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Characterization of M540 bodies in human semen: evidence that they are apoptotic bodies

S. Marchiani[1], L. Tamburrino[1], A. Maoggi[1], G.B. Vannelli[2], G. Forti[1], E. Baldi[1,3] and M. Muratori[1,3]

[1]Andrology Unit, Department of Clinical Physiopathology, Center for Research, Transfer and High Education, DENOthe, University of Florence, Viale Pieraccini 6, I-50139 Firenze, Italy; [2]Department of Anatomy Histology and Forensic Medicine, University of Florence, I-50134 Florence, Italy

[3]Correspondence address. Tel: +39 055 427 1486; Fax: +39 055 427 1371; E-mail: e.baldi@dfc.unifi.it, m.muratori@dfc.unifi.it

Our group has recently identified, in human semen, round bodies of different size and density which were termed M540 bodies due to their staining with the fluorochrome merocyanine 540. Here, we investigate the hypothesis that such structures represent apoptotic bodies. To this aim, by both fluorescence-activated cell sorting (FACS) and fluorescence microscopy, we examined the occurrence of apoptotic markers such as caspase activity, Fas, p53 and Bcl-x in M540 bodies. In addition, we evaluated their ultrastructure by transmission electron microscopy. We found that M540 bodies express all the investigated markers, strongly supporting our hypothesis. We also found that M540 bodies contain fragmented DNA, another evidence of their apoptotic derivation. We investigated also the presence of M540 bodies in the different categories of patients. With respect to normozoospermic subjects, a higher content of M540 bodies was found in oligoasthenoteratozoospermic and asthenoteratozoospermic, but not in asthenozoospermic and teratozoospermic men. Interestingly, these subjects are those whose semen shows the highest levels of apoptotic signs. The variable occurrence of apoptotic bodies in semen may thus be considered a sign of abortive apoptosis in male reproductive organs. Of interest, since M540 bodies exhibit a similar size and density to sperm, they represent a confounding factor in FACS studies on ejaculated sperm.

Keywords: apoptosis; human semen; M540 bodies

Introduction

Human semen is a complex biological fluid in which heterogeneous cell types are present, including germ cells (other than mature spermatozoa) with different degrees of maturity and somatic cells. Recently we have reported the occurrence in semen of membrane surrounded round bodies, whose levels appear to be notably higher in subjects with decreased quality of semen (Muratori *et al*., 2004). We termed such bodies as M540 bodies, due to their bright staining with merocyanine 540 (M540). M540 is a lipophilic compound, sensitive to changes in the membrane architecture such those occurring during somatic apoptosis (Aussel *et al*., 1993; Mower *et al*., 1994) and capacitation of spermatozoa from some mammalian species (Gadella and Harrison 2000; Rathi *et al*., 2001), but not human (Muratori *et al*., 2004; Martin *et al*., 2005). Since M540 bodies are promptly stained with M540, their membrane is likely to be differently packaged from normal cell membranes. In addition, the failure of the nuclear stain YOPRO-1 (Y1) to label M540 bodies suggests their virtual lack of chromatin material (Muratori *et al*., 2004). Finally, M540 bodies appear to be heterogeneous elements in both size and density as indicated by light microscopy examination and by their distribution in different fractions of discontinuous density gradients (Muratori *et al*., 2004). Later, we showed that M540 bodies are ubiquitinated and that the amount of semen ubiquitination due to M540 bodies was sharply associated to poor seminal parameters (Muratori *et al*., 2005).

Most of the characteristics of M540 bodies prompted us to originally speculate that such bodies might be apoptotic bodies derived from testis apoptosis (Muratori *et al*., 2004). Morphologically, apoptosis is characterized by the condensation of nuclear chromatin into the periphery of nuclei, the shrinkage of the cell and, finally, the formation of membrane-encased bodies containing organelles (Kerr *et al*., 1994). Physiological apoptosis in testis involves all germ cell compartments (Oldereid *et al*., 2001) and is believed to play a pivotal role in tuning the balance between germ cells and Sertoli cells (Sakkas *et al*., 1999a). Increased germ cell apoptosis has been observed in many pathological conditions, such as during maturation arrest (Tesarik *et al*., 1998a; Jurisicova *et al*., 1999), after treatment with testicular toxicants (Tesarik *et al*., 2000; Boekelheide, 2005) or after hormone withdrawal (Tesarik *et al*. 1998b; Tesarik *et al*., 2002). The presence of apoptotic signs in ejaculated sperm has been reported in concomitance with testicular and systemic diseases (Baccetti *et al*., 1996; Gandini *et al*., 2000) and infertility or subfertility (Baccetti *et al*., 1997; Shen *et al*., 2002; Agarwal and Said, 2005). Apoptotic bodies, as detected by electron microscopy, have been shown in human ejaculates from subfertile men (Baccetti *et al*., 1996; Gandini *et al*., 2000). To explain the presence of apoptotic features in semen of subfertile patients, Sakkas *et al*. (1999a) proposed that testis apoptotic pathway may derail leading to a failure of the complete cell death programme.

The aim of the present study was to further characterize M540 bodies and investigate the hypothesis that they represent apoptotic bodies by examining the presence of apoptotic markers reported previously in ejaculated sperm from subertile patients (Sakkas *et al*., 2002), including caspase activity (Weng *et al*., 2002; Paasch *et al*., 2004), Fas (Sakkas *et al*., 1999b; McVicar *et al*., 2004; Stronati *et al*., 2006), p53 (Sakkas *et al*., 2002) and Bcl-x (Sakkas *et al*., 2002; Stronati *et al*., 2006) expression as well as the presence of DNA fragmentation. Finally, we evaluated their ultrastructure by transmission electron microscopy (TEM).

Materials and Methods

Antibodies and other chemicals

The monoclonal antibodies (mouse immunoglobulins, Ig, G1) used in the present study were: ab-2, anti-Fas, used at dilution 1:40; Ab-11, anti-p53, used at dilution 1:50 (Calbiochem, Nottingham, UK) and H-5, anti-Bcl-x_L, used at dilution 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Such antibodies were revealed by a goat anti-mouse IgG, conjugated with FITC (Sigma Chemical, St Louis, MO, USA). The rabbit polyclonal antibody against Bcl-$x_{S/L}$ (Santa Cruz Biotechnology), used at dilution 1:100, was revealed by a goat anti-rabbit IgG conjugated with FITC (SouthernBiotech, Birmingham, AL, USA). DNAse I was from Pharmacia Biotech Italia (Milan, Italy). Vybrant FAM Poly caspases Assay Kit was from Invitrogen (San Giuliano Milanese, Milan, Italy). Human tubal fluid (HTF) and modified HTF media were purchased from Celbio (Milan, Italy). Mouse IgG1 Isotype Control was from Exbio (Praha, Czech Republic). Normal Rabbit Serum was from DBA (Milan, Italy). Normal Goat Serum (NGS) and the other chemicals were from Sigma Chemical.

Semen sample collection and preparation

Semen samples were collected, according to World Health Organization criteria (World Health Organization, 1999), from subjects undergoing routine semen analysis for couple infertility in the Andrology laboratory of the University of Florence. The study was approved by Hospital Committee for Investigations in Humans, and informed consent was obtained by the patients. Since the aim of the present study was to characterize M540 bodies, we chose those samples in which such bodies occurred at high level, after checking by light microscopy in May–Grunwald stained samples.

For the experiments aimed to calculate the percentages of M540 bodies versus sperm in different categories of patients, 4 asthenozoospermic (A), 14 asthenoteratozoospermic (AT), 9 normozoospermic (N), 9 oligoasthenoteratozoospermic (OAT) and 18 teratozoospermic (T) samples were included. Experiments were performed in rough semen samples after washing twice with HTF medium. After washing (500 × *g* for 10 min), samples were fixed in paraformaldehyde [PF, 200 μl, 4% in phosphate-buffered saline (PBS) pH 7.4] for 30 min at room temperature, except for the Fas detection and caspase assay (see below). For Fas detection and caspase assay, samples were fixed after the immunofluorescence staining procedure and before the addition of propidium iodide (PI, see immunofluorescence section).

Determination of percentage of M540 bodies in subfertile patients

PF fixed semen samples were washed twice in HTF medium and double stained with M540 (13.5 μM) and Y1 (25 nM) in 500 μl of modified HTF containing 1 mg/ml of PVP (Muratori *et al*., 2004). Then samples were analysed by fluorescence-activated cell sorter (FACS). For each patient, two sperm suspensions were prepared for instrumental setting and data analysis: (i) by omitting both M540 and Y1 staining (nonspecific fluorescence sample) and (ii) by omitting only the M540 staining (sample for compensation, see below). Y1 green fluorescence and M540 red fluorescence were revealed by using, respectively, an FL-1 (515–555 nm wavelength band) and an FL-2 (563–607 nm wavelength band) detector of a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 15 mW argon-ion laser for excitation. Fluorescence compensation was set by acquiring samples labelled with only Y1. For each sample, 10 000 events were recorded in a FSC/SSC (Forward Scatter/Side Scatter) region excluding debris (R1, Fig. 1A). The number of M540 bodies was determined by counting the $M540^+/Y1^-$ events in the R1 region. Sperm number was determined by counting the $Y1^+$ events in the flame shaped region characteristic of sperm (Muratori *et al*., 2004). The amount of M540 bodies was expressed as percentage versus sperm number.

To exclude the possibility that the fixation procedure modifies the percentage of M540 bodies, we also calculated the percentage of bodies before and after fixation. No difference was found between fresh and fixed samples (19.4 ± 1.9 versus 17.7 ± 3.4, mean ± SD, $P = 0.22$, $n = 3$).

Immunofluorescence for Fas, Bcl-$x_{L/S}$, Bcl-x_L and p53

For intracellular detection of p53, Bcl-x_L and Bcl-$x_{L/S}$, after washing twice with PBS/1%NGS, semen samples, adjusted to 5×10^6 spermatozoa, were incubated for 1 hour at 37°C with the primary antibodies in 100 μl of permeabilizing buffer (0.1% sodium citrate/0.1% Triton X 100). For detection of Fas, the same procedure was used but the buffer was PBS/1%NGS. After two further washes, samples were incubated with the FITC-labelled secondary antibodies

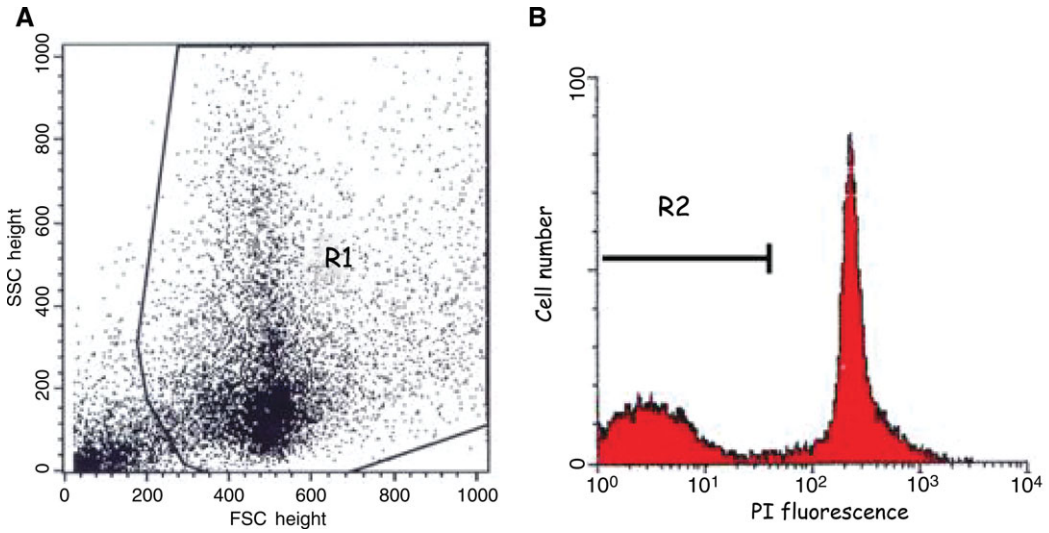

Figure 1: Strategy of analysing M540 bodies by FACS by excluding sperm and other nucleated events
(**A**) FSC/SSC dot plot obtained from a semen sample. A region (R1) is established to exclude debris. (**B**) PI fluorescence histogram obtained within R1 region. An R2 region containing the non-nucleated (PI negative) elements, M540 bodies, is set

(1:100, 1 h at room temperature) and washed again twice. Finally samples were resuspended in 400 μl of PBS and 8 μl of PI (0.6 μg/ml final concentration) were added. Fresh samples prepared for Fas detection were first fixed as described above and then labelled with PI.

Appropriate negative controls were obtained by omitting the primary antibody and by using mouse IgG1 isotype control (for Fas, p53 and Bcl-x_L detection) or normal rabbit serum (for Bcl-$x_{L/S}$ detection). For each experiment set, a sample prepared with the immunofluorescence procedure but not labelled with PI was also prepared for fluorescence compensation in the FACS analysis (see below).

Caspase assay

Caspases were detected by Vybrant FAM Poly Caspases Assay Kit, which detects active caspases by using the FAM-VAD-FMK reagent, a fluorescently labelled inhibitor of such enzymes (FLICA). Briefly, fresh semen samples were resuspended at the concentration of 1×10^6 sperm/ml in 300 μl of HTF medium. Aliquots of 10 μl of 30× 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 1× (supplied by manufacturer), samples were fixed by adding 40 μl of 10% formaldehyde solution (supplied by manufacturer) for 10 min at room temperature. Hence, sperm samples were washed again twice and resuspended in 400 μl of Wash Buffer 1× containing 8 μl of PI for FACS analysis. 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 in the FACS analysis (see below).

Terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling assay

DNA fragmentation was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick-end labelling (TUNEL) as described elsewhere (Muratori *et al*., 2003). Briefly, PF fixed semen samples adjusted to 5×10^6 spermatozoa were washed twice with 200 μl of PBS with 1% bovine serum albumin and permeabilized with 0.1% Triton X-100 in 100 μl of 0.1% sodium citrate for 2 min in ice. After washing two times, the labelling reaction was performed by incubating sperm in 50 μl of labelling solution (supplied with the *In Situ* Cell Death Detection Kit, fluorescein, Roche Molecular Biochemicals, Milan, Italy) containing the TdT enzyme for 1 h at 37°C in the dark. After labelling, two subsequent washes were performed and sperm were resuspended in 400 μl of PBS and 8 μl of PI (30 mg/ml in PBS) for FACS analysis. For each test sample, two additional samples were prepared: (i) a negative control (absence of TdT from the reaction mixture) for data analysis; (ii) a test sample labelled only by TUNEL assay (without PI staining) for fluorescence compensation in the FACS analysis (see below).

FACS analysis of DNA fragmentation, p53, Fas, Bcl-$x_{L/S}$, Bcl-xL and caspase activity in M540 bodies

Green fluorescence of the investigated markers (DNA fragmentation, p53, Fas, Bcl-$x_{L/S}$, Bcl-x_L and caspase activity) was analysed in the population formed by solely M540 bodies. To distinguish bodies from nucleated cells, before FACS analysis, we treated samples with PI (see above) that labels cells but not M540 bodies (Muratori *et al*., 2005). Hence, we established in the FSC/SSC dot plots a region excluding debris (R1, Fig. 1A) and containing both M540 bodies and nucleated cells (i.e. sperm, large cells and cell aggregates). Then, within the R1 region, a second region (R2, Fig. 1B) containing the non-nucleated events was established in the PI fluorescence histogram. Analysis of green fluorescence was performed in R2 region, thus containing only M540 bodies. FITC green fluorescence and PI red fluorescence were revealed by using, respectively, FL-1 and FL-2 detectors of the FACScan flow cytometer. Fluorescence compensation was set by acquiring samples labelled only with green fluorescence but not with the red one (i.e. PI fluorescence). For each sample, 8000 events in the R2 region (see above) were recorded. Data were processed with Lysys II software (Becton Dickinson). For each investigated marker, the percentage of labelled M540 bodies in the test sample was determined by using the fluorescence histogram of the corresponding negative sample: (i) sample incubated with isotype control for Fas, p53 and Bcl-x_L detection; (ii) sample incubated with normal rabbit serum for Bcl-$x_{S/L}$ detection; (iii) sample incubated in absence of the enzyme TdT for DNA fragmentation detection and (iv) sample incubated in absence of FLICA for caspase assay. The percentage of labelled M540 bodies was determined by setting a marker including >99% of the events in the frequency histogram of the negative control. Hence, all the events out of this region were considered positive.

In experiments aimed to compare (i) DNA fragmentation before and after nuclease treatment; (ii) expression of Bcl-$x_{L/S}$ and Bcl-x_L in the same semen samples, results were expressed as mean values of fluorescence distribution.

Fluorescence microscopy

For observation by fluorescence microscopy, double-stained sperm were smeared on slides and examined using a fluorescence microscope (type 307-148002; Leitz, Wetzlar, Germany) equipped with E4 and N2.1 filters (Leica, Milan, Italy) by an oil immersion ×100 magnification objective. Images were captured by a Canon digital camera using Remote Capture software (provided by Canon, Japan) and edited by Adobe photoshop version 5.0 (Adobe Systems Inc., CA, USA).

Transmission electron microscopy

After two washes with PBS 1×, semen samples were fixed for 1 h at room temperature in Karnovsky fixative [2.5% glutaraldehyde, 0.1% PF in 0.1 mol/l cacodylate buffer (pH 7.4)]. Then samples were postfixed with 1% buffered OsO_4 in the same buffer, dehydrated, and pellets were embedded in Epon 812 (Fluka Chemical Co., Buchs, Switzerland). Ultrathin sections were stained by uranyl acetate followed by lead citrate. Observation was performed with a Phillips 410 electron microscope.

Statistical analysis

Data were analysed with Microcal Origin software, 6.1 version (MicroCal Software Inc., Northampton, MA, USA). Results are shown as the mean ± SD. Analysis of variance and the Student's *t*-test were used to assess statistically significant differences: (i) between DNA fragmentation in M540 bodies, before and after treatment with nuclease (paired data); (ii) between the percentages of M540 bodies in the different categories of patients (independent data).

RESULTS

M540 bodies in semen from different categories of patients

In our previous paper, we reported the percentages of M540 bodies (with respect to the total population of M540 bodies + spermatozoa) in semen from subfertile subjects (Muratori *et al*., 2004). In that study, the percentage of M540 bodies in the semen samples was evaluated in the flame-shaped region of FSC/SSC dot plot characteristic of spermatozoa. Here, we re-evaluated the percentage of M540 bodies in semen samples from A, AT, N, OAT and T subjects, considering the entire population of M540 bodies in semen and referring it to the population formed by solely sperm. Indeed, M540 bodies show dimensions ranging from those similar to sperm head to much larger, as demonstrated by both FACS and fluorescence microscopy (see further sections). Figure 2A shows the population of M540 bodies ($M540^+/Y1^-$ events) as determined by double staining with M540 and the nuclear probe, Y1. In Fig. 2B, the morphometric characteristics (i.e. the FSC and SSC signals) of such population are shown. Note that M540 bodies are present both within and out of the flame-shaped region characteristic of spermatozoa (R2 in Fig. 2B). To check whether the centrifugation steps affected the composition of M540 bodies in the test sample, we collected all the supernatants and stained them with M540/Y1 together with the corresponding pellets. Then, we determined M540 bodies in the R2 region (smaller bodies) and in the R1 region (total bodies) both in the supernatants and pellet fractions. Although both bodies and spermatozoa are found in the collected supernatants, no difference was detected in the percentages of M540 bodies (versus total semen components) included in R1 and R2 regions, between supernatants and corresponding pellets (not shown).

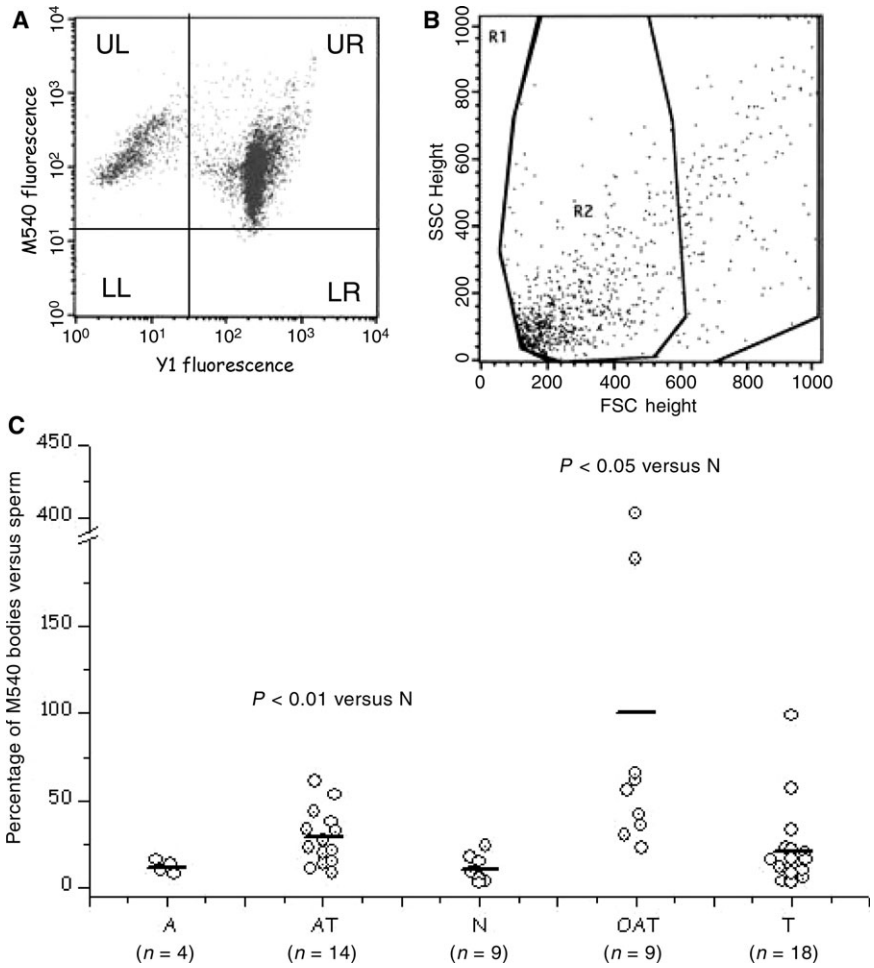

Figure 2: Strategy for evaluation and percentages of M540 bodies in semen samples from different pathologies
(**A**) M540/Y1 dot plot of fixed semen samples M540 bodies are the events positive for M540 and negative for Y1 staining, located in UL quadrant. Such events are recalled in the corresponding FSC/SSC dot plot (**B**). In the latter, an R1 region excluding debris is drawn. Note that M540 bodies are included both in the flame-shaped region characteristic of sperm (R2, Muratori *et al*., 2004) and out of it. See also for comparison Fig. 1A where the FSC/SSC signals of the whole semen sample is shown. (**C**) Percentages of M540 bodies versus sperm population in semen samples from asthenozoospermic (A), normozoospermic (N), asthenoteratozoospermic (AT), teratozoospermic (T) and oligoasthenoteratozoospermic (OAT) subjects. UL, upper left quadrant; UR, upper right quadrant; LL, low left quadrant; LR, low right quadrant

Hence, we conclude that the procedures do not affect the composition of M540 bodies in the test samples.

In Fig. 2C, percentages of M540 bodies versus spermatozoa in normal (N) subjects and subfertile patients are shown. M540 bodies are present in all categories of subjects. The highest levels of M540 bodies are found in AT ($P < 0.01$ versus N) and OAT subjects ($P < 0.05$ versus N). In the latter, the amount of M540 bodies occasionally reaches dramatic values (up to 400%).

Immunofluorescence for Fas, p53, Bcl-$x_{L/S}$ and Bcl-x_L

Detection of Fas receptor, p53 and Bcl-$x_{L/S}$ in M540 bodies was performed by immunofluorescence coupled to FACS in PI labelled samples, after the exclusion of nucleated cells (PI positive events) and debris. The expression of Fas receptor, p53 and Bcl-$x_{L/S}$ in M540 bodies was confirmed also by fluorescence microscopy. Results indicate that M540 bodies express the three markers in all the samples investigated. Figures 3, 4 and 5 show representative frequency histograms (A) and the microscopic images (B, upper panels) of green fluorescence respectively for Fas receptor, p53 and Bcl-$x_{L/S}$ in M540 bodies. The latter are easily recognizable by lack of, or poor, PI staining (B, middle panels) and round shape (B, bright fields in the lower panels). Negative control is reported in C of the three figures. In Fig. 3B, two spermatozoa are present, one negative for Fas fluorescence and the other labelled in the post-acrosomal region, as reported by other authors (McVicar *et al*., 2004). In Fig. 4B, two spermatozoa negative for p53 expression are also present, whereas of the four spermatozoa present in Fig. 5B, three show Bcl_x labelling in the post-acrosomal region. Table 1 reports the mean ($\pm$ SD) percentages of positive M540 bodies for Fas receptor, p53 and Bcl-$x_{L/S}$, as determined by FACS in several experiments. Since immunofluorescence for Bcl-x reveals both the long (x_L) and the short form (x_S), we performed FACS analysis of the long form (Bcl-x_L) in the same samples processed for Bcl-$x_{L/S}$ detection. Results indicate that the mean values of fluorescence due to Bcl-x_L in M540 bodies are lower than those due to Bcl-$x_{L/S}$ (respectively: 4.0 ± 3 versus 7.0 ± 3.6, $n = 5$), indicating that M540 bodies express both forms of Bcl-x including the pro-apoptotic one, Bcl-x_S.

Caspases activity

To detect caspase activity in M540 bodies, we double stained semen samples with the generic probe FLICA and PI and evaluated them by FACS, after the exclusion of nucleated cells (PI positive events)

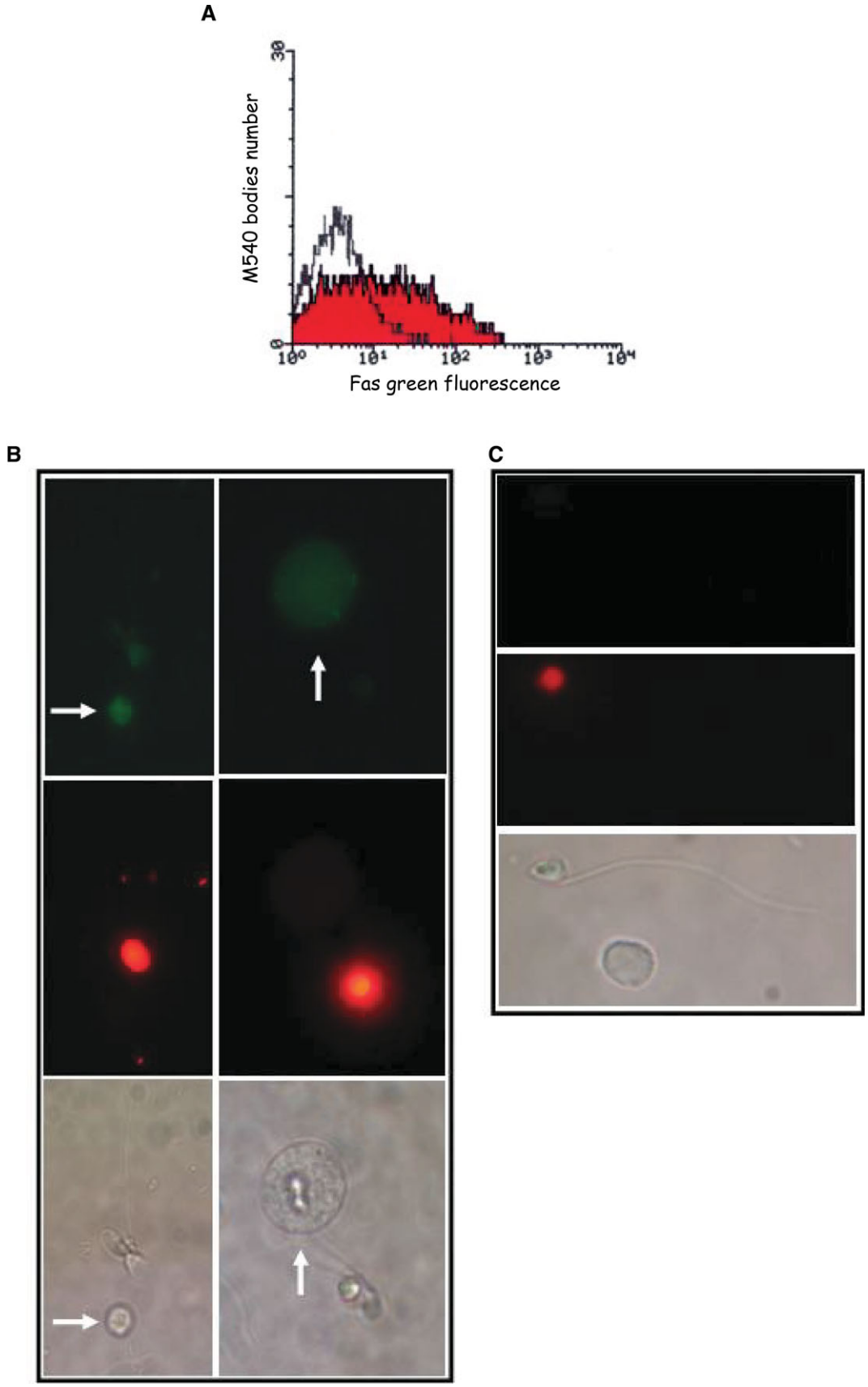

Figure 3: FAS expression in M540 bodies
(**A**) Solid histogram: frequency distributions of green fluorescence of Fas receptor in M540 bodies; open histogram represents the corresponding negative control (see below). (**B**) Micrographs of samples obtained by fluorescence microscopy after immunofluorescence for Fas receptor detection and staining with PI. Upper panels show the green fluorescence of Fas receptor; middle panels show the red fluorescence of the nuclear probe, PI; lower panels show the bright fields. Arrows indicate M540 bodies. (**C**) Negative control, incubated with isotype control IgG1 and stained by PI

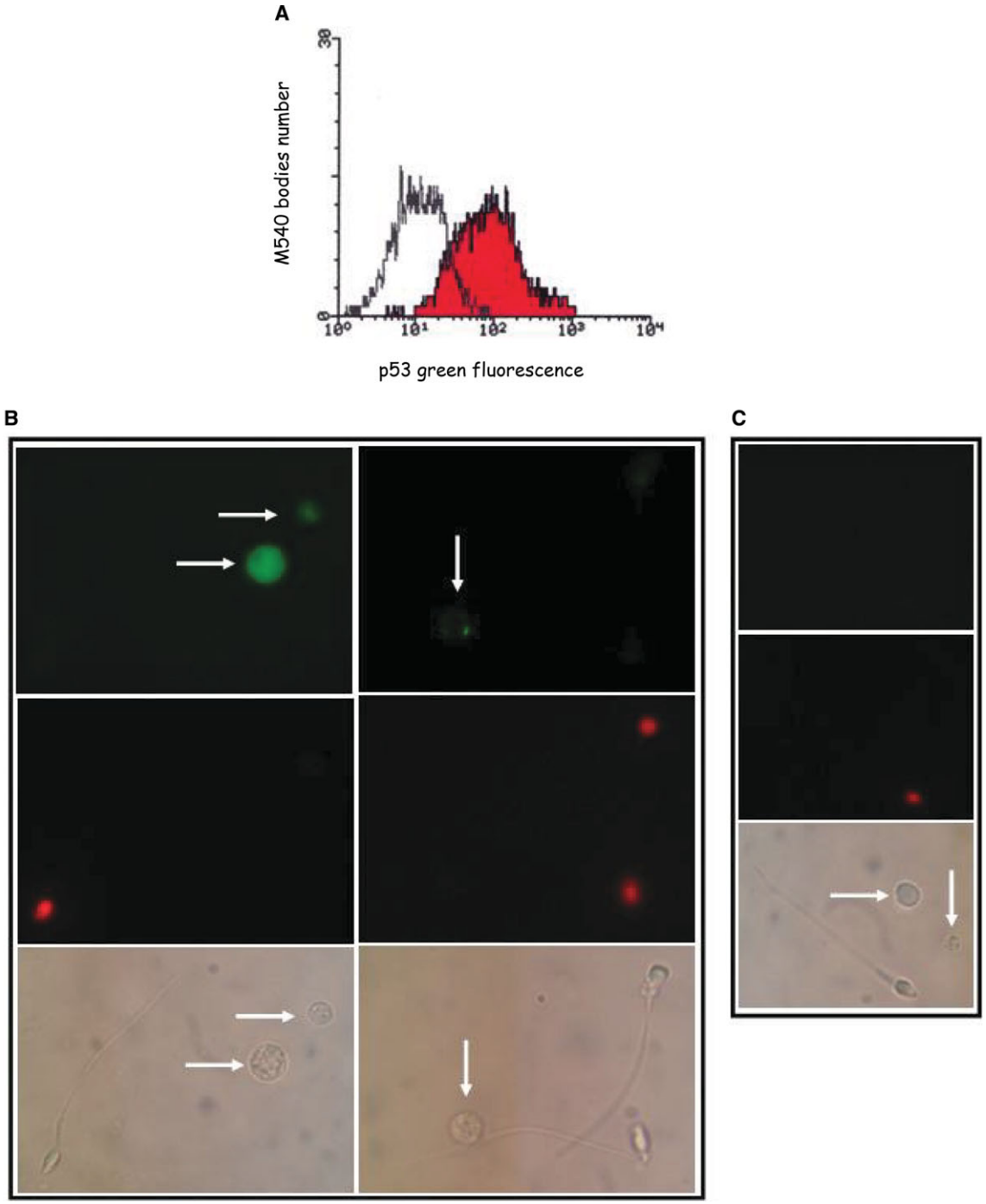

Figure 4: p53 expression in M540 bodies
(**A**) Solid histogram: frequency distributions of green fluorescence of p53 in M540 bodies; open histogram represents the corresponding negative control (see below) (**B**) Micrographs of samples obtained by fluorescence microscopy after immunofluorescence for p53 detection and staining with PI. Upper panels show the green fluorescence of p53; middle panels show the red fluorescence of the nuclear probe, PI; lower panels show the bright fields. Arrows indicate M540 bodies. (**C**) Negative control, incubated with isotype control IgG1 and stained by PI

and debris. In Fig. 6A, a representative fluorescence histogram of caspases detection is reported. Caspase activity in M540 bodies was observed also by fluorescence microscopy. Figure 6B shows the images of green fluorescence due to FLICA in M540 bodies. In the figure, fluorescence due to caspase activity is shown also in the mid-piece region and tail of sperm, as reported by other authors (Marchetti *et al*., 2004). Again, the corresponding red PI fluorescence (Fig. 6B, middle panels) and bright fields (Fig. 6B, lower panels) are reported. Images from negative controls (absence of FLICA) are reported in Fig. 6C. In Table 1, the percentages (mean $\pm$ SD) of M540 bodies exhibiting caspase activity are reported.

TUNEL assay

All the investigated apoptotic markers resulted expressed in M540 bodies, indicating that such bodies are apoptotic bodies. To further investigate this hypothesis, we tested whether M540 bodies exhibit DNA fragmentation, despite of lack of staining with nuclear probes (Muratori *et al*., 2004, 2005 and present study). DNA fragmentation

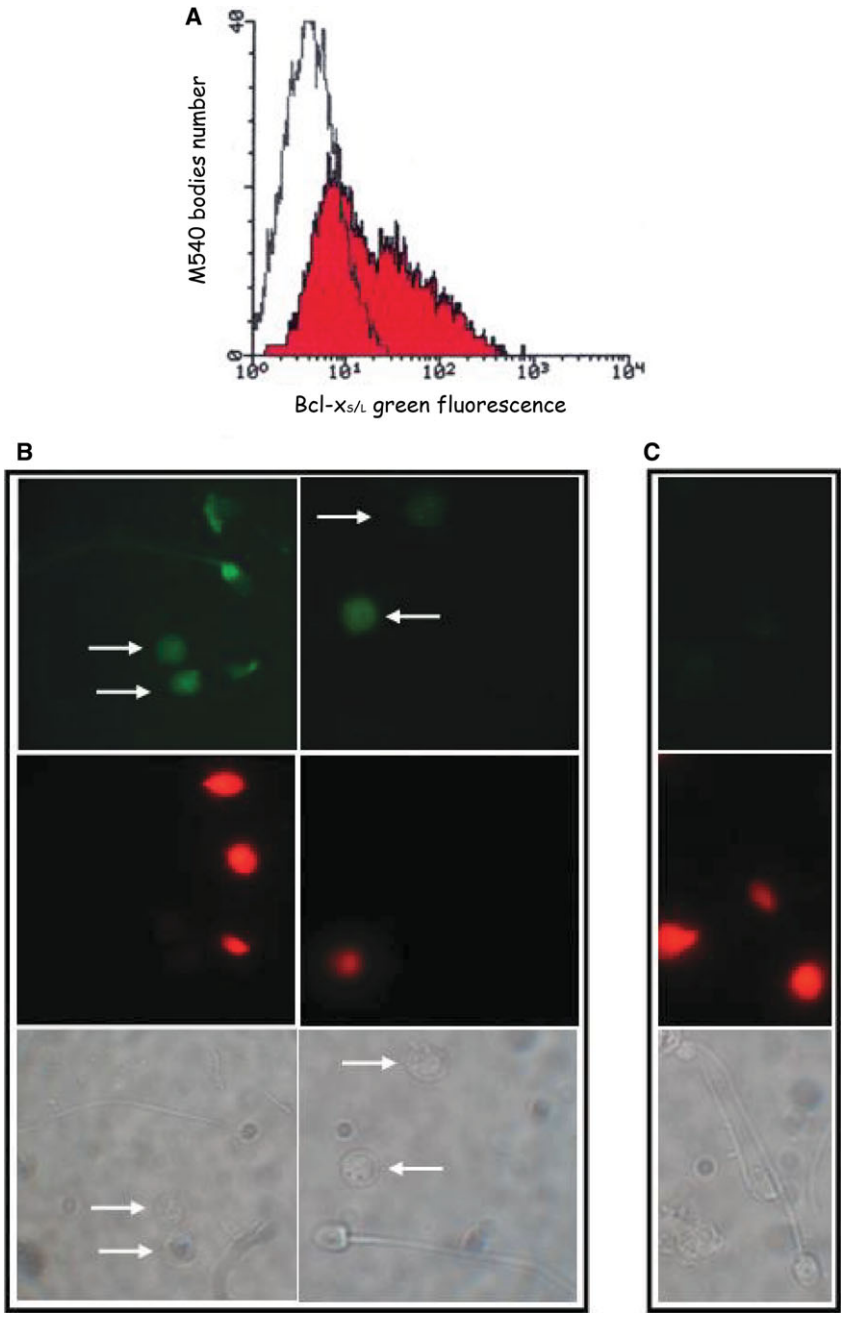

Figure 5: BCL-$x_{L/S}$ expression in M540 bodies
(**A**) Solid histogram: frequency distributions of green fluorescence of Bcl-$x_{L/S}$ in M540 bodies; open histogram represents the corresponding negative control (see below). (**B**) Micrographs of samples obtained by fluorescence microscopy after immunofluorescence for Bcl-$x_{L/S}$ detection and staining with PI. Upper panels show the green fluorescence of Bcl-$x_{L/S}$; middle panels show the red fluorescence of the nuclear probe, PI; lower panels show the bright fields. Arrows indicate M540 bodies. (**C**) Negative control, incubated with normal rabbit serum and stained by PI

in M540 bodies was investigated by TUNEL assay coupled to FACS in samples stained with PI, after the exclusion of nucleated cells (PI positive events) and debris. As shown in Fig. 7A, which reports a representative fluorescence histogram of TUNEL assay, a fraction of M540 bodies contained fragmented DNA. To further ascertain that the fluorescence signal due to TUNEL labelling in M540 bodies was really due to the occurrence of DNA fragmentation, we labelled some samples by TUNEL assay and perform FACS analysis before and after the treatment with DNAse I. Results indicate that the mean values of fluorescence distribution increase after nuclease treatment (12.0 ± 10.0 versus 22.2 ± 18.4, $P < 0.05$, $n = 6$). We conclude that a variable fraction of M540 bodies contains fragmented DNA (see Table 1). DNA fragmentation in M540 bodies was observed also by fluorescence microscopy. Fig. 7B shows the images of green fluorescence due to TUNEL labelling in M540 bodies. Again, the corresponding red PI fluorescence (Fig. 7, middle panels) and bright fields (Fig. 7, lower panels) are reported. Images from negative controls are shown in Fig. 7C.

Evaluation by TEM

We evaluated the ultrastructure of semen components by TEM in ejaculates of subjects containing high level of M540 bodies. Figure 8 shows two round bodies found in two different subjects. As shown,

Table 1: Percentages of M540 bodies exhibiting the indicated apoptotic markers

Apoptotic marker	%	SD	*n*
Fas	11.0	7.7	9
p53	43.2	13.1	5
Bcl-$x_{L/S}$	27.8	18.4	5
Caspase activity	79.1	11.4	6
DNA fragmentation	27.8	23.0	8

they closely resemble apoptotic bodies previously found in human semen from subefertile subjects (Baccetti *et al*., 1996; Gandini *et al*., 2000). Whether these elements are M540 bodies cannot be unequivocally deduced, however their dimensions, similar to sperm heads (Fig. 8), suggest this possibility.

Discussion

In this study, we report consistent evidence that M540 bodies, round structures present in semen (Muratori *et al*., 2004), represent apoptotic bodies. Indeed, we clearly show that M540 bodies express several markers, such as Fas receptor, p53, Bcl-x, caspase activity and fragmented DNA, known to have a role in testis and somatic apoptosis. In addition, we report that the levels of such bodies are much higher in semen of OAT and AT men, where the highest levels of semen apoptotic signs have been found (Sakkas *et al*., 1999b). Further, ultrastructural features of round elements from semen samples of subjects containing high levels of M540 bodies are consistent with those described for apoptotic bodies. We believe that the demonstration of occurrence of high levels of apoptotic bodies in semen has important clinical and methodological implications. Indeed, their presence in semen may be considered a sign of pathological/excessive apoptosis in the testis or, in general, in the male genital

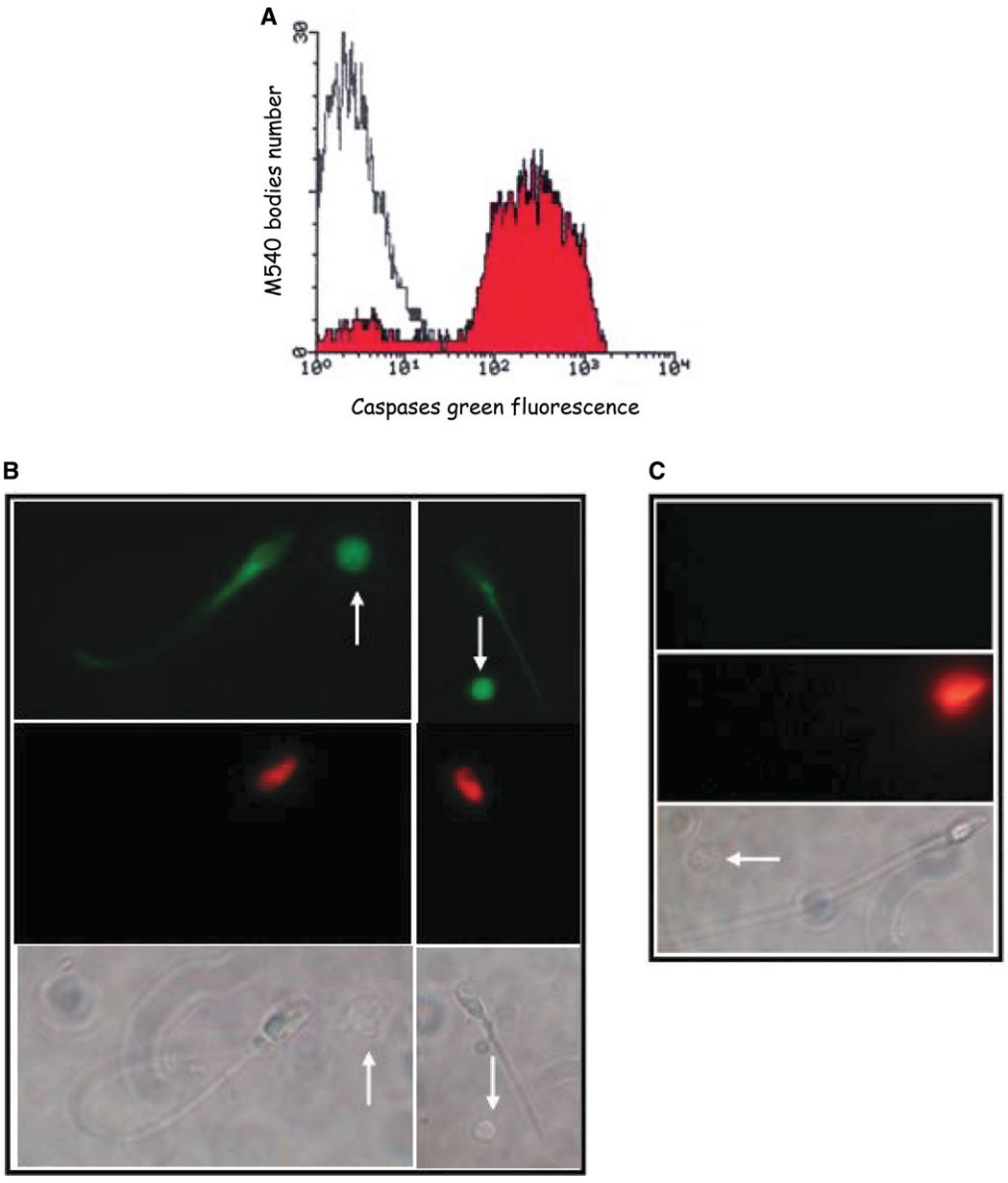

Figure 6: Caspases activity in M540 bodies
(**A**) Solid histogram: frequency distributions of green fluorescence of caspases in M540 bodies; open histogram represents the corresponding negative control (see below). (**B**) Micrographs of samples obtained by fluorescence microscopy after caspases assay and staining with PI. Upper panels show the green fluorescence of caspases; middle panels show the red fluorescence of the nuclear probe, PI; lower panels show the bright fields. Arrows indicate M540 bodies. (**C**) Negative control, incubated in absence of FLICA and stained by PI

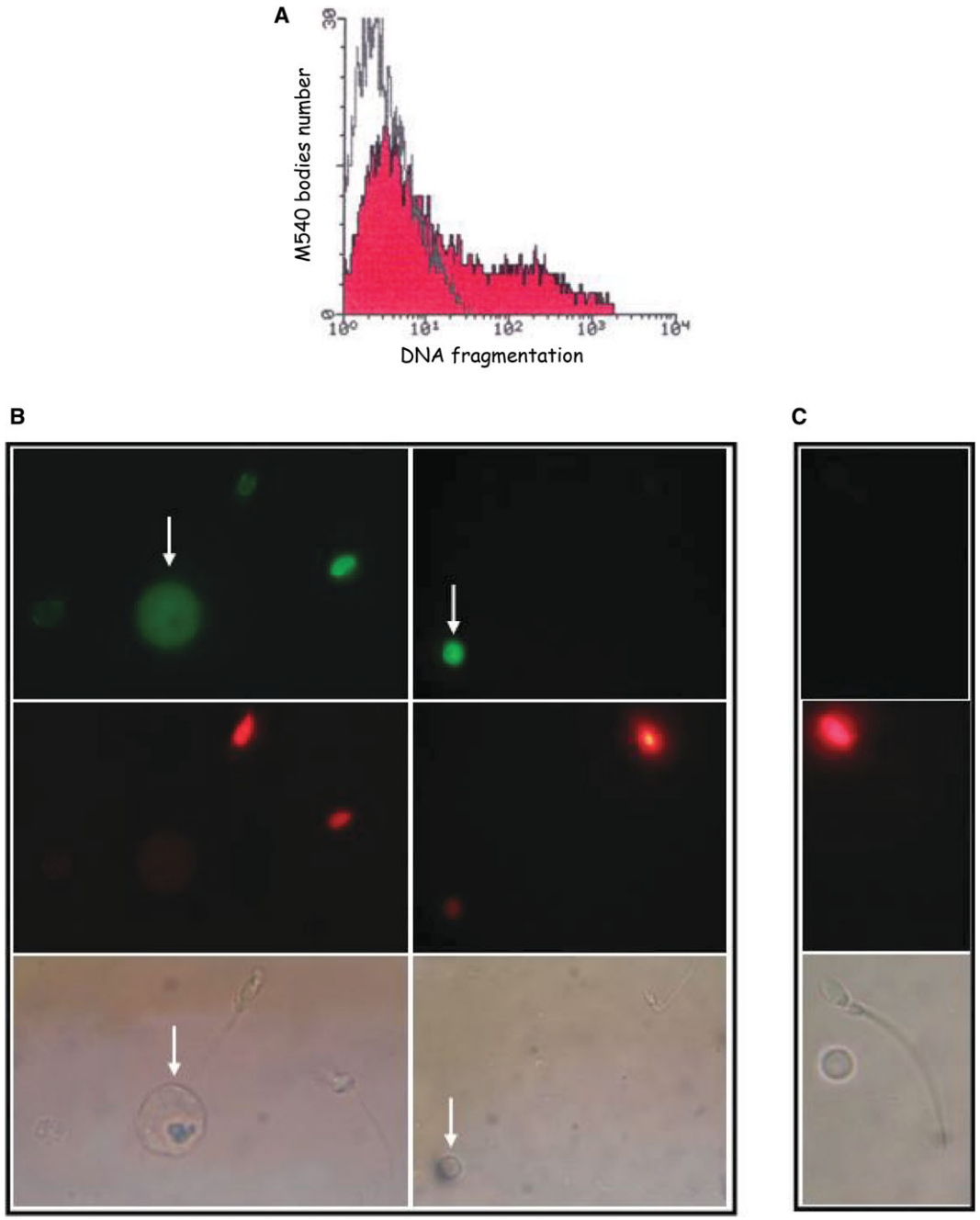

Figure 7: Occurrence of DNA fragmentation in M540 bodies
(**A**) Solid histogram: frequency distributions of DNA fragmentation in M540 bodies; open histogram represents the corresponding negative control (see below). (**B**) Micrographs of samples obtained by fluorescence microscopy after TUNEL assay and staining with PI. Upper panels show the green fluorescence due to DNA fragmentation; middle panels show the red fluorescence of the nuclear probe, PI; lower panels show the bright fields. Arrows indicate M540 bodies. (**C**) Negative control, incubated in absence of the enzyme TdT and stained by PI

tract, and at the same time, raises methodological issues in studies performed by FACS.

The molecular mechanisms responsible for testis apoptosis have not yet been completely elucidated, although they do not appear to substantially differ from apoptosis in other tissues. Pro-apoptotic stimuli in the testis are variable and can occur both in physiological and pathological conditions (Koji and Hishikawa, 2003). The different testis pro-apoptotic stimuli may induce different pathways (including the Fas-mediated and the mithochondrial-dependent pathways) to trigger the programme of cell death (Hikim *et al*., 2003). All the stimulated apoptotic pathways converge at the level of caspases, which are considered the key enzymes of apoptosis, acting both as initiators and executors of the programme of cell death. Similarly, the breakage of the double stranded DNA is an ubiquitous step in the sequence of apoptosis. Other proteins involved in the cell death programme have been demonstrated during testis apoptosis: Fas (Lee *et al*., 1997, 1999), members of the Bcl-2 family (Joshi and Dighe, 2006) and p53 (Boekelheide, 2005). We show here that M450 bodies contain all these markers to variable extents. In particular, caspase activity is detected in a very large percentage of M540 bodies, whereas the other apoptotic markers were present only in a fraction of them. This finding is not surprising, since activation of

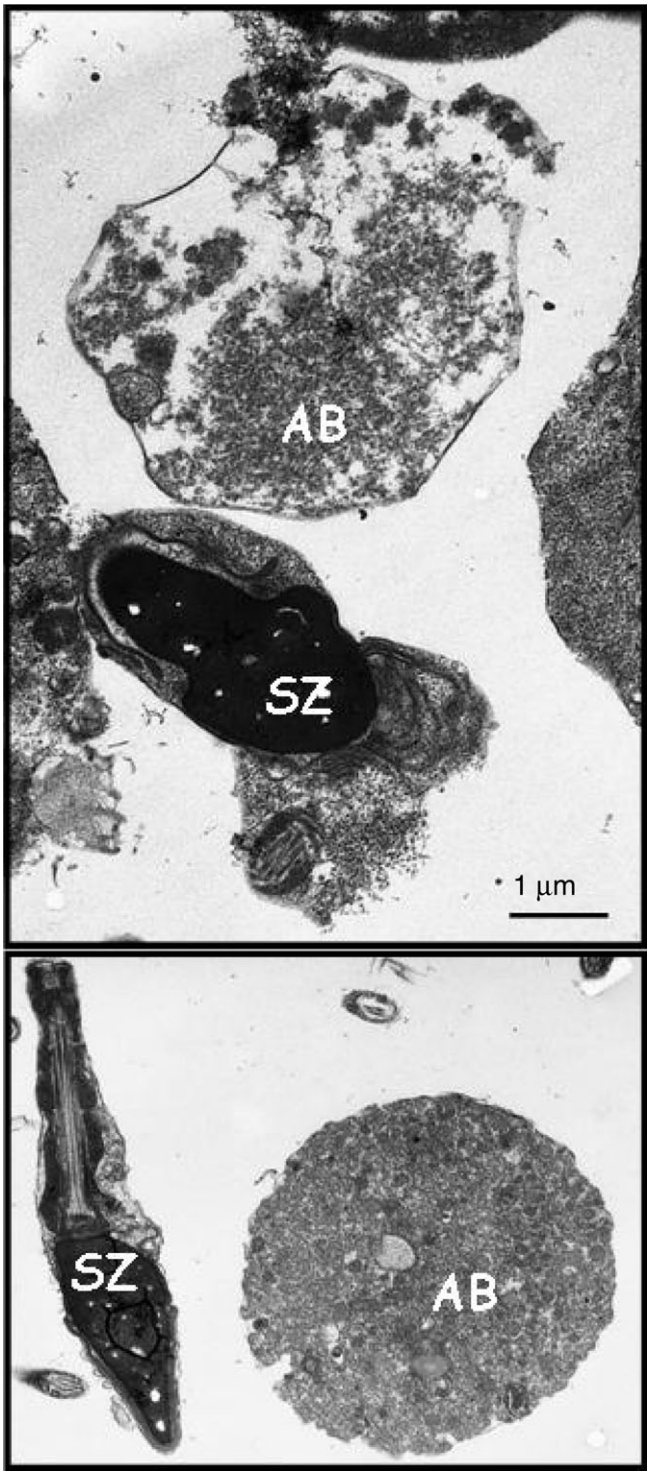

Figure 8: Ultrastructure of apoptotic bodies (AB)
Two semen samples containing a high level of M540 bodies were analysed by TEM. Two spermatozoa (SZ) are also shown. Note the similar dimension of apoptotic bodies to spermatozoa, as reported for M540 bodies (Muratori *et al*., 2004)

caspases is the final step of the process in which all the different apoptotic signalling pathways converge. In addition, the FLICA used to detect activated caspases in our study reveals most of the known caspases.

At the beginning, the finding that M540 bodies exhibit DNA fragmentation was quite surprising because, in our original study, M540 bodies were detected and distinguished from sperm in FACS, due to their lack of staining with nuclear probes (Muratori *et al*., 2004, 2005 and present study). However, in apoptotic bodies DNA may be absent or present in low amount (due to nuclei segmentation) with intensive fragmentation (due to nuclease activation). Since the intensity of PI staining is largely dependent on DNA content, whereas that of TUNEL labelling depends on the numbers of DNA breaks, it is not surprising that apoptotic bodies are poorly stained with PI and brilliantly stained with TUNEL (see e.g. Fig. 7, in which some PI staining is present in one of the shown bodies). In addition, when DNA is intensively fragmented (it occurs following apoptosis), the avidity for intercalating probes (which is elevated for coiled DNA due to the fact that binding with them reduces free energy of torsion stress) is almost completely lost.

The presence of apoptotic bodies has been already reported in human ejaculates, in TEM investigations of morphological characteristics of human spermatozoa (Baccetti *et al*., 1996; Gandini *et al*., 2000). In addition, occurrence of apoptotic signs in ejaculated sperm has been extensively reported in recent years. Such signs are mainly detected in subfertile patients and include caspase activity (Weng *et al*., 2002; Paasch *et al*., 2004), expression of key apoptotic proteins (Sakkas *et al*., 2002) and apoptosis-like ultrastructures (Baccetti *et al*., 1996). Even sperm DNA fragmentation is considered to be a sign of testicular apoptosis, although other events can explain its occurrence (Muratori *et al*., 2006). An impairment in the process of testicular apoptosis has been hypothesized and termed 'abortive apoptosis' by Sakkas *et al*. (1999a) to explain the presence of such apoptotic features in semen. Consistent with this theory, M540 bodies could represent apoptotic bodies derived from abortive testis apoptosis. However, apoptotic bodies may derive also from other cell types undergoing apoptosis in the male genital tract, such as epithelial cells of the epididymis or of the ejaculatory ducts. In this light, the high levels of apoptotic bodies found in semen of OAT and AT subjects might indicate that deregulated/excessive apoptosis of the male genital compartments, particularly of the testis, is characteristic of these subjects. However, further investigation is needed to understand whether apoptosis may be responsible for, or a consequence of, the low semen quality. Further investigation is also necessary to understand why M540 bodies (and ejaculated sperm with signs of apoptosis) are not eliminated as usually occurs in the apoptotic programme.

Occurrence of M540 bodies in human semen at variable (and sometimes high) extents and the finding that they are apoptotic residues raises methodological issues in studies on ejaculated sperm conducted by FACS. Indeed, since a fraction of M540 bodies are very similar in size and density to sperm (Muratori *et al*., 2004), they cannot be directly separated from sperm by using FSC/SSC signals, generally used to gate the population of interest. Hence, their presence may be somehow misleading when analysing sperm populations by FACS as we have recently shown (Muratori *et al*., 2005). In this light, FACS studies aimed to investigate apoptotic as well as other markers in ejaculated sperm should carefully consider that M540 bodies might also be labelled and included in the population of interest.

In conclusion, the round membrane-surrounded bodies observed in semen are likely to be apoptotic bodies. Although we are not able to unequivocally demonstrate at present that they originate from the testis, it is likely that they may derive from a process of testicular abortive apoptosis, as speculated for ejaculated sperm with apoptotic signs (Sakkas *et al*., 1999a). The variable occurrence of cell (apoptotic) residues may thus be considered a sign of deregulated apoptosis in the male reproductive organs. In addition, their presence in semen may represent a bias in FACS studies of ejaculated sperm.

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References

Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. *BJU Int* 2005;**95**:503–507.

Aussel C, Bernard G, Breittmayer JP, Pelassy C, Zoccola D, Bernard A. Monoclonal antibodies directed against the E2 protein (MIC2 gene product) induce exposure of phosphatidylserine at the thymocyte cell surface. *Biochemistry* 1993;**32**:10096–10101.

Baccetti B, Collodel G, Piomboni P. Apoptosis in human ejaculated sperm cells (notulae seminologicae 9). *J Submicrosc Cytol Pathol* 1996;**28**: 587–596.

Baccetti B, Strehler E, Capitani S, Collodel G, De Santo M, Moretti E, Piomboni P, Wiedeman R, Sterzik K. The effect of follicle stimulating hormone therapy on human sperm structure (Notulae seminologicae 11). *Hum Reprod* 1997;**12**:1955–1968.

Boekelheide K. Mechanisms of toxic damage to spermatogenesis. *J Natl Cancer Inst Monogr* 2005;**34**:6–8.

Gadella BM, Harrison RAP. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behaviour in the plasma membrane. *Development* 2000;**127**:2407–2420.

Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Pondero F, Lenzi A. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod* 2000;**5**:830–839.

Hikim AP, Lue Y, Diaz-Romero M, Yen PH, Wang C, Swerdloff RS. Deciphering the pathways of germ cells apoptosis in the testis. *J Steroid Biochem Mol Biol* 2003;**85**:175–182.

Joshi AR, Dighe RR. Expression of apoptotic genes in testicular germ cells and their modulation by luteinizing hormone. *J Steroid Biochem Mol Biol* 2006;**101**:22–30.

Jurisicova A, Lopes S, Meriano J, Oppedisano L, Casper RF, Varmuza S. DNA damage in round spermatids of mice with a targeted disruption of the Pp1cgamma gene and in testicular biopsies of patients with non-obstructive azoospermia. *Mol Hum Reprod* 1999;**5**:323–330.

Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994;**73**:2013–2026.

Koji T, Hischikawa Y. Germ cell apoptosis and its moleculare trigger in mouse testes. *Arch Histol Cytol* 2003;**66**:1–16.

Lee J, Richburg JH, Younkin SC, Boekelheide K. The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology* 1997;**138**: 2081–2088.

Lee J, Richburg JH, Shipp EB, Meistrich ML, Boekelheide K. The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. *Endocrinology* 1999;**140**: 852–858.

Marchetti C, Gallego MA, Defossez A, Formstecher P, Marchetti P. Staining of human sperm with fluorochrome-labeled inhibitor of caspases to detect activated caspases: correlation with apoptosis and sperm parameters. *Hum Reprod* 2004;**19**:1127–1134.

Martin G, Sabido O, Durand P, Levy R. Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. *Hum Reprod* 2005;**20**:3459–3468.

McVicar CM, McClure N, Williamson K, Dalzell LH, Lewis SE. Incidence of Fas positivity and deoxyribonucleic acid double-stranded breaks in human ejaculated sperm. *Fertil Steril* 2004;**81**:767–774.

Mower DA, Jr, Peckham DW, Illera VA, Fishbaugh JK, Stunz LL, Ashman RF. Decreased membrane phospholipid packing and decreased cell size precede DNA cleavage in mature mouse B cell apoptosis. *J Immunol* 1994;**152**: 4832–4842.

Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl* 2003;**24**:253–262.

Muratori M, Porazzi I, Luconi M, Marchiani S, Forti G, Baldi E. Annexin V binding and merocyanine staining fail to detect human sperm capacitation. *J Androl* 2004;**25**:797–810.

Muratori M, Marchiani S, Forti G, Baldi E. Sperm ubiquitination positively correlates to normal morphology in human semen. *Hum Reprod* 2005;**20**:1035–1043.

Muratori M, Marchiani S, Maggi M, Forti G, Baldi E. Origin and biological significance of DNA fragmentation in human spermatozoa. *Front Biosci* 2006;**11**:1491–1499.

Oldereid NB, Angelis PD, Wiger R, Clausen OP. Expression of Bcl-2 family proteins and spontaneous apoptosis in normal human testis. *Mol Hum Reprod* 2001;**7**:403–408.

Paasch U, Grunewald S, Agarwal A, Glandera HJ. Activation pattern of caspases in human spermatozoa. *Fertil Steril* 2004;**81**:802–809.

Rathi R, Colenbrander B, Bevers MM, Gadella BM. Evaluation of in vitro capacitation of stallion spermatozoa. *Biol Reprod* 2001;**65**:462–470.

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 1999a;**4**:31–37.

Sakkas D, Mariethoz E, St John JC. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp Cell Res* 1999b;**251**:350–355.

Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod* 2002;**66**:1061–1067.

Shen HM, Dai J, Chia SE, Lim A, Ong CN. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod* 2002;**17**:1266–1273.

Stronati A, Manicardi GC, Cecati M, Bordicchia M, Ferrante L, Spano M, Toft G, Bonde JP, Jonsson BA, Rignell-Hydbom A *et al.* Relationships between sperm DNA fragmentation, sperm apoptotic markers and serum levels of CB-153 and p,p′-DDE in European and Inuit populations. *Reproduction* 2006;**132**:949–958.

Tesarik J, Greco E, Cohen-Bacrie P, Mendoza C. Germ cell apoptosis in men with complete and incomplete spermiogenesis failure. *Mol Hum Reprod* 1998a;**4**:757–762.

Tesarik J, Guido M, Mendoza C, Greco E. Human spermatogenesis in vitro: respective effects of follicle-stimulating hormone and testosterone on meiosis, spermiogenesis, and Sertoli cell apoptosis. *J Clin Endocrinol Metab* 1998b;**83**:4467–4473.

Tesarik J, Mendoza C, Anniballo R, Greco E. In-vitro differentiation of germ cells from frozen testicular biopsy specimens. *Hum Reprod* 2000;**15**: 1713–1716.

Tesarik J, Martinez F, Rienzi L, Iacobelli M, Ubaldi F, Mendoza C, Greco E. In-vitro effects of FSH and testosterone withdrawal on caspase activation and DNA fragmentation in different cell types of human seminiferous epithelium. *Hum Reprod* 2002;**17**:1811–1819.

Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S, Oehninger S. Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod* 2002;**8**:984–991.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions*, 3rd edn. Cambridge, UK: University Press Cambridge, 1992.

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