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Selection of non-apoptotic, DNA intact spermatozoa

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CHAPTER 4

Advantage of Combining Magnetic Cell Separation with Sperm Preparation Techniques

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ABSTRACT

The selection of vital, non-apoptotic spermatozoa is a prerequisite for achieving optimal conception rates in assisted reproductive techniques. Magnetic cell sorting using annexin-V microbeads can effectively separate apoptotic and non-apoptotic spermatozoa. The objective of our study was to optimize the integration of magnetic cell sorting in standard sperm preparation and to correlate the effect of different sperm preparation procedures on apoptotic markers. Semen specimens collected from 15 healthy donors were prepared by either density gradient centrifugation or by one-step sperm wash technique separately and in combination with magnetic cell sorting. The preparation methods were evaluated by assessment of semen parameters (motility, viability, and morphology) as well as markers of apoptosis (levels of active caspase-3, integrity of membrane mitochondrial potential and externalization of phosphatidylserine). The apoptotic markers were measured using fluorochrome dyes coupled with flow cytometry. Our results showed that the combination of density gradient centrifugation and annexin-V magnetic cell sorting was superior to all other sperm preparation methods in terms of providing motile, viable and non-apoptotic spermatozoa. The results of our study clearly indicate the advantage of integrating magnetic cell sorting as a part of sperm preparation, which in turn may positively affect the success rates of assisted reproductive techniques.

Key words: annexin; caspase; magnetic-activated cell sorting; mitochondrial membrane potential; phosphatidylserine; sperm preparation.

INTRODUCTION

Assisted reproductive techniques (ART) have become the treatment of choice in many cases of male and female infertility; however, the current success rates of these procedures remain suboptimal (ASRM, 2004). The quality of sperm samples is one of the factors determining successful assisted reproduction (Ombelet *et al.*, 2003). A variety of sperm preparation techniques are available to select motile spermatozoa that are capable of fertilizing the female oocyte (Henkel *et al.*, 2003). The technique of double density gradient centrifugation has great potential in sperm preparation for ART (Chen *et al.*, 1999) whereas the one-step washing technique is considered a good alternative for processing certain compromised samples (Srisombut *et al.*, 1998).

The quality of spermatozoa separated by various preparation techniques can be evaluated by conventional semen analysis, determining sperm concentration, motility, viability and morphology using light microscopy. Although the conventional analysis gives considerable information, it does not assess the presence of deregulated programmed cell death (apoptosis) in spermatozoa, which may be partially responsible for the low fertilization and implantation rates seen with ART.

The role of deregulated apoptosis has been well characterized in a variety of somatic diseases but remains poorly defined in the pathogenesis of male infertility (Oehninger *et al.*, 2003). Ejaculated human spermatozoa have been shown to display characteristics that are typical of apoptosis such as caspase activation, decreased mitochondrial membrane potential (MMP) and plasma membrane translocation of phosphatidylserine (PS) (Glander *et al.*, 1999, Paasch *et al.*, 2003, Paasch *et al.*, 2004b, Weng *et al.*, 2002).

Colloidal super-paramagnetic microbeads (~50 nm in diameter) conjugated with annexin-V may be used to separate dead and apoptotic spermatozoa by magnetic-activated cell sorting (MACS). Cells with externalized PS and deteriorated plasma membranes will bind to these microbeads. When placed into a column containing iron balls and passed through a strong magnetic field, those cells remain in the separation column. On the other hand, non-apoptotic cells with intact membranes remain unlabelled and pass freely through the column (Grunewald *et al.*, 2001, Miltenyi *et al.*, 1990, Paasch *et al.*, 2004a, Paasch *et al.*, 2003).

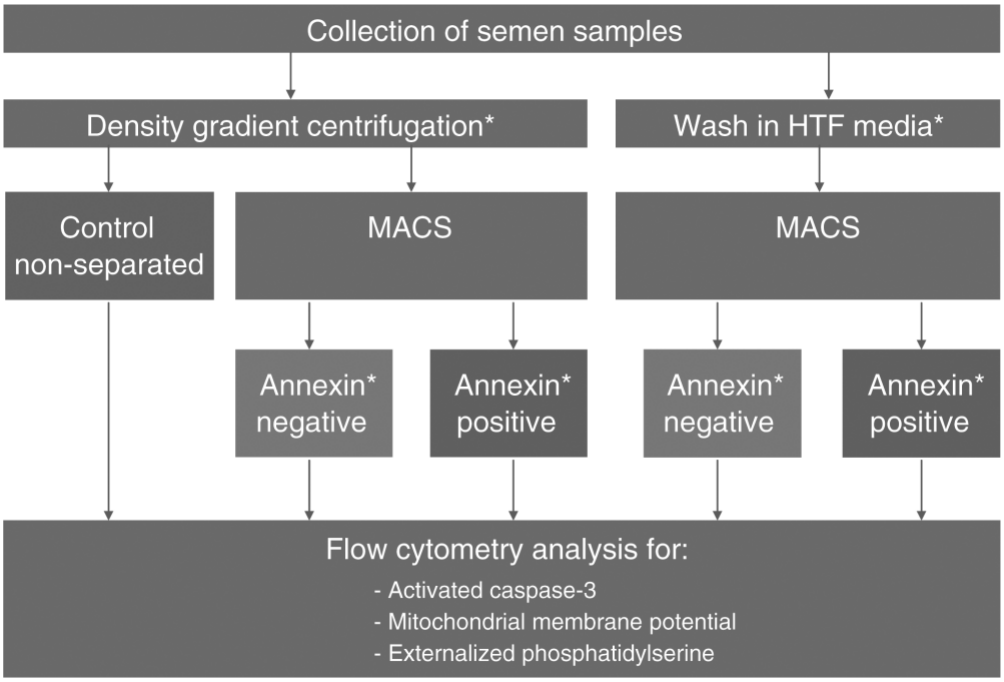
MACS separation according to the externalization of PS results in the extraction of apoptotic sperm and those with damaged membranes. Therefore, the selection of non-apoptotic spermatozoa may be used to enhance sperm quality following preparation techniques and subsequently achieve optimal conception rates in ART. The objective of our study was to evaluate and optimize the integration of MACS in sperm preparation protocols and to correlate the effect of different sperm preparation techniques on the following apoptotic markers: caspase-3, MMP and externalization of PS.

MATERIALS AND METHODS

Sample preparation

This study was approved by the Institution Review Board of the Cleveland Clinic Foundation. Semen samples were collected from 15 healthy donors. The liquefied semen samples were split into 2 equal portions. The first portion was prepared by double density gradient centrifugation (PureCeption®, SAGE BioPHARMA, Bedminster, NJ). In brief, samples were loaded onto a 40% and 80% discontinuous gradient and centrifuged at 1600 rpm for 20 minutes at room temperature. The resulting 80% pellet representing the mature fraction was washed by centrifugation for additional 7 minutes and re-suspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA). The sperm cell suspension was further divided into 2 separate fractions. The first was subjected to MACS, while the second remained unseparated to serve as a control.

The second portion was prepared by one-step sperm washing and re-suspension in HTF media followed by MACS separation. Sperm motility was assessed manually whereas viability was assessed using eosin-nigrosin stain in all samples and controls at each step of the experiment according to the World Health Organization standard protocols (WHO, 1999). The sperm morphology was assessed by both WHO standard criteria and the strict Tygerberg's (Kruger's) criteria (Kruger *et al.*, 1987) using Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL). The different steps of our experiment design are illustrated in Figure 1.



*Assessment of motility, viability and morphology

Figure 1. *Flow diagram of overall experimental design: liquefied semen samples were split into 2 equal portions, the first portion was prepared by double density gradient centrifugation, while the second was prepared by one-step sperm washing. Magnetic-activated cell sorting (MACS) was performed for both portions.*

Isolation of spermatozoa with deteriorated membranes by MACS

The sperm suspensions were passed through a magnetic field (MiniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany), and the spermatozoa were classified as either annexin-positive or annexin-negative based on the binding of the microbeads to their outer surface (Paasch *et al*., 2003).

Briefly, the washed spermatozoa were incubated with 100 µL annexin-V microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) at room temperature for 15 minutes, placed on top of the separation column containing iron balls, which was placed in a magnet. The apoptotic spermatozoa were retained in the separation column and labeled as annexin-positive whereas the spermatozoa with intact membranes passed through the column and were 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 an annexin-binding buffer (Miltenyi *et al*., 1990).

Detection and evaluation of activated caspases

Levels of activated caspases-3 were detected in viable 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 inhibitors were used with the appropriate controls according to the kit instructions provided by the manufacturers (Carboxyfluorescein FLICA, Immunochemistry Technologies, Bloomington, MN).

A 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide (DMSO) and was 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 with the rinse buffer.

Detection 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 transmembrane potential of mitochondria in spermatozoa (ApoAlert Mitosensor Kit ^{TM}, Clontech, CA, USA). 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. Mitosensor was used according to the instructions of the manufacturer. Briefly, all aliquots were incubated at 37°C for 20 min 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 (Paasch *et al*., 2004b).

Phosphatidylserine antibody

Externalisation of phosphatidylserine was examined using a monoclonal mouse anti-human phosphatidylserine antibody, clone 1H6 (Upstate cell signalling solutions, Lake Placid, NY). Spermatozoa were incubated with the phosphatidylserine-antibody at a final concentration of 0.5 µg/mL in PBSB (phosphate buffered saline containing 2% bovine serum albumin) for 20 minutes on ice, followed by addition of 150 µL PBSB and centrifugation 1600 rpm for 5 minutes at 20°C. After discarding the supernatant each sperm pellet was incubated protected from light with 50 µL of secondary antibody (goat anti-mouse IgG [H+L], fluorescein conjugate, Upstate Cell Signalling Solutions, Lake Placid, NY) on ice for 20 minutes. A second washing step in PBSB (1600 rpm for 5 minutes 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 µL PBSB.

Flow cytometric analysis of activated caspases, MMP and EPS

The extent of activated caspase-3, MMP and externalized PS were evaluated by flow cytometry analyses. All the 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 FHL-1 channel and red fluorescence (580–630 nm) in the FHL-2 channel. The percentage of positive cells and the mean fluorescence was calculated on a 1023-channel scale using the flow cytometer software CellQuest™ (version 3.3, BD Biosciences, San Jose, CA).

Statistical analysis

Each sperm parameter was submitted to a one-way analysis of variance with repeated measures. Linear contrasts were used to test the mean differences between treatment groups. Spearman's correlation between sperm parameters were calculated and tested against zero. All tests were two-tailed, and significance is indicated by $p<0.05$. The statistical analysis was done using SAS v 9.0 (SAS Institute, Cary, NC, USA).

RESULTS

Effects of preparations on sperm parameters

Prior to sample preparation, raw semen samples collected from donors exceeded the WHO criteria of normal sperm parameters (concentration: $49.75 \pm 24.12 \times 10^6$/mL; motility: $52.39 \pm 13.27\%$; viability: $65.92 \pm 6.73\%$ and morphology: $32.83 \pm 13.27\%$). The annexin-negative sperm fraction prepared by MACS following density gradient had the highest motility values, which were significantly higher than sperm prepared by density gradient only ($p=0.02$) and annexin-negative sperm prepared by MACS following one-step wash ($p<0.0001$). In addition, sperm prepared by density gradient only had significantly higher motility values when compared with annexin-negative sperm prepared by MACS following one-step wash ($p<0.0001$, Table 1). Sperm viability was also significantly higher in the annexin-negative sperm fraction prepared by MACS following density gradient compared to sperm prepared by density gradient only ($p=0.01$) and annexin-negative sperm prepared by MACS following one-step wash ($p<0.0001$). On the other hand, sperm viability was comparable in fractions prepared by density gradient only and annexin-negative sperm prepared by MACS following one-step wash (Table 1). The annexin-positive fractions separated by MACS following density gradient or following one-step sperm wash had the lowest motility and viability values compared with annexin-negative spermatozoa

and density gradient preparations. No differences in sperm morphology were observed between any of the evaluated sperm fractions (Table 1).

Sperm motility and viability values in fractions prepared by only one-step wash (55.08 ± 12.86 and 66.66 ± 8.73 respectively) were significantly lower than those obtained in spermatozoa prepared by only density gradient (p=0.0002 and p=0.0001 respectively) and annexin-negative sperm fraction prepared by MACS following density gradient (p<0.0001 and p<0.0001 respectively). On the other hand, these values were comparable to annexin-negative sperm fraction prepared by MACS following one-step wash.

Aliquot Number	**Preparation Technique**	**Sperm Fraction**	**Motility** (% Motile)	**Viability** (% Viable)	**Morphology** (% Normal by WHO criteria)	**Morphology** (% Normal by Kruger's criteria)
1	DGC	Mature sperm pellet	74.37 ± 12.32 [2, 3]	75.53 ± 10.77 [2]	21.33 ± 10.02	8.14 ± 3.23
2	DGC + MACS	Annexin-negative	82.52 ± 9.18 [1, 3]	82.91 ± 6.73 [1, 3]	21.36 ± 8.91	8.38± 3.48
3	HTF wash + MACS	Annexin-negative	49.94 ± 19.59 [1, 2]	71.58 ± 11.78 [2]	21.92 ± 8.73	7.75 ± 3.11
4	DGC + MACS	Annexin-positive	10.65 ± 9.65 [1, 2, 3]	30.22 ± 9.67 [1, 2, 3]	20.71 ± 7.14	6.79 ± 2.81
5	HTF wash + MACS	Annexin-positive	7.15 ± 9.02 [1, 2, 3]	22.67 ± 11.64 [1, 2, 3]	18.00 ± 6.93	5.42 ± 2.50

Table 1. *Descriptive statistics of routine sperm parameters assessed in all sperm fractions. Annexin-negative spermatozoa were separated by MACS either following double density gradient centrifugation (DGC) or following one-step wash and re-suspension in human tubal fluid media (HTF). Results are expressed as mean ± standard error; superscripts 1, 2, 3 indicate significantly different when compared to: 1) aliquot 1, 2) aliquot 2 and 3) aliquot 3 (p<0.05 was considered significant using paired t-test).*

Effects of preparations on apoptotic markers

Annexin-negative spermatozoa separated by MACS following density gradient expressed the least amount of apoptosis markers. In this fraction, spermatozoa revealed significantly lower activation of caspase-3 compared to sperm prepared by density gradient only (p=0.003) and annexin-negative spermatozoa prepared by MACS following one-step wash (p=0.05). Similarly, annexin-negative fraction separated by MACS following density gradient had a higher percentage of spermatozoa with intact mitochondria than sperm prepared by density gradient only (p=0.05) and annexin-negative spermatozoa prepared by MACS following one-step wash (p=0.03, Table 2).

The externalization of PS was significantly higher in the annexin-negative spermatozoa prepared by MACS following one-step wash compared to the annexin-negative spermatozoa prepared by MACS following density gradient (p=0.009) and spermatozoa prepared by density gradient only (p=0.004). Annexin-positive fractions separated by MACS following density gradient or following simple one-step wash had the highest expression of apoptotic markers compared to the annexin-negative spermatozoa and density gradient preparations (Table 2).

Aliquot Number	Preparation Technique	Sperm Fraction	Caspase-3 (% Active)	MMP (% Intact)	PS (% Externalized)
1	DGC	Mature sperm pellet	20.75 ± 10.46 [2]	73.63 ± 16.33	4.56 ± 3.30 [3]
2	DGC + MACS	Annexin-negative	6.79 ± 4.87 [1]	83.54 ± 11.50 [3]	4.56 ± 3.92 [3]
3	HTF wash + MACS	Annexin-negative	17.49 ± 16.47	75.15 ± 7.82 [2]	8.90 ± 5.65 [1, 2]
4	DGC + MACS	Annexin-positive	47.43 ± 20.87 [1, 2, 3]	34.94 ± 17.33 [1, 2, 3]	44.00 ± 26.86 [1, 2, 3]
5	HTF wash + MACS	Annexin-positive	49.51 ± 12.93 [1, 2, 3]	31.82 ± 12.62 [1, 2, 3]	40.95 ± 15.37 [1, 2, 3]

Table 2. *Descriptive statistics of apoptotic markers detected in all sperm fractions. Annexin-negative spermatozoa were separated by MACS either following double density gradient centrifugation (DGC) or following one-step wash and re-suspension in human tubal fluid media (HTF). Caspase-3= percentage of sperm with activated caspase-3; MMP=percentage of sperm with intact mitochondria; PS=percentage of sperm with externalized phosphatidylserine. Results are expressed as mean ± standard error, superscripts 1, 2, 3 indicate significantly different when compared to: 1) aliquot 1, 2) aliquot 2 and 3) aliquot 3 ($p<0.05$ was considered significant using paired t-test).*

Correlations of apoptotic markers

The percentage of spermatozoa exhibiting signs of apoptosis such as activated caspase-3 and PS expression had significant negative correlations with sperm motility and viability. Additionally, non-apoptotic spermatozoa with intact mitochondria had significant positive correlations with sperm motility and viability (Table 3). The sperm morphology did not seem to correlate significantly with any of the apoptotic markers. Only a slight correlation was detected between the percentage of sperm with intact mitochondria and the extent of phosphatidylserine externalization with the sperm morphology assessed by the Kruger's criteria.

The apoptotic markers evaluated in our experiment showed significant correlations with each other (Figure 2). The percentage of spermatozoa with activated caspase-3 negatively correlated

with the percentage of spermatozoa with intact mitochondria (r=-0.74, p<0.0001) and positively correlated with the percentage of spermatozoa with externalized phosphatidylserine (r=0.77, p<0.0001). Similarly, the percentage of spermatozoa with intact mitochondria negatively correlated with the percentage of spermatozoa with externalized phosphatidylserine (r=-0.72, p<0.0001).

Sperm parameter	Apoptotic marker	Spearman's r value	p value
Motility (% Motile)	Caspase-3	-0.68	<0.0001
	MMP	0.88	<0.0001
	PS	-0.72	<0.0001
Morphology (% normal by WHO criteria)	Caspase-3	-0.018	0.89
	MMP	0.13	0.32
	PS	-0.20	0.11
Morphology (% normal by Kruger's criteria)	Caspase-3	-0.17	0.23
	MMP	0.28	0.037
	PS	-0.34	0.005
Viability (% Viable)	Caspase-3	-0.74	<0.0001
	MMP	0.86	<0.0001
	PS	-0.64	<0.0001

Table 3. *Correlation relationships between standard sperm parameters and apoptotic markers in all prepared samples; Caspase-3= percentage of sperm with activated caspase-3; MMP=percentage of sperm with intact mitochondrial membrane potential; PS=percentage of sperm with externalized phosphatidylserine. p<0.05 was considered significant.*

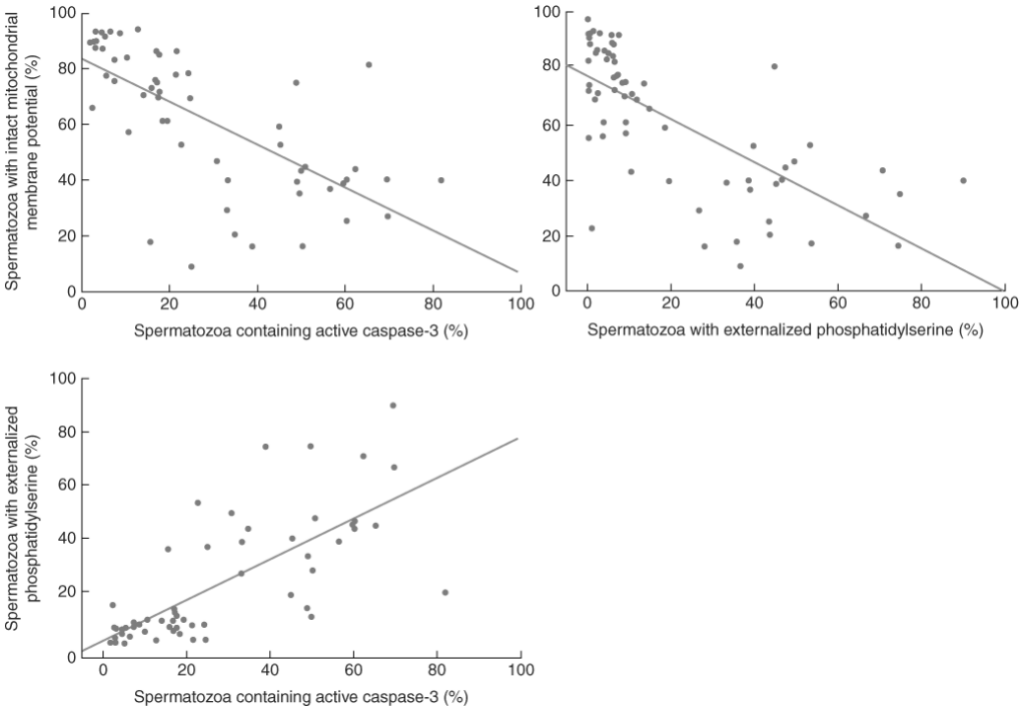


Figure 2. *Correlations of apoptotic markers in various sperm preparations: percentage of sperm with activated caspase-3, percentage of sperm with intact mitochondrial membrane potential (MMP) and percentage of sperm with externalized phosphatidylserine.*

DISCUSSION

Mitochondria are known to play a central role during the execution phase of apoptosis as a decrease in their membrane potential occurs and opening of mitochondrial pores leads to the subsequent release of pro-apoptotic factors (Ravagnan *et al*., 2002). In the cytoplasmic compartment, the pro-apoptotic factors—the caspases family (cysteine proteases)—are subsequently activated, leading to cellular degradation (Thornberry *et al*., 1998). Phosphatidylserine, which is normally sequestered in the plasma membrane inner leaflet, appears in the outer leaflet, where it triggers non-inflammatory phagocytic recognition of the apoptotic cell (Martin *et al*., 1995). Annexin-V has a high affinity for PS, and cannot pass through an intact sperm membrane. Therefore, annexin-V binding to spermatozoa denotes that the integrity of the membrane has been disturbed (Glander *et al*., 2002).

Superparamagnetic microbeads can effectively separate cells. The beads may be used for immunomagnetic separation of membrane-intact and non-apoptotic spermatozoa (Glander *et al*., 2002, Paasch *et al*., 2003). The superparamagnetic annexin V- conjugated microbeads

were previously reported to eliminate spermatozoa with externalized PS (apoptotic cells) and disintegrated plasma membranes from cryopreserved semen samples (Grunewald *et al*., 2001, Paasch *et al*., 2004a, Paasch *et al*., 2004b).

In our study, we attempted to evaluate the effect of integrating MACS in sperm preparation protocols on sperm quality. MACS, when performed after density gradient centrifugation, resulted in the separation of a sperm population that displayed the highest quality, which was reflected by higher motility and viability values as well as lower expression of apoptotic markers. The results obtained were significantly different compared with the values detected in sperm prepared by density gradient only – a standard sperm preparation technique. This implies that the current standard protocols for sperm preparation can still be improved by technical additions such as MACS.

We have previously reported that apoptotic markers are more evident in immature sperm, which is increased in annexin-positive fraction separated by MACS (Paasch *et al*., 2004b). In our current experiment we have only used the pellet resulting from double density gradient centrifugation for the processing of half of each semen sample. Although this approach eliminates the majority of immature spermatozoa, still some spermatozoa showing signs of immaturity could be included. The magnetic separation process was in our study however based on the externalization of phosphatidylserine to the outer layer of sperm membrane; which could be manifested in apoptotic as well as immature sperm. Therefore, our separation protocol (double density gradient followed by MACS) can effectively eliminate the presence poor quality spermatozoa (either apoptotic or immature).

The one-step wash technique was the least effective method for sperm preparation in terms of motility and viability values. In addition, MACS following one-step wash did not make any significant contribution to the sperm quality. The annexin-negative (non-apoptotic) sperm separated by MACS following one-step wash had lower quality than those prepared by density gradient combined with MACS in all the assessed parameters and even compared to those prepared by density gradient only in terms of motility and PS externalization. Therefore, it appears that one-step wash is not an optimal option for sperm preparation regardless of the MACS integration. One limitation of our study is the lack of assessment of apoptotic markers in spermatozoa prepared by one-step wash only, which was due to the lack of a sufficient number of cells to conduct the assays.

We were able to detect significant correlations between the presence of apoptotic markers such as caspase activation, mitochondrial membrane potential and externalization of PS and the sperm quality in terms of motility and viability values, which is consistent with reported findings (Liu *et al*., 2004, Marchetti *et al*., 2002, Pena *et al*., 2003, Shen *et al*., 2002, Weng *et al*., 2002). In addition, we also found that all 3 apoptotic markers correlated well with each other in

spermatozoa, which is also consistent with previous reports from our group (Paasch *et al.*, 2004a, Paasch *et al.*, 2003) and those of other researchers (Marchetti *et al.*, 2004).

In the context of male reproduction, apoptosis controls the overproduction of male gametes. Animal studies have shown that apoptosis is a key regulator of spermatogenesis in normal and pathological conditions (Furuchi *et al.*, 1996). A number of studies have documented the implication of deregulated apoptosis in the pathogenesis of male infertility (Oosterhuis *et al.*, 2000, Sakkas *et al.*, 2003). Most important, there is a likelihood that some sperm selected for ART will display features of apoptosis despite their normal appearance, which may impact their fertilization potential (Lopes *et al.*, 1998, Sakkas *et al.*, 2004, Tesarik *et al.*, 2004).

Our results clearly indicate that integrating MACS as a part of sperm preparation techniques is advantageous and eliminates apoptotic sperm in donor samples characterized by normal sperm parameters. The combination of density gradient centrifugation and MACS is superior to other sperm preparation methods in terms of providing motile, viable and non-apoptotic spermatozoa. The significance of our study lies in separating non-apoptotic sperm, which is expected to enhance the efficiency of ART. Future research should be directed towards the validation of these findings using samples from infertile male patients characterized by poor sperm quality. In addition, the fertilization potential of the non-apoptotic spermatozoa separated by MACS remains to be assessed.

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