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Characterization and sorting of flow cytometric populations in human semen

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SUMMARY

Human semen is a complex biological matrix. It contains mature spermatozoa, immature germ cells, residual apoptotic bodies and, in some cases, epithelial cells and leucocytes. Hence, one of the challenges in applying flow cytometry in spermatology is the correct recognition of spermatozoa and their separation from signals of other semen cells/elements. In this study, we show that semen spermatozoa are included in a well-defined, flame-shaped FSC/SSC region (FR), by demonstrating that the count of the spermatozoa contained in such region overlaps that obtained by microscopy in the same samples. In FR, nuclear staining of semen samples reveals three different populations: unstained, brighter and dimmer. Unstained elements were previously characterized as apoptotic bodies of testis origin and the brighter elements represent the majority of semen spermatozoa, whereas the composition and the origin of the population with a lower nuclear staining is less clear, albeit we have previously shown that all the elements constituting it are positive for TUNEL. In this study, we sorted all the elements contained in FR region and demonstrated that the dimmer elements are spermatozoa. To further characterize dimmer spermatozoa, we evaluated apoptotic caspases and chromatin immaturity, the latter detected by aniline blue (AB) and chromomycin A (CMA3) staining. We found that caspases were much more expressed in the dimmer spermatozoa (71.4 ± 18.8%) than in the brighter (46.7 ± 15.1%), whereas similar amounts of spermatozoa with chromatin immaturity were found in both populations (brighter, AB: 48.2 ± 19.5%; CMA3: 48.5 ± 20.4% and dimmer, AB: 43.4 ± 19.8%; CMA3: 36.1 ± 18.0%). Hence, the role of apoptosis in generating dimmer spermatozoa and their DNA fragmentation appears clear, whereas the involvement of defects during the chromatin packaging remains elusive.

INTRODUCTION

In western countries, infertility is becoming an emerging problem as one of five couples may fail to conceive within 1 year of attempts. Male factor is involved in about 50% of cases of couple infertility and up to now routine semen analysis is the main male diagnostic test. Routine semen analysis measures sperm concentration, motility and morphology by visually examining few hundreds of cells and using reference values for normality, as established by WHO (2010). However, the conventional determination of standard semen parameters is affected by the subjectivity of the operator and by a high intra- and interobserver variability (Jørgensen *et al.*, 1997; Gandini *et al.*, 2000; Cooper *et al.*, 2002; Filimberti *et al.*, 2013). In addition, it is recognized that this test poorly identifies infertile men (Lewis, 2007), except for few cases, such as azoospermia, severe oligozoospermia, necrozoospermia or presence of antisperm antibodies in over 50% spermatozoa (WHO, 2010). Indeed, this test is not able to unveil the many sperm attributes necessary to successfully reach the oocyte and properly deliver an intact paternal genome (Muratori *et al.*, 2009). In principle, the simultaneous assessment of all these sperm attributes would provide the determination of the true number of spermatozoa able to fertilize in the ejaculate, that is, the spermatozoa exhibiting all the needed features for a proper function (Rijsselaere *et al.*, 2005). Flow cytometry (FCM) appears the promising technology to fitful this requirement because of its ability to simultaneously detect many parameters at the single cell level and on a statistically relevant number of observations. Flow cytometric applications in clinical andrology have been delayed in comparison with other medical areas (e.g. immunology), even if this technology has been extensively

employed for research purposes in reproductive biology and many assays were developed to investigate sperm traits and functions (Hossain *et al.*, 2011).

Human semen is a complex biological matrix containing both somatic and germ cells, the latter represented by immature elements and mature spermatozoa, as well as, in pathological conditions, leucocytes, bacteria and prostatic corpuscles. FCM has the potential to reflect the complexity of human semen, by depicting semen components on bases of size and density properties in FSC/SSC cytograms. With samples prepared by swim up and gating back spermatozoa previously stained by propidium iodide (PI), our group established a flame-shaped region (FR) that excludes every other semen cell different from spermatozoa (Muratori *et al.*, 2000). However, in a later study, using neat semen samples of subfertile men, we observed that such region contains also round bodies that we termed M540 bodies (Muratori *et al.*, 2004) and that are apoptotic bodies (Marchiani *et al.*, 2007) of testicular origin (Lotti *et al.*, 2012). Not surprisingly, the inclusion of M540 bodies in the fluorescence analyses of spermatozoa yields incorrect results (Muratori *et al.*, 2005, 2008), also confirmed by other Authors (Petrunkina & Harrison, 2010). As mentioned, FR was originally established using spermatozoa prepared by swim-up (Muratori *et al.*, 2000) that it is supposed to select the male gametes according to maturity and morphology beside to motility (Jakab *et al.*, 2003). However, it is possible that spermatozoa with a certain degree of immaturity and/or morphological abnormality are not included in FR and thus that the region is not representative of the entire ejaculate.

Beside the presence of M540 bodies (Muratori *et al.*, 2004; Marchiani *et al.*, 2007), the PI staining of membrane permeabilized or fixed spermatozoa unveils the occurrence, in FR, of two subpopulations that differ for the intensity of labelling with PI, indicated as PI brighter and PI dimmer (Muratori *et al.*, 2008). PI brighter spermatozoa represents the bulk of semen spermatozoa and its nuclear staining overlaps that of spermatozoa selected by both swim up and density gradient centrifugation. Conversely, the PI dimmer population is less expressed (on average it represents the 15% of the PI stained elements in semen from sub/infertile patients) although it may show very high levels in some subjects (Muratori *et al.*, 2008). In addition, its composition is less clear, although it was supposed to be formed by spermatozoa with pale nuclear staining observed in neat semen by fluorescence microscopy (Muratori *et al.*, 2008). Finally, PI dimmer population is highly negatively related to semen quality and completely labelled by TUNEL (Muratori *et al.*, 2008). Given that, investigation of the mechanisms responsible for PI dimmer DNA fragmentation would help to clarify the origin of this population as well as the mechanisms for its lower nuclear staining. Since PI dimmer population does not show any sign of oxidative DNA damage (Cambi *et al.*, 2013), the attack of DNA by reactive oxygen species does not seem to be involved and other mechanisms, including the failure of sperm chromatin maturation and/or the occurrence of abortive apoptosis (Sakkas *et al.*, 1999a) could be responsible for the DNA damage in this population (Muratori *et al.*, 2006). Given these premises, the first aim of this study was to separate, by cell sorting, the different populations within the FR to directly observe them by microscopy. After demonstrating that all the PI stained elements contained in FR are spermatozoa, we verified whether FR is a reliable selection for flow cytometric studies on neat semen. In addition, we investigated the maturity of chromatin in sorted PI dimmer and PI brighter cells by aniline blue (AB) and chromomycin A (CMA3) staining. Finally, the presence of caspase activity as sign of apoptosis was also studied by FCM.

MATERIAL AND METHODS

Human tubal fluid (HTF) medium was purchased from Celbio (Milan, Italy) and bovine serum albumin (BSA) was from ICN Biomedicals, Irvine, CA, USA. BD Trucount Tubes were purchased from Becton-Dickinson, San Jose, CA, USA. Stain-coated Testsimplets slides were from Waldeck GmbH & Co. KG, Munster, Germany. Paraformaldehyde (PFA) was obtained from Merck Chemicals, Milan, Italy. The other chemicals were from Sigma Chemical (St. Louis, MO, USA) unless otherwise indicated.

Samples

Semen samples were collected, according to WHO criteria (WHO, 2010), from men undergoing routine semen analysis for couple infertility in the Andrology laboratory of the University of Florence after the approval of the Hospital Committee for Investigations in Humans and after informed patients consent. Semen samples with any detectable leucocytes, evaluated by assessing non-sperm components of semen after Diff-Quik staining, were excluded from the study. Sperm fixation was performed by PFA [500 μL, 4% in phosphate-buffered saline (PBS) pH 7.4] for 30 min at room temperature (RT), after washing neat semen twice with HTF medium.

Sperm count by light microscopy and FCM

Fixed spermatozoa ($0.25–10 \times 10^5$/250 μL), from 20 subjects, were stained by 4.5 μL of PI solution (50 μg/mL in PBS) in PBS/BSA 1% (Brando *et al.*, 2001) and incubated at RT in the dark for 15 min. Then spermatozoa were added to Trucount Tubes, containing a calibrated number of fluorescent beads. Beads and spermatozoa were counted by microscopy by deposing 10 μL of the sample in a Neubauer improved haemocytometer according to the WHO guidelines (WHO, 2010). In addition, samples were acquired by a FACScan flow cytometer (Becton–Dickinson) and green fluorescence of the beads and red fluorescence of PI were detected by, respectively, the FL-1 (515–555 nm wavelength band) and the FL-2 (563–607 nm wavelength band) detectors. Flow cytometric analysis was stopped when 1000 beads were acquired. Beads were counted by gating their peak in the histograms of green fluorescence, within the FSC/SSC region containing them. Spermatozoa were counted in a gate including PI stained events within FR. The ratio: number of spermatozoa/number of beads was determined by both light microscopy and FCM and the number of spermatozoa in the sample was calculated by multiply such ratio by the total number of beads contained in the Trucount tube.

Fluorescence-activated cell sorting of semen subpopulations

Fixed spermatozoa from 10 subjects were washed twice by PBS/BSA 1% and stained by PI. After adjusting sperm concentration to 10^7 cells/mL, the samples were filtered by 50-μm Syringe Filcons (Becton Dickinson) and immediately sorted by a BD FACSAria II cell-sorting system (Becton–Dickinson) equipped with a 15-mW argon laser with an excitation wavelength of 488 nm, and with a FACSSort fluid sorting module. For sorting

we used the followings settings: laser power: 13 mW; nozzle: 70 μm; sort setup: low; sheath pressure: 34.50 p.s.i.; frequency: 60.0 kHz; flow rate: 1–3 μL/min (maximum of 7000 events/sec); and precision – 0160. BD FACSDiva Software (Becton–Dickinson) was used for acquisition and data analysis. Regions around PI brighter, PI dimmer and unstained elements were drawn within the FR of FSC/SSC dot plots and the three subpopulations were collected in tubes containing PBS. To verify the purity of collected PI brighter and PI dimmer populations, the sorted samples were stained by PI and acquired by FCM again. For observation by light microscopy, 10 μL of sorted samples was placed on the Testsimplets slides, covered with the coverslip and visualized at 100× magnification with oil immersion.

AB staining in sorted PI brighter and dimmer populations

To evaluate sperm immaturity we performed AB staining that selectively stains lysine-rich histones (Auger *et al.*, 1990). Briefly, after sperm sorting as described above and washing with PBS/BSA 1%, 100 000 spermatozoa were smeared on slide, air-dried and then stained with 5% aqueous AB (Sigma Aldrich, St Louis, MO, USA) mixed with 4% acetic acid (pH 3.5) for 5 min (Franken *et al.*, 1999) at RT. Two hundred spermatozoa were analysed on each slide from five subjects under a light microscope (Leica DM LS; Leica, Wetzlar, Germany). Spermatozoa showing dark-blue staining were considered as AB positive, whereas those stained only weakly or not at all were considered as AB negative (Franken *et al.*, 1999).

CMA3 staining in sorted PI brighter and dimmer populations

To detect protamine deficiency in sperm chromatin we performed CMA3 staining (Bianchi *et al.*, 1993). CMA3 is a fluorochrome which competes with the protamines for binding to the minor groove of GC-rich DNA (Kazerooni *et al.*, 2009). After sperm sorting and washing with PBS/BSA 1%, spermatozoa (400 000) were stained with 100 μL of CMA3 (Sigma Aldrich) solution [0.25 mg/mL in McIlvane's buffer (0.2 M Na_2HPO_4, 0.1 M citric acid), pH 7.0, containing 10 mM $MgCl_2$] for 20 min at RT in the dark. Then cells were washed and resuspended in 10 μL of McIlvane's buffer, pH 7.0, containing 10 mM $MgCl_2$, smeared on slide, air-dried and mounted with PBS: glycerol (1 : 1). Two hundred spermatozoa were analysed on each slide from five subjects by fluorescence microscope (Axiolab A1 FL; Carl Zeiss, Milan, Italy), equipped with Filter set 49 and an oil immersion 100× magnification objective. Two types of staining patterns were identified: bright green fluorescence of the sperm head (abnormal chromatin packaging) and weak green staining (normal chromatin packaging) (Lolis *et al.*, 1996).

Detection of caspase activity

Caspases 3 and 7 were detected by Vybrant FAM Caspases 3 and 7 Assay Kit (Life Technologies, Carlsbad, CA, USA), which detects active caspases by using the FAM-DEVD-FMK reagent, a fluorescently labelled inhibitor of such enzymes (FLICA), as described previously (Marchiani *et al.*, 2007). Briefly, 10 fresh semen samples were resuspended at the concentration of 1×10^6 spermatozoa/mL in 300 μL of HTF medium. Aliquots of 10 μL of 30X FLICA working solution were added and sperm suspensions were incubated for 1 h at 37 °C and 5% CO_2, in the dark. After two washes with Wash Buffer 1X (supplied by the Kit), samples were fixed by adding 40 μL of 10% formaldehyde solution (supplied by the Kit) for 10 min at RT. Hence, sperm samples were washed again twice and resuspended in 400 μL of Wash Buffer 1X containing 8 μL of PI solution (50 μg/mL in PBS) for FACS analysis. Samples were acquired by a FACScan flow cytometer equipped with a 15-mW argon-ion laser for excitation. For each test sample, two additional samples were prepared: (i) a negative control (absence of FLICA working solution) for data analysis; (ii) a test sample labelled only with FLICA (without PI staining) for fluorescence compensation. Green fluorescence of caspases and red fluorescence of PI were revealed by the FL-1 and FL-2 detector respectively. For each sample, 8000 events were recorded within the FR region in the FSC/SSC dot plot (Muratori *et al.*, 2003, 2004). We determined caspase activity within FR region, after gating PI positive events of both PI brighter and PI dimmer population. A marker was established in the histogram of fluorescence distribution, including all the events of the negative control. All the events beyond the marker were considered positive for caspases. CellQuest-Pro software program (Becton–Dickinson) was used for acquisition and analysis.

Determination of PI dimmer and M540 bodies by FCM

After fixation, staining with PI and acquisition by FACScan, semen samples from 29 men were used to determine the percentage of both M540 bodies and PI dimmer population. M540 bodies were calculated as unstained elements within a region that excluded debris and containing both M540 bodies and nucleated cells (see fig. 1A in Marchiani *et al.*, 2007). PI dimmer population was calculated within FR region, after gating PI dimmer events. The amounts of M540 bodies and PI dimmer were both expressed as percentages of total spermatozoa.

Statistical analysis

Each variable was checked for normal distribution. To assess statistically significant differences, Student's *t*-test (paired data) was used for percentages of caspase activity in PI brighter and PI dimmer populations, whereas Wilcoxon test was used for statistical analysis of AB and CMA3 staining. We considered the *p*-value of 0.05 as statistically significant. Bivariate correlation between PI dimmer population and M540 bodies was evaluated by calculating the Spearman's correlation coefficient. Data were analysed with Microcal Origin software, 6.1 version (MicroCal Software Inc., Northampton, MA, USA) and SPSS version 20 software for Windows (SPSS, Inc., Chicago, IL, USA). Results are shown as mean ± SD.

RESULTS

Sorting PI brighter, PI dimmer populations and M540 bodies

After sorting of PI brighter, PI dimmer and unstained elements contained in FR (Fig. 1A) by FACSAria II instrument, we verified the successful separation of the two PI stained populations (Fig. 1B) and observed the sorted fractions by light microscopy (Fig. 1C). In all the semen samples (n = 10), both the flow cytometric populations PI brighter and PI dimmer resulted formed by spermatozoa (Fig. 1C, upper and middle), whereas, as expected (Muratori *et al.*, 2004; Marchiani *et al.*, 2007), the unstained events were identified as M540 bodies (Fig. 1C, lower).

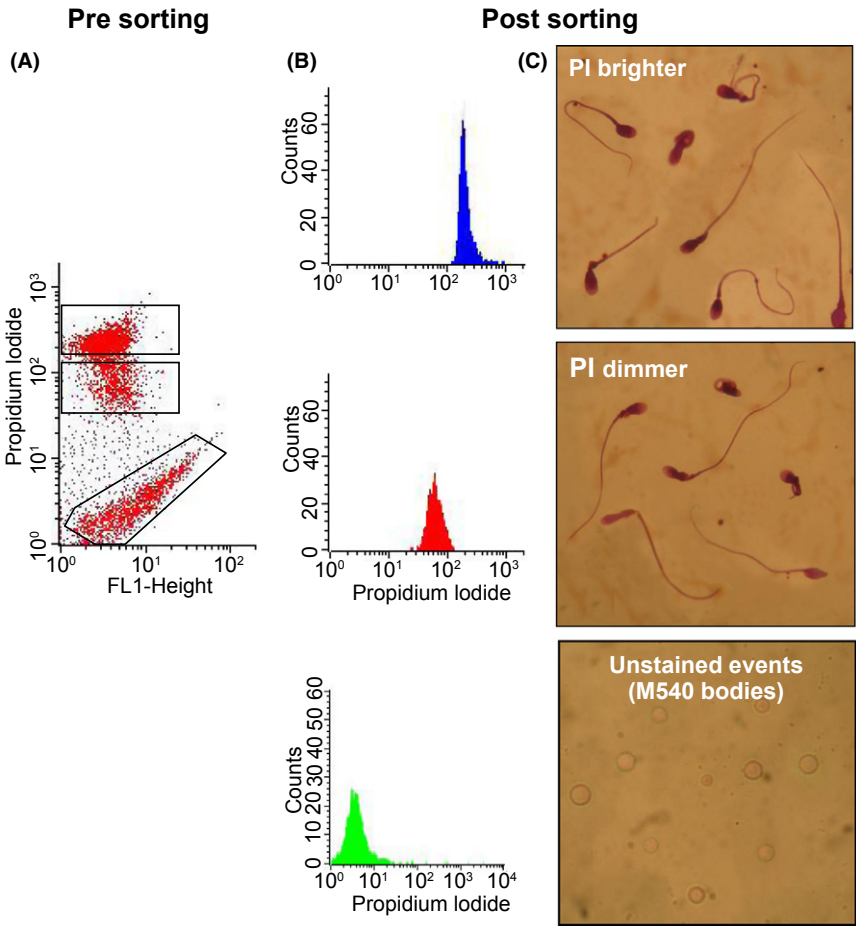


Figure 1 Sperm sorting by FACSAria II. (A) Regions drawn around propidium iodide (PI) brighter (upper), PI dimmer (middle) and unstained elements (lower) in the pre-sorted samples. (B) After sorting, the successful separation of the populations was checked by repeating PI staining and flow cytometry acquisition. From top to bottom the peaks of PI fluorescence, respectively, related to PI brighter, PI dimmer and M540 bodies. (C) Micrographs of PI brighter (upper) PI dimmer (medium) and M540 bodies (lower) populations, after sorting and staining in TestSimplets slides.

Comparing semen spermatozoa and PI stained events in FR

The comparison of the counts of semen spermatozoa and PI stained events in FR was conducted in 20 samples showing values for standard parameters that are representative of the semen quality found in male partners of infertile couples attending our laboratory for semen analysis (Table 1). In these samples, we verified whether the FR set in the FSC/SSC dot plots (Fig. 2A, left side) includes or not all the spermatozoa present in the sample, by counting them with FCM (Fig. 2A) and Neubauer improved haemocytometer (Fig. 2B), both respect to fluorescent beads serving as volume standard (Brando *et al.*, 2001). The results demonstrated that there was no statistical difference between the two types of count ($p > 0.05$) that resulted virtually overlapping (Fig. 2C). Consistently, the mean value of the ratio between sperm counts determined by FCM and those determined by microscopy was 1.04 ± 0.3 ($n = 20$). Only in few cases, the score of PI-stained events by FCM was different by sperm count with microscopy, being, however, both higher and lower and thus excluding a systematic error.

AB and CMA3 staining after sorting PI brighter and dimmer populations

To investigate sperm chromatin immaturity, we sorted PI brighter and dimmer populations, verified the successful separation (see above) and stained the two fractions with AB and CMA3. Examples of AB labelling in PI dimmer spermatozoa are reported in Fig. 3A, showing stained (immature) and unstained (mature) spermatozoa. After scoring, we found no difference between the mean percentage of AB staining in PI dimmer (43.4 ± 19.8%) and in PI brighter (48.2 ± 19.5%) populations ($p = 0.5$, $n = 5$). Concerning CMA3 staining, examples of PI dimmer sperm brightly (immature) and weakly stained (mature) spermatozoa with this nuclear probe are shown in Fig. 3B. Consistent with the results obtained with AB, the mean percentage of spermatozoa showing CMA3 staining in PI dimmer (36.1 ± 18.0%) was not significantly different with that found in PI brighter (48.5 ± 20.4%) population ($p = 0.1$, $n = 5$).

Table 1 Semen parameters and age (mean ± SD) of subjects ($n = 20$) included in the experiments of counting spermatozoa by Neubauer improved haemocytometer and flow cytometry

Volume (mL)	pH	Age (years)	Concentration (10^6/mL)	Number (10^6/ejaculate)	Progressive motility (%)	Non-progressive motility (%)	Immotile spermatozoa (%)	Normal morphology (%)
4.1 ± 1.7	7.6 ± 0.2	34.7 ± 6.8	42.2 ± 38.1	144.2 ± 125.5	43.0 ± 21.7	13.3 ± 7.1	44.5 ± 17.6	4.9 ± 4.5

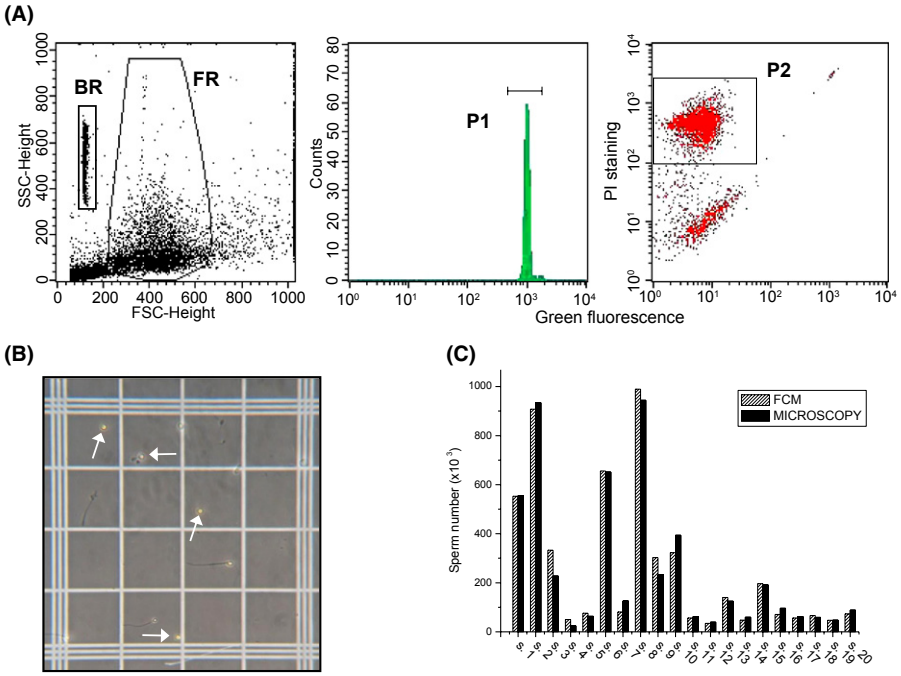
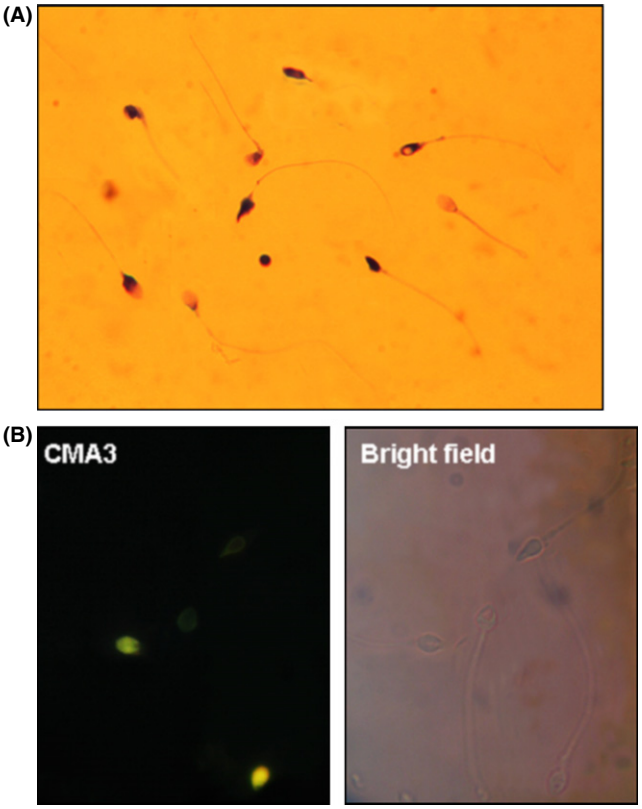


Figure 2 Spermatozoa were added to Trucount Tubes and counted with flow cytometry (FCM) (A) and Neubauer improved haemocytometer (B) by (number of counted spermatozoa)/(number of counted beads) × (total number of beads in the Trucount tube). For FCM, propidium iodide stained events (P2) were counted within the flame-shaped region drawn in the FSC/SSC dot plots (FR), whereas the beads were counted in their green fluorescence peak (P1), within the BR region in the FSC/SSC dot plots. (C) Sperm counts obtained by the Neubauer improved haemocytometer (black columns) and FCM (hashed columns) in 20 semen samples. White arrows in B indicate the Trucount beads.

Figure 3 (A) Aniline blue (AB) staining. Images of propidium iodide (PI) dimmer spermatozoa stained or not with AB obtained by light microscope. Sperm nuclei with intense dark-blue staining were considered to be immature. (B) Chromomycin A (CMA3) staining. Left side shows images of PI dimmer spermatozoa brightly or weakly stained with CMA3 and right side shows the corresponding bright fields, obtained by fluorescence microscope. Sperm nuclei with bright staining were considered to be immature.



Detection of caspase activity and correlation between PI dimmer and M540 bodies amounts

To determine whether PI dimmer spermatozoa derive from an apoptotic process, we assessed the occurrence of caspase 3 and 7 (the main executors of apoptotic cascade) activities. Histograms representing the fluorescence of caspase activity in PI dimmer and PI brighter populations are shown in Fig. 4A. We calculated the mean percentage of PI dimmer spermatozoa showing caspases activity on total PI dimmer population. The same evaluation was performed in PI brighter population. We found that 71.4 ± 18.8% of PI dimmer and 46.7 ± 15.1% of PI brighter spermatozoa exhibited caspase activity ($p < 0.0001$, $n = 10$, Fig. 4B).

To further verify the involvement of apoptosis in the origin of PI dimmer population, we evaluated the correlation between the percentage of PI dimmer spermatozoa (on average 19.7 ± 10.6 and the apoptotic elements, M540 bodies, present in semen (Marchiani *et al.*, 2007; Lotti *et al.*, 2012). A strict association between the two parameters was found ($r = 0.5$, $p < 0.01$, $n = 29$, Fig. 5). To note, a subject characterized by a high percentage of M540 bodies (circled dot), considered an outlier, was excluded from the above analysis. However, its inclusion does not substantially change the correlation between the two parameters ($r = 0.4$, $p < 0.02$, $n = 28$).

DISCUSSION

In the past decades, FCM has been greatly advancing, becoming an invaluable tool for cell analyses in many fields of research. In addition, FCM has progressively extended from basic research to clinical laboratories, where it has become important for both diagnosis and prognosis of patients, in particular those affected by blood disorders. Despite that spermatozoa are eligible for flow analysis as like as blood cells, the application of FCM in clinical andrology has been delayed respect to other medical fields. Because of the complexity of semen fluid, one of the

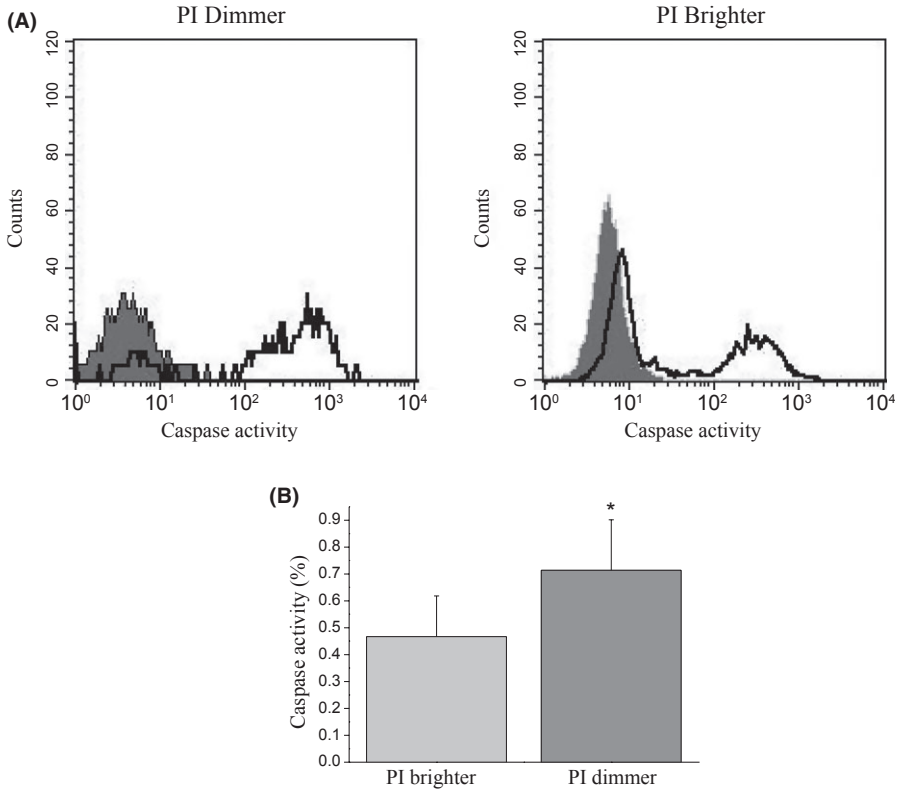


Figure 4 (A) Caspases 3 and 7 activity detected by flow cytometry. Typical histograms of caspases activity fluorescence showing overlay of the test sample (open) and the corresponding negative control (absence of FLICA, shaded) related to propidium iodide (PI) dimmer population (left side) and PI brighter population (right side). (B) Percentages (mean ± SD) of spermatozoa with caspases activity in PI dimmer and PI brighter populations; *$p < 0.0001$.

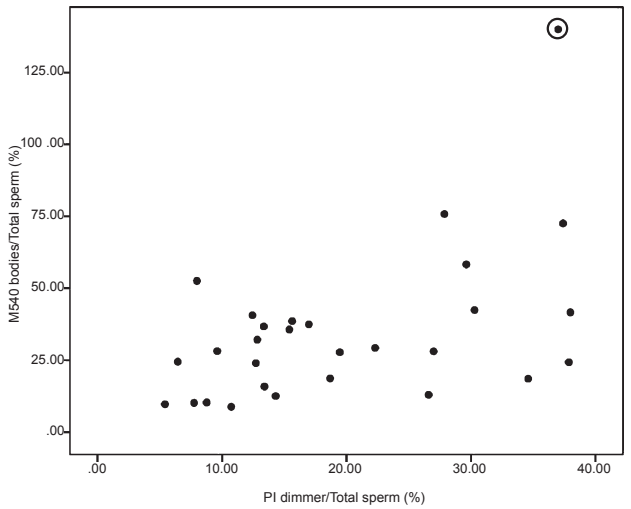


Figure 5 Correlation between the percentages of propidium iodide (PI) dimmer population and apoptotic M540 bodies both calculated on total spermatozoa. The circled dot shows a subject with a high percentage of M540 bodies, considered an outlier.

challenges in applying FCM in spermatology has been the correct recognition of spermatozoa and their separation from signals of other cells/elements present in the sample.

The nuclear staining is routinely used in our laboratory to exclude M540 bodies from sperm analyses, because of their virtual lack of chromatin material (Muratori *et al.*, 2004; Marchiani *et al.*, 2007). As mentioned above, in the FR region there are two PI stained populations termed by us PI brighter and PI dimmer (Muratori *et al.*, 2008) and here, by sorting, we directly demonstrated that they are both composed by spermatozoa. In addition, counting such stained events in the FR and the semen spermatozoa by Neubauer improved hemocytometer, we demonstrate that FR virtually includes all the semen spermatozoa of the sample and thus it is a reliable gate for defining semen spermatozoa in flow cytometric studies. Such conclusion is based on the use of fixed samples and we cannot exclude that fresh samples would yield different results. However, although FR is well defined also in such samples (Muratori *et al.*, 2004), so far we are not able to distinguish spermatozoa from bodies without nuclear staining of all (dead and viable) spermatozoa, obtained by fixing or permeabilizing the cells. PI dimmer spermatozoa represent another example of the heterogeneity in the DNA status of human spermatozoa, beside the already reported high DNA stainability (HDS) sperm population (Evenson, 2013) and the DNA degraded spermatozoa (DDS; Enciso *et al.*, 2006). HDS population is detected by Sperm Chromatin Structure Assay and consists of spermatozoa with a high staining with acridine orange, because of a poor protamine–histone replacement (Evenson, 2013), whereas DDS are spermatozoa revealed by Sperm Chromatin Dispersion test and show a ghostlike morphology because of a massive depletion of DNA and protein (García-Peiró *et al.*, 2012). HDS population and DDS do not overlap (García-Peiró *et al.*, 2012). Similarly, although we show here that PI dimmer population contains many immature spermatozoa, it cannot correspond to the HDS fraction, as the latter has no significant DNA breaks (Evenson, 2013). Conversely, it is possible that PI dimmer population includes DDS. Indeed, we reported in a previous study the occurrence of spermatozoa with pale PI staining with similar characteristics of DDS (see fig. 1D in Muratori *et al.*, 2008 and fig. 1C in García-Peiró *et al.*, 2012). In addition, it has been recently reported that DDS contains large amounts of double- and single-strand DNA breaks

(Gosálvez *et al.*, 2013), similar to PI dimmer spermatozoa (Muratori *et al.*, 2008). However, although all PI dimmer spermatozoa are DNA fragmented, they do not seem to exactly overlap DDS as a part of them show a normal appearance of the nucleus (present study and Muratori *et al.*, 2008). Consistently, the amount of DDS was not different between normozoospermic and men with abnormal semen parameter (Enciso *et al.*, 2006), at variance with PI dimmer population that is strictly associated with poor semen quality (Muratori *et al.*, 2008). Furthermore, the percentage of DDS population is lower (about 11% in patients with abnormal semen parameters, García-Peiró *et al.*, 2012) than that of PI dimmer (on average about 15–20% in semen of male partners of infertile couples, Muratori *et al.*, 2008 and present study). Enciso *et al.* (2006), who first reported the occurrence of DDS, speculated that such degraded spermatozoa originate after a strong and prolonged exposure to oxidative stress, considered an important cause of sperm DNA damage (Aitken *et al.*, 2010). However, detection of 8-OHdG by an immunofluorescence method demonstrated a variable amount of the oxidized base in PI brighter sperm nuclei, whereas PI dimmer spermatozoa did not show this sign of oxidative DNA damage (Cambi *et al.*, 2013), suggesting that the oxidative attack can play a role in brighter but not in dimmer population. Conversely, we show here that a very high percentage of PI dimmer spermatozoa express the apoptotic caspase enzymes and that there is a clear correlation between PI dimmer spermatozoa and testis apoptotic bodies. These results suggest a key role of apoptosis in generating DNA fragmentation in this population, in agreement with other studies reporting the association between DNA breakage and apoptotic markers (Marchetti *et al.*, 2004; Said *et al.*, 2006). PI dimmer spermatozoa could be apoptotic cells stemming in the testis but escaping removal because of the failure of the apoptotic pathway (Sakkas *et al.*, 1999a). This conclusion is consistent with the fact that the percentages of both PI dimmer spermatozoa and other markers of abortive apoptosis are strictly associated with an abnormal spermatogenesis (Sakkas *et al.*,1999b; Muratori *et al.*, 2008). The long time and travel through the male genital tract after the trigger of the apoptotic DNA cleavage in the testis could provoke the loss of DNA fragments, similar to what occurs during apoptosis of somatic cells (Jerzak *et al.*, 1998), or even of chromatin material, explaining the lower PI staining in these spermatozoa.

The derailment of sperm chromatin maturation could be another mechanism contributing to generate DNA fragmentation in PI dimmer, by preventing the re-ligation of the DNA breaks occurring to favour the protamine–histone replacement (Marcon & Boissonneault, 2004) or, as suggested by the prevailing presence of caspase in this population (present study) by triggering the apoptotic pathway (Sakkas *et al.*, 2004).

Detection of chromatin immaturity by both AB and CMA3 staining in sorted PI dimmer and brighter spermatozoa revealed similar percentages of immature spermatozoa in the two populations. Considering the different extent of DNA fragmentation in brighter and dimmer spermatozoa (Muratori *et al.*, 2008), this finding is quite surprising as immaturity is believed to cause and/or favour the detection of DNA damage (Aitken *et al.*, 2013). However, it is also possible that the score of AB/CMA3 staining is underestimated in the dimmer population owing to the depletion of chromatin material in these spermatozoa (see above). In such a case, the association between chromatin immaturity and DNA fragmentation in PI dimmer population could be masked. It should be also mentioned that experiments on sperm immaturity have been performed in only five sorted samples, possibly representing a limitation of our study.

In conclusion, the results of this study are important for the use of FCM for research and clinical purposes in spermatology. Indeed, we definitively establish the region containing spermatozoa in the complex FSC/SSC cytograms of human semen by showing that all ejaculated spermatozoa are included in it and that all the nuclear stained events (brighter and dimmer) of such region correspond to spermatozoa. Gating spermatozoa in FR in combination with the use of a nuclear stain allows a reliable use of FCM in spermatology. In addition, our study indicates that apoptosis is the main mechanism generating (DNA fragmentation of) PI dimmer sperm population, which is highly present in pathological semen samples representing another index of impaired spermatogenesis as like as the occurrence of semen apoptotic bodies (Lotti *et al.*, 2012).

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AUTHORS CONTRIBUTION

SM collected and analysed the data and took part in writing the manuscript. LT performed experiments and participated in analysis of data. BO and LB performed flow cytometric acquisition and sperm sorting. CA provided the flow cytometric instrumentation for sperm sorting and contributed to the final review of the manuscript. GF critically revised the manuscript. EB participated in design of the study and critically revised the manuscript. MM designed the study and wrote the manuscript.

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