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Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations

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Abstract. In the present study, we have related mitochondrial membrane potential ($\Delta\Psi_m$) and forward scatter (FSC) to apoptotic-related changes in spermatozoa. Thawed red deer spermatozoa were incubated in synthetic oviductal fluid medium (37°C, 5% CO_2), with or without antioxidant (100 μM trolox). At 0, 3, 6 and 9 h, aliquots were assessed for motility and were stained with a combination of Hoechst 33342, propidium ioide (PI), YO-PRO-1 and Mitotracker Deep Red for flow cytometry. The proportion of spermatozoa YO-PRO-1+ and PI+ (indicating a damaged plasmalemma; DEAD) increased, whereas that of YO-PRO-1− and PI− (INTACT) spermatozoa decreased. The proportion of YO-PRO-1+ and PI− spermatozoa (altered plasmalemma; APOPTOTIC) did not change. Both DEAD and APOPTOTIC spermatozoa had low $\Delta\Psi_m$. Most high-$\Delta\Psi_m$ spermatozoa were INTACT, and their proportion decreased with time. The FSC signal also differed between different groups of spermatozoa, in the order APOPTOTIC > DEAD > INTACT/low $\Delta\Psi_m$ > INTACT/high $\Delta\Psi_m$; however, the actual meaning of this difference is not clear. APOPTOTIC spermatozoa seemed motile at 0 h, but lost motility with time. Trolox only slightly improved the percentage of INTACT spermatozoa ($P < 0.05$). The population of APOPTOTIC spermatozoa in the present study may be dying cells, possibly with activated cell death pathways (loss of $\Delta\Psi_m$). We propose that the sequence of spermatozoon death here would be: (*1*) loss of $\Delta\Psi_m$; (*2*) membrane changes (YO-PRO-1+ and PI−); and (*3*) membrane damage (PI+). INTACT spermatozoa with low $\Delta\Psi_m$ or altered FSC may be compromised cells. The present study is the first that directly relates membrane integrity, apoptotic markers and mitochondrial status in spermatozoa. The results of the present study may help us understand the mechanisms leading to loss of spermatozoon viability after thawing.

Additional keywords: cell volume, flow cytometry, membrane changes, mitochondrial membrane potential, sperm death, YO-PRO-1.

Introduction

Even though spermatozoa may lack a complete apoptotic mechanism (Weil *et al.* 1998), many studies have confirmed that at least part of the apoptotic pathway could be active, or potentially active, in spermatozoa (Paasch *et al.* 2004*a*; Grunewald *et al.* 2008; Marti *et al.* 2008). In fact, active caspases, proteases functioning as apoptotic mediators, have been detected in spermatozoa under various conditions (Paasch *et al.* 2003, 2004*a*, 2004*b*). Previous studies have described the effects of apoptosis in spermatozoa in terms of membrane changes, loss of mitochondrial membrane potential ($\Delta\Psi_m$) and volume deregulation. Changes in membrane permeability have been described as a typical apoptotic event in many cell types (Idziorek *et al.* 1995). Although spermatozoa can undergo physiological changes on the plasmalemma, due to capacitation, it was soon discovered that some of these changes were linked to apoptotic markers (Waterhouse *et al.* 2004; Martin *et al.* 2005). Spermatozoa affected by these apoptotic-like events exhibit membrane changes that apparently herald cell death (Peña *et al.* 2005).

Loss of $\Delta\Psi_m$ associated with apoptosis has been found in spermatozoa (Martin *et al.* 2005; Aziz *et al.* 2007; Grunewald *et al.* 2008). The presence of active mitochondria is important because they participate in many regulatory and maintance processes (Aitken *et al.* 2007). Therefore, we can consider that apoptotic spermatozoa may eventually lose viability because

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of a lack of mitochondrial activity. Furthermore, mitochondria may play a key role in spermatozoon apoptosis (Paasch *et al.* 2004*a*; Martin *et al.* 2007), containing some factors necessary to elicit it. Therefore, in the present study, we characterised $\Delta\Psi_m$ in our samples using Mitotracker Deep Red, a relatively new mitochondrial probe with a fluorescence profile compatible with the simultaneous assessment of other probes, such as YO-PRO-1 (used to detect apoptosis-related membrane changes) and propidium ioide (PI; Hallap *et al.* 2005).

Cell shrinkage is another feature of apoptosis. Studies on spermatozoa have confirmed that these cells have a system to regulate their volume so that they can cope with changes in external osmolality in the female genital tract (Petrunkina and Topfer-Petersen 2000; Yeung *et al.* 2002; Petrunkina *et al.* 2005*a*, 2007). The ability to withstand these changes, which includes recovery of their original volume after osmotic swelling or shrinkage, is of considerable importance for both traversing the female reproductive tract and surviving cryopreservation. This ability to withstand changes depends on many transport systems attached to the plasma membrane and on a balanced energy system (Petrunkina *et al.* 2004). Therefore, changes to them membrane or loss of mitochondrial activity may affect the ability of spermatozoa to regulate their volume and, thus, induce changes in volume. Indeed, Petrunkina *et al*. (2005*b*) have reported that necrotic volume increase (NVI), a process involving normotonic uptake of ions, occurs in spermatozoa. This process produces cell swelling and, eventually, leads to cell death. Petrunkina *et al*. (2005*b*) did not distinguish between apoptotic and necrotic spermatozoa, but these findings indicate that it possible that apoptotic spermatozoa undergo characteristic volume changes either because of loss of $\Delta\Psi_m$ or other processes.

The present study was designed to directly relate apoptotic membrane changes, decreases in $\Delta\Psi_m$ and possible volume changes in spermatozoa. To this end, we used flow cytometry, a technology that allows the analysis of many parameters in a large population of cells. Previously, several studies have investigated this issue, relating membrane or mitochondrial changes to apoptotic markers using flow cytometry. However, membrane permeability and mitochondrial status have been related indirectly and not determined simultaneously in the same spermatozoon. We hypothesised that plasmalemma changes, loss of $\Delta\Psi_m$ and loss of volume regulatory capacity would occur concurrently in apoptotic spermatozoa but that some spermatozoa would exhibit only some of these characteristics, thus enabling us to define the order in which these changes occurred. In the present study, we used the forward light scattering signal (FSC) as a gross measure of changes in cell morphometry. We must caution against relating the FSC data we present here with cell volume; rather, the FSC data should be used as an indicator of changes in the size or structure of the cells (Shapiro 2003). Moreover, some authors have suggested that reactive oxygen species (ROS), which could be generated during the incubation period, may elicit membrane and apoptotic changes (Taylor *et al.* 2004). Thus, we also tested the hypothesis that the antioxidant vitamin E (Ball and Vo 2002) would modify the timing or occurrence of apoptotic changes in spermatozoa.

Materials and methods

Reagents and media

Both YO-PRO-1 and Mitotracker Deep Red were purchased from Invitrogen (Barcelona, Spain). Flow cytometry equipment, software and consumables (including the sheath fluid, BD FACSFlow) were purchased from BD Biosciences (San Jose, CA, USA). The remaining fluorescence probes and chemicals (reagent or higher grade) were obtained from Sigma (Madrid, Spain). The synthetic oviductal fluid (SOF) medium was prepared according to Takahashi and First (1992) and had a composition of 107 mM NaCl, 7.17 mM KCl, 1.19 mM KH_2PO_4, 1.71 mM $CaCl_2$, 0.49 mM $MgCl_2$, 25.07 mM $NaHCO_3$, 3.3 mM sodium lactate, 0.30 mM sodium pyruvate and 14.1 μM phenol red in milli-Q water. This solution was supplemented with 5 mL L^{-1} of 200 mM glutamine solution, 20 mL L^{-1} Sigma M7145 essential amino acids solution (BME) and 10 mL L^{-1} Sigma B6766 non-essential amino acids solution (MEM) and then filtered through a 0.2-μm pore. Immediately prior to use, 3 mg mL^{-1} bovine serum albumin (BSA) was added and the solution was equilibrated in a 95% air/5% CO_2 atmosphere. The final osmolality was 280 mOsmol kg^{-1}, with pH 7.2–7.3.

Experimental protocol

The spermatozoa used in this experiment were obtained from our cryobank of red deer semen. We used samples from six males, treating each male separately and performing experiments in triplicate for each male. Thawed semen was split into two aliquots of 200 μL each, which were diluted in 800 μL SOF medium (final concentration 20×10^6 spermatozoa mL^{-1}); 1 μL of 100 mM trolox in ethanol (final concentration 100 μM vitamin E) was added to one of the aliquots, whereas 1 μL ethanol was added to the other.

Once diluted, spermatozoa were incubated in 0.25-mL drops in different wells of four-well sterile plates (Nunc, Roskilde, Denmark) covered with mineral oil. Plates were covered with their lids and placed in an incubator holding a water-saturated atmosphere of 95% air and 5% CO_2. At 15 min and 3, 6 and 9 h, drops with and without trolox were assessed for sperm motility and apoptotic changes. For the detection of apoptotic changes (membrane status and $\Delta\Psi_m$), samples were stained with a combination of YO-PRO-1, PI and Mitotracker Deep Red and run through a flow cytometer (see below).

Sperm collection and freezing

Spermatozoa were recovered from the cauda epididymides of six mature stags (*Cervus elaphus hispanicus* Helzheimer; age: 4.5–8.5 years; weight approximately 130 kg) that were legally culled and hunted in their natural habitat during the rutting season (September–October). Gamekeepers collected the complete male genitalia and recorded the time of death. Hunting was in accordance with the harvest plans of the game reserves, following Spanish Harvest Regulation (Law 2/93 of Castilla-La Mancha), which conforms to European Union Regulations. The use of these animal samples was approved by the ethics committee of University of Castilla-La Mancha, Spain. Immediately upon removal, the testes (with epididymides attached)

were placed into plastic bags and transported to the laboratory at room temperature (approximately 22°C) within 3 h of removal.

Spermatozoa were collected immediately according to the method described by Soler *et al*. (2003*b*). Briefly, testes and epididymides were removed from the scrotal sac and the sperm mass was collected several cuts were made to the cauda epididymis. Spermatozoa were frozen according to the protocol described by Soler *et al*. (2003*a*). Just after collection, sperm samples were diluted to a final concentration of 200×10^6 spermatozoa mL^{-1} using Tryladyl (Minitüb, Tifenbach, Germany) with 20% egg yolk and 6% glycerol. Diluted spermatozoa were cooled slowly to 5°C, equilibrated for 2 h at that temperature and then loaded into 0.25-mL plastic straws (IMV, L'Aigle Cedex, France). The straws were frozen in nitrogen vapour 4 cm above the surface of liquid nitrogen for 10 min and then plunged into and stored in liquid nitrogen. The straws were frozen in liquid nitrogen for a minimum of 1 year. Samples were thawed by immersing the straws in a 37°C water bath for 30 s.

Assessment of sperm motility

A 5-μL drop of the sample was put on a prewarmed slide, covered with a coverslip and examined under phase-contrast microscope with a warming stage at 37°C (Nikon (Kanagawa, Japan) Eclipse 80i; negative contrast optics). We calculated a sperm motility index (SMI), as described by Comizzoli *et al*. (2001), assessing the percentage of motile spermatozoa (MI) and the quality of movement (QM; on a scale of 0–5, where 0 is no motility and 5 is rapid and progressive motility) using the following formula:

$$SMI = \frac{MI + (20 \times QM)}{2}$$

Flow cytometry analysis

The staining solution consisted of flow cytometer sheath fluid (phosphate buffered saline; BD FACSFlow) with 36 nM Hoechst 33342 (stock solution 9 μM in milli-Q water), 50 nM YO-PRO-1 (stock solution 100 μM in dimethylsulfoxide (DMSO)), 15 μM PI (stock solution 7.5 mM in milli-Q water) and 100 nM Mitotracker Deep Red (stock solution 1 mM in DMSO). Spermatozoa were diluted in 0.5 mL staining solution in polypropylene tubes for flow cytometry (final concentration 8×10^5 spermatozoa mL^{-1}). Samples were incubated in the dark for 15 min and run through an LSR-I flow cytometer (BD Biosciences, San Jose, CA, USA). Hoechst 33342 was excited at 325-nm using a helium–cadmium ultraviolet laser, YO-PRO-1 and PI were excited 488-nm argon using an ion laser and Mitotracker Deep Red was excited at 633-nm using a helium–neon laser. From each spermatozoon, we recorded forward and side scatter (FSC and SSC, respectively) and the emission of each fluorochrome using four photodetectors, as follows: FL1 for YO-PRO-1 (530/28BP filter); FL3 for PI (670 LP filter); FL5 for Hoechst 33342 (424/44BP filter); and FL6 for Mitotracker Deep Red (670/40BP filter). Acquisition was controlled using Cell Quest Pro 3.1 software (BD Biosciences). All parameters were read using logarithmic amplification. We discarded events with low Hoechst 33342 fluorescence as artefacts (Hallap *et al.* 2006). The filtered events were displayed in dot plots showing FL1/FL3 (YO-PRO-1 *v.* PI), FL6/FL3 (Mitotracker Deep Red *v.* PI) and FSC/SSC to monitor the correct acquisition of the parameters being investigated. Thus, in the subsequent analysis, each spermatozoon was characterised by four parameters, namely YO-PRO-1 (apoptotic changes to the plasmalemma), PI (necrotic spermatozoa), Mitotracker Deep Red ($\Delta\Psi_m$) and FSC (putatively related to particle size). We acquired 10 000 spermatozoa per sample.

Data for YO-PRO-1/PI were interpreted as described previously (Peña *et al.* 2005), identifying three subpopulations: (*1*) YO-PRO-1−/PI− spermatozoa (INTACT), which were considered viable; (*2*) YO-PRO-1+/PI− spermatozoa (APOPTOTIC), which were considered apoptotic; and (*3*) YO-PRO-1+/PI+ spermatozoa (DEAD), which were considered necrotic (loss of plasmalemma integrity). Data for Mitotracker Deep Red were interpreted as described by Hallap *et al*. (2005), regarding spermatozoa with high fluorescence as having a high $\Delta\Psi_m$. The FSC signal depends on many factors and interpretation of the results can vary between devices and experiments (Shapiro 2003). This is considered further in the Discussion.

Data processing and statistical analysis

The R statistical environment (R Development Core Team 2007) was used to process flow cytometry data and to perform subsequent statistical analysis. We processed the data belonging to the three YO-PRO-1/PI subpopulations separately, obtaining the following data for each: (*1*) proportion of spermatozoa in each subpopulation; (*2*) first quartile, median and third quartile of the FSC and FL6 (Mitotracker Deep Red) signals for each subpopulation (in order to identify changes in distribution); and (*3*) the percentage of cells with high $\Delta\Psi_m$ (FL6 higher than channel 450). Our hypotheses were tested using linear mixed-effects models, with males and replicates within males as random effects. Pairwise comparisons were performed using Holm's correction for multiple comparisons. The YO-PRO-1−/PI− subpopulation (unaltered membrane; termed INTACT) was split into two subpopulations (high and low $\Delta\Psi_m$) for further analysis. Numeric results are expressed as the mean ± s.e.m.

Results

Effect of incubation time and antioxidant treatment on general parameters

Using the YO-PRO-1/PI stain, we were able to separate the three subpopulations, as described in the Material and Methods, namely INTACT (YO-PRO-1−/PI−), APOPTOTIC (YO-PRO-1+/PI−) and DEAD (YO-PRO-1+/PI+). Samples with vitamin E had a slightly higher proportion of INTACT spermatozoa ($P < 0.01$), but the average improvement in the proportion of INTACT spermatozoa compared with control was very low (~2%). Vitamin E had no significant effect on the proportion of the APOPTOTIC subpopulation, mitochondrial activity or FSC signal.

The SMI was 56.4 ± 4.8% just after thawing, decreasing to 21.8 ± 4.1% after 9 h incubation ($P < 0.001$; Fig. 1). Sperm viability also decreased (Fig. 1*a*). Although the proportion of DEAD spermatozoa increased from 37.9 ± 4.8% at 0 h to 51.2 ± 4.2% at 9 h ($P < 0.001$), the proportion of INTACT spermatozoa decreased from 30.9 ± 3.5% at 0 h to 17.5 ± 2.3% at

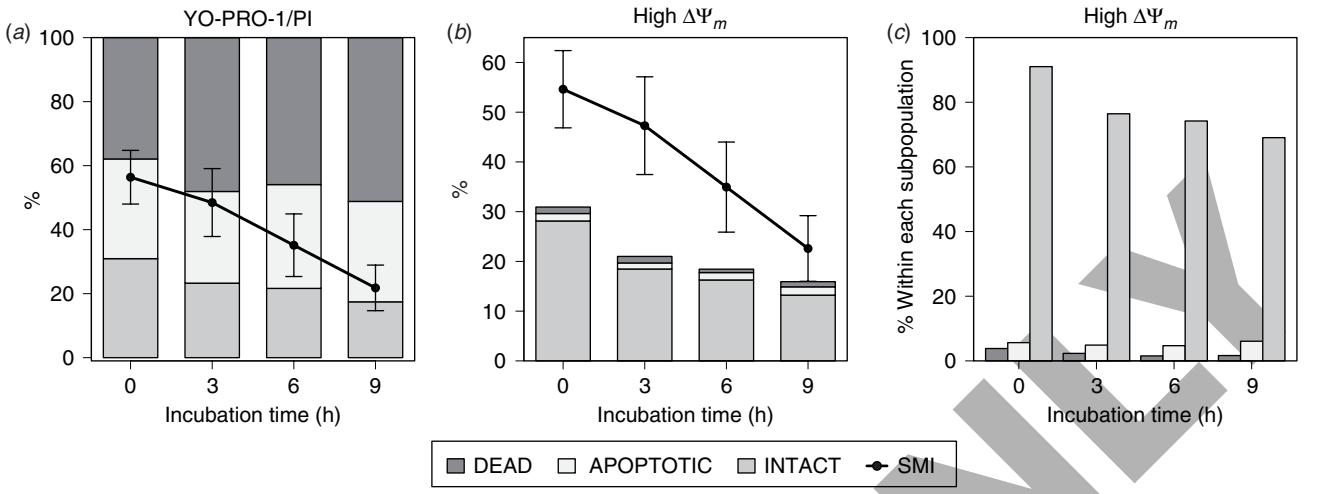

Fig. 1. Variations in some of the parameters analysed over time (0, 3, 6 and 9 h). (*a*) Proportion of each spermatozoan subpopulation as defined by YO-PRO-1 and propidium iodide (PI) staining: INTACT (YO-PRO-1−/PI−), APOPTOTIC (YO-PRO-1+/PI−) and DEAD (YO-PRO-1+/PI+). (*b*) Proportion of spermatozoa with high mitochondrial membrane potential ($\Delta\Psi_m$; Mitotracker Deep Red staining), showing the contribution of each YO-PRO-1/PI subpopulation. (*c*) Results for each subpopulation are shown separately to clearly show the decreasing proportion of high $\Delta\Psi_m$ in the INTACT group. (*a*, *b*) The sperm motility index (SMI) is shown as an overplotted line (mean ± s.e.m.), highlighting the changing relationship between motility in the INTACT and APOPTOTIC subpopulations and $\Delta\Psi_m$.

9 h ($P < 0.001$). The proportion of APOPTOTIC spermatozoa did not change significantly over time ($P > 0.05$), but the ratio of APOPTOTIC : INTACT spermatozoa increased linearly from 1.1 to 2.2 ($P < 0.001$). Dot-plots of YO-PRO-1/PI from flow cytometry analysis indicated that the flux between populations was INTACT → APOPTOTIC → DEAD.

The percentage of spermatozoa with high $\Delta\Psi_m$ (active mitochondria) fell from 28.1 ± 3.2% at 0 h to 12.9 ± 2.3% at 9 h. Figure 1*c* shows that most spermatozoa with high $\Delta\Psi_m$ belonged to the INTACT (YO-PRO-1−) subpopulation. Moreover, the percentage of spermatozoa characterised as having high $\Delta\Psi_m$ within the DEAD and APOPTOTIC subpopulations was less than 5% on average (Fig. 1*b*) and this did not change over time ($P > 0.05$). It is possible this result was due to fluorescence spill over rather than respresenting spermatozoa with an authentic high $\Delta\Psi_m$. In the INTACT subpopulation, 90.6 ± 1.2% of spermatozoa had a high $\Delta\Psi_m$ at 0 h, which declined to 67.8 ± 4.6% by the end of the experiment ($P < 0.001$). Figures 2 and 3 show Mitotracker Deep Red staining at 15 min and 9 h of incubation, indicating that the APOPTOTIC and DEAD subpopulations almost completely consisted of spermatozoa with low $\Delta\Psi_m$.

Distribution of Mitotracker Deep Red fluorescence in each YO-PRO-1/PI subpopulation

Incubation time did not change the distribution of Mitotracker Deep Red fluorescence in the DEAD and APOPTOTIC subpopulations ($P > 0.05$), which also overlapped (Fig. 3). However, Mitotracker Deep Red fluorescence in the INTACT subpopulation did change over time (Fig. 1*b*, *c*). In this case, $\Delta\Psi_m$ was significantly higher ($P < 0.001$) for the INTACT subpopulation compared with the DEAD and APOPTOTIC subpopulations. The distribution of INTACT for this fluorescence changed with time, resulting in a skew towards the left (median fluorescence channel 523 ± 5 at 0 h *v.* 512 ± 21 at 9 h; $P < 0.05$). In fact, a second peak formed with time in the low $\Delta\Psi_m$ region (Fig. 3*b*).

When INTACT spermatozoa with high and low $\Delta\Psi_m$ were analysed separately, we found that Mitotracker Deep Red fluorescence of the INTACT/low $\Delta\Psi_m$ subpopulation was still higher than for the APOPTOTIC subpopulation at 0, 3 and 6 h, but similar at 9 h (when the low $\Delta\Psi_m$ peak of INTACT was more conspicuous). In any case, the INTACT/high $\Delta\Psi_m$ subpopulation had higher Mitotracker Deep Red fluorescence than any other subpopulation ($P < 0.001$).

Distribution of FSC data for each YO-PRO-1/PI subpopulation

The distribution of FSC data for each of the three YO-PRO-1/PI subpopulations overlapped completely (Fig. 4). However, there were several differences in the location and shift of each FSC distribution. Thus, the median value of the FSC signal was higher for the INTACT (YO-PRO-1−/PI−) subpopulation in all cases ($P < 0.001$). Moreover, its distribution was markedly skewed towards the right. In contrast, the FSC distribution for the DEAD (YO-PRO-1+/PI+) and APOPTOTIC (YO-PRO-1+/PI−) subpopulations was more symmetrical, showing slightly lower values for APOPTOTIC than DEAD spermatozoa ($P < 0.001$). The distribution for both DEAD and APOPTOTIC subpopulations underwent a shift towards the left over time ($P < 0.001$). A lower FSC signal was observed for the INTACT/low $\Delta\Psi_m$ subpopulation compared with the INTACT/high $\Delta\Psi_m$ subpopulation and was shited to left with time ($P < 0.001$), similar to observations for the DEAD and APOPTOTIC subpopulations. Thus, after 9 h incubation, median FSC values were 502 ± 4 for DEAD, 490 ± 2 for APOPTOTIC, 507 ± 6 for INTACT/low $\Delta\Psi_m$ and 551 ± 5 for INTACT/high $\Delta\Psi_m$ subpopulations ($P < 0.001$ in pairwise comparisons).

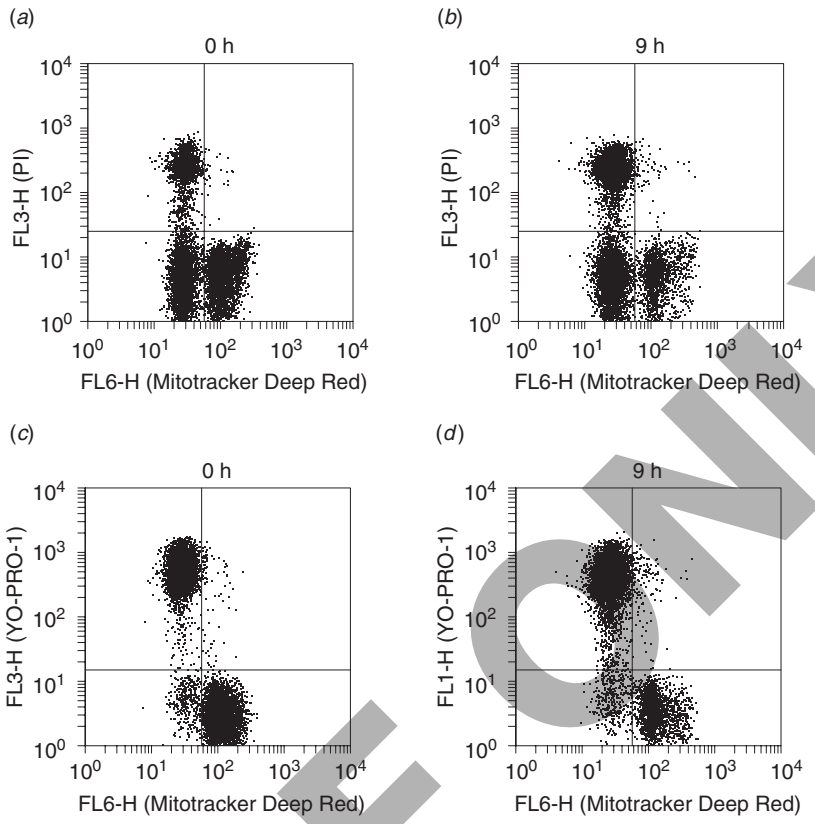

Fig. 2. Flow cytometry analysis of mitochondrial membrane potential ($\Delta\Psi_m$). Data show flow cytometry dot-plots of Mitotracker, YO-PRO-1 and propidium iodide (PI) staining at 0 and 9 h. (*a*, *b*) FL6 *v.* FL3 (Mitotracker *v.* PI) for three subpopulations: viable spermatozoa (undamaged plasmalemma) with active mitochondria in the lower-right quadrant (Mitotracker+/PI−); viable spermatozoa with inactive mitochondria in the lower-left quadrant (Mitotracker−/PI−); and dead spermatozoa in the upper-left quadrant (Mitotracker−/PI+). (*c*, *d*) FL6 *v.* FL1 (Mitotracker *v.* YO-PRO-1) for three subpopulations: spermatozoa with intact plasmalemma and active mitochondria in the lower-right quadrant (Mitotracker+/YO-PRO-1−); spermatozoa with intact plasmalemma and inactive mitochondria in the lower-left quadrant (Mitotracker−/YO-PRO-1−); and spermatozoa with either altered or damaged plasmalemma (and inactive mitochondria) in the upper-left quadrant (Mitotracker−/YO-PRO-1+; also PI+). The vertical line is situated on channel 450 of the horizontal axis, used to discriminate between high and low $\Delta\Psi_m$ events. Events in the upper-right quadrant are possibly due to PI fluorescence spillage to the FL6 detector, not to real Mitotracker+ events. Note the continuity between the PI−/low $\Delta\Psi_m$ and PI+ events, as well as between the YO-PRO-1−/low $\Delta\Psi_m$ and YO-PRO-1+ events.

Discussion

The difference in YO-PRO-1 compared with other supravital stains, such as PI or Hoechst 33258, is its capacity to label spermatozoa that have membrane changes that do not necessarily imply a loss of continuity. In the present study, we have shown that YO-PRO-1+/PI− (APOPTOTIC) spermatozoa exhibit several characteristics that allow them to be distinguised from YO-PRO-1− spermatozoa. These characteristics indicate that the 'apoptotic' phenomenon in spermatozoa partly resembles apoptosis in other cell types, while presenting some differences. Although other studies have already used YO-PRO-1 staining in different experiments with spermatozoa (Waterhouse *et al.* 2004; Martin *et al.* 2005; Peña *et al.* 2005), we are the first to focus on its relationship with mitochondrial status and FSC signal. What makes our study original is that we have assessed simultaneously membrane condition (YO-PRO-1/PI), mitochondrial status and FSC for each single spermatozoon, characterising each intact/apoptotic/necrotic subpopulation. Our results suggest that the information provided by the supravital stains used until now may be reviewed, considering the use of improved stains that inform more realistically on the physiological status of the spermatozoon. It is remarkable that Harrison *et al*. (1996)

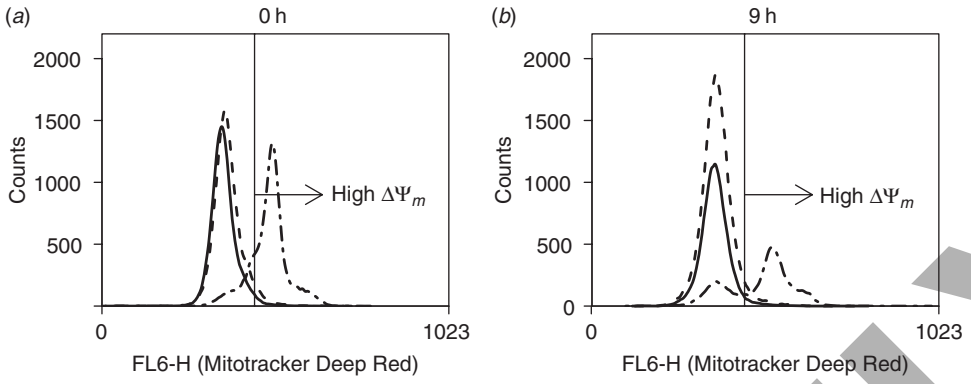

Fig. 3. Flow cytometry analysis of mitochondrial membrane potential ($\Delta\Psi_m$; see Fig. 2). Data show overlapping histograms for the three YO-PRO-1 and propidium iodide (PI) subpopulations at 0 and 9 h for FL6 fluorescence (the scale is given in fluorescence channels, 0–1023): INTACT (YO-PRO-1−/PI−; –·–·–), APOPTOTIC (YO-PRO-1+/PI−; —) and DEAD (YO-PRO-1+/PI+; -----). The vertical line is situated on channel 450 of the horizontal axis, used to discriminate between high and low $\Delta\Psi_m$ events. The histograms show that almost all DEAD and APOPTOTIC spermatozoa were in the low $\Delta\Psi_m$ region, whereas INTACT spermatozoa were mostly in the high $\Delta\Psi_m$ region.

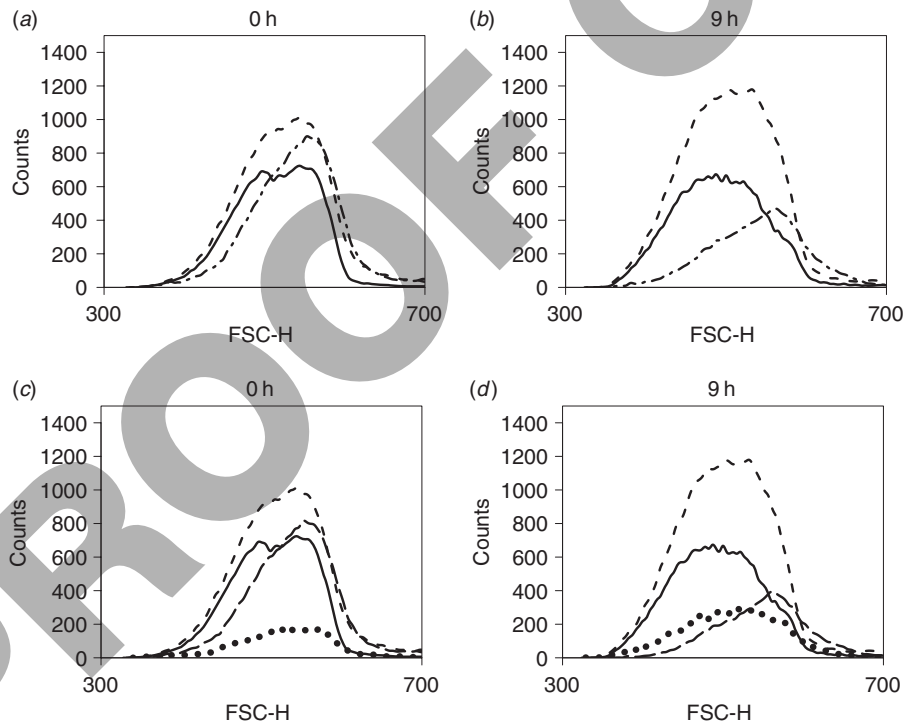

Fig. 4. Histograms showing forward scatter (FSC) data distribution; the scales are given in fluorescence channels (0–1023). (*a*, *b*) Overlapping histograms for the three YO-PRO-1 and propidium iodide (PI) subpopulations at 0 and 9 h: INTACT (YO-PRO-1−/PI−; –·–·–), APOPTOTIC (YO-PRO-1+/PI−; —) and DEAD (YO-PRO-1+/PI+; -----). The histograms show a considerable degree of overlapping, but the differences between the three distributions were significant. (*c*, *d*) The INTACT (viable and with unaltered membrane) subpopulatioin was broken down into a high-$\Delta\Psi_m$ subpopulation (– – –) and a low-$\Delta\Psi_m$ subpopulation (· · · · ·; the height of the low $\Delta\Psi_m$ was increased to better show its distribution). The FSC distributions of these two subpopulations differed significantly.

first used YO-PRO-1 on spermatozoa in 1996 but, after almost 12 years, apparently fewer than 20 spermatology studies have reported using it.

Our results support the predominant idea that a viable spermatozoon entering a degenerative process would not undergo membrane breakage directly, but would exhibit changes in the plasmalemma reflecting increased permeability while maintaining its continuity. That is, spermatozoa would first be stained with YO-PRO-1 and, eventually, PI would stain these YO-PRO-1+ spermatozoa. In the present study, antioxidant treatment did not have a significant protective effect on the samples, although it delayed membrane changes in a low percentage of spermatozoa, preventing YO-PRO-1 staining. We hypothesised that vitamin E supplementation would decrease the negative effects of post-thawing and incubation stresses caused by the formation of ROS. Several studies have reported that ROS scavengers could protect mitochondrial function and blocking apoptotic pathways, therefore inhibiting apoptosis (Lemasters *et al.* 1999; Shimizu *et al.* 2004). Grizard *et al*. (2007) found that spermatozoa treated with the herbicide alachlor exhibited increased ROS production associated with decreased $\Delta\Psi_m$ and phosphatidylserine (an apoptotic marker) externalisation. Because we could not detect an improvement in mitochondrial activity following vitamin E supplementation, either the antioxidant protection was not sufficient or the processes underway in the spermatozoa were mostly independent of ROS (more necrosis like than apoptosis like). It is possible that vitamin E would be more effective in other types of stress processes, such as freeze–thawing (Peña *et al.* 2007). Nevertheless, a previous study showed no improvement of deer sperm cryopreservation following vitamin E supplementation (Fernandez-Santos *et al.* 2007).

In the present study we tested whether the mitochondrial activity of APOPTOTIC spermatozoa was similar to that of DEAD (YO-PRO-1+/PI+) spermatozoa. Our results showed that $\Delta\Psi_m$, as measured with Mitotracker Deep Red, was very low in both populations. Although high $\Delta\Psi_m$ may be not important for the maintenance of motility, the presence of active mitochondria could be fundamental for many sperm functions, including osmotic regulation, membrane integrity, maturation and capacitation (Silva and Gadella 2006; Aitken *et al.* 2007). Therefore, APOPTOTIC spermatozoa, which lacked mitochondrial activity, could be considered as early non-viable cells. This idea is also supported by findings from other studies. For instance, Peña *et al*. (2005) showed, in a multiple stain experiment, that the YO-PRO-1− subpopulation was stained by SNARF-1, an indicator of esterase activity, whereas this activity was absent from YO-PRO-1+ spermatozoa (even if PI−). The YO-PRO-1− spermatozoa may be really 'viable' as opposed to the classic criteria of membrane-intact/membrane-damaged spermatozoa used with other supravital stains. It is important to consider that, although the SNARF-1 staining seems to be quite specific, some other stains that are based on esterase activity may not be. For example, the stain carboxyfluorescein diacetate (CFDA) is also cleaved by intracellular esterases, but the CFDA/PI stain yields some cells as esterase active (CFDA+) while also labelling them as membrane damaged (PI+).

In a recent study, de Vries *et al*. (2003) incubated fresh human spermatozoa in capacitating conditions for up to 4 h, analysing $\Delta\Psi_m$ with the combination YO-PRO-1/Mitotracker Deep Red. These authors found a slight decrease in the percentage of spermatozoa with high $\Delta\Psi_m$ over time and that all spermatozoa with low $\Delta\Psi_m$ were YO-PRO-1+ too. However, these authors did not use PI and interpreted all YO-PRO-1+ events as membrane-damaged cells, suggesting that membrane damage caused the loss of $\Delta\Psi_m$. However, our data indicate that the loss of $\Delta\Psi_m$ precedes the membrane changes that lead to YO-PRO-1 staining. In fact, other studies have shown that stress in cell types other than spermatozoa can lead to mitochondrial depolarisation, which constitutes a key event for either apoptosis or necrosis (Lemasters *et al.* 1999). Martin *et al*. (2007) studied apoptosis (using a caspase marker) in bovine frozen–thawed spermatozoa, finding that after thawing less than 5% of spermatozoa had high $\Delta\Psi_m$, whereas 45% of spermatozoa were still YO-PRO-1−. Unfortunately, these authors could not combine YO-PRO-1 and the mitochondrial or caspase-labelling probe in the same analysis, but it is interesting to note that they found a coincidence between the proportions of caspase+/PI− and YO-PRO-1+/PI− spermatozoa. However, their results on mitochondrial activity are difficult to compare with those of the present study, because Martin *et al*. (2007) used a different staining technique and different criteria to separate subpopulations with high and low $\Delta\Psi_m$. Nevertheless, the results of that study agree with those of the present study in that loss of $\Delta\Psi_m$ would not be caused by plasmalemma damage, but rather the former would precede the latter.

According to our results, the dynamics of sperm changes would occur in the following order: membrane intact and high $\Delta\Psi_m$ (YO-PRO-1−/PI−/Mitotracker+); membrane intact and low $\Delta\Psi_m$ (YO-PRO-1−/PI−/Mitotracker−); membrane altered and low $\Delta\Psi_m$ (YO-PRO-1+/PI−/Mitotracker−); membrane damaged and low $\Delta\Psi_m$ (YO-PRO-1+/PI+/Mitotracker−). According to the results of Martin *et al*. (2007) and others, caspase activation may be concurrent or immediately precede the membrane changes conducive to YO-PRO-1 staining. Moreover, Grunewald *et al*. (2008) showed an association between the presence of apoptotic markers and mitochondrial disruption. Therefore, taking into account the information provided by these previous studies and combining it with the results of the present study, it seems that the probable order of occurrence of these events at a subcellular level may be as follows: (*1*) stress induces the formation of the mitochondrial permeability transition pore (Lemasters *et al.* 1999) or other mechanisms cause mitochondrial depolarisation (YO-PRO-1− spermatozoa with low $\Delta\Psi_m$), possibly liberating many factors (e.g. the pro-apoptotic cytochrome *c*); (*2*) activation of apoptotic and/or necrotic pathways (Paasch *et al.* 2004*a*; Petrunkina *et al.* 2005*b*); (*3*) plasmalemma alterations resulting in increased permeability and/or loss of functionality (YO-PRO-1+); and (*4*) increased loss of cell functions, finally reflecting plasmalemma destabilisation and breakage (PI+). However, there is still a lot of information lacking regarding apoptosis and necrosis in spermatozoa; many processes could occur in parallel, presenting different cause–effect relationships, and some may or may not occur depending on the initial conditions.

In the present study, we have analysed the FSC signal of each spermatozoon in an attempt to determine whether the

YO-PRO-1/PI subpopulations differed in this parameter. FSC has been related to particle size, but this idea is misleading (Shapiro 2003). Forward scatter is not a monotonic function of particle size in many flow cytometers and can be affected by factors other than particle size, such as refractive index, membrane folding or cell shape (McGann *et al.* 1988). Nevertheless, this allows us to hypothesising that the decrease in FSC of PI+ spermatozoa could be due to the loss of the membrane as a permeability barrier, with cells thus having a refractive index more similar to that of the medium, resulting in decreased light scatter. In the case of APOPTOTIC spermatozoa, the FSC shift may be an indicator of apoptotic changes. In fact, Scherer *et al*. (1999) noted that alterations of lipid packing, such as those related to apoptosis, decreased FSC without changes to cell volume or refractive index. Nevertheless, we cannot discount the possibility that volume changes are occurring in APOPTOTIC spermatozoa that result in changes to the FSC. Petrunkina *et al*. (2005*b*) found that samples with an increasing proportion of necrotic spermatozoa exhibited increased cell volume, compatible with the NVI observed in other cell types. Come of the changes in the plasmalemma associated with apoptotic or necrotic pathways in spermatozoa (YO-PRO-1+) may include the opening of Na^+ and Cl^- channels under isotonic conditions and the influx of ions would cause changes in spermatozoon volume.

The INTACT/low $\Delta\Psi_m$ subpopulation yielded an interesting FSC distribution, located between that of the APOPTOTIC and INTACT/high $\Delta\Psi_m$ subpopulations. This observation has not been recorded before and could give an indication of the underlying processes in spermatozoon death. It suggests that mitochondrial depolarisation may be related to very early membrane changes and/or volume deregulation and that the induction of apoptosis and/or necrosis would occur even before plasmalemma changes (YO-PRO-1+) were detectable. Indeed, mitochondrial activity is important for maintaining ATP levels in the sperm head and midpiece and, consequently, the activity of the Na^+/K^+-ATPase and other systems implied in membrane stability and volume regulation (Silva and Gadella 2006). Dissipation of $\Delta\Psi_m$ could be associated with the first steps of spermatozoon death, but the $\Delta\Psi_m$ of some spermatozoa may drop temporally in response to stress and recover after a return to physiological conditions (Martin *et al.* 2007). If volume changes really occur in these spermatozoa, what type of membrane changes take place and whether $\Delta\Psi_m$ recovery is possible are topics deserving further investigation.

We have defined the APOPTOTIC spermatozoa as lacking $\Delta\Psi_m$. However, this did not seem to suppress sperm motility immediately. It is known that spermatozoa can sustain motility by using solely the ATP produced by glycolisis (Marin *et al.* 2003). In our experiment, motility at 0 and 3 h approximately matched the proportion of PI− spermatozoa, supporting the notion that APOPTOTIC spermatozoa are able to maintain motility (approximately half the PI− spermatozoa were YO-PRO-1+) and that motility is largely independent of high $\Delta\Psi_m$. However, motility almost matched the YO-PRO-1− subpopulation at 9 h, suggesting that APOPTOTIC spermatozoa would not be able to maintain physiological functions for a long time. Paasch *et al*. (2004*a*) observed that a drop in $\Delta\Psi_m$ (induced by betulinic acid) did not affect the motility of human spermatozoa in the short term; however, after 1 h incubation, motility dropped. The reasons for this drop may include depletion of ATP, loss of cell functions or the activation of apoptotic factors; it must be noted that these options are not mutually exclusive.

In conclusion, we have found that the occurrence of apoptotic spermatozoa after thawing (as defined by membrane changes) is accompanied, and possibly preceded, by the loss of $\Delta\Psi_m$ and a decrease in FSC. For the first time, we assessed all these parameters simultaneously in each spermatozoon, allowing us to establish a direct relationship among them. At the time of writing this manuscript, we can report similar findings for red deer spermatozoa submitted to oxidative stress (F. Martínez-Pastor *et al*., unpubl. data) and on frozen–thawed ram spermatozoa (A. J. Soler, pers. comm.). The meaning of the FSC changes were beyond the scope of the present study and deserve further research. Moreover, the decreasing FSC in YO-PRO-1− spermatozoa with low $\Delta\Psi_m$ suggests that the processes in the dying spermatozoa may be activated much earlier than they were detectable by YO-PRO-1. The correct identification of these processes and their discrimination from physiological events (such as capacitation) may help to eventually improve our ability to freeze spermatozoa and consequent fertility through the use of blockers of sperm death pathways, such as mitochondrial protectors of caspase inhibitors.

In the present study, more than 50% of the PI− spermatozoa were YO-PRO-1+ in the initial analysis. Therefore, if not using YO-PRO-1, we would have greatly overestimated the proportion of viable spermatozoa in the sample. It would be advisable to develop and spread the use of improved stains that would not only detect plasmalemma damage, but would also detect subtle changes in the plasmalemma. An increase in the knowledge of the processes causing a spermatozoon to be labelled by YO-PRO-1 would help us understand the underlying processes related to apoptosis.

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