Fas receptor is not present on ejaculated human sperm

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BACKGROUND: Apoptosis appears to have an essential role in the control of testis germ cell number and Fas expression has been reported in apoptotic spermatocytes and spermatids. We investigated if Fas (CD95) was present on ejaculated human sperm and any relationship between Fas on sperm and the apoptotic marker Syto16. METHODS: Semen samples from 77 male partners of infertile couples were evaluated. Each sample was analysed both before and after semen preparation by conventional microscopical procedures and by flow cytometry (FC). A multiparameter FC analysis to assess simultaneously sperm concentration, sperm viability, sperm apoptosis, CD45 positive (leukocyte) and CD95 (Fas) positive cell concentration was carried out. A further 10 samples were studied by indirect immunofluorescence to confirm results. RESULTS: The mean concentration of CD95 positive cells was very low (<1%), with no significant difference between normozoospermic and non-normozoospermic men. There was no correlation between apoptotic sperm and CD95 positive cell concentration. A linear correlation was found between CD95 positive cell and leukocyte (CD45 positive) concentration (r = 0.9946, P < 0.0001). CD95 mean fluorescence intensity of leukocytes was 10-fold greater than that of sperm and of isotypic control. Both incubation with activating anti-Fas antibody and betulinic acid induced apoptosis in leukocytes. Incubation with betulinic acid, but not with activating anti-Fas antibody, induced apoptosis in sperm. Pre-incubation with neutralizing anti-Fas antibody suppressed CD95 expression on leukocytes, whereas it did not change sperm CD95 peak fluorescence. CONCLUSIONS: There is no detectable quantity of Fas on human ejaculated sperm.

Keywords: apoptosis; flow cytometry; Fas; sperm; Syto 16

Introduction

Apoptosis appears to have an essential role in the control of germ cell number in testis (Lee et al., 1997). During spermatogenesis, germ cell death via apoptosis has been estimated to result in the loss of up to 75% of the potential number of mature sperm cells (Dunkel et al., 1997). This apoptotic wave appears necessary for normal spermatogenesis to develop, probably because it maintains a proper cell number ratio between maturing germ cell stages and Sertoli cells (Rodriguez et al., 1997). The Fas (CD95) system is considered to be one of the key regulator systems of testicular germ-cell apoptosis (Lee et al., 1997). The expression of Fas has been reported in the apoptotic spermatocytes and spermatids (Pentikainen et al., 1999; Francavilla et al., 2000, 2002; Eguchi et al., 2002; Kim et al., 2007; O’Neill et al., 2007) and also in Sertoli cells (Sugihara et al., 1997). The presence of Fas on ejaculated sperm was first described by Sakkas et al. (1999) and it was explained by the abortive apoptosis theory. This theory suggests that in some cases of infertility, the normal apoptotic mechanisms have misfunctioned, have been overridden or have not been completed and Fas positive sperm have failed to be eliminated (Sakkas et al., 1999). It was also shown that men with abnormal semen had a higher percentage of Fas positive sperm than men with normal semen (Sakkas et al., 1999). These findings were also confirmed in subsequent studies (Grunewald et al., 2001; McVicar et al., 2004). Using immunomagnetic cell separation, it was reported that 40.6% of sperm with phosphatidylserine (PS) externalization showed CD95 on their surface, whereas only 0.1% of sperm without PS externalization were CD95 positive (Grunewald et al., 2001). In morphologically abnormal sperm, Fas immunoreactivity was observed and a marked difference in Fas immunofluorescence between fertile and infertile men was found, supporting further the role of the Fas pathway in regulating sperm apoptosis (McVicar et al., 2004). However, almost simultaneously, another study reached apparently opposite conclusions. Using flow cytometry (FC), no presence of Fas protein was detected on the ejaculated sperm of normozoospermic and non-normozoospermic men (Castro et al., 2004).
These conflicting results might be explained by the different methodologies employed to detect Fas receptor on sperm (Castro et al., 2004). In some studies, sperm were fixed and/or permeabilized before analysis (Grunewald et al., 2001; McVicar et al., 2004). It is known that the use of fixation and permeabilization agents may cause non-specific uptake of probes and increased auto-fluorescence, leading to a significant interference in the fluorescence analysis (Wing et al., 1990; O’Brien and Bolton, 1995). Furthermore, no relationship between Fas presence and other apoptosis indicators has been observed (Sakkas et al., 2002; McVicar et al., 2004). It has also been shown that treatment of sperm with anti-Fas antibodies does not induce any change in apoptosis markers (Taylor et al., 2004; Grunewald et al., 2005), hence, it has been suggested that Fas receptor might be non-functional after ejaculation (Taylor et al., 2004). Therefore, the presence of Fas receptor on ejaculated sperm is still an unsolved question.

Recently, we have proposed a new multiparameter FC method for semen analysis, which offers the possibility of a simultaneous, single, rapid, reproducible and accurate assessment of several semen parameters (Perticarari et al., 2007). Moreover, we have demonstrated that the combination of Syto 16/7-AAD (7-amino-actinomycin D) provides a valuable assay to investigate sperm apoptosis (Perticarari et al., 2007). We have compared Syto 16/7-AAD with activated caspases method, a known sperm apoptosis detection method, and we have shown that it is equally able to detect both spontaneous and induced sperm apoptosis (Perticarari et al., 2007). In comparison with other technologies available to investigate sperm pathophysiology, FC allows simultaneous evaluation of a high number of cells (nearly 100,000 cells instead of 100–400 counted by optical microscopy). Multiparameter FC allows detecting and analysing the expression patterns of multiple proteins; therefore, it is possible to better understand their combined role in biological processes. Furthermore, in cytometry, indirect immunofluorescence has been progressively replaced with directly conjugated monoclonal antibody (mAb) reagents, permitting multiple mAb-colour combinations in a single tube (O’Gorman and Nicholson, 2000).

The aims of this study were: (i) to evaluate the presence of Fas on ejaculated sperm and (ii) to investigate the relationship between the presence of Fas on ejaculated sperm and the apoptotic marker Syto16, by using the multiparameter FC method.

Materials and Methods

Subjects

The study was approved by the Institutional Review Board of the Institute of Child Health IRCCS Burlo Garofolo. Semen samples were obtained from 77 men (mean age 36.0 ± 5.9 years) undergoing routine infertility investigations at the Assisted Reproduction Unit of the Institute of Child Health IRCCS Burlo Garofolo and University of Trieste. These men were normozoospermic, asthenozoospermic, asthenoteratozoospermic or oligoasthenoteratozoospermic. Additional semen samples from 10 infertile men were used to perform confirmatory tests using indirect immunofluorescence. They were not included in the statistical analyses. All subjects were Caucasians and were the partners of women who failed to conceive after 24 months of unprotected intercourse. All subjects were asymptomatic for genitourinary infections. Semen samples were collected by masturbation into sterile containers after 3–4 days of sexual abstinence. Routine analysis was performed according to World Health Organization standard guidelines (World Health Organization, 1999). A leukocyte count was carried out by using standard peroxidase test.

Sperm preparation

The sperm preparation was carried out using a 40–80% double density gradient (PureSperm, Nidacon International, AB, Göteborg, Sweden). Double density gradient was brought to a temperature of 37 °C. Using a sterile pipette, 0.5 ml of liquefied semen sample was placed on top of the upper layer in a conical 5 ml centrifuge tube which was centrifuged at 300 g for 20 min. The supernatant was then removed and the pellet was suspended in a volume of 1 ml of medium and again centrifuged at 500 g for 10 min. The pellet was resuspended in a volume of 0.5 ml of medium. An aliquot was used for light microscopic analysis and another aliquot was used for the multiparameter FC analysis.

Multiparameter FC analysis

A multiparameter FC analysis to assess simultaneously sperm concentration, sperm viability, sperm apoptosis, CD45 positive (leukocyte) and CD95 (Fas) positive cell concentration was carried out, as previously described (Perticarari et al., 2007), with minor modifications. Sperm before and after semen preparation have been investigated. Briefly, 100 μl of whole semen or prepared sperm were stained for 20 min in the dark at room temperature using 2 μl of a 10 μM solution of Syto 16 Green-Fluorescent nucleic acid stain from Molecular Probes (Eugene, OR, USA) (final concentration 200 nM), 10 μl of 7-AAD (Via-Probe, BD Pharmingen, San Diego, CA, USA) and 10 μl of allophycocyanin conjugated anti-CD45 mAb and phycoerythrin (PE)-conjugated anti-CD95 mAb clone DX2 (BD Biosciences Pharmingen, San Diego, CA, USA). The DX2 clone specifically reacts with murine L cells, murine L1210 leukaemia cells and murine P815 mastocytoma cells transfected with human Fas complementary DNA but not with untransfected parental cell lines. Cross-linking with DX2 delivers an apoptotic signal indicating that DX2 recognizes a functional epitope of the CD95 antigen. The sperm in whole semen were counted and diluted in medium to reach approximately the same concentration as prepared sperm (1–10 × 10^6/ml). A Flow-Count™ fluorospheres vial (Beckmann-Coulter, Fullerton, CA, USA, lot 754863), at a concentration of 1016 beads/μl, was gently mixed for 10–12 s. Immediately prior to analysis, 100 μl of fluorospheres were added accurately to each tube by precision reverse pipetting with wet tip. After the 20 min incubation period, 1 ml of cold phosphate-buffered saline (PBS) was added to each tube, and the samples were analysed by FC.

FC analysis was performed by using a FACS Calibur four-colour (Becton Dickinson, San José, CA, USA) equipped with a 488 nm argon laser with 530 nm (FL1), 585 nm (FL2) and 670 nm (FL3) band-pass fluorescence filters and a 635 nm red diode laser with a 661 nm band-pass filter (FL4). One hundred thousand events were collected in list mode and analysed with CELLQuest Pro software. A gating strategy was used to allow the identification of viable, dead and apoptotic sperm, as well as of CD45 positive cells (Ricci et al., 2000), CD95 positive cells and of fluorospheres. A gate for improving the detection of the entire sperm population was determined on scattering measurements [(forward-angle scatter (FSC) versus side-angle scatter (SSC)].

Because the gate for sperm on SSC and FSC could also include cells or debris with similar sizes and granularities as sperm, the method...
based also on Syto 16 staining was applied to allow more precise identification of sperm population. A unique region was set to include both Syto 16<sup>low</sup> and Syto 16<sup>high</sup> sperm. Such a gated population was then analysed in another cytogram, Syto 16 versus 7-AAD, where the Syto 16 population displays different expression of 7-AAD. By using this gating strategy, it was possible to distinguish between viable, apoptotic and dead sperm. Syto 16<sup>high</sup>/7-AAD<sup>neg</sup> sperm were defined as viable, Syto 16<sup>low</sup>/7-AAD<sup>neg</sup> sperm as apoptotic, Syto 16<sup>low</sup>/7-AAD<sup>pos</sup> sperm as necrotic.

Two gates were set, on FL1 versus SSC and on FSC versus SSC, to identify accurately the fluorosphere population.

Sperm, CD45 and CD95 positive cell concentrations were calculated according to the formula:

\[
\text{cell concentration} = \frac{\text{number of cells counted} \times \text{concentration of fluorospheres}}{\text{number of fluorospheres counted}}
\]

Concentration of fluorospheres indicates the number of fluorospheres per microlitre (known concentration) as given by the manufacturer, referred to the volume pipetted per sample.

**Fas presence on sperm and leukocytes**

Sperm samples were stained with Syto16/7-AAD and CD45 to identify sperm and leukocyte regions and exclude other necrotic cells, such as epithelial, prostate, red blood cells, bacteria and cellular debris. Sperm and leukocyte gating was necessary because many leukocyte subpopulations show Fas positivity. Samples were also stained either with PE-conjugated anti-Fas/CD95 (Clone DX2) or with isotypic control to evaluate CD95/Fas presence on sperm and leukocytes. The FC analysis was also carried out separately in the leukocyte subpopulations. All analyses were performed on each sample both before and after semen preparation.

**Induction of apoptosis**

Semen samples were washed and resuspended in human tubal fluid (HTF)—HEPES medium (Quinn’s Advantage Medium w/HEPES, SAGE BioPharma<sup>TM</sup>, Bedminster, NJ, USA), supplemented with 0.5% human serum albumin (HSA, SAGE Assisted Reproduction Products<sup>TM</sup>, CooperSurgical, Trumbull, CT, USA) to a concentration of 5–10 × 10<sup>6</sup> sperm/ml. An aliquot of 0.5 ml was then incubated for 4 h at room temperature with 1 μg/ml of agonistic anti-Fas antibody [mAb immunoglobulin (Ig)M apoptosis-activating clone CH-11, Immunotech Coulter, Marseille Cedex, France] according to the manufacturer’s instructions. CD95 clone CH-11 specifically reacts with human Fas but not with mouse Fas. This antibody induces apoptosis of some CD95 expressing cell lines in vitro. A second aliquot was incubated with betulinic acid (BA; Alexis, Grünberg, Germany) at final concentration of 60 μg/ml, for 10 min at room temperature to induce mitochondria-derived apoptosis (Grunewald et al., 2005). For each treated sample, another aliquot without stimuli was incubated under identical conditions (10 min of incubation for BA, 4 h for Fas) and served as a negative control (non-induced). The samples were then centrifuged at 400 g for 5 min, resuspended in 1 ml of PBS and prepared for multiparameter FC analysis.

**Blocking treatment with anti-Fas and presence of CD95**

To demonstrate the presence/absence of CD95 receptor on sperm and leukocytes, we added to each suspension of sperm or leukocytes a saturating amount (5 μg/ml) of blocking anti-Fas mAb unconjugated clone ZB4 (Immunotech Coulter, Marseille Cedex, France). CD95 antibody ZB4 clone has been demonstrated to block the apoptotic activity of other anti-CD95 antibodies that are known to induce apoptosis. After 1 h incubation at 4°C and two washes in PBS, cells were incubated for 30 min at 4°C with anti-CD95 clone DX2 PE-conjugated (BD Biosciences Pharmingen), or isotypic control (BD Biosciences Pharmingen). Isotypic control is defined as a mAb of the same Ig subclass as the test antibody and conjugated to the same fluorochrome. In all cases, 50 000 cells were analysed by FC.

**Indirect immunofluorescence staining for Fas**

Indirect immunofluorescence staining for Fas was performed as described by Sakkas et al. (2002). After the initial wash, sperm were resuspended in 200 μl PBS with 0.5% HSA and 20 μl of the primary antibody (clone DX2) was added. Then samples were incubated for 1 h at 4°C. After incubation, samples were washed, resuspended in 100 μl of PBS plus 0.5% HSA containing the fluorescein isothiocyanate (FITC) labelled secondary detection antibody (FITC conjugated goat anti-mouse IgG, Dako, Milan, Italy) diluted as indicated by manufacturer, and incubated for a further 1 h as previously described. Samples were then washed with PBS. Ten microlitres of the suspension was mounted in PBS–glycerol and the slide was viewed and photographed with a Zeiss Axiosplan microscope 2 with plan-neofluar 40× objective (Zeiss, Oberkochen, Germany).

**Statistical analysis**

Statistical analysis of results from the 77 samples was carried out using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA). Between-group differences were analysed by the Kruskal–Wallis analysis of variance test followed by Dunn’s multiple comparisons post hoc test. Within-group comparisons were performed using Wilcoxon signed rank test. Significant relationships between the various parameters were evaluated using the Pearson correlation test. All statistical tests were two-sided and a P-value of <0.05 was considered to be statistically significant.

<table>
<thead>
<tr>
<th>Table I. Sperm, CD95 (Fas) positive and CD45 positive (leukocyte) cell concentration in whole semen.</th>
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<tr>
<td>Sperm (×10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
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<tr>
<td>CD-95 positive cells (×10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
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<tr>
<td>CD-45 positive cells (×10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
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</tbody>
</table>

Values are mean ± SD.

*Kruskal–Wallis analysis of variance test.

Dunn’s multiple comparisons post hoc test: * versus d, P < 0.001; b versus d, P < 0.001; c versus d, P < 0.001.
Results

In whole semen, the mean concentration of CD95 positive cells was very low (<1%), with no significant difference between normozoospermic and non-normozoospermic men (Table I, n = 77). The same results were found after semen processing (Table II). In samples with very low leukocyte concentration (<5 × 10⁴ ml) CD95 positive cells were either absent or present at very low concentration (mean value: 0.014 × 10⁶/ml). There was no correlation between apoptotic sperm concentration (Syto 16) and CD95 positive cells (r = -0.047, P = 0.6901) whereas a linear correlation was found between CD95 (Fas) positive cell and leukocyte (CD45 positive) concentration (r = 0.9946, P < 0.0001; n = 77): the same results were obtained in prepared sperm (r = -0.0472, P = 0.7269, n = 77; r = 0.9247, P < 0.0001, n = 77, respectively). These findings prompted us to test the hypothesis that CD95 positive cells in semen are represented by leukocytes only. Sperm samples were stained with Syto 16 and CD45 to identify sperm and leukocyte regions and exclude other necrotic cells, such as epithelial, prostate, red blood cells, bacteria and cellular debris. This analysis was necessary to avoid non-specific antibody binding to debris or to other damaged cells. Samples were also stained either with PE-conjugated anti-Fas/CD95 (Clone DX2) or with isotypic control to

### Table II. Sperm, CD95 (Fas) positive and CD45 positive (leukocyte) cells concentration in prepared semen.

<table>
<thead>
<tr>
<th></th>
<th>Normozoospermic (n = 21)</th>
<th>Asthenozoospermic (n = 19)</th>
<th>Asthenoteratozoospermic (n = 17)</th>
<th>Oligoasthenoteratozoospermic (n = 20)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm (× 10⁶/ml)</td>
<td>44.0 ± 22.8a</td>
<td>18.3 ± 9.2b</td>
<td>14.3 ± 16.1c</td>
<td>4.3 ± 3.2d</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD95 positive cells (× 10⁶/ml)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.3</td>
<td>0.05 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>CD45 positive cells (× 10⁶/ml)</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.3</td>
<td>0.1 ± 0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

*Kruskal–Wallis analysis of variance test.

Dunn’s multiple comparisons post hoc test: *versus a, P < 0.001; *versus d, P < 0.001; b versus d, P < 0.001.

Figure 1: Cytogram A displays a leukocyte-rich semen sample stained with Syto 16 and CD45 to identify leukocytes and sperm. Leukocytes (CD45 positive) are shown in R1 (red region) and sperm (CD45 negative) are shown in R2 (green region). Graph B represents the two cellular populations gated in cytogram A, which were also stained with CD95 PE-conjugated clone DX2 or with isotypic negative control, to evaluate CD95/Fas expression. Sperm CD95 fluorescence intensity (green histogram), expressed as log relative fluorescence, is <1 log unit and is considered as negative (autofluorescence). In fact, sperm fluorescence coincides with that of isotypic negative control (gray histogram). Leukocyte fluorescence intensity (red histogram) ranges from 1 to 2 log units and is considered as positive internal control. Cytogram C and Graph D are representative of the FC analysis of a sample with a low leukocyte concentration.
evaluate CD95/Fas presence. Two separate CD95 fluorescence peaks, corresponding to sperm and leukocyte populations, were observed (Fig. 1). Sperm CD95 fluorescence intensity, expressed as log relative fluorescence, was < 1 log unit and was considered as negative and corresponding to the autofluorescence level of the sperm. In fact, peak sperm fluorescence coincided with that of isotypic negative control. The sperm mean fluorescence intensity (MFI) was 3.90 ± 0.30, and it was not significantly different from that of the isotypic negative control (MFI = 3.64 ± 0.54), indicating the absence of Fas on the sperm. Leukocyte fluorescence intensity ranged from 1 to 2 log units and it was considered as a positive internal control. In fact, the leukocyte MFI was ~10-fold greater (31.65 ± 2.95) than that measured for sperm or isotypic control. No differences were detected gating viable and non-viable or damaged sperm. In viable and non-viable sperm subpopulations, CD95 peak fluorescence coincided with that of isotypic negative control (Fig. 2).

The presence of Fas on the surface of spermatozoa was also investigated using indirect immunofluorescence in semen samples from non-normozoospermic men (n = 10). Fas immunoreactivity was observed on the surface of the seminal leukocytes, whereas there was no evidence of Fas immunofluorescence on the sperm (Fig. 3).

In order to evaluate Fas-mediated apoptosis induction, sperm were incubated with anti-Fas activating mAb, clone CH11. A fraction was incubated with BA which served as positive control for caspase activation. As a positive control for apoptosis induction, peripheral blood mononuclear cells were used. Apoptosis was detected by using FC Syto 16/7AAD staining. Both incubation with anti-Fas CH-11 and BA induced apoptosis in leukocyte samples, as shown by an increase in the Syto 16low/7-AADneg leukocyte fraction (Fig. 4). No increase in the apoptotic sperm (Syto 16low/7-AADneg) fraction was detected after incubation with anti-Fas CH11, whereas an increase in the apoptotic sperm fraction was detected after incubation with BA (Fig. 4).

Finally, in order to test the hypothesis that Fas receptor might be expressed, but is either non-functional or non-operative after ejaculation (Taylor et al., 2004), samples were incubated with anti-Fas neutralizing mAb, clone ZB4: i.e. the CD95-neutralizing antibody which binds Fas receptor but does not induce apoptosis. The samples were then stained either with PE-conjugated anti-Fas/CD95 (Clone DX2) or with isotypic control. Neither before nor after treatment with

Figure 2: Representative dot plot of the FC analysis of Fas expression on viable or non-viable or damaged sperm. The viable sperm that do not incorporate 7-AAD are gated in region 1 (R1, in green, in the left cytogram). The damaged or non-viable sperm that incorporate 7-AAD are gated in region 2 (R2, in red, in the left cytogram). The right graph shows CD95 expression on the two sperm populations. Histogram overlay indicates that both viable and damaged or non-viable sperm do not express CD95/Fas. In fact, viable (green peak) and damaged or non-viable sperm fluorescence (red peak) coincide with that of isotypic negative control (dotted line).

Figure 3: Fas on spermatozoa investigated using indirect immunofluorescence in samples from non-normozoospermic men (n = 10). Light microscopy (A) displaying sperm and seminal leukocytes. Fluorescence microscopy (B) of the same sample showing that leukocytes but not sperm show Fas positivity (green) on their surface.
ZB4 was CD95 presence detected on the sperm. In fact, CD95 fluorescence of sperm was unchanged with anti-Fas neutralizing mAb pre-incubation and it coincided with that of isotype negative control (Fig. 5). A comparison of CD95 MFI of untreated sperm, sperm pre-incubated with anti-Fas antibody ZB4 and negative isotype control showed no differences between them (Fig. 6). In contrast, ZB4 pre-incubation was able to significantly reduce the CD95 peak fluorescence of the seminal leukocytes (Figs 5 and 6).

Discussion

Over the past few years, several studies have investigated apoptosis in ejaculated sperm. The presence of Fas, a somatic apoptotic marker, on human ejaculated sperm was reported and it was hypothesized that in some men an ‘abortive apoptosis’ had taken place (Sakkas et al., 1999). Other somatic apoptotic markers, such as some morphological characteristics (Baccetti et al., 1996), DNA fragmentation (Gorczyca et al., 1993; Barroso et al., 2000; Donnelly et al., 2000; Gandini et al., 2000) and PS exposure (Glander and Schaller, 1999; Oosterhuis et al., 2000; Ricci et al., 2002) have been used to detect sperm apoptosis. However, subsequent studies provided controversial results (Muratori et al., 2000; de Vries et al., 2003; Martin et al., 2005). DNA fragmentation has been shown to be more likely a sign of defective sperm maturation (Muratori et al., 2000), due to problems in the nuclear remodelling process (Sakkas et al., 2002). Under experimental conditions, PS exposure has been found to be related to sperm capacitation and/or acrosome reaction rather than to apoptosis (de Vries et al., 2003; Martin et al., 2005). However, in these studies only mature sperm selected by discontinuous Percoll gradient have been investigated (de Vries et al., 2003; Martin et al., 2005). Therefore, theoretically, these studies would not be able to detect ‘abortive apoptosis’. More recent studies have investigated other apoptotic markers. The existence of a caspase-dependent apoptotic pathway in ejaculated human sperm has been demonstrated and the evaluation of caspase activation has been suggested as a sensitive assay for detection of sperm apoptosis (Said et al., 2004; Marchetti and Marchetti, 2005). In a previous work (Perticarari et al., 2007), we have demonstrated that the combination of Syto 16/7-AAD, commonly used for the assessment of somatic cell apoptosis (Wlodkowic et al., 2007), provides a valuable cytometric tool for the detection of apoptosis in human sperm.

**Figure 4:** Syto 16/7-AAD staining upon induction of apoptosis. The upper cytograms represent an untreated sperm sample (A), after incubation with anti-Fas antibody CH11 (B), or after incubation with betulinic acid (C). No significant increase in apoptotic sperm (Syto 16<sup>low</sup>/7-AAD<sup>neg</sup>) fraction was detected after incubation with anti-Fas CH11. A significant increase in apoptotic sperm fraction was detected after incubation with betulinic acid. The lower cytograms represent a leukocyte sample, not treated (D), after incubation with anti-Fas antibody CH11 (E), or after incubation with betulinic acid (F). Significant increases in apoptotic leukocytes (Syto 16<sup>low</sup>/7-AAD<sup>neg</sup>) were detected after incubation either with anti-Fas CH11 (E) or betulinic acid (F). Plots are representative of three independent experiments.
The assay also for detection of both spontaneous and induced sperm apoptosis.

The objectives of the present study were to evaluate the presence of Fas on ejaculated sperm and to correlate the presence of Fas with apoptosis, as detected by Syto 16 staining. The role of Fas system in regulating testicular germ cell apoptosis has been demonstrated, although the significance of Fas in ejaculated sperm is an open question. Sakkas et al. (1999) reported that in men with normal sperm parameters the percentage of Fas positive sperm is low, whereas in men with abnormal sperm parameters, this can be as high as 50%.

McVicar et al. (2004) found that no sperm from fertile men displayed Fas positivity, whereas in abnormal semen 70% of sperm were Fas positive. In both studies, there was no relationship between Fas positivity and DNA damage. Grunewald et al. (2001) used the binding of paramagnetic Annexin V-conjugated microbeads to eliminate apoptotic sperm and observed that in the unlabelled sperm fraction (sperm without ability to bind Annexin V-conjugated microbeads) only 0.1% of sperm showed Fas on their surface, whereas 40.6% of sperm with bound microbeads were Fas positive. The total percentage of Fas positive sperm was 2.5%.

Using a multiparameter cytometric method in the present study, we failed to detect the presence of Fas on the ejaculated sperm both in normal and in abnormal semen samples. To confirm our results, we replicated the original experiment described by Sakkas et al. (1999) using indirect immunofluorescence. Fas immunofluorescence was found only on the seminal leukocytes. The sperm population did not show Fas immunoreactivity. We concluded that CD95 positive cells in ejaculate are represented by leukocytes only. Our findings agree with results of Castro et al. (2004) who used a methodology similar to ours and did not find the presence of Fas in ejaculated sperm of normozoospermic and non-normozoospermic men.

However, in order to obtain more reliable results, we used as positive control not only peripheral leukocytes in a separate analysis, but also the seminal leukocytes by gating in semen CD45 positive cells. The recently developed multiparameter cytometric method (Petricarari et al., 2007) allows the precise identification of regions of sperm and leukocytes, excluding other necrotic cells, such as epithelial, prostate, red blood cells, bacteria and cellular debris, and to quantify absolute number of CD45 and CD95 positive cells.

The methodology that we have adopted may explain the differences with the results obtained in previous studies (Sakkas et al., 1999; Grunewald et al., 2001; McVicar et al., 2004) (see Table III for details). First, in such studies indirect immunofluorescence employing a second step antibody carrying the fluorochrome probe was used to detect CD95 positive cells. Indeed, over the last years, the use of direct
Comparison of the methods used to investigate the presence of Fas on sperm.

<table>
<thead>
<tr>
<th>Method to detect Fas</th>
<th>Selective flow cytometry gating of sperm and leukocytes</th>
<th>Negative control</th>
<th>External positive control</th>
<th>Internal positive control</th>
<th>Experimental Fas-mediated apoptosis induction</th>
<th>Fas presence tested by anti-Fas neutralizing monoclonal antibody</th>
</tr>
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<tbody>
<tr>
<td>Sakkas et al., 1999</td>
<td>Indirect immunofluorescence and flow cytometry</td>
<td>No</td>
<td>Isotypic monoclonal antibody</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Grunewald et al., 2001</td>
<td>Indirect immunofluorescence and flow cytometry</td>
<td>No</td>
<td>Isotypic monoclonal antibody</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>McVicar et al., 2004</td>
<td>Indirect immunofluorescence and flow cytometry</td>
<td>No</td>
<td>Isotypic monoclonal antibody</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Castro et al., 2004</td>
<td>Indirect immunofluorescence and flow cytometry</td>
<td>Yes (identified subpopulations using propidium iodide staining)</td>
<td>Isotypic monoclonal leukocytes antibody</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Perticarari et al. (present study)</td>
<td>Direct immunofluorescence and multiparameter flow cytometry</td>
<td>Yes (identified subpopulations using Syto 16/7-amino-actinomycin D combination and anti-CD45 monoclonal antibody staining)</td>
<td>Isotypic Peripheral monoclonal leukocytes antibody</td>
<td>Seminal leukocytes</td>
<td>Yes</td>
<td>Yes</td>
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fluorochrome-conjugated, rather than indirect, immunofluorescence analysis has been increased. Indirect staining, in comparison with direct staining, may sometimes amplify the fluorescent signal, and, consequently, the background fluorescence may be increased. Furthermore, false positives may occur due to potential binding of the secondary conjugated antibody to the primary antibody target (Nijkamp and Parnham, 2005). Secondly, in the previous studies, viable sperm and leukocytes were not gated (Sakkas et al., 1999; Grunewald et al., 2001; McVicar et al., 2004). Therefore, leukocytes and other dead cells were not excluded from the cell population analysed. Monocytes, granulocytes and subpopulations of lymphocytes express the Fas antigen (Mincheff et al., 1998). If they are not excluded, they may give false sperm Fas positivity. Leukocytes can be gated only by using a specific mAb. We used an anti-CD45 mAb that we demonstrated could detect seminal leukocytes (Ricci et al., 2000). Dead cells may cause analytical errors. In fact, non-specific uptake of fluorescent probes by dead cells may occur, leading to overestimated percentages of positive cells, increased autofluorescence and, consequently, incorrect assessment of antigen expression (Rothe and Schmitz, 1996). We used a gating strategy to identify live, dead and apoptotic sperm and leukocytes. A gate to improve the detection of the whole sperm population was determined on scattering measurements (FSC versus SSC). Because the gate for sperm based on SSC and FSC could also include other cells or debris with similar sizes and granularities as sperm, the method based on Syto 16 staining was also applied to allow a precise identification of the sperm population. A unique region (R1) was set in order to include both Syto 16\textsuperscript{low} and Syto 16\textsuperscript{high} sperm. Such a gated population was then analysed in another cytogram, Syto 16 versus 7-AAD, where the Syto 16 population displays different expression of 7-AAD. By using this gating strategy, it was possible to distinguish between viable, dead or apoptotic cells. Separate analysis of viable and non-viable or damaged sperm showed that both of these sperm subpopulations do not express Fas on their surface. Thirdly, in order to test the reliability of our findings, we used a double control: an isotypic mAb of the same Ig subclass as the test antibody and conjugated to the same fluorochrome was used as negative control; peripheral leukocytes and seminal leukocytes were used as internal positive control.

Using this methodology, we also performed functional studies to assess whether Fas-mediated sperm apoptosis could be induced. Taylor et al. (2004) investigated the apoptosis pathways in ejaculated sperm and demonstrated that various stimuli, as well staurosporine and H\textsubscript{2}O\textsubscript{2}, increased caspase activity or PS translocation, but treatment with anti-Fas receptor did not result in any changes in apoptosis markers. They did not evaluate the presence of Fas on the sperm, but concluded that the Fas receptor, if present, might be either non-functional or non-operative after ejaculation. Similar results were obtained from Grunewald et al. (2005) who were unable to detect caspase activation in human sperm in response to Fas induction. We confirmed that incubation with BA, but not with anti-Fas antibody CH-11, induces apoptosis in sperm samples.

In order to test the hypothesis that Fas receptor might be expressed but non-functional or non-operative, we pre-incubated sperm and leukocytes with the antagonistic ZB4 anti-Fas antibody unconjugated, which blocks Fas receptor and, consequently, prevents binding of the conjugated antibody. We observed a down-regulation of CD95 expression on leukocytes, which confirmed the block of Fas receptor by the anti-Fas neutralizing antibody. In contrast, pre-incubation with ZB4 anti-Fas antibody did not change sperm peak fluorescence, suggesting that Fas was not constitutively expressed on sperm.

In conclusion, the results of the present study confirm that multiparameter FC is a useful method to investigate sperm pathophysiology. One of the main characteristics is the possibility to easily
extend the parameters to be investigated. Our findings show that there is no detectable quantity of Fas on human ejaculated sperm. The presence of apoptotic sperm in semen, as detected by Syto 16 and by other apoptotic markers, suggests that apoptosis can take place in the human spermatozoa. Further studies are required to assess whether this process starts in the testis and can be, consequently, an expression of ‘abortive apoptosis’ or it may occur even during sperm transit through the male genital tract in response to other stimuli.

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References

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