomed Online* 2007;14:746–57.
50. Aitken RJ, De Iuliis GN. Origins and consequences of DNA damage in male germ cells. *Reprod Biomed Online* 2007;14:727–33.
51. Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. *Curr Opin Obstet Gynecol* 2006;18:325–32.
52. Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, et al. A comparison of BoviPure and Percoll on bull sperm separation protocols for IVF. *Anim Reprod Sci* 2006;91:237–47.
53. Morrell JM, Moffatt O, Sakkas D, Manicardi GC, Bizzaro D, Tomlinson M, et al. Reduced senescence and retained nuclear DNA integrity in human spermatozoa prepared by density gradient centrifugation. *J Assist Reprod Genet* 2004;21:217–22.
54. Moskovtsev SI, Willis J, White J, Mullen JB. Sperm survival: relationship to age-related sperm DNA integrity in infertile men. *Arch Androl* 2007;53:29–32.
55. Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S, et al. Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod* 2002;8:984–91.
56. Chen Z, Hauser R, Trbovich AM, Shifren JL, Dorer DJ, Godfrey-Bailey L, et al. The relationship between human semen characteristics and sperm apoptosis: a pilot study. *J Androl* 2006;27:112–20.
57. Stronati A, Manicardi GC, Cecati M, Bordicchia M, Ferrante L, Spano M, et al. Relationships between sperm DNA fragmentation, sperm apoptotic markers and serum levels of CB-153 and p,p'-DDE in European and Inuit populations. *Reproduction* 2006;132:949–58.
58. Kajstura M, Halicka HD, Pryjma J, Darzynkiewicz Z. Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete “sub-G1” peaks on DNA content histograms. *Cytometry A* 2007;71:125–31.
59. Muriel L, Goyanes V, Segrelles E, Gosalvez J, Alvarez JG, Fernandez JL. Increased aneuploidy rate in sperm with fragmented DNA as determined by the sperm chromatin dispersion (SCD) test and FISH analysis. *J Androl* 2007;28:38–49.
60. Slotter ED, Marchetti F, Eskenazi B, Weldon RH, Nath J, Cabrerros D, et al. Frequency of human sperm carrying structural aberrations of chromosome 1 increases with advancing age. *Fertil Steril* 2007;87:1077–86.
61. Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW, et al. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proc Natl Acad Sci U S A* 2006;103:9601–6.
62. Lachaud C, Tesarik J, Canadas ML, Mendoza C. Apoptosis and necrosis in human ejaculated spermatozoa. *Hum Reprod* 2004;19:607–10.