Study of apoptosis-related markers in ram spermatozoa

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Abstract

Certain features of capacitated or frozen–thawed spermatozoa have been considered to be an apoptosis-like phenomenon, and, it has been suggested that the presence of apoptotic sperm in seminal doses could be one of the reasons for poor fertility. The objective of this study was to determine whether phosphatidylserine (PS) translocation, caspase activity and DNA fragmentation, which are considered to be apoptotic markers in somatic cells, occur in ram sperm. Fresh ejaculates and sperm samples in different physiological state (cold-shocked, in vitro capacitated and acrosome-reacted (AR)) were compared. Simultaneous staining with 6-carboxyfluorescein diacetate (6-CFDA) and Annexin V-Cy3.18 (AnnV) revealed four different sperm subpopulations in ejaculates. The main subpopulation was composed of viable cells without PS exposure (CFDA+/AnnV−). A total of 40.8% of sperm showed inverted PS, with two levels of alteration: CFDA+/AnnV+ in midpiece (“type I AnnV+”), and in acrosome and midpiece (“type II AnnV+”). The fewest subpopulation contained non-viable cells showing Annexin labelling in the entire cell (CFDA-/AnnV+). Labeling of caspases-3 and -7 by immunocytochemistry revealing different sperm subtypes depending on their localization in apical, equatorial, post-acrosomal regions and tail. The results obtained by western-blot showed, for the first time to our knowledge, that caspase-like proteins are present in fresh ram semen as both inactive and active forms. The proportion of sperm with fragmented DNA [terminal transferase-mediated dUDP nick end-labeling (TUNEL)-positive] were found rarely (2.7 ± 0.5%) in all fresh ejaculates involved in this study. The analysis of total activity of both caspases by a fluorometric method showed a decrease in vitro capacitated and acrosome-reacted samples as well as in cryoinjured samples. However, the percentage of TUNEL-positive sperm demonstrating DNA fragmentation was significantly increased after in vitro induced capacitation and acrosome reaction, as well as after cold-shock although this augment was not significant. PS exposure is not totally dependent on caspases in ram spermatozoa as the addition of a caspase

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1 These authors contributed equally to this article and share senior co-authorship.
inhibitor prevented the increase in PS inversion due to incubation in capacitating conditions but not to the ionophore-induced acrosome reaction or cold-shock.

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1. Introduction

Cell death can, in general, occur through two distinct ways, necrosis and apoptosis. Necrosis is a passive process that results from injury and causes cell swelling and membrane rupture. During necrosis, the cellular contents are released uncontrolled into the cell’s environment which results in damage of surrounding cells and a local inflammatory response; in contrast, apoptosis is an active reaction that follows a sequence of controlled steps leading to locally and temporally defined self-destruction without causing an inflammatory reaction (Lockshin, 1964; Kerr et al., 1972; Wyllie et al., 1980). Apoptotic cells are characterized by morphological changes, especially on the cell surface (shrinkage of the cell, exposure of membrane phosphatidylserine (PS), blebbing), and in the nucleus (fragmentation of the nucleus, hypercondensation of chromatin and DNA degradation).

Apoptosis can be initiated by many stimuli from outside (extrinsic apoptosis pathways) or inside (intrinsic apoptosis pathways) the cell, including binding to the cell surface receptors, treatment with cytotoxic agents or irradiation, DNA damage or oxidative stress. In most cases, a central component of the apoptotic machinery involves a family of aspartic acid-directed cysteine proteases, called caspases (cysteinyl aspartate-specific proteases). They are expressed as catalytically inactive proenzymes that are activated by proteolytic cleavage. Caspases involved in apoptosis are divided into two groups, initiator caspases, such as caspase-2, -8, -9 and -10 (in mammals), and effector or executioner caspases, such as caspase-3, -6 and -7. In most cell types, caspases are involved in the apoptotic machinery responsible for DNA fragmentation (Scaffidi et al., 1998), which is one of the essential endpoints of apoptosis (Compton, 1992). The terminal transferase-mediated dUDP nick end-labeling (TUNEL) method is commonly used for detection of DNA fragmentation (Li and Darzynkiewicz, 1995). It takes advantage of the multiple free DNA ends generated by activated endonucleases to insert labelled dUTP that can be later detected by light or fluorescence microscopy. The simplicity and sensitivity of this technique together with its quantitative power have led to its wide acceptance and use.

Reproductive cells, such as somatic cells, can undergo apoptosis. Thus, germ cell apoptosis appears necessary for normal mature spermatogenesis to develop, reducing germ cells to a number that can be effectively supported by the existing population of Sertoli cells (Rodriguez et al., 1997; Blanco-Rodriguez, 1998; Kierszenbaum, 2001). Likewise, ejaculated spermatozoa have been shown to exhibit certain characteristics of apoptotic somatic cells such as DNA fragmentation or phosphatidylserine translocation, especially in human (Gorczyca et al., 1993) and bull (Anzar et al., 2002) semen. Likewise, we have recently analyzed the PS translocation and caspase-3 and -7 activities in ram sperm samples washed by different methods (Martí et al., 2006). The reasons why apoptotic sperm are present in ejaculated semen are not very clear. Some authors attribute it to the existence of immature sperm (Paasch et al., 2004a), others to a phenomenon of abortive testicular apoptosis (Sakkas et al., 2004), and some to pathologic causes (Oehninger et al., 2004). Whatever the cause, the presence of apoptotic spermatozoa in seminal doses could also be one of the reasons for poor fertility, as has been reported in humans (Taylor et al., 2004; Said et al., 2004).
2006) and bulls (Anzar et al., 2002). Furthermore, apoptosis in sperm would be activated as a mechanism of elimination of abnormal spermatozoa, or in response to environmental stress. Thus, some authors have described certain features of frozen–thawed spermatozoa as an apoptosis-like phenomenon (Martin et al., 2004).

Sperm capacitation is a complex set of modifications undergone in the female reproductive tract whereby sperm acquire the ability to suffer the acrosome reaction and, finally, to fertilize the oocyte (Yanagimachi, 1994). One of these described modifications is the increased lipid disorder in the membrane and external exposure of phosphatidylserine. The translocation of PS is considered by some authors to be a physiological event during the capacitation process (Gadella and Harrison, 2002; de Vries et al., 2003), while for others it represents a sign of cellular damage, a feature of the apoptotic phenomenon (Muratori et al., 2004; Kurz et al., 2005).

Although several studies on apoptosis have been carried out with human (Schuffner et al., 2001; Weng et al., 2002), rat (Brinkworth et al., 1995; Blanco-Rodriguez and Martinez-Garcia, 1999) or bull (Anzar et al., 2002; Martin et al., 2004) sperm, the pathway of such putative apoptosis in sperm cells is not completely known, and the presence of caspases in ejaculated sperm is still a subject of controversy. While some authors have demonstrated the presence of caspase enzymatic activity in human (Weng et al., 2002; Paasch et al., 2003; Wang et al., 2003a, 2003b; Grunewald et al., 2005a, 2005b) and bull (Martin et al., 2004) ejaculated sperm, other results have restricted the presence of caspases only to immature sperm (de Vries et al., 2003).

This study was undertaken to analyse certain features related to apoptosis, such as PS translocation, caspase activity and DNA fragmentation comparing fresh ram ejaculates and in vitro capacitated and acrosome-reacted (AR) sperm samples. In addition, we have studied these apoptotic-related markers in cold-shocked spermatozoa, to compare the implication of apoptosis in these situations as many authors have suggested that post-cooling spermatozoa have the features of premature capacitation (Watson, 1995; Maxwell and Watson, 1996; Gillan et al., 1997; Bailey et al., 2003). This information is of great relevance due to the especially high sensitivity of ram spermatozoa to cryoinjury (Watson, 1981; Holt and North, 1991), which might be related to cold-shock-induced apoptotic-related features. Finally, to investigate further the mechanism of apoptosis, we analyze the effect of a broad-spectrum caspase inhibitor on the PS translocation after different treatments.

2. Materials and methods

2.1. Sample collection and sperm preparation

All experiments were performed using fresh ram spermatozoa. Semen was collected from eight mature Rasa Aragonesa ram using an artificial vagina. The rams, which belonged to the national association of Rasa Aragonesa breeding (ANGRA), ranged from two to four years of age, and were kept at the faculty of veterinary medicine under uniform nutritional conditions. The sires were divided into two groups, and two successive ejaculates were collected every third day to avoid deterioration of spermatozoa (Ollero et al., 1996). For every experiment, the second ejaculates from each group (four rams) were pooled and used for each assay in order to eliminate individual differences (Ollero et al., 1996).

Sperm cells were separated from seminal plasma by a dextran/swim-up procedure (García-López et al., 1996). The medium used was composed of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM Hepes, 10 mM KCl, 2.8 mM glucose, 0.4 mM MgSO₄, 0.3 mM sodium pyruvate, 0.3 mM K₂HPO₄, 1.5 UI/ml penicillin, and 1.5 μg/ml streptomycin, pH 6.5 with
capacitating agents (+3 mM Ca$^{2+}$ + 4 mM HCO$_3^-$ medium) for the induction of capacitation and acrosome reaction, or devoid of capacitating agents (−Ca$^{2+}$–HCO$_3^-$ medium) for cold-shock treatment.

In the experiments of inhibition of caspases, samples were incubated with z-VAD-fmk (a broad-spectrum caspase inhibitor, BD Biosciences, San José, CA) diluted in dimethyl sulphoxide (DMSO) and added to samples to a final concentration of 100 μM inhibitor and 0.26% DMSO.

2.2. In vitro capacitation

The swim-up obtained sperm samples (2 × 10$^7$ cells/ml) were incubated for 4 h at 39°C in a humidified incubator with 5% CO$_2$ in air. Sperm capacitation was evaluated using the chlortetracycline (CTC) assay that we previously validated for the evaluation of capacitation and acrosome reaction like changes in ram spermatozoa (Grasa et al., 2006) following the procedure already reported (Pérez-Pé et al., 2002). For the evaluation of CTC patterns, the samples were observed under a Nikon Eclipse E-400 microscope under epifluorescence illumination using a V-2A filter (excitation filter 380–425 nm) at 1000× magnification. At least 200 cells were counted in duplicate for each sample. Three sperm types were estimated (Gillan et al., 1997): not capacitated (NC, even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (C, with fluorescence in the anterior portion of the head) and acrosome-reacted cells (showing no fluorescence on the head).

2.3. In vitro acrosome reaction

For the ionophore-induced acrosome reaction, a sample obtained by swim-up was diluted in a Hepes-glucose buffer (149 mM NaCl, 2.5 mM KCl, 10 mM glucose, 20 mM Hepes and 3 mM CaCl$_2$; pH 7.4) to a final concentration of 2 × 10$^7$ cells/ml (Shams-Borhan and Harrison, 1981; Martí et al., 2000). Calcium ionophore A23187 was dissolved in DMSO and added to the sample in a final concentration of 3 μM A23187 and 0.26% DMSO. Control tubes had DMSO added but no ionophore. The samples were incubated at 39°C for 1 h, and the acrosome reaction status was assessed by the chlortetracycline staining.

2.4. Cold-shock treatment

For cold-shock, the swim-up obtained sperm samples (2 × 10$^7$ cells/ml) were incubated for 5 min at 25°C, then transferred to 5°C for 10 min, and replaced at 25°C for a further 5 min. All operations were carried out in a water bath.

2.5. Viability staining

Cell viability is defined here as both intact plasma and acrosomal membranes. It was assessed by the fluorescent staining with 6-carboxyfluorescein diacetate (6-CFDA) and propidium iodide (Harrison and Vickers, 1990). The cells were then examined under Nikon Labophot-2 fluorescence microscope with a B-2A (excitation filter 450–490 nm) and G-2A (excitation filter 510–560 nm) filter at 400× magnification. The numbers of fluorescein-positive (plasma membrane-intact) and propidium iodide-positive (plasma membrane-damaged) spermatozoa per 100 cells were estimated and recorded. At least 200 cells were counted in duplicate for each sample.
2.6. Detection of membrane phosphatidylserine translocation

Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for PS. The translocation of PS residues to the outer layer of the plasma membrane was detected with the Annexin V-Cy3.18 (Apoptosis detection kit, Sigma, Madrid, Spain). In order to differentiate between viable cells with or without PS translocation and non-viable cells, we used 6-CFDA along with Annexin V-Cy3.18 (AnnV). The non-fluorescent 6-CFDA enters the cell and is converted to the green fluorescent compound 6-carboxyfluorescein (6-CF). This conversion is a function of the esterases present only in living cells. Thus, only red fluorescence can be observed in non-viable cells.

Sperm supernatants (50 μl of each sample) were diluted with 450 μl of 1 × binding buffer (commercial kit) and stained with 5 μl 6-CFDA (1 mM in DMSO) and 2 μl Annexin V-Cy3.18 (commercial antibody provided in the kit). Each sample was placed on a slide and analyzed at 1000× magnification by epifluorescence microscopy. Viable sperm (6-CFDA+) were visualized in green with a standard fluorescein (Nikon B-2A) filter, and AnnV+ sperm (labelling PS exposure, Annexin V-Cy3.18+) in red with a rhodamine (Nikon G-2A) filter. A total of 400 spermatozoa were counted per slide.

2.7. Protein extraction and caspase activity measurement

Activated caspase-3 and -7 were detected in cellular extracts using a specific substrate (Molecular Probes Inc., Eugene, OR, USA) according to the manufacturer’s instructions. Each sample was washed with PBS and centrifuged at 30,000 × g for 15 min at 4 °C. Supernatants were discarded, and pellets were stored frozen at −80 °C until analysis. Each pellet was then resuspended in the lysis buffer provided by the manufacturer, and electropermeabilized in predetermined conditions (One pulse at 2 kV, 200 Ω and 50 μF). Lysed cells were centrifuged at 11,000 × g for 5 min at 4 °C, supernatants were collected and protein concentration was determined by the Bradford assay (Bradford, 1976).

The EnzChek caspase-3 assay kit#2 (Molecular Probes Inc., Eugene, OR, USA) allows the detection of apoptosis by assaying the increase in caspase-3 and another DEVD-specific protease activities. As caspase-7 recognizes the same aminoacidic sequence, the result of the measurement with this kit will provide us with the total activity of both caspases together.

The basis for the assay is rhodamine 110-bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110). This substrate is a derivative of rhodamine 110 containing DEVD peptides covalently linked to each of R110’s amino groups, thereby suppressing the dye’s visible absorption and its fluorescence. Upon enzymatic cleavage, the non-fluorescent bisamide substrate is converted into fluorescent R110. The assay was used according to the manufacturer’s instructions and the activity was monitored using a fluorometer (Tecan Spectrafluor Plus). Fluorescence units were converted to nM R110/min mg protein.

2.8. Western-blotting

Sperm aliquots containing 8 × 10^7 cells were washed once by centrifugation in 1 × phosphate buffered saline (PBS) (15 min, 4 °C, 30,000 × g) and the supernatants were discarded. Each pellet was resuspended in a lysis buffer (10 mM Tris [Tris–hydroxymethyl-aminomethane] pH 7.5, 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.01% Triton X-100) and a protease inhibitor cocktail was added. Samples were electropermeabilized in predetermined conditions
(One pulse at 2 kV, 200 Ω and 50 μF). Lysed cells were centrifuged at 11,000 × g for 5 min at 4°C. The supernatants were collected and protein concentration was analyzed by the Bradford assay (Bradford, 1976).

Samples were diluted with the sample buffer (25% glycerol, 2% SDS [sodium dodecyl sulfate] 0.027 M Tris–HCl, pH 6.8, 5% 2-mercaptoethanol, 0.018% EDTA, and 0.1% bromophenol blue), boiled for 5 min and loaded on 17% (w/v) SDS-PAGE gels. Proteins were separated by standard SDS-PAGE (Laemmli, 1970) and transferred onto immobilon-P (PVDF membrane) (Millipore, Bedford, MA) using a wet transfer unit (Mini Trans Blot Electrophoretic Transfer Cell Unit, Bio-Rad). After blocking of non-specific sites on the membrane with 5% dry milk in 10 mM Tris–HCl (pH 8), 120 mM NaCl and 0.5% Tween-20, proteins were immunodetected by incubating with a polyclonal primary antibody, (1:3000) anti-caspase-3 (Calbiochem, EMD BioSciences, Darmstadt, Germany) or (1:140) anti-caspase-7 (Alexis Biochemicals, Lausen, Switzerland).

Membranes were then incubated with a secondary mouse antirabbit alkaline phosphatase-conjugated IgG (Sigma, Madrid, Spain). Bound antibodies were visualized, after extensive washing, by incubation with a substrate mixture of nitro blue tetrazolium (0.4 mM), and 5-bromo-4-chloro-3-indolyl phosphate (0.38 mM) in 0.2 M Tris, 5 mM MgCl₂ and 0.1 M NaCl (pH 9.6).

2.9. Immunocytochemistry assays

An aliquot of fresh semen (about 6 × 10⁸ sperm) was permeabilized and fixed with three volumes of an ethanol:acetic acid (3:1) solution at room temperature for 15 min. Samples, processed in triplicate, were placed and incubated on a poly-l-lysine slide (Sigma Chemical Co., Madrid, Spain) for 20 min. Slides were washed and incubated with PBS containing 5% bovine serum albumin (BSA) for 45 min at 37°C to block non-specific sites. After washing, cells were incubated for 90 min at 37°C with a primary antibody and then, 90 min with a secondary antibody, both diluted in antibody buffer (PBS containing 1% BSA). The primary antibodies used were anticaspase-3 (Calbiochem, EMD BioSciences, Darmstadt, Germany) and anti-caspase-7 (Alexis Biochemicals, Lausen, Switzerland), and the secondary antibody was a fluorescein-labeled IgG (Molecular Probes, Inc., Eugene, OR, USA). Slides were washed and desiccated in darkness at room temperature. After addition of 2 μl of anti-fading solution (0.22 M triethilendiamine [DABCO] in 9:1 glycerol:PBS), the samples were covered with a coverslip and sealed. Samples were assessed on a Nikon Eclipse E-400 microscope under epifluorescence illumination using a B-2A filter at 1000× magnification. At least 100 spermatozoa per slide were scored. As a control for the specificity of the primary antibodies, all immunofluorescent-labelling experiments were also carried out without using the primary antibodies.

2.10. Evaluation of DNA fragmentation

The presence of apoptosis-related DNA strand breaks in ram sperm was evaluated by TUNEL assay using the in situ cell death detection kit with fluorescein isothiocyanate (FITC)-labelled dUTP (Roche, Mannheim, Germany). Previously, washed sperm samples were fixed with 4% paraformaldehyde in PBS at room temperature for 1 h and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The rest of the procedure was carried out according to the manufacturer’s instructions.

Samples were observed in a fluorescent microscope with a standard fluorescein (Nikon B-2A) filter, at 1000× magnification. Two slides were prepared from each sample and at least 200 spermatozoa per slide were evaluated. The negative and the positive control were performed, respectively,
by omitting the enzyme terminal transferase and by pre-incubating fixed and permeabilized sperm samples with DNase I (20 UI) for 10 min at room temperature.

2.11. Statistical analysis

Results were expressed as the mean ± S.E.M. of the number of samples. Means were compared by analyses of variance tests to determine whether there was any significant difference between samples, using INSTAT for Windows (version 3.01, license GTA-31516-136). \( P < 0.05 \) was considered to be statistically significant.

3. Results

3.1. Apoptosis-related markers in fresh ram semen

PS translocation was analyzed using fresh ram ejaculates. Simultaneous staining with 6-CFDA and Annexin V-Cy3.18 resulted in four different ram sperm subpopulations in relation to membrane functionality and PS exposure (Fig. 1). The main subpopulation in fresh semen (48.7 ± 3.1%) was composed of viable cells without PS exposure (CFDA+/AnnV−, Fig. 1 A and B), and was labelled “intact cells”. A total of 40.8 ± 2.9% of sperm showed fluorescein labelling and PS translocation, with two different levels of alteration: the labelled “type I AnnV+” (CFDA+/AnnV+ in midpiece, Fig. 1C and D), and the “type II AnnV+” (CFDA+/AnnV+ in acrosome and midpiece, Fig. 1C and D) that accounted for 24.7 ± 0.7% and 16.14 ± 2.7% of total sperm in ejaculate, respectively. The fewest subpopulation (10.4 ± 3.3%) contained “non-viable cells” (CFDA−/AnnV+, Fig. 1E and F) showing Annexin labelling in the entire cell. These dead cells could have died by either an apoptotic or a necrotic process.

Apart from PS translocation, another marker commonly used to study the apoptotic phenomenon is the presence of active caspases. As a first approach, we tried to identify sperm with caspase-3 and -7 by immunocytochemistry. Fig. 2 shows representative results of numerous immunostaining assays. Three different sperm types were found in fresh ejaculates when anti-caspase-3 antibody was used (Fig. 2A). One showed positive immunostaining in the apical region and tail (Fig. 2A, panel 1), and another that also showed fluorescence in the equatorial region (Fig. 2A, panel 2). In both cases, labelling in tail did not include the midpiece region. Finally, a third group of spermatozoa with apical and post-acrosomal regions staining was observed (Fig. 2A, panel 3). Mean percentage value (±S.E.M.) of each group was 36.5 ± 4.5%, 62.5 ± 5.5% and 1.5 ± 0.5%, respectively.

When anti-caspase-7 antibody was used, only two different sperm subpopulations were observed, both exhibiting intense fluorescence in the neck region (Fig. 2B). The first group also showed fluorescence in the apical region and tail (Fig. 2B, panel 1), and the second had also a positive signalling in the post-acrosomal region (Fig. 2B, panel 2). Mean percentage value (±S.E.M.) of each group was 67.0 ± 1.0% and 33.0 ± 1.0%, respectively. Negative controls without primary antibodies showed no detectable fluorescent labelling.

Given that these two antibodies recognize both caspase forms, proenzyme (inactive) and cleaved (active), we cannot distinguish whether the fluorescence belongs to the active enzyme, the proenzyme or a mixture of both. To find out whether both enzyme forms, active and inactive, were present in fresh ram spermatozoa, we analysed sperm lysates by western-blot using anti-caspase-3 (Fig. 3A) and anti-caspase-7 (Fig. 3B) antibodies. As described in antibody manufacture specifications, anti-caspase-three antibody detects a ~34 kDa inactive form of caspase-3 as well as a 20
Fig. 1. Representative patterns of ram sperm as observed with 6-CFDA and Annexin V-Cy3.18 staining. Panels A and B, intact cells; C and D, apoptotic cells (types I and II); E and F, dead cells. Left panels, membrane functionality observed in green with a standard fluorescein (Nikon B-2A) filter. Right panels, PS exposure observed in red with a rhodamine (Nikon G-2A) filter. Magnification 1000×.

and 18 kDa processed (active) forms of the enzyme, and also indicate the possibility of the appearance of a weak, unrelated ~30 kDa band in some samples. All these bands can be seen in Fig. 3A, lane 1, that corresponds to control Jurkat cells treated with camptothecin. Similarly, in ram sperm we detected a reactive band of ~30–32 kDa, and a minor one, of ~15–17 kDa (Fig. 3A, lane 2).

Regarding caspase-7 antibody, manufacture specifications describe the detection of bands of ~34 kDa (full length caspase-7) and ~20 kDa (processed caspase-7) that we found in control
samples (Jurkat cells treated with camptothecin: Fig. 3B, lane 1). In ram sperm, we observed one or two reactive bands of $\sim 32–34$ kDa, a band of $\sim 15$ kDa, and one or two intermediate bands of $\sim 25$ kDa (Fig. 3B, lane 2).

Sperm with fragmented DNA (TUNEL-positive) were found rarely in all fresh ejaculates involved in this study. Only $2.7 \pm 0.5\%$ (medium value $\pm$ S.E.M. of six experiments) of fresh sperm showed TUNEL labelling (data not shown).

3.2. Effect of in vitro induced capacitation

To assess the relationship between in vitro capacitation changes and apoptotic markers, we made a double assessment using both CTC and CFDA/AnnV staining. The proportion of

![Fig. 3. Western blot analysis of caspase-3 and -7 in ram semen. (A) Anti-caspase-3 antibody showing inactive ($\sim 30$ kDa) and active ($\sim 15$ kDa) caspase-3. (B) Anti-caspase-7 antibody showing inactive ($\sim 30$ kDa) and active ($\sim 10–15$ kDa) caspase-7. Lanes 1: positive control (Jurkat cells treated with camptothecin); Lanes 2: fresh ram semen.](image)
non-capacitated cells in control samples (0 h) decreased ($P<0.001$) after 4 h incubation under capacitating conditions (55.3 ± 4.8% and 26.0 ± 1.2%, respectively) (Fig. 4A). Likewise, significant differences in Annexin V patterns were observed after capacitation (Table 1). The proportion of sperm with inverted PS was significantly ($P<0.05$) higher in capacitated samples than in controls (63.0 ± 4.7% and 45.6 ± 4.2%, respectively). Simultaneously, a diminished membrane integrity value (CFDA/PI) was observed in capacitated samples relative to controls (Table 1).

After incubation under capacitating conditions, a positive correlation was found between the proportion of non-capacitated (CTC assay) and intact (without PS exposure) spermatozoa ($r=0.65$, $P<0.05$), and between capacitated and PS-inverted spermatozoa ($r=0.73$, $P<0.05$).

To find whether there was any relationship between PS exposure and other factors usually described as apoptotic markers, we determined caspase activity in control and capacitated sperm samples. In order to analyse changes in active caspases in a more quantitative way than western-blot, we used a fluorometric method to measure caspase-3 and -7 activity. The analysis of total activity of caspases-3 and -7 in cellular extracts (Fig. 5) showed no significant differences in caspase activity in capacitated samples relative to control (4.1 ± 0.7 nM versus 10.8 ± 5.2 nM substrate/min mg protein, respectively).

Unlike caspase activity, the percentage of TUNEL-positive sperm demonstrating DNA fragmentation after in vitro induced capacitation was significantly increased related to control (27.2 ± 1.8% versus 9.3 ± 1.4%, $P<0.001$, Fig. 6). The proportion of TUNEL positive sperm in the control sample (swim-up sample obtained with a Ca$^{2+}$/HCO$_3^-$ containing media, 9.3 ± 1.4%) was not significantly different to that in fresh ejaculated semen (2.7 ± 0.5%).
Table 1
Sperm viability value (%) and CFDA/Annexin V sperm subtypes (%) \(^*\) in control, capacitated (CA), acrosome-reacted (AR), DMSO-containing and cold-shocked (CS) sperm samples in the absence or presence of a caspase inhibitor (I)

<table>
<thead>
<tr>
<th></th>
<th>Viability (%)</th>
<th>CFDA/Annexin V staining</th>
<th>Intact</th>
<th>Apoptotic I**</th>
<th>Apoptotic II**</th>
<th>Dead</th>
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<tr>
<td><strong>Capacitation (n = 8)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>70.3 ± 2.9</td>
<td>54.3 ± 3.3a</td>
<td>14.3 ± 1.5a</td>
<td>20.3 ± 2.5a</td>
<td>11.0 ± 2.5a</td>
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<tr>
<td>CA</td>
<td>58.3 ± 5.5</td>
<td>37.0 ± 3.7b</td>
<td>26.0 ± 2.3b</td>
<td>13.0 ± 1.9b</td>
<td>24.0 ± 4.9b</td>
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<tr>
<td>CA + I</td>
<td>58.3 ± 5.2</td>
<td>50.3 ± 2.0a</td>
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<td>17.0 ± 1.1ab</td>
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<td><strong>Acrosome reaction (n = 6)</strong></td>
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<tr>
<td>Control</td>
<td>60.1 ± 4.4a</td>
<td>49.0 ± 5.2a</td>
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<td>DMSO</td>
<td>57.3 ± 3.6a</td>
<td>42.0 ± 7.2a</td>
<td>12.0 ± 1.1a</td>
<td>34.3 ± 7.8a</td>
<td>11.7 ± 0.9a</td>
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<tr>
<td>AR</td>
<td>26.7 ± 4.4b</td>
<td>5.3 ± 0.9b</td>
<td>5.7 ± 1.3b</td>
<td>65.3 ± 6.8b</td>
<td>23.7 ± 6.1b</td>
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<tr>
<td>AR + I</td>
<td>21.0 ± 4.8b</td>
<td>5.7 ± 0.5b</td>
<td>5.3 ± 1.5b</td>
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<td><strong>Cold-shock (n = 7)</strong></td>
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<tr>
<td>Control</td>
<td>61.7 ± 1.9a</td>
<td>57.7 ± 2.7a</td>
<td>12.7 ± 0.4a</td>
<td>14.3 ± 2.7a</td>
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<tr>
<td>CS</td>
<td>20.3 ± 0.2b</td>
<td>15.0 ± 3.5b</td>
<td>17.3 ± 1.6</td>
<td>52.7 ± 2.7b</td>
<td>15.0 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>CS + I</td>
<td>21.3 ± 2.9b</td>
<td>16.3 ± 3.0b</td>
<td>21.0 ± 3.3</td>
<td>46.7 ± 6.1b</td>
<td>16.0 ± 2.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) Values are means ± S.E.M. of the number of samples indicated between brackets.

** Apoptotic I and II are referred to type I and II apoptotic cells described above. Different letters (a, b) in columns indicate significant differences (\(P < 0.05\)).
Fig. 5. Caspase activity on ram sperm, expressed as nM substrate/min mg protein in: (□) control, (■) capacitated (n = 8), acrosome-reacted (n = 4) or cold-shocked samples (n = 6), and (☑) control samples incubated with DMSO (n = 4). * Significant differences relative to control, P < 0.05.

3.3. Effect of in vitro induced acrosome reaction

Assessment of the induced-acrosome reaction with calcium ionophore by CTC staining showed that the proportion of lost acrosomes increased (P < 0.05) from 12.7% in control to 50.7% in acrosome-reacted samples (Fig. 4B). In addition, an acute and significant (P < 0.01) decrease in the percentage of intact spermatozoa (without PS translocation) compared to control was found after acrosome reaction (5.3 ± 0.9% versus 49.0 ± 5.2%) (Table 1). The huge increment in the percentage of sperm with PS exposure was mainly due to the increase in “type II AnnV+” (65.3 ± 6.8 versus 23.3 ± 2.9 in control samples, P < 0.05). Incubation with only DMSO did not exert any of these effects. A positive correlation was found between the proportion of non-capacitated (CTC assay) and intact (without PS exposure) spermatozoa (r = 0.97, P < 0.0001) in ionophore-reacted samples.

Furthermore, caspase activity was significantly lower (P < 0.05) in DMSO-control and ionophore-reacted samples (6.2 ± 0.2 nM and 2 ± 0.4 nM substrate/min mg protein, respectively) than control extracts (11.9 ± 1.9 nM substrate/min mg protein) (Fig. 5).

As expected, the in vitro induction of acrosome reaction also caused a significant decrease in membrane integrity (60.1 ± 4.4% in control samples to 26.7 ± 4.4%, P < 0.05, Table 1).
Fig. 7. Maintenance (%) of intact sperm (without PS exposure) relative to control after capacitation, acrosome-reaction or cold-shock treatment, in the absence (□) or presence (■) of a caspase inhibitor. Mean values ± S.E.M. (n = 4).

effect was not due to the toxicity of DMSO as no significant differences were found between control and DMSO-containing samples as we have already reported (Martí et al., 2000).

Once more, the percentage of TUNEL-positive sperm was significantly increased (24.5 ± 2.7% versus 9.7 ± 1.0%, P < 0.001) after in vitro induced acrosome reaction (Fig. 6).

3.4. Effect of cold-shock

As previously shown, the cold-shock treatment produces important membrane alterations in ram spermatozoa with a significant decrease in membrane integrity (Barrios et al., 2000) and induced protein tyrosine phosphorylation (Pérez-Pé et al., 2002).

The study of PS translocation in cold-shocked samples (Table 1) also showed a significant (P < 0.001) decrease in the proportion of intact spermatozoa (15.0 ± 3.5% versus 57.7 ± 2.7%) and an acute increment (P < 0.001) in the “type II AnnV+” sperm compared with control samples (52.7 ± 2.7% versus 14.3 ± 2.7%). Likewise, a great part of caspase activity was lost in cold-shocked samples related to controls (7.39 ± 2.35 nM versus 21.73 ± 5.8 nM substrate/min mg protein, respectively, P < 0.05) (Fig. 5).

The percentage of sperm with DNA fragmentation visualized by TUNEL increased after cold-shock (17.7 ± 1.6% versus 7.8 ± 1.2%) although it was not significant (Fig. 6).

3.5. Effect of the addition of a caspase inhibitor before treatments

The effect of the addition of z-VAD-fmk (a broad-spectrum caspase inhibitor) was dependent on the treatment (induction of in vitro capacitation, acrosome-reaction or cold-shock) although no significant effect on membrane integrity was shown during any treatment (Table 1).

The addition of z-VAD-fmk to the capacitation medium counteracted the PS inversion due to capacitation as the percentage of intact spermatozoa (without PS inversion) increased (P < 0.05) up to the same value of control samples (50.3 ± 2.0 versus 37.0 ± 3.7) (Table 1). Furthermore, the proportion of sperm that maintained intact PS orientation after capacitation with z-VAD-fmk was 93.7 ± 2.8%, (relative to control), whereas in the absence of the inhibitor only 67.3 ± 5.9% of sperm related to control were intact (Fig. 7). Comparative analysis of CTC patterns between
capacitated samples in the presence or absence of z-VAD-fmk showed no significant effect of this compound on capacitation. The proportions of the four subpopulations were very similar with or without z-VAD-fmk (Fig. 4A).

Surprisingly, the inhibition of caspases during in vitro induction of acrosome reaction and cold-shock did not affect PS translocation (Table 1), and the percentage of maintained intact sperm relative to control samples was no different either with or without inhibitor (Fig. 7). Similarly, no significant differences were found in CTC patterns of acrosome-reacted samples (Fig. 4B).

4. Discussion

Normal spermatogenesis is modulated by apoptotic events that adjust the number of germ cells to the number of Sertoli cells removing the abnormal gametes. Failure to eliminate apoptotic (defective) germ cells, results in a high number of abnormal sperm in semen and low fertility (Yin et al., 1998). Thus, although it is known that a large proportion of spermatogonias normally die by apoptosis in testis (De Rooij and Lok, 1987; Billig et al., 1995), the role of apoptosis in ejaculated sperm remains controversial. However, it is widely accepted today that the presence of apoptotic sperm in seminal doses could also be one of the reasons for poor fertility (Gorczyca et al., 1993; Baccetti et al., 1996; Barroso et al., 2000). Therefore, the evaluation of apoptotic sperm could be useful together with other semen quality assays to assess the potential fertility of a given sample.

Features usually evaluated in sperm as indicators of apoptosis are DNA fragmentation, PS translocation and caspase activation, known characteristics of apoptotic somatic cells. Thus, our first aim was the investigation of these typical apoptotic markers in ram semen.

The use of Annexin V (with a high affinity for PS) in combination with 6-CFDA allows a clear distinction of three sperm types: live-intact cells, viable cells with PS translocation, and non-viable cells (Schuffner et al., 2001; Weng et al., 2002). In this study, using this CFDA/Annexin V staining with ram sperm, we not only observed the described types, but also were able to distinguish two different types of viable sperm with externalized PS, some cells with a slight red fluorescence only at the midpiece, and others showing also an intense labelling on the acrosome. Given that non-viable cells showed Annexin labelling over their entire surface, our results could suggest that the exposure of PS in ram spermatozoa could be a sequential process initiating in the midpiece, later affecting to the acrosome region and finally the entire surface of the cell.

The second apoptotic marker considered in this study was the presence of caspases. As a first approach, we tried to detect caspase-3 and -7 by immunocytochemistry, and the obtained results revealed different sperm labeling depending on their localization in apical, equatorial, post-acrosomal regions and tail. It is worth pointing out that caspase-7 was clearly detected in the neck of all labelled spermatozoa, and that no spermatozoa showed labelling in the midpiece region. These results differ from those described for caspase-3 and other caspases in human (Weng et al., 2002). This different distribution could be attributed to the higher percentage of spermatozoa with cytoplasmic droplets in ejaculated human sperm (Cooper et al., 2004) in comparison to domestic animal species where the presence of residual cytoplasm is associated with infertility (Aman et al., 2000; Thundathil et al., 2001). The presence of active caspases (1–9) has been reported in the postacrosomal region and in cytoplasmic residues of abnormally shaped human spermatozoa (Paasch et al., 2003). Furthermore, Weng et al. (2002) suggested that caspase-dependent apoptosis might be sequestered in a region where remnants of the cytoplasmic droplet would be located in abnormal and/or immature sperm. This might be the reason for a higher presence of caspases in human sperm concomitant to an intense immunofluorescence signal near to the midpiece region. However, in our study we used semen collected from breeding rams maintained in a continuous
extraction system, with standard sperm quality and where the cytoplasmic droplets are scarce (Ollero et al., 1996).

With the immunocytochemistry experiments, we revealed the presence and localization of caspase-3 and -7 in ram sperm, but we could not conclude whether these enzymes were present as active or inactive forms. With the results of western-blot we can state, for the first time to our knowledge, that caspase-like proteins are present in fresh ram semen as both inactive and active forms. According to that reported in human sperm where 32 kDa inactive and 17 kDa active forms of caspase-3 were found (Weng et al., 2002), we could suggest that the approximately 30–32 kDa band identified by the anti-caspase-3 antibody would correspond to the proenzyme (inactive form), and the \( \sim 15–17 \) kDa processed band would be the active form. Likewise, the \( \sim 32–34 \) kDa band of caspase-7 could correspond to the inactive form of the enzyme, and the band of \( \sim 15 \) kDa could be related to the active form, similar to that described in human sperm (Taylor et al., 2004).

After the detection of apoptosis-related markers in ram sperm, we focussed on analysing how induction of certain in vitro treatments that try to reproduce physiological situations, such as capacitation and acrosome-reaction, affected the cited markers. Likewise, since it has been suggested that post-cooling spermatozoa have the features of premature capacitation (Maxwell and Watson, 1996; Pérez et al., 1996; Cormier et al., 1997), we also studied the effect of cold-shock treatment. Capacitation and acrosome reaction require a rebuilding of the lipid phase of the plasma membrane in order to desestabilize and to prepare it for membrane fusion with the oocyte (Wolf et al., 1986; Martinez and Morros, 1996; Nolan and Hammerstedt, 1997). The increase in PS translocation observed following capacitation in boar (Gadella and Harrison, 2002) and human (de Vries et al., 2003), and acrosome reaction in human (Martin et al., 2005) sperm is a subject of controversy. It seems to be an important physiological event in the capacitation process (Gadella and Harrison, 2002), or an early signal for the elimination of impaired or dead spermatozoa (Kurz et al., 2005). Our data confirm an increase in PS exposure after capacitation and acrosome reaction of ram sperm. It is worth pointing out that capacitation is a transient process, and each individual sperm gets to this stage at different time points, resulting in a continuous replacement of the capacitated sperm population within the semen sample. This replacement could be a useful mechanism, developed in such species, as in ovine, in which there is a variable and unpredictable interval between mating or artificial insemination and ovulation, maximizing the probabilities that an ovulated egg will meet spermatozoa in the most suitable functional state (Giojalas et al., 2004). As proposed (Eisenbach, 2003), sperm that have ceased to be functional (“post-capacitated” spermatozoa) must be destroyed without producing any damage to the surrounding cells. Therefore, the PS exposure could be the signal to mark the non-functional sperm to be removed by phagocytosis in the female genital tract.

Our data indicated that the addition of a caspase inhibitor before inducting capacitation diminished the percentage of spermatozoa with PS exposure although it did not affect the proportion of capacitated sperm (CTC patterns). The CTC technique was recently validated for the evaluation of capacitation and acrosome reaction-like changes in ram sperm, carrying out a double estimation of the acrosome status after treatment with lysophosphatidylcholine, by using FITC-RCA/EthD-1 and CTC/EthD-1 (Grasa et al., 2006). Based on this validation assay, it can be deduced that the CTC patterns changes could correlate with the capacitation state. Therefore, we could hypothesize that PS exposure must be more closely related to an apoptotic phenomenon rather than to capacitation alterations.

As we previously reported (Pérez-Pé et al., 2002), the cold-shock treatment induces certain molecular changes in ram sperm related to those caused by capacitation. So, as occurs in capacitated samples, we observed an increment in PS exposure as a consequence of cryoinjury. However,
this increment, as well as that caused by the acrosome reaction, was not prevented by the addition of a caspase inhibitor, which is in concordance with results of several authors that have found neither inhibition of PS translocation (Gadella and Harrison, 2002) nor any beneficial effect of caspase inhibitors in canine (Peter and Linde-Forsberg, 2003) or equine (Peter et al., 2005) sperm cryopreservation. One possible explanation would be that cold-shock and the ionophore-induced acrosome reaction not only produce apoptosis, but a necrotic phenomenon. However, the possibility that caspase-independent mechanisms may be operative in ram sperm, or that sperm do not utilize the same mechanism for cell death as somatic cells, cannot be ruled out (Tesarik et al., 2004). The possibility also exists that some of the modifications induced by cryoinjury or ionophore (PS externalization) may represent changes that ram sperm undergo both under in vivo and in vitro conditions, according to previous observations (Gadella and Harrison, 2002).

A processed caspase (the short fragment in the western-blot) is not necessarily catalytically active since processing and activation are under control of different protein factors, such as IAPs (Inhibitor of apoptosis proteins) and HSPs (Heat shock proteins) (Kohler et al., 2002). Therefore, to carry out a more accurate quantitative analysis of active caspases, we measured directly caspase-3 and -7 activity by a fluorometric method. The low caspase activities found in ram sperm are in accordance with those reported in human supposedly due to the small sperm cytoplasmic volume relative to somatic cells (Weng et al., 2002).

Our results indicated that caspase activities decreased after capacitation and acrosome reaction, unlike that reported in human sperm in which no relationship between capacitation (de Vries et al., 2003) or acrosome-reaction (Martin et al., 2005) and caspase activity has been found. Surprisingly, cold-shocked sperm also showed a diminished caspase activity unlike several studies that revealed an increment following sperm cryopreservation (Martin et al., 2004; Paasch et al., 2004a, 2004b; Grunewald et al., 2005b). This difference could be due to the effect of the cold-shock treatment that only mimics the first steps of the cooling stage.

As the caspase activity found in treated ram sperm was very low, we believed at first that the difference could lie in a special putative susceptibility of ram spermatozoa to centrifugation, given that the measurement of the caspase activity method included a preliminary centrifugation step with supernatant removal. This mechanical damage on sperm already subjected to a previous stress such as capacitation, acrosome-reaction or cold-shock could lead to the breaking of the plasma membrane and the release of all cellular content, including caspases. As sperm samples were centrifuged and the first supernatant was discarded, caspases might have been removed in this step. Furthermore, samples already subjected to any treatment would be more liable to suffer membrane alterations than controls. Therefore, the maintained caspase activity in the pellet of treated samples would be lower than in controls that should retain higher cytoplasmic content. In order to corroborate this hypothesis, we measured caspase activity in all supernatants, but despite many attempts, we could not detect any activity at all (data not shown).

A second explanation that we could hypothesize is that caspases, once activated, would be rapidly eliminated. This would be in agreement with several studies that demonstrated that the active form of caspases, particularly caspase-3, is extremely weak and rapidly turned over to its proenzyme form (Granville et al., 1998; Luo et al., 1998; Tawa et al., 2004). The rapid degradation of caspase-3 probably reflects the cell’s need to ensure the timely suppression of a destructive proteolytic activity (Tawa et al., 2004) that constitutes an efficient strategy to eliminate induced or even spontaneously generated caspase activity, and thereby, helps to protect cells. Thus, our data would indicate that in ram sperm caspase-like proteins are activated in the first wave of the apoptotic process, while the PS translocation would be restricted to a later step of the apoptosis in which the caspase activity will be lost or at least reduced.
Analysis of DNA fragmentation corroborates the PS translocation data because the proportion of TUNEL-positive sperm significantly increased after capacitation and acrosome reaction as well as after cold-shock, although in this case the difference was not significant. It is worth pointing out the high incidence of TUNEL-positive sperm in the DMSO-control sample. This could suggest that, despite the used concentration of DMSO did not affect the plasma membrane integrity it could have an effect on DNA fragmentation, even higher than that together with the ionophore.

The data presented in this paper open new interesting perspectives to the study of an important cellular regulatory mechanism in ram spermatozoa. The understanding of these mechanisms may provide the means to control apoptosis which in turn could help in the formulation of better diluents for preserving spermatozoa during freezing or storage for reproductive technologies.

5. Conclusion

In conclusion, we have evidenced certain apoptotic-related markers in fresh ram sperm such as PS translocation, the presence of active caspase-3 and -7, and DNA fragmentation. Cold-shock, and in vitro capacitation and acrosome reaction increased PS translocation and DNA fragmentation although caspase activities were decreased. PS exposure is not totally dependent on caspases as the addition of a caspase inhibitor prevented the increase in PS inversion due to incubation in capacitating conditions but not to the ionophore-induced acrosome reaction or cold-shock.

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