Article

Advantage of combining magnetic cell separation with sperm preparation techniques

Professor Hans-Jürgen Glander completed his studies in biochemistry in 1972 and in medicine in 1974 at the University of Leipzig, Germany. Since 1993, he has been a full Professor in Andrology and the Head of the Department of Andrology at the University of Leipzig, which has been certified as a Training Centre of the European Academy of Andrology since 1996. Professor Glander was among the first to describe annexin binding to human spermatozoa. Recently, he and his team investigated apoptotic signal transduction, lipid changes at the membrane, and related sperm selection methods in close co-operation with the group lead by Professor Ashok Agarwal at The Cleveland Clinic Foundation, Cleveland, Ohio, USA.

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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 the present study was to optimize the integration of magnetic cell sorting in standard sperm preparations 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. The 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. This study clearly shows 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.

Keywords: annexin, caspase, magnetic-activated cell sorting, mitochondrial membrane potential, phosphatidylserine, sperm preparation

Introduction

Assisted reproductive techniques 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 and Schill, 2003). The technique of double density gradient centrifugation has great potential in sperm preparation for assisted reproduction (Chen and Bongso, 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 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 assisted reproduction.

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 and Schaller, 1999; Weng et al., 2002; Paasch et al., 2003, 2004b).

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 (Miltenyi et al., 1990; Grunewald et al., 2001; Paasch et al., 2003, 2004a).

MACS separation according to the externalization of PS results in the extraction of apoptotic spermatozoa 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 assisted reproduction. The objective of this 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 two equal portions. The first portion was prepared by double density gradient centrifugation (PureCeption®; Sage BioPharma, Bedminster, NJ, USA). In brief, samples were loaded onto a 40 and 80% discontinuous gradient and centrifuged at 300 g for 20 min at room temperature. The resulting 80% pellet, representing the mature fraction, was washed by centrifugation for an additional 7 min and re-suspended in human tubal fluid media (HTF; Irvine Scientific, Santa Ana, CA, USA). The sperm cell suspension was further divided into two 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). 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, USA). The different steps of the experimental design are illustrated in Figure 1.

![Flow diagram of overall experimental design](Image)

**Figure 1.** Flow diagram of overall experimental design: liquefied semen samples were split into two 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) at room temperature for 15 min, 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 labelled as annexin-positive, whereas the spermatozoa with intact membranes passed through the column and were labelled 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 caspase-3 were detected in viable spermatozoa using fluorescein-labelled inhibitor of caspase (FLICA), which is cell permeable, non-cytotoxic, and binds covalently to active caspase-3 (Ecket al., 1999). The inhibitors were used with the appropriate controls according to the kit instructions provided by the manufacturers (carboxyfluorescein FLICA; Immunochemistry Technologies, Bloomingston, MN, USA).

A 150-fold stock solution of the inhibitor was prepared in dimethyl sulphoxide (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 h 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; Clontech, Pasadena, 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 spermatozoa with a disrupted mitochondrial membrane. The 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

Externalization of phosphatidylserine was examined using a monoclonal mouse anti-human phosphatidylserine antibody, clone 1H6 (Upstate Cell Signalling Solutions, Lake Placid, NY, USA). 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 min on ice, followed by addition of 150 µl PBSB and centrifugation 300 g for 5 min 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] on ice for 20 min. A second washing step in PBSB (300 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 µl PBSB.

Flow cytometric analysis of activated caspases, MMP and externalized PS

The extent of activated caspase-3, MMP and externalized PS were evaluated by flow cytometry analyses. All the fluorescence signals of labelled spermatozoa were analysed by the flow cytometer FACSscan (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 spermatozoa was examined for each assay at a flow rate of <100 cells/s. 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 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, USA).

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 performed using SAS v9.0 (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 ± 24.12 × 10^6/ml; motility: 52.39 ± 13.27%; viability: 65.92 ± 6.73% and morphology: 32.83 ± 13.27%). The annexin-negative sperm fraction prepared by MACS following density gradient had the highest motility values, which were significantly higher than spermatozoa prepared by density gradient only (P = 0.02) and annexin-negative spermatozoa prepared by MACS following one-step wash (P < 0.0001). In addition, spermatozoa prepared by density gradient only had significantly higher motility values when compared with annexin-negative spermatozoa 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 with spermatozoa prepared by density gradient.
only \(P = 0.01\) and annexin-negative spermatozoa 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 spermatozoa prepared by MACS following one-step wash \(\text{Table 1}\).

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 \(\text{Table 1}\).

Sperm motility and viability values in fractions prepared by only one-step wash \(55.08 \pm 12.86\) and \(66.66 \pm 8.73\) 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.

**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 showed significantly lower activation of caspase-3 compared with spermatozoa 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 spermatozoa prepared by density gradient only \(P = 0.05\) and annexin-negative spermatozoa prepared by MACS following one-step wash \(P = 0.03\), \(\text{Table 2}\).

The externalization of PS was significantly higher in the annexin-negative spermatozoa prepared by MACS following one-step wash compared with the annexin-negative spermatozoa prepared by MACS following density gradient \(P = 0.009\) and spermatozoa prepared by density gradient only \(P = 0.05\).

**Table 1.** Descriptive statistics of routine sperm parameters assessed in all sperm fractions. Annexin-negative spermatozoa were separated by magnetic-activated cell sorting (MACS) either following double density gradient centrifugation (DGC) or following one-step wash and re-suspension in human tubal fluid media (HTF).

<table>
<thead>
<tr>
<th>Aliquot no.</th>
<th>Preparation technique</th>
<th>Sperm fraction</th>
<th>Motility (% motile)</th>
<th>Viability (% viable)</th>
<th>Morphology (% normal by WHO criteria)</th>
<th>Morphology (% normal by Kruger's criteria)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DGC</td>
<td>Mature sperm pellet</td>
<td>74.37 ± 12.32abc</td>
<td>75.53 ± 10.77b</td>
<td>21.33 ± 10.02</td>
<td>8.14 ± 3.23</td>
</tr>
<tr>
<td>2</td>
<td>DGC + MACS</td>
<td>Annexin-negative</td>
<td>82.52 ± 9.18abc</td>
<td>82.91 ± 6.73abc</td>
<td>21.36 ± 8.91</td>
<td>8.38 ± 3.48</td>
</tr>
<tr>
<td>3</td>
<td>HTF wash + MACS</td>
<td>Annexin-negative</td>
<td>49.94 ± 19.59ab</td>
<td>71.58 ± 11.78b</td>
<td>21.92 ± 8.73</td>
<td>7.75 ± 3.11</td>
</tr>
<tr>
<td>4</td>
<td>DGC + MACS</td>
<td>Annexin-positive</td>
<td>10.65 ± 9.65ab,abc</td>
<td>30.22 ± 9.67ab,abc</td>
<td>20.71 ± 7.14</td>
<td>6.79 ± 2.81</td>
</tr>
<tr>
<td>5</td>
<td>HTF wash + MACS</td>
<td>Annexin-positive</td>
<td>7.15 ± 9.02ab,abc</td>
<td>22.67 ± 11.64ab,abc</td>
<td>18.00 ± 6.93</td>
<td>5.42 ± 2.50</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; superscripts a, b, c indicate significantly different values when compared with aliquot 1 (a), aliquot 2 (b), and aliquot 3 (c). \(P < 0.05\) was considered significant using paired \(t\)-test.

**Table 2.** Descriptive statistics of apoptotic markers detected in all sperm fractions. Annexin-negative spermatozoa were separated by magnetic-activated cell sorting (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 spermatozoa with activated caspase-3; MMP = percentage of spermatozoa with intact mitochondrial membrane potential; PS = percentage of spermatozoa with externalized phosphatidylserine.

<table>
<thead>
<tr>
<th>Aliquot no.</th>
<th>Preparation technique</th>
<th>Sperm fraction</th>
<th>Caspase-3 (% active)</th>
<th>MMP (% intact)</th>
<th>PS (% externalized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DGC</td>
<td>Mature sperm pellet</td>
<td>20.75 ± 10.46b</td>
<td>73.63 ± 16.33</td>
<td>4.56 ± 3.30c</td>
</tr>
<tr>
<td>2</td>
<td>DGC + MACS</td>
<td>Annexin-negative</td>
<td>6.79 ± 4.87a</td>
<td>83.54 ± 11.50c</td>
<td>4.56 ± 3.92c</td>
</tr>
<tr>
<td>3</td>
<td>HTF wash + MACS</td>
<td>Annexin-negative</td>
<td>17.49 ± 16.47</td>
<td>75.15 ± 7.82b</td>
<td>9.70 ± 5.65ab,abc</td>
</tr>
<tr>
<td>4</td>
<td>DGC + MACS</td>
<td>Annexin-positive</td>
<td>47.43 ± 20.87ab,abc</td>
<td>34.94 ± 17.33ab,abc</td>
<td>44.00 ± 26.86ab,abc</td>
</tr>
<tr>
<td>5</td>
<td>HTF wash + MACS</td>
<td>Annexin-positive</td>
<td>49.51 ± 12.93ab,abc</td>
<td>31.82 ± 12.62ab,abc</td>
<td>40.95 ± 15.37ab,abc</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; superscripts a, b, c indicate significantly different values when compared with aliquot 1 (a), aliquot 2 (b), and aliquot 3 (c). \(P < 0.05\) was considered significant using paired \(t\)-test.
= 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 with the annexin-negative spermatozoa and density gradient preparations (Table 2).

**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 ($P < 0.0001$). Additionally, non-apoptotic spermatozoa with intact mitochondria had significant positive correlations with sperm motility and viability ($P < 0.0001$, 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 spermatozoa with intact mitochondria and the extent of phosphatidylserine externalization with the sperm morphology assessed by Kruger’s criteria.

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

<table>
<thead>
<tr>
<th>Sperm parameter</th>
<th>Apoptotic marker</th>
<th>Spearman's r-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (% motile)</td>
<td>Caspase-3</td>
<td>-0.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>MMP</td>
<td>0.88</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>-0.72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Morphology (% normal by WHO criteria)</td>
<td>Caspase-3</td>
<td>-0.018</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>MMP</td>
<td>0.13</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>-0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Morphology (% normal by Kruger’s criteria)</td>
<td>Caspase-3</td>
<td>-0.17</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>MMP</td>
<td>0.28</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>-0.34</td>
<td>0.005</td>
</tr>
<tr>
<td>Viability (% viable)</td>
<td>Caspase-3</td>
<td>-0.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>MMP</td>
<td>0.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>-0.64</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

$P < 0.05$ was considered significant. NS = not significant.

**Figure 2.** Correlations of apoptotic markers in various sperm preparations: percentage of spermatozoa with activated caspase-3, percentage of spermatozoa with intact mitochondrial membrane potential (MMP) and percentage of spermatozoa with externalized phosphatidylserine.
The apoptotic markers evaluated in this 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 \((r = -0.72, P < 0.0001)\).

**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 caspase family (cysteine proteases), are subsequently activated, leading to cellular degradation (Thornberry and Lazebnik, 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,b).

In this study, it was 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.

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

It was possible 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 \((P < 0.0001)\), which is consistent with reported findings (Marchetti et al., 2002; Shen et al., 2002; Weng et al., 2002; Pena et al., 2003; Liu et al., 2004). In addition, it was also found that all three apoptotic markers correlated well with each other in spermatozoa, which is consistent with previous reports from our group (Paasch et al., 2003, 2004a) 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, it is likely that some spermatozoa selected for assisted reproduction will display features of apoptosis despite their normal appearance, which may impact upon their fertilization potential (Lopes et al., 1998; Sakkas et al., 2004; Tesarik et al., 2004).

These results clearly indicate that integrating MACS as a part of sperm preparation techniques is advantageous and eliminates apoptotic spermatozoa 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 this study lies in separating non-apoptotic spermatozoa, which is expected to enhance the efficiency of assisted reproduction. 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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