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# Correlation between aneuploidy, apoptotic markers and DNA fragmentation in spermatozoa from normozoospermic patients

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**Abstract** Genetic and biochemical sperm integrity is essential to ensure the reproductive competence. However, spermatogenesis involves physiological changes that could endanger sperm integrity. DNA protamination and apoptosis have been studied extensively. Furthermore, elevated rates of aneuploidy and DNA injury correlate with reproductive failures. Consequently, this study applied the conventional spermiogram method in combination with molecular tests to assess genetic integrity in ejaculate from normozoospermic patients with implantation failure by retrospectively analysing aneuploidy (chromosomes 18, X, Y), DNA fragmentation, externalization of phosphatidylserine and mitochondrial membrane potential status before and after magnetic activated cell sorting (MACS). Aneuploid, apoptotic and DNA-injured spermatozoa decreased significantly after MACS. A positive correlation was detected between reduction of aneuploidy and decreased DNA damage, but no correlation was determined with apoptotic markers. The interactions between apoptotic markers, DNA integrity and aneuploidy, and the effect of MACS on these parameters, remain unknown. In conclusion, use of MACS reduced aneuploidy, DNA fragmentation and apoptosis. A postulated mechanism relating aneuploidy and DNA injury is discussed; on the contrary, cell death markers could not be related. An 'apoptotic-like' route could explain this situation.



**KEYWORDS:** aneuploidy, apoptosis, cell death, DNA fragmentation, MACS, spermatozoa

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## Introduction

Nowadays, molecular tests are being requested to complete the andrological evaluation and evaluate sperm DNA integrity. In this context, apoptotic markers such as caspase activation, externalization of phosphatidylserine (ePS), alteration of mitochondrial membrane potential ($\Delta\psi_m$) and DNA fragmentation are the most common markers tested. Particularly, there are increasing clinical data showing a negative correlation between elevated DNA fragmentation index (DFI) and assisted reproduction outcomes (Borini et al., 2006; Bungum et al., 2004, 2007; Duran et al., 2002; Evenson and Wixon, 2006; Larson et al., 2000; Larson-Cook et al., 2003; Meseguer et al., 2011; Muriel et al., 2006a,b; Simon et al., 2011). Several clinical indications have been proposed: idiopathic prolonged infertility (Sakkas and Alvarez, 2010), poor embryo quality (Benchaib et al., 2007; Zini et al., 2005), recurrent miscarriage (Benchaib et al., 2007; Borini et al., 2006; Carrell et al., 2003; Khadem et al., 2014; Robinson et al., 2012; Zini et al., 2008), advanced paternal age (Vagnini et al., 2007; Wyrobek et al., 2006), varicocele and other inflammatory diseases (Mieusset and Bujan, 1995; Smith et al., 2006) and fever episodes (Sergerie et al., 2007).

On the other hand, the aneuploidy rate of spermatozoa measured by fluorescent in-situ hybridization (FISH) is another clinical marker evaluated in infertile males. Direct correlation has been reported between high levels of sperm aneuploidy and poor IVF outcomes. In this context, abnormal FISH results have been strongly associated with oligozoospermia (Calogero et al., 2001; Durak Aras et al., 2012; Finkelstein et al., 1998; Martin et al., 2003; McAuliffe et al., 2012; Mougou-Zerelli et al., 2011; Rives et al., 1999; Sarrate et al., 2010; Shi et al., 2001; Tempest et al., 2010; Vegetti et al., 2000) but there is not a clear correlation with asthenozoospermia or teratozoospermia (Brahem et al., 2012; Calogero et al., 2001; Mehdi et al., 2012; Mougou-Zerelli et al., 2011; Rives et al., 1999; Rubio et al., 2001; Sarrate et al., 2010; Shi et al., 2001; Tempest et al., 2010). Furthermore, pathological aneuploidy rates have been correlated with implantation failure, spontaneous abortion (Burrello et al., 2003; Carrell et al., 2003; Nicopoullos et al., 2008; Petit et al., 2005; Rubio et al., 2001; Vialard et al., 2008) and previous aneuploid conceptions (Blanco et al., 1998; Carrell et al., 2001; Martínez-Pasarell et al., 1999). Some authors have postulated a causal connection between aneuploidy and DNA integrity. Increased aneuploidy levels have been observed in spermatozoa with fragmented DNA in infertile patients with abnormal semen parameters (Brahem et al., 2012; Muriel et al., 2007; Perrin et al., 2011a,b). Muriel et al. (2007) suggested a possible causative effect of aneuploidy on DNA integrity leading to DNA-fragmented spermatozoa during sperm maturation. However, little is known concerning the biological basis of this correlation. On the contrary, other authors have not found correlations (Balasuriya et al., 2011; Bronet et al., 2012). Additionally, the simultaneous relationship between apoptotic markers, aneuploidy and DNA fragmentation remains to be elucidated.

Considering the link between poor assisted reproduction results and high levels of sperm injury, different strategies have been suggested to select healthy spermatozoa: testicular sperm extraction (Moskovtsev et al., 2010), selection of nonvacuolated spermatozoa using high magnification (intracytoplasmic morphologically selected sperm injection; Hammoud et al., 2013), preselection of non fragmented spermatozoa for intracytoplasmic sperm injection (ICSI; Jakab et al., 2005; Vozdova et al., 2012) and the combination of density-gradient centrifugation (DGC) with magnetic activated cell sorting (MACS) (Said et al., 2006, 2008). The combination of ICSI and MACS-selected spermatozoa has been used successfully (Dirican et al., 2008; Polak de Fried and Denaday, 2010; Rawe et al., 2010).

The aims of this study were two-fold. First, the postulated correlation between aneuploidy and ploidy rates, DNA fragmentation and early apoptotic markers such as ePS and $\Delta\psi_m$ was retrospectively analysed in ejaculated spermatozoa from infertile males. Secondly, the possible effects of the use of MACS on sperm parameters was investigated.

## Materials and methods

### Patient recruitment

This prospective study included patients from the Assisted Reproduction Medical Centre CREA's fertility programme. In this programme, all males from couples with at least two previous idiopathic implantation failures received an evaluation of sperm aneuploidy by FISH and DFI by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL). Specifically, 57 patients from this group with normozoospermic semen parameters (WHO 2010 criteria; WHO Department of Reproductive Health and Research, 2010), normal blood karyotype and pathological values of sperm aneuploidy and DFI were contacted. All patients were visited by an experienced andrologist to assess the existence of any infectious, inflammatory, autoimmune, endocrine or transmissible diseases or ultrasound alterations. These criteria, especially the inclusion of only normozoospermic patients, permitted this work to discard confounding factors that could mask the analysis of the FISH and/or DFI test results, and the presence of high numbers of spermatozoa in the ejaculates allowed all of the scheduled experiments to be performed on all of the samples.

Six normozoospermic patients were included after signing the consent. The age range was 38—52 years (mean ± standard deviation, 44.3 ± 5.85 years). The study was approved by the Institutional Research Ethics Committee and was supported by the Health Authority of Local Government included in the Official Program for the Promotion of Clinical Research (project reference AP-203/11, approved 27 September 2011).

### Study design

Participants attended CREA's andrology laboratory on the date of sample collection. Semen samples were obtained by masturbation on site after 2—3 days of sexual abstinence (WHO, 2010). Routine semen parameters were analysed following WHO guidelines. Two aliquots were taken from each sample, one for aneuploidy determination by FISH of chromosomes X, Y and 18 (aliquot no. 1) and one for DGC (aliquot 2). A schematic representation of the study design

is presented in Figure 1. After DGC, aliquot 2 was subdivided to study aneuploidy, $\Delta\psi_m$, ePS and DFI and to apply MACS. The fraction eluted after MACS was divided into aliquots to study aneuploidy, $\Delta\psi_m$, ePS and DFI.

## DGC and MACS

Samples for DGC were prepared using EDTA-free medium (Isolate; Irvine Scientific, CA, USA) at 300*g* for 20 min. The pellet from lower fraction was resuspended in binding buffer (Annexin-V Microbead kit; Miltenyi Biotec, Germany) and centrifuged at 500*g* for 10 min.

MACS was performed using a previously published protocol (Rawe et al., 2010). Basically, a cell suspension was incubated in the dark and at room temperature for 20 min with annexin-V conjugated microbeads (Annexin-V Microbead kit). The quantity of beads was adjusted to 100 μl for each $10 \times 10^6$ cells, according to the manufacturer's instructions. After incubation, cell were eluted using a magnetic column (Miltenyi Biotec, Germany).

## Aneuploidy and diploidy assessment

Semen samples were washed in phosphate-buffered saline (PBS; Gibco; Life Technologies, Madrid, Spain) with 0.1% polyvinyl alcohol (PVA; Sigma–Aldrich, Madrid, Spain), fixed in Carnoy's solution (methanol/acetic acid, 3:1; Merck, Madrid, Spain) and air dried. For sperm membrane permeabilization and nuclei decondensation, fixed samples were washed twice in 2× SSC (Gibco) for 3 min, dehydrated in an ethanol series (70%, 90%, 100%) for 1 min each, air dried and incubated in 5 mmol dithiothreitol with 0.1% Triton X-100 (Sigma–Aldrich) for 15 min at 37°C. The, slides were washed twice in 2× SSC, dehydrated for 1 min and air dried.

Hybridization with centromeric probes for chromosomes X, Y and 18 was performed using a commercial kit (AneuVysion; Vysis, Abbott Laboratories, USA). Decondensed and permeabilized slides were denatured (75°C for 4 min) and hybridized (37°C for 4 h) using the Thermobryte Slide Processing System (Iris International, USA). Post-hybridization washes were performed to remove unbound DNA probe, according to the manufacturer's instructions. Finally, slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI-II; Abbott Laboratories).

Triple-colour FISH analysis was carried out using an automated system. Scanning, relocation, capture and fluorescence analysis was performed using a Imager.Z2 fluorescence microscope (Zeiss, Germany) connected to a Metafer-Metacyte system (Metasystems International, Germany). The functionality of the software for evaluating sperm nuclei has been previously validated (Carrell and Emery, 2008). Cells with abnormal scores were manually reanalysed by an experienced technician applying strict criteria (Blanco et al., 1996). In brief, the accepting criteria were hybridization efficiency >95%, intact nuclei of similar size, and clearly located, nonoverlapping, similarly intense and nonfragmented signals. Disomy was defined as two clearly separated signals (at least one signal distance apart) for the same chromosome. Diploidy was defined as two signals from gonosomes and two signals from chromosome 18 in the same nucleus. Nullisomy was defined as no signal for one chromosome and a signal for the other simultaneously analysed chromosome.

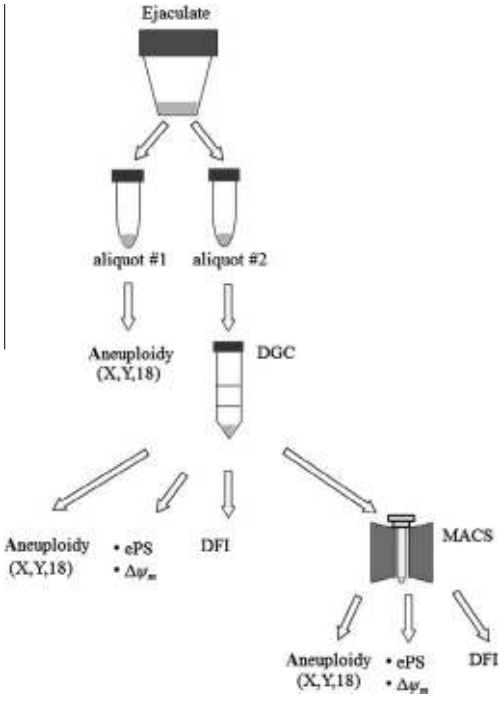


**Figure 1** Study design. DGC, density-gradient centrifugation; ePS, externalized phosphatidylserine; $\Delta\psi_m$, mitochondrial membrane potential; DFI, DNA fragmentation index; MACS, magnetic activated cell sorting.

## Apoptotic markers analysis

Multichannel flow cytometry was used to evaluate apoptotic cell status in both post-DGC and post-MACS fractions. To interrogate intact cells, ePS was detected with a combined annexin-V/propidium iodide (PI) assay (Invitrogen, Life Teccnologies, USA). Disruption of $\Delta\psi_m$ was assessed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide dye (Mitoprobe JC-1 Assay Kit; Life Technologies, USA). DFI was calculated by TUNEL.

### Annexin-V/PI staining

Annexin-V is a protein with a high affinity for PS. Detection of fluorescein isothiocyanate-conjugated annexin-V (Miltenyi Biotec) permitted the identification of ePS on the outer layer of the plasma membrane. Cell permeability to PI was used to discriminate necrotic cells. The combination of detection of ePS with PI permeability allowed the differentiation of sperm subpopulations according to grades of vitality and apoptotic stage: i.e. necrotic ($PI^+/ePS^-$ and $PI^+/ePS^+$), early apoptotic ($PI^-/ePS^+$) and intact ($PI^-/ePS^-$) subpopulations.

### JC-1 mitochondrial membrane potential assay

Alteration of $\Delta\psi_m$ was determined using JC-1 dye, which selectively penetrates and accumulates in mitochondria depending on their membrane potential. With intact (healthy) cells with high $\Delta\psi_m$, JC-1 has an aggregate conformation in the mitochondrial matrix visible by intense red

fluorescence. With apoptotic cells, with altered $\Delta\psi_m$, JC-1 has a monomeric form and shows only green fluorescence. Flow cytometry was used to analyse the ratio of green to red fluorescence allowing comparison of membrane potential and differentiation between intact (nondisrupted $\Delta\psi_m$: $\Delta\psi_m^-$) and unhealthy (disrupted $\Delta\psi_m$: $\Delta\psi_m^+$) cell populations.

Annexin-V/PI and JC-1 assays were performed according to manufacturers' instructions. In brief, sample concentration was adjusted to $1 \times 10^6$ total cells. After washing in 1 ml binding buffer and centrifugation at 300*g* for 10 min, pellets were resuspended in 100 μl binding buffer. For the annexin-V/PI assay, 10 μl fluorescein isothiocyanate-conjugated annexin-V was added and for the JC-1 assay, the final concentration of JC-1 was 10 μg/ml. Samples were incubated for 15 min in the dark, pelleted and washed twice and then the final pellets were resuspended in 500 μl binding buffer. For annexin-V/PI assay, 5 μl PI was added immediately prior to fluorescence detection by flow cytometry. Negative controls were used to assess samples' autofluorescence emissions. All samples were analysed using a MACSQuant VYB Analyzer with an argon laser operating at 488 nm (Miltenyi Biotec). A total of 50,000 events/μl were experienced in each parameter. Data were then recorded and processed with MACS Quantify software (Milteny Biotec).

### TUNEL

The presence of DNA single- and double-strand breaks was evaluated by TUNEL using an *In situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, USA). TUNEL was chosen because it quantifies direct cellular DNA breakage and does not require denaturation, unlike indirect methods such as the sperm chromatin structure assay, which determines the extent of cellular DNA denaturation more than real DNA breakages. Samples were prepared for staining as previously described (Domínguez-Fandos et al., 2007). Briefly, semen samples were washed in PBS/PVA before fixation (4% formaldehyde in PBS) and permeabilization (Triton X-100 in 0.1% sodium citrate in PBS). Pellets were resuspended in 50 μl staining solution containing TdT for 1 h at room temperature. Negative and positive controls were performed. Two rounds of washing and centrifugation were done before flow cytometry.

## Statistical analysis

Data were analysed using the R statistical platform (http://cran.r-project.org/). The nonparametric test Wilcoxon signed rank one tail for paired samples was employed for statistical contrast of aneuploidy and disomy values between fresh, post-DGC and post-MACs fractions. Relationships between DFI, ePS and $\Delta\psi_m$ and aneuploidy was studied using Pearson's correlation coefficient. Statistical significance was defined as $P < 0.05$.

## Results

The study population included six patients. Individual sperm parameters and age are shown in Table 1.

## Aneuploidy and diploidy assessment

Triple-colour FISH was performed on each aliquot analysed (Figure 1). Three aliquots were tested: fresh, post-DGC and post-MACS. A total of 84,908 spermatozoa were evaluated for aneuploidy and diploidy in chromosomes 18, X and Y. For each patient, 14,151 ± 2721 spermatozoa (mean ± standard deviation) were assessed.

The results are summarized in Table 2. A statistically significant decrease in total aneuploidy was detected between fresh and post-DGC samples (7.10 ± 2.92 versus 5.13 ± 2.22) and between post-DGC and post-MACS samples (5.13 ± 2.22 versus 4.22 ± 2.52; both $P < 0.05$). The same results were observed in chromosome 18 total aneuploidy decrease between fresh and post-DGC (2.69 ± 1.59 versus 1.66 ± 1.81) and between post-DGC and post-MACS samples (1.66 ± 1.81 versus 1.37 ± 1.91; both $P < 0.05$). Chromosome 18 nullisomy significantly decreased between fresh and post-DGC samples (2.30 ± 1.54 versus 1.42 ± 1.79) and between post-DGC and post-MACS samples (1.42 ± 1.79 versus 1.15 ± 1.91; both $P < 0.05$). Chromosome 18 disomy significantly decreased between fresh and post-MACS samples (0.39 ± 0.26 versus 0.22 ± 0.13; $P < 0.05$). Concerning the X and Y chromosomes, no statistically differences were established for nullisomy; however, there was a statistically significant decrease in disomy between fresh and post-MACS samples (0.63 ± 0.23 versus 0.40 ± 0.19; $P < 0.05$). Finally, a significant decrease in total diploidy was detected between

**Table 1** Individual sperm parameters.

| *Patient* | *Age (years)* | *Ejaculated volume (ml)* | *Sperm concentration (million/ml)* | *Progressive motility (%)* | *Normal morphology (%)* |
|---|---|---|---|---|---|
| 1 | 38 | 2.9 | 95 | 59 | 10 |
| 2 | 52 | 2.1 | 71 | 54 | 7 |
| 3 | 48 | 2.1 | 63 | 37 | 8 |
| 4 | 38 | 2.4 | 48 | 33 | 4 |
| 5 | 42 | 1.9 | 53 | 42 | 8 |
| 6 | 48 | 1.8 | 80 | 51 | 11 |
| Overall[a] | 44.3 ± 5.9 | 2.2 ± 0.4 | 68.3 ± 17.5 | 46.0 ± 10.2 | 8.0 ± 2.5 |

[a]Values are mean ± standard deviation.

**Table 2** Sperm aneuploidy and diploidy for chromosomes X, Y and 18.

| *Chromosome* | *Fresh* | *Post-DGC* | *Post-MACS* |
|---|---|---|---|
| Chromosome 18 | | | |
| Nullisomy | 2.30 ± 1.54[a] | 1.42 ± 1.79[b] | 1.15 ± 1.91[c] |
| Disomy | 0.39 ± 0.26[a] | 0.25 ± 0.08[a,b] | 0.22 ± 0.13[b] |
| Aneuploidy | 2.69 ± 1.59[a] | 1.66 ± 1.81[b] | 1.37 ± 1.91[c] |
| Sex chromosomes | | | |
| Nullisomy | 1.86 ± 1.50 | 0.93 ± 0.97 | 1.26 ± 0.74 |
| Disomy | 0.63 ± 0.23[a] | 0.75 ± 0.62[a,b] | 0.40 ± 0.19[b] |
| Aneuploidy | 2.49 ± 1.66 | 1.67 ± 0.7 | 1.66 ± 0.78 |
| Diploidy | 1.91 ± 0.75[a] | 1.79 ± 1.11[a,b] | 1.19 ± 0.48[b] |
| Total aneuploidy | 7.10 ± 2.92[a] | 5.13 ± 2.22[b] | 4.22 ± 2.52[c] |

Values are mean ± standard deviation.
Different superscript letters within a row indicate statistically significant differences ($P < 0.05$).
Aneuploidy = nullisomy plus disomy; DGC = density-gradient centrifugation; MACS = magnetic activated cell sorting.

fresh and post-MACS samples (1.91 ± 0.75 versus 1.19 ± 0.48; $P < 0.05$). Figure 2 shows the gradual decrease in aneuploidy and diploidy during the processing of samples.

## ePS, $\Delta\psi_m$ and DNA fragmentation

Measures of ePS and $\Delta\psi_m$ revealed an increased rate of intact spermatozoa post-MACS (Figure 3). Specifically, the intact ($PI^-/ePS^-$) and nondisrupted $\Delta\psi_m$ ($\Delta\psi_m^-$) subpopulations increased significantly after MACS (15.79 ± 17.89 versus 78.72 ± 25.26 and 23.82 ± 27.70 versus 63.53 ± 26.21; both $P < 0.05$; Table 3). TUNEL revealed a significant decrease in DFI post-MACS (75.77 ± 14.73; 2.84 ± 3.09; $P < 0.05$).

## Correlation analysis of aneuploidy, apoptotic markers and DNA fragmentation

Correlation analysis detected relationships between the increase/decrease in each parameter and MACS. Decrease in total aneuploidy was correlated with decrease in DFI ($R = 0.75$; $P < 0.05$). Weak inverse correlations were detected between aneuploidy and the increase in the intact subpopulation: $PI^-/ePS^-$ and $\Delta\psi_m^-$ ($R = -0.43$ and $R = -0.36$; both $P < 0.05$). In these cases, $R$ could be expressed as absolute values (Table 4). Concerning DNA fragmentation and apoptotic markers, no correlation was found between DFI and $PI^-/ePS^-$ ($R = -0.04$; $P < 0.05$) but a weak correlation was observed between DFI and $\Delta\psi_m^-$ ($R = -0.39$; $P < 0.05$). No correlation was found between $PI^-/ePS^-$ and $\Delta\psi_m^-$ ($R = 0.09$; $P < 0.05$).

## Discussion

This study observed a clear reduction on aneuploidy rates and fragmented DNA in spermatozoa after DGC and MACS in normozoospermic patients with previous IVF failures, abnormal FISH results and fragmented sperm DNA. Conversely, the presence of early apoptotic markers showed no clear correlation with aneuploidy or DNA fragmentation. These results are in agreement with data from a previous report in which a significant increase in aneuploidy in spermatozoa with fragmented DNA was found (Muriel et al., 2007). There is an attractive hypothesis that proposes that aneuploid spermatozoa can trigger DNA fragmentation, blocking sperm development and producing an ineffective sperm cell. This selective mechanism of genomic labelling would prevent the production of aneuploid conceptus. In the same sense, Enciso et al. (2013) recently showed a positive correlation between aneuploidy and DNA fragmentation using a combined analysis of aneuploidy and DNA integrity. Previously, the correlation was documented particularly in carriers of chromosomal structural abnormalities (Brugnon et al., 2006; Perrin et al., 2009, 2011a,b). On the contrary, other authors have failed to find correlations (Balasuriya et al., 2011; Bronet et al., 2012), which could make the present results controversial. However, prior to establishing a controversy, careful analysis of the technical approaches and designs used by different authors is needed. The use of inappropriate techniques to assess aneuploidy and indirect homemade DNA fragmentation tests (Balasuriya et al., 2011) leads to incomparable results; similarly, the use of nonstandardized protocols makes this task difficult. Also, patient selection and sample preparation methods are highly relevant. These factors may introduce significant bias and may explain differing results. The results of current study are in agreement with those of Enciso et al. (2013), whilst contradictory results were reported by Bronet et al. (2012) in patients with no abnormally high aneuploidy rates. Also, Bronet et al. (2012) showed a paradoxical increase in DFI in the post-DGC fraction. In this scenario, comparison between studies should be performed with caution.

Aneuploidy has been established as the first cause of pregnancy loss and mental retardation in human. Most cases have a maternal origin; however, 5–10% of autosomal aneuploidies and 50–100% of sex chromosome aneuploidies have paternal origin (Hassold et al., 1993; Nicolaidis and Petersen, 1998). Basal levels of sperm aneuploidy have been registered in fertile men, affecting chromosomes 21, 22, X and Y (Tempest and Griffin, 2004), hence it is clear that constitutional levels of nondisjunction persist throughout the

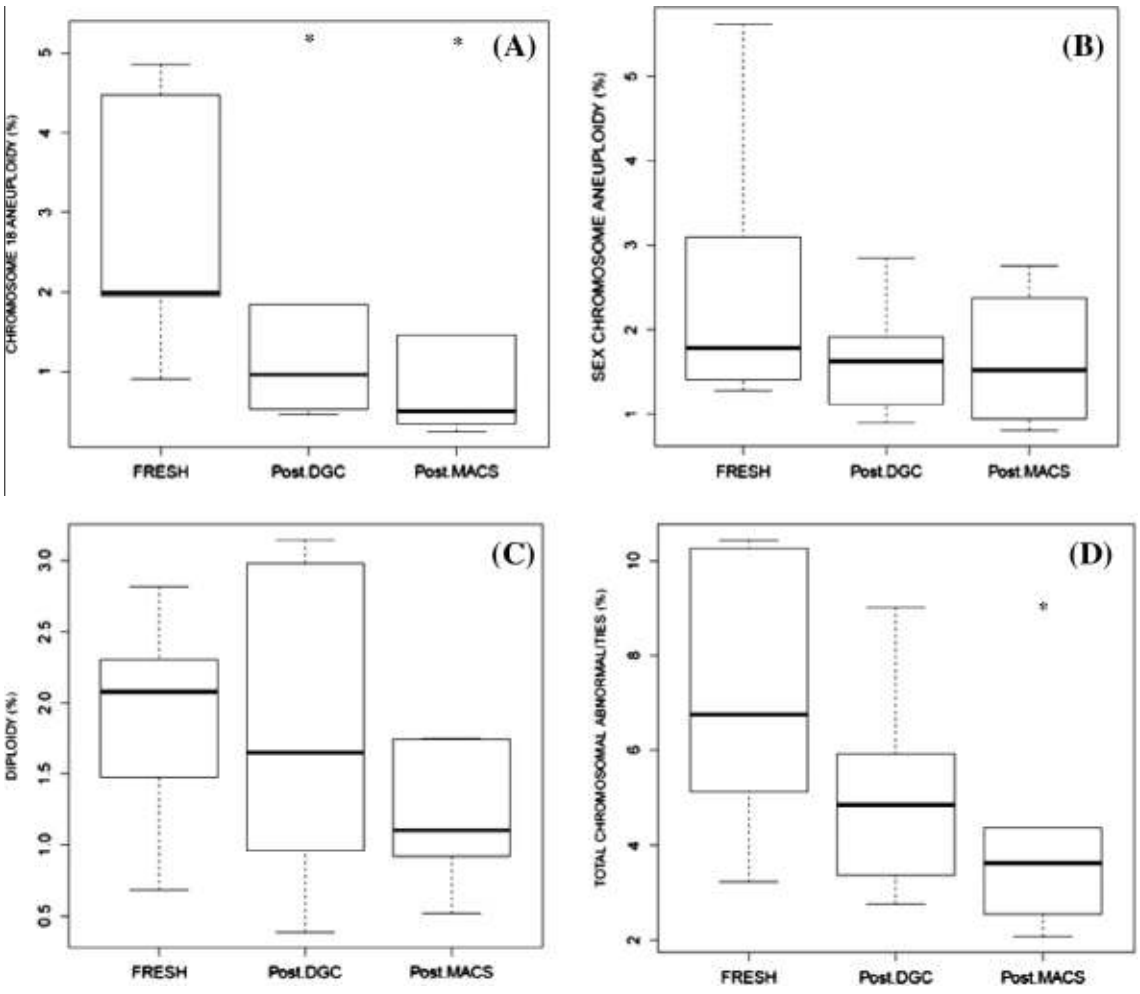


**Figure 2** Aneuploidy and diploidy for chromosomes X, Y and 18 in fresh, post-DGC and post-MACS fractions: (A) aneuploidy (nullisomy plus disomy) for chromosome 18; (B) aneuploidy for sex chromosomes; (C) total diploidy; (D) total aneuploidy. Horizontal lines indicate mean, boxes indicate interquartile range, whiskers indicate minimum and maximum values and asterisks indicate atypical values. DGC = density-gradient centrifugation; MACS, magnetic activated cell sorting.

formation of spermatozoa. On the other hand, significantly elevated levels of sperm aneuploidy are related with poor assisted reproduction outcomes.

Similarly, highly fragmented sperm DNA has been correlated with lower pregnancy and implantation rates. Biologically, it is difficult to explain the association between DNA damage and poor reproduction outcomes. A varying percentage of adult sperm cells suffer DNA damage. Hypothetical causes, grade and kind of DNA injury have been reviewed (Agarwal and Said, 2003; González-Marín et al., 2012; Sakkas and Alvarez, 2010). Apoptosis has been one of the most studied mechanisms. More than a decade ago, the detection of apoptotic markers on ejaculated spermatozoa suggested a process by which a subpopulation of programmed-to-cell-death spermatozoa could escape this programme by undergoing a process called abortive apoptosis (Sakkas et al., 1999). This idea has been continuously revisited. It is well known that a variable number of ejaculated mature spermatozoa present membrane, cytoskeletal, nuclear and organelle abnormalities associated with apoptosis (reviewed in Print and Loveland, 2000). In the testis, this process plays a key role related to homeostatic maintenance of the regulatory capacity of Sertoli cells, maintaining the right ratio of Sertoli to germ cells, eliminating defective germ cells and ensuring correct control of sperm production (Shukla et al., 2012). Nevertheless, the relationship between apoptosis and DNA fragmentation in the mature sperm cell may not be strictly direct. Paradoxically, instead of what occurs in other cell types, no direct link between apoptotic markers and DNA damage measured by TUNEL has been highlighted in spermatozoa (Sakkas et al., 2002). Consequently, the potential deleterious effect of apoptotic signals on sperm DNA integrity remains unclear. Some authors have suggested that damage to sperm DNA and apoptosis could respond to separate multifactorial mechanisms, probably related to chromatin remodelling, protamination and the absence of DNA repair in mature spermatozoa (Muriel et al., 2004; Sakkas et al., 2002).

In this sense, it is highly interesting to observe the evolution of apoptotic markers by jointly analysing the hypothetical relationship between DNA fragmentation and aneuploidy. The alteration of $\Delta\psi_m$ and ePS could be good indicators of early apoptosis. Decreases in $\Delta\psi_m$ at the beginning of apoptosis has been reported in several studies (Ly

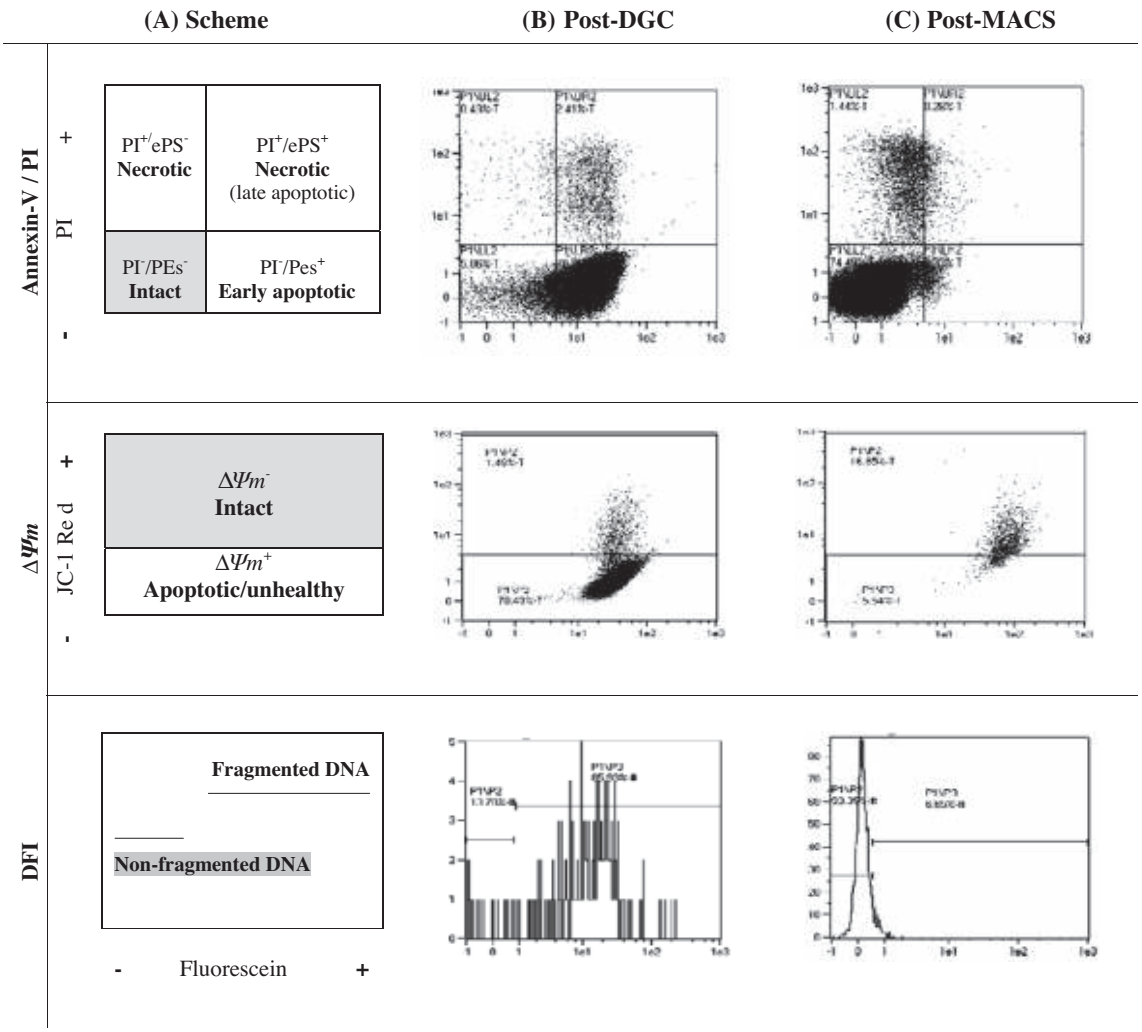


**Figure 3** Multichannel flow cytometry charts, showing subpopulation distributions in analysed parameters (annexin-V/PI, $\Delta\psi_m$ and DFI) in patient 1. (A) Schemes explaining the distribution of the subpopulations: grey indicates the subpopulation selected by MACS. (B) Cell distribution post-DGC. (C) Cell distribution post-MACS. Abscises and ordinates axis correspond to fluorescence intensity. DGC = density-gradient centrifugation; MACS, magnetic activated cell sorting.

**Table 3** ePS, $\Delta\psi_m$ and DNA fragmentation in post-DGC and post-MACS fractions.

| *Flow cytometry parameter* | *Post-DGC* | *Post-MACS* |
|---|---|---|
| $PI^-/ePS^-$ | 15.79 ± 17.89[a] | 78.72 ± 25.26[b] |
| $\Delta\psi_m^-$ | 23.82 ± 27.70[a] | 63.53 ± 26.21[b] |
| DFI | 75.77 ± 14.73[a] | 2.84 ± 3.09[b] |

Values are mean ± standard deviation.
Different superscript letters within a row indicate statistically significant differences ($P < 0.05$).
$\Delta\psi_m^-$ = nondisrupted mitochondrial membrane potential; DFI = DNA fragmentation index; DGC = density-gradient centrifugation; MACS = magnetic activated cell sorting; $PI^-/ePS^-$ = nonpermeable to PI and nonexternalized phosphatidylserine.

et al., 2003; Suzuki et al., 2013). The depolarization of mitochondria occurs as an initial event and is considered a prerequisite for cytochrome c release. Particularly, mitochondrial outer membrane permeabilization constitutes a point of no return for intrinsic apoptosis (Kroemer et al., 2007). In turn, ePS has been used as marker for both necrotic and apoptotic cells. In the apoptosis framework, ePS occurs at the initial stages in spermatozoa and other cells. The transposition of PS from the inner side of the plasma membrane to the outer layer serves as a specific recruitment signal for phagocyte attachment, subsequent engulfment and degradation (Fadok et al., 2000; Savill and Fadok, 2000). The combined analysis of both parameters is enormously interesting to study selective death of spermatozoa, and the inclusion of MACS is novel and allows the comparison of pre-MACS and post-MACS sperm populations and detection of changes in sperm parameters.

In this study population, significant decreases in aneuploidy and DNA fragmentation after MACS were observed. Simultaneously, MACS yielded increasing intact ($PI^-/ePS^-$ and $\Delta\psi_m^-$) cells. This finding suggests that aneuploidy and diploidy and DNA breakage, $\Delta\psi_m$ and ePS could have a common factor. It is interesting to remark the high correlation between decrease in DNA fragmentation and total

**Table 4** Correlation analysis of aneuploidy, ePS, $\Delta\psi_m$ and DFI.

| | *Total aneuploidy* | *PI⁻/ePS⁻* | *$\Delta\psi_m^-$* | *DFI* |
|---|---|---|---|---|
| Total aneuploidy | 1.00 | | | |
| PI⁻/ePS⁻ | [0.43] | 1.00 | | |
| $\Delta\psi_m^-$ | [0.36] | 0.09 | 1.00 | |
| DFI | 0.75 | [0.04] | [0.39] | 1.00 |

Values are Pearson's correlation coefficient with $P < 0.05$. Values in square brackets are absolute $R$ values.
$\Delta\psi_m^-$ = nondisrupted mitochondrial membrane potential; DFI = DNA fragmentation index; PI⁻/ePS⁻ = nonpermeable to PI and nonexternalized phosphatidylserine.

aneuploidy ($R = 0.75$). In contrast, a significantly but weak correlation was observed between the decrease in aneuploidy and the increase in PI⁻/ePS⁻ ($R = -0.43$) and $\Delta\psi_m^-$ ($R = -0.36$). Similarly, no correlation was observed between decrease in DNA fragmentation and increase in PI⁻/ePS⁻ ($R = -0.04$; $P < 0.05$). Thus, the results suggest that ePS, altered $\Delta\psi_m$, aneuploidy and DNA breakages coexist in ejaculated sperm cells and could be reduced by sample processing.

These data argue for the hypothetical connection between incorrect chromosomal segregation, DNA injury and activated cell death. However, chromosomal segregation and DNA injury do not exhibit clear connections with early apoptosis. Decreased $\Delta\psi_m$ is an earlier marker than ePS and the action mechanisms of MACS could permit elution of mitochondrially affected but not yet ePS cells, and this could probably explain the poor correlation. On the basis of aneuploidy and $\Delta\psi_m$ disruption, this work suggests that there is an early destabilized stage. This first phase would not exhibit ePS and these spermatozoa would not be retained by MACS. The results also show that early cell injury and DNA damage are not correlated, which suggests a hypothetical sequence of events (**Figure 4**) that begins in abnormal segregation, as proposed by Muriel et al. (2007). Interestingly, novel cell death modes that show irreversible $\Delta\psi_m$ dissipation but not ePS have been reported and are considered to be a kind of regulated necrosis and/or autophagic cell death (reviewed in Galluzzi et al., 2012).

In addition, concerning genomic labelling that has been postulated to eliminate aneuploid (defective) cells, a conceptually analogous mechanism has been proposed for somatic cells (Vitale et al., 2011). This route, called 'mitotic catastrophe', is activated with gains or losses of chromosomes. Perturbations of segregation trigger an executioner pathway that culminates in cell death or senescence (Galluzzi et al., 2012). This does not constitute a strict apoptotic trail. In this sense, in the context of sperm death, discrepancies between 'programmed', 'regulated' and 'accidental' cell death must be clarified. Most probably, a holistic perspective must be established. On the basis of disrupted biochemical parameters, it is difficult to determine which cell death subroutine is acting. Besides, it is

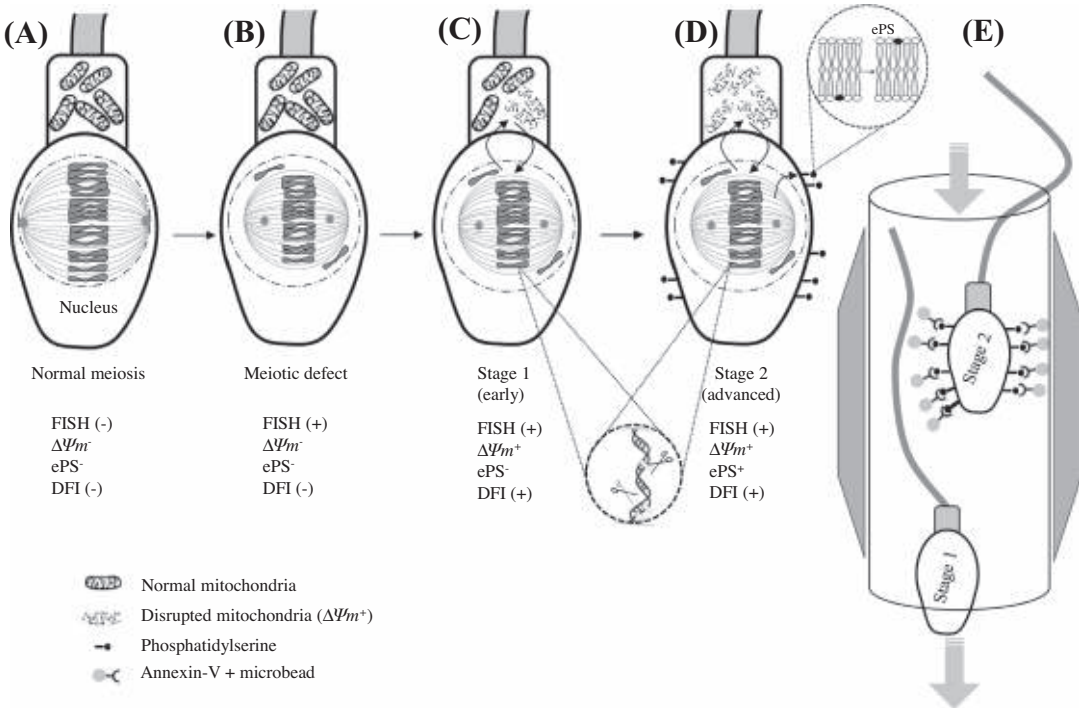


**Figure 4** Postulated sequence of events in spermatogenesis. (A) Normal meiosis. (B) Meiotic defect: abnormal chromosome segregation should trigger initial apoptotic events. (C) Stage 1: the primary destabilizing phenomenon is $\Delta\psi_m$ disruption; this early damage could occur concomitantly with DNA fragmentation and the starting signals for PS externalisation; stage-1 damaged spermatozoa should not be retained by the annexin-V column and would be found in the fraction eluted after MACS. (D) Stage 2: advanced injured spermatozoa; these most-affected spermatozoa have ePS and should be retained by MACS. (E) MACS: sperm retention dependent on the suggested stages. $\Delta\psi_m$ = mitochondrial membrane potential; DFI = DNA fragmentation index; ePS = externalized phosphatidylserine; FISH = fluorescent in-situ hybridization; MACS = magnetic activated cell sorting.

important to remark that the reduced sample size represents a limitation of this study, and in-vivo and/or systems biology studies would help to recognize which routes, where pro-survival and pro-death pathways coexist, are interrelated. These studies could elucidate which factors trigger cell death in sperm subpopulations.

The methodological aspects of this study should be noted. First of all, normozoospermic infertile men were recruited. It is difficult to segregate strict phenotypes (i.e. pure oligozoospermy). When trying to isolate a biological effect in a clinical context, the pathology can often mask the phenomenon *per se*. Consequently, the study of normozoospermic males offered two advantages: enough sperm cells to interrogate all the parameters in separate aliquots, and the baseline analysis of aneuploidy, fragmentation and apoptosis not associated with alterations of sperm parameters analysed by conventional spermiogram or karyotyping. Furthermore, concerning aneuploidy, three chromosomes (X, Y, 18) were analysed in an automated device. This offers a qualitative perspective and serves to infer that there are abnormalities in the pairing, recombination or segregation of meiotic chromosomes, indicating that spermatogenesis is not always optimal (Sarrate et al., 2010). This work's approach allowed the study of specific errors in the meiotic process and the identification of at-risk patients. Using automated platforms offers important advantages versus manual analysis (Carrell and Emery, 2008). Fundamentally, the capture of large number of nuclei (up to 10,000/probe) allows an accurate estimate of disomies and diploidies, and nullisomies can also be considered.

In conclusion, healthy spermatozoa are crucial to obtain reproductive success. The study of genetic and biochemical factors that affect sperm competence is clinically relevant. Moreover, selective sperm demise is poorly understood and precise subroutines should be clarified. As far as is known, this is the first study to have simultaneously analysed cell death markers, aneuploidy and DNA fragmentation. In accordance with previous findings, there was a clear correlation between DNA fragmentation and aneuploidy. Additionally, the beneficial role of MACS on aneuploidy and DNA fragmentation rates indicates that MACS is a promising tool to select healthy spermatozoa for use in IVF programmes. However, the use of selected spermatozoa does not totally eliminate the genetic risk; the majority of embryo aneuploidy has an oocyte origin. In this sense, MACS does not replace other options, such as preimplantation genetic screening, in order to diminish the transmission of aneuploidy to the offspring. Despite the sample size as a limiting factor, this study has provided a deeper understanding of the mechanisms of abnormal reproduction in the sterile male.

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