

Selection of Nonapoptotic Spermatozoa As a New Tool for Enhancing Assisted Reproduction Outcomes: An In Vitro Model¹

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ABSTRACT

Magnetic cell sorting (MACS) using annexin V-conjugated microbeads eliminates apoptotic spermatozoa based on the externalization of phosphatidylserine residues. The procedure delivers two sperm fractions: annexin V-negative (nonapoptotic) and annexin V-positive (apoptotic). Our aim was to determine whether the sperm fertilizing potential can be improved by selecting a nonapoptotic fraction using MACS. Semen samples (n = 35) were subjected to separation on a density gradient followed by MACS. Extent of apoptosis was assessed by measuring levels of activated caspase 3 using fluorescein-labeled inhibitors of caspase, alterations in mitochondrial membrane potential (MMP) using a lipophilic cationic dye, and DNA fragmentation using terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling assay. The sperm fertilization potential was assessed using hamster oocyte penetration assay and hamster oocyte-intracytoplasmic sperm injection (ICSI). Annexin V-negative sperm displayed superior quality in terms of high motility, low caspase 3 activation, MMP integrity, and small extent of DNA fragmentation. Annexin V-negative sperm demonstrated higher oocyte penetration capacity but comparable sperm chromatin decondensation (SCD) following ICSI. Conversely, the annexin V-positive sperm presented with poor quality and fertilization potential. The oocyte penetration rate was negatively correlated with apoptotic marker expression, whereas SCD following ICSI was only associated with apoptosis on sperm-damaged membranes. We conclude that apoptosis appears to impact sperm-oocyte penetration rate; however, it does not seem to affect early stages of fertilization such as SCD in spermatozoa of healthy donors. The selection of nonapoptotic sperm by MACS may be used to enhance results of in vitro fertilization by increasing sperm-oocyte penetration.

apoptosis, in vitro fertilization, sperm

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INTRODUCTION

Apoptosis is an ongoing physiological phenomenon that maintains the number of germ cells within the supportive capacity of the Sertoli cells [1]. As opposed to somatic and testicular germ cells, the presence and significance of apoptosis in ejaculated spermatozoa remains elusive [2]. Although ejaculated spermatozoa display several apoptosis-like characteristics, as in somatic cells, these apoptosis-related features do not necessarily indicate apoptosis death functions [3]. Nevertheless, apoptosis-like phenotype in ejaculated sperm has been associated with the presence of abnormal spermatozoa in semen [4–6]. The failure to eliminate these abnormal spermatozoa during spermatogenesis—also termed “abortive apoptosis”—may be the reason for their presence in semen [4, 6].

Activation of caspases, disruption of mitochondrial membrane potential (MMP), and increased DNA fragmentation are some of the apoptotic features that have been identified in ejaculated spermatozoa [3, 7–9]. Another apoptotic event reported in human spermatozoa is the externalization of the phospholipid phosphatidylserine (PS), which is normally present on the inner leaflet of the sperm plasma membrane [10, 11]. Annexin V is a phospholipid-binding protein that has high affinity for PS and lacks the ability to pass through an intact sperm membrane [12]. Therefore, binding of annexin V to spermatozoa indicates that the membrane integrity has been compromised [13].

Magnetic-activated cell sorting (MACS) using annexin V-conjugated superparamagnetic microbeads can effectively separate nonapoptotic spermatozoa from those with deteriorated plasma membranes based on the externalization of PS. MACS separation of sperm yields two fractions: annexin V-negative (intact membranes, nonapoptotic) and annexin V-positive (externalized PS, apoptotic) [14, 15]. We have recently established a sperm preparation protocol that combines MACS with double-density centrifugation. This novel combination provides spermatozoa of higher quality in terms of motility, viability, and apoptosis indices compared with other conventional sperm preparation methods [16]. The protocol can also be used to improve cryosurvival rates following freezing and thawing [17].

The last decade has witnessed a rapidly increasing trend in the application of assisted reproductive technology for the treatment of infertile couples. Although the indications of assisted reproductive techniques (ART) have expanded, a defined pathophysiological diagnosis is often missed [18]. Moreover, current pregnancy and live birth success rates remain unsatisfactory [19]. The inclusion of apoptotic sperm during in vitro fertilization (IVF) may be one of the reasons for

these suboptimal rates [20, 21]. Similarly, there is likelihood that some sperm selected for intracytoplasmic sperm injection (ICSI), despite appearing normal, may contain fragmented DNA, thus affecting the outcome of the procedure [22]. Thus, it appears that the selection of nonapoptotic spermatozoa is one of the prerequisites for achieving optimal conception rates following ART. MACS using annexin V microbeads may yield a sperm population that displays superior functional abilities and in turn higher fertilization potential. In this study, we investigated the possible effects of apoptosis and DNA damage on the sperm function following MACS, using in vitro models that test the sperm-oocyte penetration and chromatin decondensation upon oocyte entry. Our aim was to assess the fertilization potential of annexin V-negative (nonapoptotic) sperm to determine whether the use of MACS before ART procedures can enhance success rates.

MATERIALS AND METHODS

Experimental Design

This study was approved by the Institution Review Boards of the Cleveland Clinic Foundation and the Faculty of Medicine, University of Leipzig. Semen samples were obtained from 35 healthy donors following a period of 3–5 days of sexual abstinence. Semen analysis was performed according to World Health Organization guidelines [23]. Samples with $\geq 20 \times 10^6$ spermatozoa/ml and at least 50% progressive sperm motility were selected for the study.

Semen samples were prepared by double-density-gradient centrifugation (PureCepition; SAGE BioPharma). Samples were loaded onto a 40% and 80% discontinuous gradient and centrifuged at $300 \times g$ for 20 min at room temperature (25°C). The resulting 80% pellet was washed by centrifugation for an additional 7 min and resuspended in human tubal fluid media (HTF; Irvine Scientific).

One aliquot of the sperm suspension served as control, and the other aliquot was subjected to MACS. Activated caspase 3 levels, integrity of the MMP, and extent of DNA fragmentation were assessed as markers of apoptosis in the annexin V-negative and -positive aliquots following MACS as well as in the control aliquot. The extent of PS externalization in the annexin V-negative and -positive aliquots was evaluated and served as a control for MACS separation. The zona-free hamster oocyte penetration assay and the extent of sperm chromatin decondensation (SCD) following hamster oocyte intracytoplasmic injection were used as in vitro models to test the sperm fertilization potential (Fig. 1).

Isolation of Spermatozoa with Deteriorated Membranes by MACS

Spermatozoa were incubated with annexin V-conjugated microbeads (Miltenyi Biotec) for 15 min at room temperature. One hundred microliters

of microbeads was used for each 10 million separated cells. The sperm/microbeads suspension was loaded in a separation column containing iron globes, which was fitted in a magnet (MiniMACS; Miltenyi Biotec). The fraction composed of apoptotic spermatozoa was retained in the separation column and labeled as annexin V-positive, whereas the fraction with intact membranes that was eluted through the column was labeled as annexin V-negative. The power of the magnetic field was measured as 0.5 T between the poles of the magnet and up to 1.5 T within the iron globes of the column. After the column was removed from the magnetic field, the retained fraction was eluted using annexin V-binding buffer (Miltenyi Biotec).

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 and noncytotoxic and which binds covalently to active caspase 3 [24]. The inhibitor was used with the appropriate controls according to the kit instructions provided by the manufacturer (Carboxyfluorescein FLICA; Immunochemistry Technologies). A 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide and further diluted in PBS to yield a 30-fold working solution. All test aliquots and controls (with 100 μ l PBS) were incubated at 37°C for 1 h with 10 μ l 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; Clontech). Spermatozoa with intact mitochondria excite an intense red fluorescence because of 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 min in 1 μ g 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 μ l PBS.

Evaluation of DNA Fragmentation

Sperm DNA strand breaks were evaluated using a TUNEL assay kit (Apo-Direct; Chemicon) as established earlier [25]. Briefly, spermatozoa were washed twice in PBS, resuspended in 1% paraformaldehyde (Electron Microscopy Sciences) at a concentration of $1-2 \times 10^6$ sperm/ml, and placed on ice for 30–60 min. These spermatozoa were again washed by centrifugation at $300 \times g$ for 5 min and resuspended in 70% ice-cold ethanol.

Following a second wash in PBS to remove ethanol, sperm pellets were resuspended in 50 μ l of the staining solution for 60 min at 37°C. The staining solution contained terminal deoxytransferase (TdT) enzyme, TdT reaction buffer, fluorescein tagged deoxyuridine triphosphate nucleotides, and distilled water. All cells were further washed in rinse buffer, resuspended in 0.5 ml of propidium iodide/RNase solution, and incubated for 30 min in the dark at room temperature. Somatic cells were used without (negative control) and after induction of apoptosis (positive control) for each run according to the manufacturer's instructions. An additional sperm negative control was processed identically for each fraction except that the TdT enzyme was replaced with an equal volume of PBS.

Phosphatidylserine Externalization

Externalization of PS was examined using a monoclonal mouse anti-human PS antibody, clone 1H6 (Upstate Cell Signaling Solutions). Spermatozoa were incubated with the PS antibody at a final concentration of 0.5 μ g/ml in PBS containing 2% bovine serum albumin (PBSB) for 20 min on ice, followed by addition of 150 μ l PBSB and centrifugation at $300 \times g$ for 5 min at 20°C. After the supernatant was discarded, each sperm pellet was incubated with 50 μ l of secondary antibody (goat anti-mouse IgG, fluorescein conjugate; Upstate Cell Signaling Solutions) on ice for 20 min and protected from direct light. A second washing step in PBSB ($300 \times g$ for 5 min at 20°C) was performed to remove excess antibody that was not bound to the spermatozoal surface. For assessment by flow cytometry, sperm pellets were diluted in 400 μ l PBSB.

Flow Cytometry Analysis

The extent of activated caspase 3, intact MMP, externalized PS, and sperm DNA damage were evaluated by flow cytometric analyses. All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan

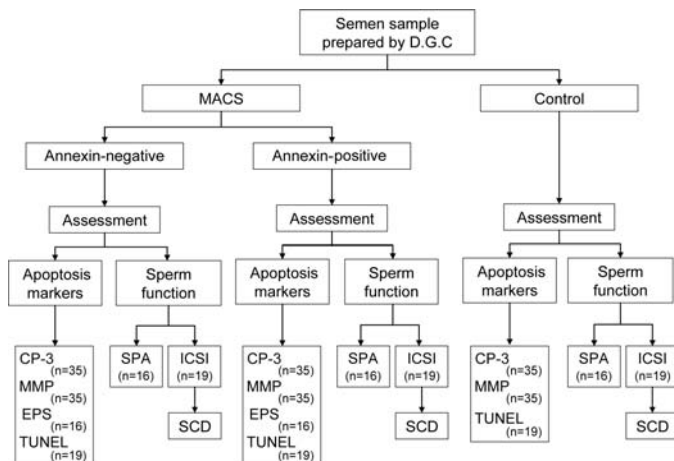


FIG. 1. Overall experimental design showing the different steps performed in our study. DGC, Double density gradient centrifugation; CP 3, activated caspase 3; EPS, externalized phosphatidylserine.

TABLE 1. Results of sperm motility and apoptotic markers in annexin V-negative and -positive sperm and controls.

	Motility (% motile) ^a (n = 35)	Caspase 3 (% activated) ^a (n = 35)	MMP (% intact) ^a (n = 35)	TUNEL (% DNA damaged) ^a (n = 19)
Annexin V-negative fraction (aliquot A)	78.1 ± 11.3	8.8 ± 5.9	88.6 ± 7.8	9.7 ± 10.6
Annexin V-positive fraction (aliquot B)	12.0 ± 9.8	70.5 ± 18.8	30.0 ± 16.5	21.0 ± 13.6
Control (aliquot C)	74.8 ± 9.4	15.2 ± 10.5	78.0 ± 15.1	14.4 ± 13.2
<i>P</i> value ^b				
A vs. B	<0.001	<0.001	<0.001	<0.001
A vs. C	0.007	<0.001	<0.001	0.007
B vs. C	<0.001	<0.001	<0.001	0.002

^a Results are expressed as mean ± SD.

^b *P* < 0.05 was considered significant using one-way ANOVA with repeated measures.

(Becton Dickinson). 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° 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).

Zona-Free Hamster Oocyte Penetration Assay

The assay was performed as described by Johnson et al. [26] with slight modifications to mimic the human IVF procedure. Sperm aliquots used for the evaluation of sperm penetration assay were subjected to capacitating conditions (incubation in HTF media containing 3% bovine serum albumin for 3.5 h at 37°C and 5% carbon dioxide). Frozen-thawed hamster oocytes (Embryotech) were briefly exposed to acidified Tyrode's media (Irvine Scientific) to remove the zona pellucida and in turn their species specificity. The zona-free hamster oocytes were placed in 50-μl sperm droplets (concentration adjusted to 3–5 × 10⁶ sperm/ml), overlaid with mineral oil (Sigma) and incubated for 3 h at 37°C, 5% CO₂. A total of 20 oocytes (five oocytes per sperm droplet) was used for each of the three experiment aliquots. Following incubation, oocytes were washed in PBS + 10% BSA to remove the excess sperm attached to their surface and examined by 40× phase-contrast microscopy (BH2-PC; Olympus). The presence of decondensed nucleus with an attached tail was taken to represent a positive penetration. The number of oocytes scored for each of the three experiment aliquots ranged from 15 to 20 depending on the number of oocytes damaged during the assay. Results were evaluated as the percentage of oocytes penetrated by sperm (SPA) and the average number of sperm penetrated per oocytes (sperm capacitation index [SCI]).

Intracytoplasmic Sperm Injection

Micromanipulation was carried out under a 400× inverted microscope (Nikon) using Hoffman optics. The microscope was equipped with two hydraulic micromanipulators (M0-102M) and two microinjectors (IM-4 and IM-6; Narishige Co. Ltd.).

ICSI was carried out as described by Palermo et al. [27]. In brief, 1 μl of sperm suspension was diluted with 4 μl of 7% polyvinyl pyrrolidone (Irvine Scientific) and placed in the center of an injection dish. Each frozen-thawed hamster oocyte (Embryotech) was placed in 5 μl of HTF media + 10% BSA surrounding the central drop containing the sperm suspension and covered with mineral oil. A total of 10 oocytes was injected for each of the three experiment aliquots. The selected sperm was aspirated into the injector micropipette and introduced through the zona into the ooplasm. The micropipette was then slowly withdrawn and the injected oocytes were kept at 37°C, 5% CO₂ for 18–22 h.

To evaluate the occurrence of SCD, oocytes were fixed by incubation in 2% formaldehyde + 0.02% triton-X (Bio-Rad) for 30 min at 37°C. Following incubation, oocytes were washed in PBS + 10% BSA and stained with Hoechst 33258 (10 μg/ml; Sigma) for 45 min at 37°C. Washing was repeated to remove the excess stain and oocytes were mounted on glass slide using Slow Fade mounting medium (Molecular Probes) [28]. Images were collected using a Leica AOBSP2 confocal microscope (Leica Microsystems) using the 351-nm line of an argon laser for excitation. Emitted light between 400 and 500 nm was collected using a 63× lens, and the Z-series was collected using a step size of 0.4 μm. The number of oocytes scored for each of the three experiment aliquots ranged from 5 to 10 depending on the number of oocytes damaged during the assay.

Statistical Analysis

One-way ANOVA with repeated measures was used to compare various parameters among the three experiment aliquots. Pairwise Pearson correlation coefficients were used to study the association between different parameters. The common correlation coefficient, which can be regarded as a measure of the average correlation across treatment groups, was calculated when necessary from a weighted average of the Fisher Z transformations of the aliquot-specific correlations. All tests were two-tailed, and significance was indicated by *P* < 0.05. The statistical analysis was done using SAS, version 9.0 (SAS Institute).

RESULTS

Semen Sample Evaluation

The mean sperm concentration and percentage motility in the raw ejaculates were 93.4 ± 76.6 × 10⁶/ml and 57.3 ± 9.4%. Following separation on a double-density gradient, the sperm recovery rate was 55.2 ± 24.0%, resulting in an average sperm concentration of 83.8 ± 57.6 × 10⁶/ml and an average motility of 73.9 ± 8.7%. The sperm motility values were significantly higher in the annexin V-negative fraction following MACS compared to the annexin V-positive fraction (*P* < 0.001) as well as controls (*P* = 0.007). On the other hand, the annexin V-positive sperm presented with significantly lower motility compared to the controls (*P* < 0.001, Table 1).

Activated Caspase 3, MMP, and DNA Fragmentation

Overall, a significantly large difference was seen between the three aliquots in caspase 3 activation, MMP, and DNA fragmentation (*P* < 0.001, *P* < 0.001, and *P* < 0.001, respectively). The extent of spermatozoa displaying activated caspase 3 was significantly lower in the annexin V-negative fraction in comparison to the annexin V-positive fraction (*P* < 0.001) as well as the controls (*P* < 0.001). The integrity of the mitochondrial membrane potential was more preserved in the annexin V-negative fraction (*P* < 0.001 vs. annexin V-positive fraction; *P* < 0.001 vs. controls). Results of the TUNEL assay revealed that the percentage of sperm with fragmented DNA was significantly lower in the annexin V-negative fraction (*P* < 0.001 vs. annexin V-positive fraction; *P* = 0.007 vs. controls). The annexin V-positive fraction had higher caspase 3 activation and DNA fragmentation and lower MMP integrity compared with the controls (*P* < 0.001, *P* = 0.002, and *P* < 0.001, respectively; Table 1). The externalization of PS was significantly lower in the annexin V-negative fraction compared to the annexin V-positive fraction following MACS separation (3.4 ± 1.7% vs. 54.9 ± 18.1%, *P* < 0.001; Fig. 2).

Caspase 3 activation was significantly and negatively correlated with MMP and sperm motility. Both MMP and sperm motility showed a significant positive correlation. On the other hand, the percentage of spermatozoa with fragmented

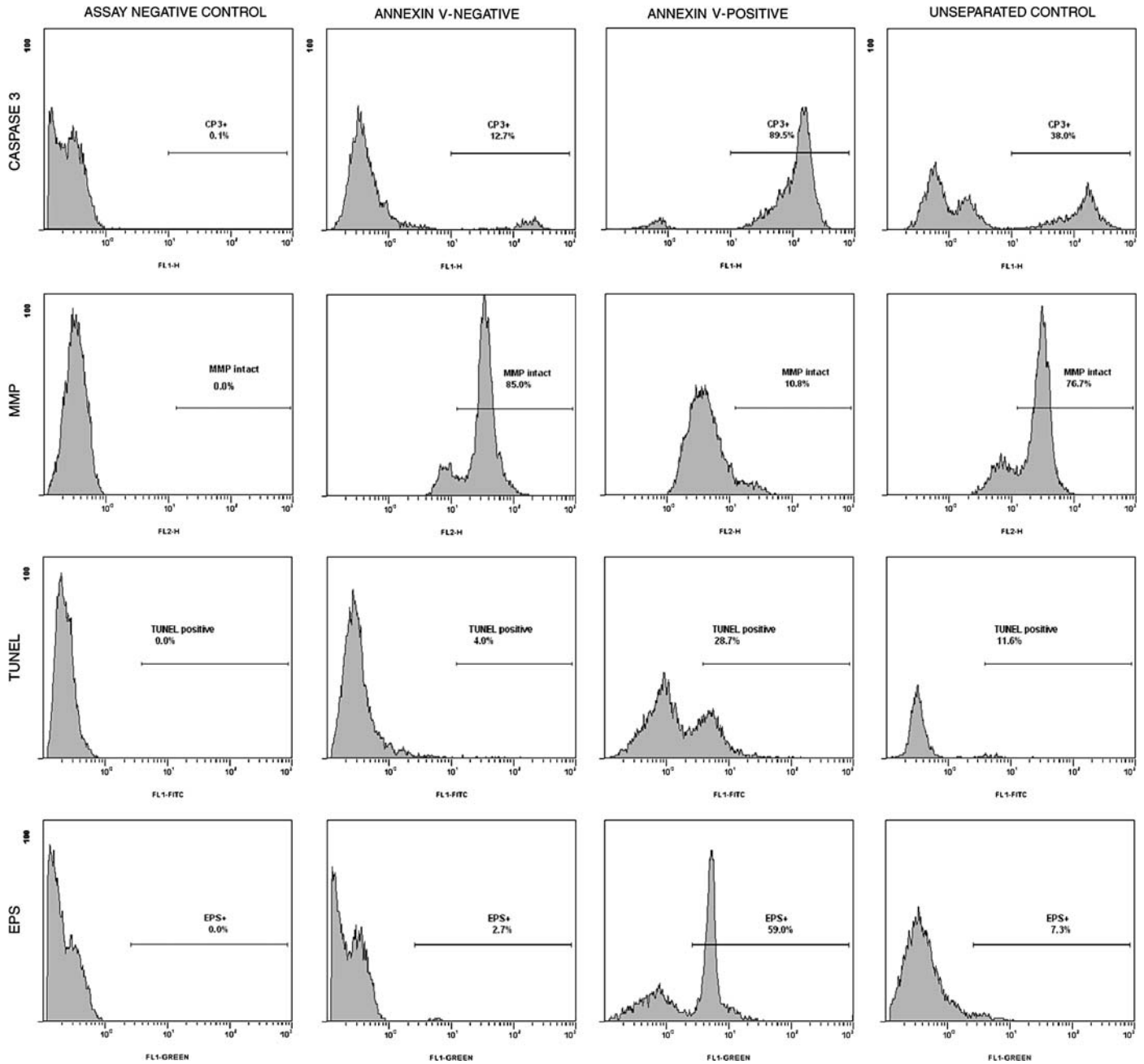


FIG. 2. Results of flow cytometric analyses. The X-axis of each histogram depicts the intensity of fluorescence in either the green spectrum (FITC-FL1) channel for the analysis of caspase 3, TUNEL and externalized phosphatidylserine (EPS), or in the red spectrum (rhodamine-FL2) channel. The Y-axis depicts the frequency in terms of the number of cells.

DNA showed a weak positive correlation with caspase 3 activation and a negative correlation with sperm motility but not with MMP (Table 2).

Models for Evaluating Sperm Fertilization Potential

Following the sperm penetration assay, a significantly higher percentage of penetrated oocytes were detected in the annexin V-negative fraction compared to the annexin V-positive sperm ($P < 0.001$) as well as the controls ($P = 0.001$). Similarly, SCI values were significantly higher in the annexin V-negative fraction compared to the annexin V-positive sperm ($P < 0.001$) and the controls ($P < 0.04$; Table 3). The annexin V-positive spermatozoa had lower percentage of penetrated

oocytes and SCI values when compared with the controls ($P < 0.001$ and $P = 0.01$, respectively).

The percentage of penetrated oocytes had a significant negative correlation with caspase 3 activation ($r = -0.61$, $P < 0.001$) and significant positive correlations with MMP and sperm motility ($r = 0.67$ and $r = 0.70$, $P < 0.001$) in samples for which the sperm penetration assay was performed ($n = 16$). To a lesser extent, SCI values had a significant negative correlation with caspase 3 activation ($r = -0.44$, $P < 0.001$) and significant positive correlations with MMP and sperm motility ($r = 0.48$ and $r = 0.52$, $P < 0.001$).

A sample of SCD following ICSI is presented in Figure 3. Both annexin V-negative sperm and controls showed a higher percentage of SCD following ICSI compared to the annexin V-

TABLE 2. Sperm motility and markers of apoptosis showing the correlation and the significance (in parentheses) with each other.^a

	Motility (n = 35)	Caspase 3 (n = 35)	MMP (n = 35)	TUNEL ^b (n = 19)
Motility (n = 35)	1.00	-0.58 (<0.001)	0.54 (<0.001)	-0.55 (<0.001)
Caspase 3 (n = 35)		1.00	-0.54 (<0.001)	0.37 (<0.008)
MMP (n = 35)			1.00	-0.21 (0.144)
TUNEL (n = 19)				1.00

^a The average correlations are derived from the weighted averages of the Fisher Z transformations of the group specific Pearson correlations; $P < 0.05$ is considered significantly different from zero using a Wald test.

^b The correlation between TUNEL and others were based on the 19 samples available to both measures.

positive sperm ($P = 0.02$ and $P = 0.01$, respectively). However, SCD rates were identical in the annexin V-negative sperm and controls ($P = 0.98$; Table 3). In samples evaluated by hamster ICSI (n = 19), the SCD had a weak negative correlation with caspase 3 activation ($r = -0.31$, $P = 0.01$) and weak positive correlations with MMP and sperm motility ($r = 0.29$, $P = 0.03$, and $r = 0.31$, $P = 0.02$, respectively). No significant correlation was found between the percentage of DNA-fragmented sperm and the percentage of spermatozoa showing chromatin decondensation following ICSI ($r = 0.05$, $P = 0.57$).

DISCUSSION

Effect of MACS on Sperm Quality

The increase in ART applications with concurrent low success rates has intensified the need to develop an ideal sperm preparation technique. Novel approaches such as electrophoretic separation and magnetic cell separation have demonstrated encouraging results for the isolation of spermatozoa with superior quality [16, 29]. Among other available procedures, density gradient centrifugation has become a standard for sperm preparation [30]. Although the technique has been reported to provide spermatozoa with higher MMP and DNA integrity [31, 32], its mode of action does not include the identification of apoptotic markers in spermatozoa. On the other hand, MACS using annexin V-conjugated microbeads specifically targets spermatozoa with deteriorated membranes that display externalized PS as a manifestation of apoptosis [13, 15]. Therefore, MACS acts on the sperm molecular level, as opposed to routine sperm preparation techniques that rely

solely on sperm density and motility. In this context, MACS may be considered a unique molecular preparation technique that complements conventional sperm preparation protocols.

In the current experiment, the recovery rate of motile sperm following density-gradient centrifugation was relatively high because of the nature of our study population, which consisted of men with normal semen parameters. Nevertheless, the percentage of motile sperm was further significantly increased following MACS separation, as noted in the annexin V-negative fraction. This clearly indicates that sperm motility can still be improved by integrating MACS as a part of the sperm preparation protocol, which is in agreement with our earlier studies [16, 17]. The enhancement of sperm motility following MACS separation is not surprising because functional assays of sperm plasma membrane integrity are good indicators of sperm quality [13]. Moreover, sperm motility has been negatively correlated with several apoptotic indices, such as caspase 3 activation [3, 9] and PS externalization [5, 11].

Impact of MACS on Apoptosis Markers

The annexin V-negative fraction separated by MACS displayed the lowest percentage of caspase 3 activation as well as the highest mitochondrial membrane integrity. Conversely, the annexin V-positive fraction demonstrated the highest expression levels of these apoptotic markers. MACS coupled with annexin V microbeads has been repeatedly shown to efficiently isolate apoptotic spermatozoa [14, 33–36]. Because PS externalization is considered an apoptotic event, other features of apoptosis would be expected to manifest concomitantly. In our earlier study, MACS separation resulted in the depletion of spermatozoa with activated caspases [34]. Moreover, mitochondrial changes typical of apoptosis coincided with PS externalization and annexin V binding [2]. The strong correlation detected in the present study between PS externalization, caspase 3 activation, and MMP further supports this observation.

Alterations in the sperm phospholipid bilayer are not always considered manifestations of cell death but may be a part of the normal sperm physiology. During sperm capacitation, protein kinases have been identified as mediators for signaling pathways that lead to externalization of PS and phosphatidylethanolamine (PE) [37–39]. However, species-specific differences in sperm capacitation as well as different methodologies for capacitation induction restricted the validation of these findings [40]. In our study, the significantly reduced sperm motility in the annexin V-positive fraction is an indicator that annexin V binding is more associated with cell death rather than sperm capacitation.

TABLE 3. In vitro models showing the impact of magnetic cell separation on sperm fertilization potential of various sperm fractions using SPA and SCD following ICSI.

	SPA (n = 16)		SCD (n = 19)
	Oocytes penetrated (%) ^a	Sperm capacitation index ^a	Decondensed sperm (%) ^a
Annexin V-negative fraction (aliquot A)	44.5 ± 12.6	1.8 ± 0.3	34.2 ± 13.7
Annexin V-positive fraction (aliquot B)	20.8 ± 5.3	1.3 ± 0.4	24.6 ± 13.0
Control (aliquot C)	33.8 ± 6.9	1.5 ± 0.6	34.0 ± 13.0
<i>P</i> value ^b			
A vs. B	<0.001	<0.001	0.02
A vs. C	0.001	0.04	0.98
B vs. C	<0.001	0.01	0.01

^a Results are expressed as mean ± SD.

^b $P < 0.05$ was considered significant using one-way analysis of variance with repeated measures.

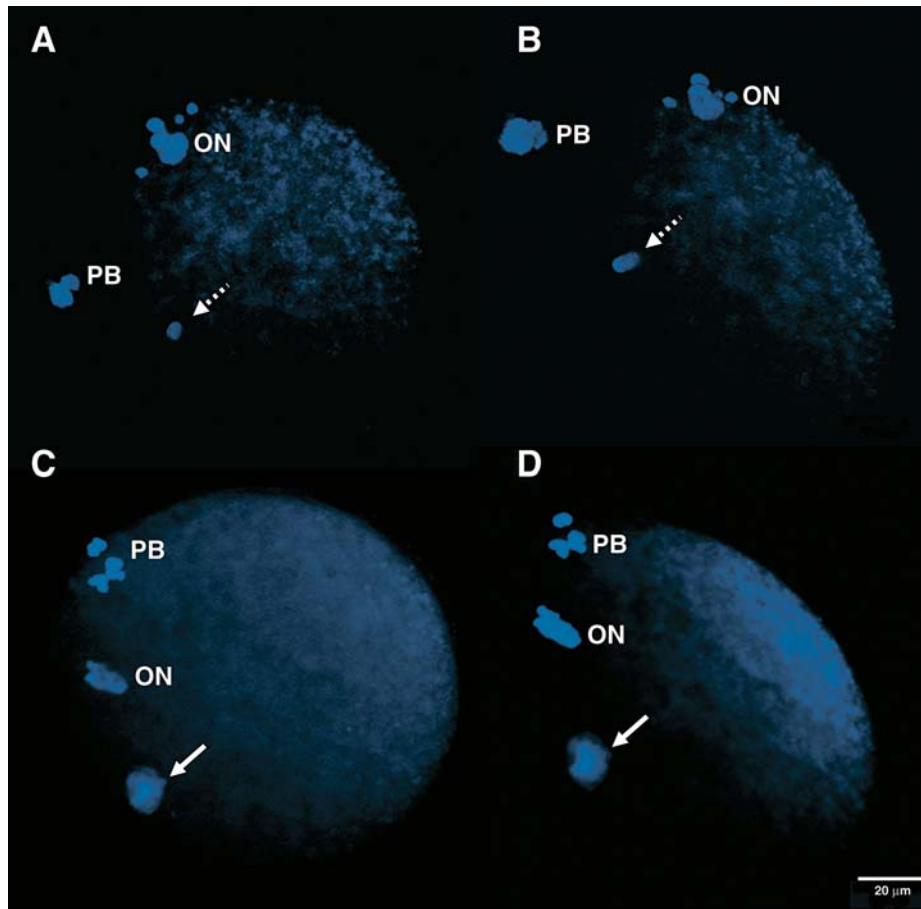


FIG. 3. Sperm chromatin decondensation following hamster oocyte-human sperm intracytoplasmic injection. **A** and **B** show condensed sperm chromatin (dotted arrow), and **C** and **D** show decondensed sperm chromatin (solid arrow). **A** and **C** are cross-sectional views; **B** and **D** are 3-D views with 90° angle rotation around the Z-axis. PB, Polar body; ON, oocyte nucleus. Original magnification $\times 63$.

We compared PS externalization between the annexin V-negative and -positive fractions separated by MACS as an internal control measure to assess the efficiency of MACS separation. The technique appears to be adequate because the number of PS-positive sperm was lower in the annexin V-negative fraction than in the annexin V-positive fraction. Minimal PS externalization was noted in the annexin V-negative fraction, whereas a considerable number of spermatozoa that stained negative for PS were found in the annexin V-positive fraction. The absence of PS externalization in some spermatozoa in the annexin V-positive fraction may be because beads have already blocked the PS binding sites. In addition, annexin V can also bind to other enzymes such as protein kinases, and phospholipids such as PE, despite high affinity for PS [41, 42].

Defective chromatin packaging and failure of replacement of histones with protamines during spermiogenesis, along with oxidative stress, have been linked to DNA damage in male germ cells [43, 44]. Alternatively, DNA fragmentation in ejaculated human spermatozoa may be one of the characteristics of programmed cell death [7, 43, 44]. In the present study, annexin V-negative sperm following MACS had the lowest incidence of DNA fragmentation. However, the extent of this fragmentation did not correlate with the percentage of motile spermatozoa or other markers of apoptosis. This may be attributed to the fact that DNA integrity is an independent measure of sperm quality that is not necessarily associated with other sperm parameters [45]. Moreover, markers of apoptosis do not always occur at the same time [3, 4]. Another possible explanation would be that TUNEL assessment for DNA damage correlates better with necrotic rather than apoptotic sperm [46].

Models for Sperm Fertilization Potential

Although there is an established consensus on the implication of apoptosis in male infertility, the exact mechanisms of its involvement remain to be elucidated [47]. In our study, we used in vitro models to assess whether the selection of nonapoptotic spermatozoa may be used as a strategy to enhance ART outcomes. The hamster oocyte penetration assay has been well standardized to measure the ability of the acrosome-reacted spermatozoa to fuse with the vitelline membrane of the oocyte and initiate nuclear decondensation [26]. On the other hand, human SCD following the hamster oocyte ICSI provides a method to test the sperm fertilizing capacity and can be used as a measure for training and proficiency testing [48, 49]. The preliminary data generated from these two models may justify the future validation and application of MACS in human clinical ART.

In the present study, annexin V-negative sperm had the highest SPA and SCI, followed by the controls, and annexin V-positive sperm demonstrated the weakest penetration ability. The low expression of apoptotic markers in the annexin V-negative fraction indicates that the increased oocyte penetration is associated with and may be caused by decreased incidence of sperm apoptosis. Moreover, the increased oocyte penetration potential was directly correlated with expression of apoptotic markers (the lower the better), which further supports that apoptosis plays a role in preventing sperm oocyte penetration. A previous report has documented the correlation between zona-free hamster egg penetration and annexin V staining [50]. This correlation supports our separation method, which is based on annexin V-conjugated microbeads.

In general, SCD rates following ICSI were lower than those found in published reports [49, 51, 52]. This may be attributed to the use of cryopreserved hamster oocytes, which are of lower quality than fresh oocytes, in our experiment. Although annexin V-negative sperm had higher SCD following ICSI, these values were comparable to the controls. This may be attributed to the design of the experiment, in which semen samples from healthy donors were used. The weak correlation of caspase 3 activation and MMP, as well as the lack of correlation of DNA damage with the results of SCD, may indicate a weak impact of apoptosis on early fertilization stages of healthy donor sperm. Therefore, it is evident that apoptosis and DNA damage do not directly affect the sperm fertilization rate following ICSI. This finding is in accordance with other studies that reported a lack of association between sperm DNA integrity and fertilization rates in clinical IVF programs [53–55]. However, this may not hold true for later stages of the fertilization process such as embryo development, blastocyst development rate, or clinical pregnancy rates [20, 56, 57]. The assessment of embryonic development was not possible in the present study because of the limitation of our animal model.

Significant gene expression in human embryos is observed after the 4-cell stage [58–60]. Thus, disorders of the paternal genome such as sperm DNA fragmentation are not expected to directly affect the rate of pronucleus formation, which represents the fertilization rate. Nevertheless, other functional abnormalities could be concurrently present in the DNA damaged sperm and may be the reason for the decreased fertilization rate. For instance, the annexin V-positive fraction in the present study had the lowest percentage of motile spermatozoa in addition to the highest incidence of deregulated apoptosis. Therefore, the significantly decreased SCD following ICSI in this fraction may be attributed to the decrease in motility as well as deregulated apoptosis. In a study by Lopes et al. [61], abnormal sperm with low motility had an increased incidence of DNA fragmentation, which correlated with low fertilization rates following ICSI.

Our results indicate that MACS separation enhances the sperm-oocyte penetration potential; therefore, it may be of potential benefit during ART procedures such as intrauterine insemination (IUI) or IVF. On the other hand, MACS does not seem to be a useful measure for enhancing early fertilization following ICSI. Whether or not it will affect the results of later fertilization stages requires further study, which was not possible because of the limitation of our animal model. Our findings are in agreement with multiple published reports that document the association of apoptosis and DNA damage with the fertilization rates following IUI and IVF but not with ICSI [22, 62, 63]. The discrepancy may be because of the technical nature of ICSI that plays an important role in minimizing the impact of sperm preparation methods. During ICSI, a motile and, as far as possible, morphologically normal sperm is selected for injection [62]. Therefore, there is an increased chance of using a sperm with intact DNA. On the other hand, during IVF, a larger number of spermatozoa are incubated with the oocyte, and a natural selection process takes place to control sperm penetration.

In conclusion, apoptosis appears to have a negative impact on sperm-oocyte penetration. Subsequently, the separation of a nonapoptotic fraction using MACS further improves the sperm quality and oocyte penetration capacity. Based on these conclusions, including an additional step of sperm preparation involving the isolation of apoptotic sperm using MACS may significantly enhance the outcome of some assisted reproductive techniques such as IUI or IVF. Although our data support the association of apoptosis with DNA fragmentation in human

spermatozoa, both phenomena do not seem to impact early stages of fertilization such as SCD. Therefore, MACS can be used to isolate spermatozoa with compromised genomic integrity, but whether or not it will have the ability to increase fertilization rates following ICSI requires further investigation in a clinical ART program.

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REFERENCES

1. Sinha Hikim AP, Swerdloff RS. Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod* 1999; 4:38–47.
2. Oehninger S, Morshedi M, Weng SL, Taylor S, Duran H, Beebe S. Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa. *Reprod Biomed Online* 2003; 7:469–476.
3. Taylor SL, Weng SL, Fox P, Duran EH, Morshedi MS, Oehninger S, Beebe SJ. Somatic cell apoptosis markers and pathways in human ejaculated sperm: potential utility as indicators of sperm quality. *Mol Hum Reprod* 2004; 10:825–834.
4. Sakkas D, Mariethoz E, St John JC. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp Cell Res* 1999; 251:350–355.
5. Barroso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 2000; 15:1338–1344.
6. 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; 66:1061–1067.
7. Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z. Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. *Exp Cell Res* 1993; 207:202–205.
8. Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S, Hoshi K. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. *Asian J Androl* 2002; 4:97–103.
9. Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S, Oehninger S. Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod* 2002; 8:984–991.
10. 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.
11. Oosterhuis GJ, Mulder AB, Kalsbeek-Batenburg E, Lambalk CB, Schoemaker J, Vermes I. Measuring apoptosis in human spermatozoa: a biological assay for semen quality? *Fertil Steril* 2000; 74:245–250.
12. van Heerde WL, de Groot PG, Reutelingsperger CP. The complexity of the phospholipid binding protein Annexin V. *Thromb Haemost* 1995; 73:172–179.
13. Glander HJ, Schaller J. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. *Mol Hum Reprod* 1999; 5:109–115.
14. Grunewald S, Paasch U, Glander HJ. Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. *Cell Tissue Bank* 2001; 2:127–133.
15. Glander HJ, Schiller J, Suss R, Paasch U, Grunewald S, Arnhold J. Deterioration of spermatozoal plasma membrane is associated with an increase of sperm lyso-phosphatidylcholines. *Andrologia* 2002; 34:360–366.
16. Said TM, Grunewald S, Paasch U, Glander H-J, Baumann T, Kriegel C, Li L, Agarwal A. Advantage of combining magnetic cell separation with sperm preparation techniques. *Reprod Biomed Online* 2005; 10:740–746.
17. Said TM, Grunewald S, Paasch U, Rasch M, Agarwal A, Glander HJ. Effects of magnetic-activated cell sorting on sperm motility and cryosurvival rates. *Fertil Steril* 2005; 83:1442–1446.
18. Katz P, Nachtigall R, Showstack J. The economic impact of the assisted reproductive technologies. *Nat Cell Biol* 2002; 4(suppl):s29–32.
19. Society for Assisted Reproductive Technology, American Society for Reproductive Medicine. Assisted reproductive technology in the United

- States: 2000 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. *Fertil Steril* 2004; 81:1207–1220.
20. Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril* 2004; 82:378–383.
 21. Henkel R, Hajimohammad M, Staff T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB, Kruger TF. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 2004; 81: 965–972.
 22. Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod* 1997; 56:602–607.
 23. WHO. World Health Organization Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4th ed. Cambridge: Cambridge University Press; 1999.
 24. Ekert PG, Silke J, Vaux DL. Caspase inhibitors. *Cell Death Differ* 1999; 6: 1081–1086.
 25. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993; 53:1945–1951.
 26. Johnson A, Bassham B, Lipshultz LI, Lamb DJ. A quality control system for the optimized sperm penetration assay. *Fertil Steril* 1995; 64:832–837.
 27. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992; 340:17–18.
 28. Gook D, Osborn SM, Bourne H, Edgar DH, Speirs AL. Fluorescent study of chromatin and tubulin in apparently unfertilized human oocytes following ICSI. *Mol Hum Reprod* 1998; 4:1130–1135.
 29. Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. *Hum Reprod* 2005; 20: 2261–2270.
 30. Centola GM, Herko R, Andolina E, Weisensel S. Comparison of sperm separation methods: effect on recovery, motility, motion parameters, and hyperactivation. *Fertil Steril* 1998; 70:1173–1175.
 31. Donnelly ET, O'Connell M, McClure N, Lewis SE. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. *Hum Reprod* 2000; 15:1552–1561.
 32. Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod* 2002; 17:1257–1265.
 33. Paasch U, Grunewald S, Fitzl G, Glander HJ. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. *J Androl* 2003; 24:246–252.
 34. Paasch U, Grunewald S, Agarwal A, Glander HJ. Activation pattern of caspases in human spermatozoa. *Fertil Steril* 2004 81 Suppl1802–809.
 35. Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ Jr., Glander HJ, Agarwal A. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod* 2004; 71:1828–1837.
 36. Paasch U, Grunewald S, Wuendrich K, Jope T, Glander HJ. Immunomagnetic removal of cryo-damaged human spermatozoa. *Asian J Androl* 2005; 7:61–69.
 37. Kotwicka M, Jendraszak M, Warchol JB. Plasma membrane translocation of phosphatidylserine in human spermatozoa. *Folia Histochem Cytobiol* 2002; 40:111–112.
 38. Gadella BM, Harrison RA. Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. *Biol Reprod* 2002; 67:340–350.
 39. de Vries KJ, Wiedmer T, Sims PJ, Gadella BM. Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. *Biol Reprod* 2003; 68:2122–2134.
 40. Muratori M, Porazzi I, Luconi M, Marchiani S, Forti G, Baldi E. AnnexinV binding and merocyanine staining fail to detect human sperm capacitation. *J Androl* 2004; 25:797–810.
 41. Stuart MC, Reutelingsperger CP, Frederik PM. Binding of annexin V to bilayers with various phospholipid compositions using glass beads in a flow cytometer. *Cytometry* 1998; 33:414–419.
 42. Russo-Marie F. Annexin V and phospholipid metabolism. *Clin Chem Lab Med* 1999; 37:287–291.
 43. Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001; 122:497–506.
 44. Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online* 2003; 7:428–432.
 45. Spano M, Kolstad AH, Larsen SB, Cordelli E, Leter G, Giwercman A, Bonde JP. The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. *Asclepius. Hum Reprod* 1998; 13:2495–2505.
 46. Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl* 2003; 24:253–262.
 47. Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. *BJU Int* 2005; 95:503–507.
 48. Ahmadi A, Bongso A, Ng SC. Intracytoplasmic injection of human sperm into the hamster oocyte (hamster ICSI assay) as a test for fertilizing capacity of the severe male-factor sperm. *J Assist Reprod Genet* 1996; 13: 647–651.
 49. Gvakharia MO, Lipshultz LI, Lamb DJ. Human sperm microinjection into hamster oocytes: a new tool for training and evaluation of the technical proficiency of intracytoplasmic sperm injection. *Fertil Steril* 2000; 73: 395–401.
 50. Sion B, Janny L, Boucher D, Grizard G. Annexin V binding to plasma membrane predicts the quality of human cryopreserved spermatozoa. *Int J Androl* 2004; 27:108–114.
 51. Perreault SD, Barbee RR, Elstein KH, Zucker RM, Keefer CL. Interspecies differences in the stability of mammalian sperm nuclei assessed in vivo by sperm microinjection and in vitro by flow cytometry. *Biol Reprod* 1988; 39:157–167.
 52. Ahmadi A, Ng SC. Destruction of protamine in human sperm inhibits sperm binding and penetration in the zona-free hamster penetration test but increases sperm head decondensation and male pronuclear formation in the hamster-ICSI assay. *J Assist Reprod Genet* 1999; 16:128–132.
 53. Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, Guerin JF. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod* 2003; 18:1023–1028.
 54. Larson-Cook KL, Brannian JD, Hansen KA, Kasperon KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril* 2003; 80:895–902.
 55. Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, Leter G, Ciriminna R, Culasso F, Dondero F, Lenzi A, Spano M. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Hum Reprod* 2004; 19:1409–1417.
 56. Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 2004; 19:611–615.
 57. Viro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril* 2004; 81:1289–1295.
 58. Tesarik J, Kopečný V, Plachot M, Mandelbaum J. Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing in vitro. *J Reprod Fertil* 1986; 78:463–470.
 59. Tesarik J, Kopečný V, Plachot M, Mandelbaum J. Early morphological signs of embryonic genome expression in human preimplantation development as revealed by quantitative electron microscopy. *Dev Biol* 1988; 128:15–20.
 60. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988; 332:459–461.
 61. Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril* 1998; 69:528–532.
 62. Host E, Lindenberg S, Smidt-Jensen S. The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. *Acta Obstet Gynecol Scand* 2000; 79:559–563.
 63. Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod* 2004; 19:1401–1408.