Journal of Andrology, Vol. 27, No. 6, November/December 2006

Comparative Study of Four Different Sperm Washing Methods Using Apoptotic Markers in Ram Spermatozoa

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ABSTRACT: The accurate measurement of semen fertilizing potential is of great importance in determining the acceptability of processed semen for breeding purposes. A good sperm preparation technique results in a sample with high viability and motility and also takes into account other parameters such as the capacitation and apoptotic state which could compromise the ability to fertilize an oocyte. In this study, we investigate the effects of 4 sperm preparation techniques (a dextran/swim-up procedure, discontinuous Percoll density gradient centrifugation, sucrose washing, and filtration) on ram sperm quality parameters. Besides the evaluation of viability and the capacitation state, we also analyzed the apoptotic status of the sperm samples by assessing the phosphatidylserine translocation and caspase-3 and -7 activities. This is the first report, to our knowledge, that evidences the presence of active caspases in ram sperm. The results confirm the better ability of the dextran/swim-up procedure to select nonapoptotic spermatozoa, in addition to viable and noncapacitated sperm, compared with other sperm preparation methods. This should be considered to enhance results of artificial insemination techniques in ovine reproduction.

Key words: Swim-up, caspase activity, annexin V.

J Androl 2006;27:746–753

Introduction

Prolonged exposure of spermatozoa to seminal plasma has been found to have adverse effects on sperm function. Although seminal plasma protects the spermatozoa from certain harmful conditions such as oxidative stress (Saleh and Agarwal, 2002), ejaculated semen contains senescent sperm and particle debris that can reduce sperm survival. Therefore, the separation of mammalian spermatozoa from seminal plasma is a practice routinely used in the laboratory and in assisted reproductive technology applications. This separation process is commonly referred to as "sperm washing," in which spermatozoa are removed from the seminal plasma and resuspended in culture medium. Most of these methods include filtration (Graham and Graham, 1990; Cisale et al, 2001) or centrifugation steps (Alvarez et al, 1993). Other techniques are more than simple washing methods since they are able to separate populations of spermatozoa differing in motility and viability, resulting in a high-quality sample. These selective techniques include density gradient centrifugation (Pousette et al, 1986), swim-up (Alvarez et al, 1993), and the more recent immunomagnetic separation procedure (Paasch et al, 2005), among others. However, these selection and washing techniques can affect sperm during the development of the process, reducing viability (Drobnis et al, 1991) or motility (Drobnis et al, 1991; Tucker et al, 1991). Moreover, a good sperm preparation technique requires not only providing a sample with high viability and motility but also taking into account other parameters such as the capacitation and apoptotic state which could comprise the ability to fertilize an oocyte (Anzar et al, 2002; Marchar et al, 2002; Oosterhuis and Vermes, 2004; Said et al, 2006).

Although apoptosis is a phenomenon which has been known and studied in somatic cells for a long time, it is still a subject of controversy in ejaculated sperm (Gadella and Harrison, 2002; Oehninger et al, 2003; Martin et al, 2004; Said et al, 2006). Thus, germ cell apoptosis plays an important role in sperm production during normal spermatogenesis (Sakkas et al, 1999). Apoptosis in sperm would then be activated as a mechanism of elimination of abnormal or senescent spermatozoa or in response to environmental stress or injures. Ejaculated spermatozoa of humans (Gorczyca et al, 1993) and bulls (Anzar et al, 2002) have been shown to exhibit certain characteristics of apoptotic somatic cells.

Supported by grants CICYT-FEDER AGL 2004-02882, INIA RZ03-035, CICYT-FEDER AGL 2005-02614, and DGA A-26/2005.

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Received for publication March 13, 2006; accepted for publication June 18, 2006.

DOI: 10.2164/jandrol.106.000109

Apoptosis can be initiated by many stimuli from outside (extrinsic apoptosis pathways) or inside the cell (intrinsic apoptosis pathways), including ligation of cell surface receptors, treatment with cytotoxic agents or irradiation, DNA damage, or oxidative stress. A central component of the apoptotic machinery involves, in most cases, 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 mammalian apoptosis are divided into 2 groups: initiator caspases, such as caspase-2, -8, -9, and -10, and effector or executioner caspases, such as caspase-3, -6, and -7. All initiator caspases are activators of downstream caspases, which execute the disassembly of the cell by cleaving a variety of cell structure proteins and generation of DNA strand breaks. Translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is an early feature of the terminal phase of apoptosis (Koopman et al, 1994), thus conveying cells to phagocytosis.

In the present study, we investigated the effects of 4 sperm preparation techniques (a dextran/swim-up procedure, discontinuous Percoll density gradient centrifugation, sucrose washing, and filtration) on ram sperm quality parameters. Besides evaluation of the viability and capacitation state, we also analyzed the apoptotic status of the sperm samples by assessing the PS translocation and caspase-3 and -7 activities to determine which method is the least harmful for spermatozoa.

Materials and Methods

Sample Collection

All experiments were performed using fresh ram spermatozoa. Semen was collected from 8 mature Rasa Aragonesa rams using an artificial vagina. The rams, which belonged to the National Association of Rasa Aragonesa Breeding, ranged from 2 to 4 years and were kept at the Faculty of Veterinary Medicine under uniform nutritional conditions. The sires were divided into 2 groups, and 2 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 (4 rams) were pooled and used for each assay to eliminate individual differences (Ollero et al, 1996).

Dextran Swim-Up Procedure

The swim-up procedure was performed following the method we developed previously for ram semen (García-López et al, 1996). A 4-step procedure was carried out. Briefly, aliquots of 0.5 mL of unprocessed semen were pipetted into round-bottomed, 15-mm diameter tubes under 0.5 mL of dextran solution (30 mg/mL) and then overlaid with 1.5 mL of SM medium (200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K_2HPO_4, 1.5 UI/mL penicillin, and 1.5 μg/mL streptomycin (pH 6.5, 323 mOsm/kg) containing 5 mg/mL bovine serum albumin. The tubes were kept at 37°C in a vertical position for 15 minutes. The top 0.75 mL of the layer containing the spermatozoa was removed and replaced by careful addition of 0.75 mL of fresh medium. The incubation sequence was then repeated 3 more times so that 4 supernatants were obtained. The first supernatant was rejected because it contained lower-quality spermatozoa and plasma component contamination (García-López et al, 1996). The 3 top layers obtained from the final 3 consecutive swim-ups were then combined to give 2.25 mL of sperm suspension.

Discontinuous Percoll Density Gradient Centrifugation

The 70% and 35% working density solutions were prepared by diluting commercial Percoll with a saline buffer (v/v) (Vincent and Nadeau, 1984) composed of 135 mM NaCl, 10 mM glucose, and 20 mM HEPES (pH 7.6). A discontinuous 2-layer Percoll density gradient was created by carefully pipetting 4 mL of 35% solution over 2 mL of 70% Percoll solution into a round-bottomed, 15-mm diameter tube. An aliquot of 0.3 mL of raw semen was layered onto the top of the Percoll discontinuous gradient, and the tube was centrifuged for 5 minutes at 200 × *g* followed by a second centrifugation at 1200 × *g* for 15 minutes at 20°C. The pellet was washed twice by resuspension in a dilution buffer (0.25 M sucrose, 0.1 mM EGTA, 4 mM sodium phosphate [pH 7.5], and 10% [v/v] of 10× buffer stock HEPES [composed of 50 mM glucose, 100 mM HEPES, and 20 mM KOH]; the osmotic pressure was 330 mOsm/kg) and centrifugation for 5 minutes at 700 × *g*.

Sucrose Washing

Raw semen was diluted in 4 volumes of SM medium, and 1 mL of the diluted sample was carefully pipetted over 7.5 mL of sucrose buffer (232 mM sucrose, 2.5 mM KOH, 20 mM HEPES, 10 mM glucose, 0.5 mg/mL polyvinyl alcohol, and 0.5 mg/mL polyvinylpyrrolidone (PVP); the osmotic pressure was 305 mOsm/kg; Harrison et al, 1982) into a conical-bottomed tube. The sample was centrifuged for 5 minutes at 200 × *g* followed by a second centrifugation at 750 × *g* for 10 minutes. The supernatant was removed and the pellet was collected in a conical tube.

Filtration

The filtration process was carried out by diluting the sample twice with 20 volumes of a dilution buffer (0.25 M sucrose, 0.1 mM EGTA, 4 mM sodium phosphate [pH 7.5], and 10% [v/v] of 10× buffer stock HEPES) and filtering through a 5-μm pore size Millipore disk (Millipore Ibérica, Madrid, Spain). Sperm were recovered by sweeping the last mL of the sample across the surface of the filter (Pérez-Pé et al, 2001).

Viability Staining

Cell viability was defined as intact plasma and acrosomal membranes. It was assessed by fluorescent staining with 6-carboxyfluorescein diacetate (6-CFDA) and propidium iodide (Sigma Chemical Co, Madrid, Spain) (Harrison and Vickers, 1990). Sperm samples were diluted to 5×10^6 sperm/mL.

The cells were then examined under a Nikon Labophot-2 fluorescence microscope with a B-2A filter at 400× magnification. The number of fluorescein-positive (plasma membrane–intact) and propidium iodide–positive (plasma membrane–damaged) spermatozoa per 100 cells was estimated and recorded. At least 200 cells were counted in duplicate for each sample.

Assessment of Capacitation Status

Sperm capacitation was evaluated using the chlortetracycline (CTC) assay as previously reported (Pérez-Pé et al, 2002) using the technique described by Ward and Storey (Ward and Storey, 1984). For the evaluation of CTC patterns, the samples (5×10^6 sperm/mL) were observed through a Nikon Eclipse E-400 microscope under epifluorescent illumination using a V-2A filter at 1000× magnification. At least 200 cells were counted in duplicate for each sample.

Annexin V Staining

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). To differentiate between live cells (with or without PS translocation) and dead cells, we used 6-CFDA along with Ann V-Cy3.18. The nonfluorescent 6-CFDA enters the cell and is converted to the green fluorescent compound 6-carboxyfluorescein. This conversion is a function of the esterases that are present only in living cells. Thus, no green fluorescence can be observed in dead cells.

Sperm samples were diluted with 1× binding buffer (commercial kit) up to 500 μL (5×10^6 sperm/mL) and stained with 5 μL 6-CFDA (1 mM in DMSO) and 2 μL Ann V-Cy3.18 (commercial antibody provided in the kit).

Each sample was placed on a slide and analyzed at 1000× magnification by epifluorescence microscopy. Live sperm (6-CFDA+) were visualized in green with a standard fluorescein (Nikon B-2A) filter, and apoptotic sperm (Ann V-Cy3.18+) in red with an ultraviolet (Nikon G-2A) filter. Three patterns of fluorescence were observed: 1) CFDA+/AnnV−: Live cells without PS exposure—this subpopulation was labeled "intact cells"; 2) CFDA+/AnnV+: Live cells with PS exposure—this subpopulation was labeled "apoptotic cells"; and 3) CFDA−/AnnV+: "Dead cells"—this subpopulation showed annexin labeling in the entire cell. These dead cells could have died either by an apoptotic or necrotic process. A total of 400 spermatozoa were counted per slide.

Protein Extraction and Caspase Activity Measurement

Each sample (2×10^7 cells) was washed with phosphate-buffered saline and centrifuged at 30 000 × *g* for 15 minutes 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 (1 pulse at 2 Kv, 200 Ω, and 50 μF). Lysed cells were centrifuged at 11 000 × *g* for 5 minutes at 4°C, and supernatants were collected and stored at −80°C until analysis. Protein concentration in supernatants was determined by the Bradford assay (Bradford, 1976).

We used a commercial kit (EnzChek Caspase-3 Assay Kit #2; Molecular Probes Inc, Eugene, Ore) that allows the detection of apoptosis by assaying the increase in caspase-3 and other DEVD-specific protease activities. As caspase-7 recognizes the same amino acid sequence, the result of the measurement with this kit represents the total activity of both caspases together.

The substrate used in this test is rhodamine 110-bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide), which 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 nonfluorescent bisamide substrate is converted into fluorescent R110. The substrate is a synthetic product, and the enzyme recognizes a synthetic sequence. Therefore, it is not a species-specific recognition. We confirmed that the observed fluorescence signal was specifically due to the activity of caspase-3–like proteases using an inhibitor provided with the kit (Ac-DEVD-CHO inhibitor) (data not shown). The assay was used according to the manufacturer's instructions, and the activity was monitored using a fluorometer (Tecan Spectrafluor Plus; Tecan Ibérica, Barcelona, Spain). Fluorescence units were converted to nM R110/min·mg protein.

Statistical Analysis

Results were expressed as the mean ± standard error of the mean (SEM) of the number of samples. Means were compared by analyses of variance (ANOVA) tests to determine whether there were any significant differences between samples using INSTAT for Windows (version 3.01). P less than .05 was considered to be statistically significant. If ANOVA was appropriated for data type and distribution was investigated by the Kolmogorov-Smirnov test.

Results

Viability

Sperm viability values were different in samples obtained following the 4 washing methods (Table 1). The highest value was found in the swim-up samples. In fact, the swim-up procedure was the only method that accounted for an increase in viability compared with the raw sample (67.4 ± 2.7% vs 62.2 ± 2.3%, respectively). The other 3 methods, Percoll density gradient centrifugation, sucrose washing, and filtration, caused some sperm membrane alteration, slightly

Table 1. *Viable spermatozoa and caspase activity of fresh semen and semen after 4 sperm preparative techniques*

Sperm sample	Viability (%)	Caspase activity*
Fresh	62.2 ± 2.3	31.1 ± 6.1†
Swim-up	67.4 ± 2.7	23.9 ± 4.1†
Percoll gradient	55.9 ± 4.8	0.0 ± 1.1‡
Sucrose washing	55.7 ± 1.7	16.1 ± 2.7†
Filtration	55.0 ± 2.6	22.1 ± 2.5†

* Activity of caspase-3 and -7 expressed as nM R110 substrate/min·mg protein; †,‡ indicate significant differences ($P < .05$); values are means ± SEM (n = 8).

diminishing sperm viability (55.9%, 55.7%, and 55.0%, respectively).

Capacitation State

Evaluation of the capacitation state by CTC staining confirmed the higher quality of the swim-up sample (Table 2). The highest percentage of noncapacitated spermatozoa was recovered by the swim-up procedure (62.2 ± 2.3%), thus increasing the initial proportion present in fresh semen (55.5 ± 2.1%), although differences between both were not significant. Consequently, swim-up samples contained a lower percentage of capacitated and acrosome-reacted spermatozoa than fresh semen. With the other 3 washing procedures, the percentage of noncapacitated spermatozoa was significantly lower ($P < .05$) than that in fresh semen and the swim-up sample (32.5%, 35.5%, and 27.0% in Percoll density gradient, sucrose washing, and filtration samples, respectively).

Phosphatidylserine Translocation

Changes in sperm membrane involving PS translocation after the washing processes were analysed using Annexin V/6-CFDA staining. As shown in Table 3, the population of apoptotic cells decreased in all washed samples compared with fresh semen. It is worth pointing out that only the swim-up procedure accounted for a significant effect, with a decrease ($P < .05$) in the proportion of apoptotic cells of 19.1 ± 5.1%. The decreases after Percoll density gradient and sucrose washing (13.0 and 2.8%, respectively) were not significant. Moreover, filtration hardly reduced the presence of apoptotic sperm (0.6 ± 1.0%).

Caspase Activity

To analyze whether other apoptotic markers were modified after these preparative techniques, caspase activity was determined. The mixed activity of caspase-3 and -7 was comparatively studied in cellular extracts of fresh and treated samples (Table 1). Decreases in caspase activity were found in the samples obtained by swim-up (23.9 ± 4.1 nM substrate/min·mg protein), sucrose washing (16.1 ± 2.7 nM substrate/min·mg protein), and filtration (22.1 ± 2.5 nM substrate/min·mg protein) procedures compared with fresh semen (31.1 ± 6.1 nM substrate/min·mg protein), although the differences were not significant. Surprisingly, caspase activity in the Percoll density gradient sample was hardly detected (0.0 ± 1.3 nM substrate/min·mg protein). To clarify this result, an additional experiment was carried out to test the effect of Percoll on caspase activity. Increasing amounts of Percoll were mixed with a solution of a commercial caspase-3 (0.025 μg/mL of recombinant human caspase-3; Sigma), and enzyme activity was determined. The concentration of caspase used in this assay was chosen on the basis of its activity to obtain a value similar to that in our samples. As shown in the Figure, the higher the Percoll concentration, the lower the enzyme activity was found. With a Percoll concentration similar to that used in the gradient (70%), caspase activity was almost zero.

Discussion

Artificial insemination (AI) in sheep has not been widely adopted, probably due to the relatively poor fertility obtained (Robinson et al, 1989; Maxwell et al, 1999; Grasa et al, 2004). The development of enhanced sperm selection procedures might help to achieve improvements in the results of ovine AI.

Sperm preparative techniques used in animal reproduction are usually directed towards maintaining

Table 2. *Capacitation status of fresh semen and semen after 4 sperm preparative techniques, expressed as percentages of noncapacitated, intact capacitated, capacitated, and acrosome-reacted spermatozoa (chlortetracycline staining)**

Sperm samples	Noncapacitated	Intact capacitated	Capacitated	Acrosome reacted
Fresh	55.5 ± 2.1†	5.2 ± 0.7†	20.7 ± 3.3	18.5 ± 2.5
Swim-up	62.2 ± 2.3†	10.0 ± 0.7†	12.5 ± 4.2	15.2 ± 3.3
Percoll gradient	32.5 ± 4.6‡	18.7 ± 2.5‡	33.0 ± 7.5	15.8 ± 2.7
Sucrose washing	35.5 ± 3.0‡	18.0 ± 1.7‡	25.5 ± 5.5	21.0 ± 3.2
Filtration	27.0 ± 3.7‡	27.5 ± 2.5§	18.5 ± 3.9	27.0 ± 2.2

* Values are means ± SEM (n=8); †,‡,§ indicate significant differences ($P < .05$).

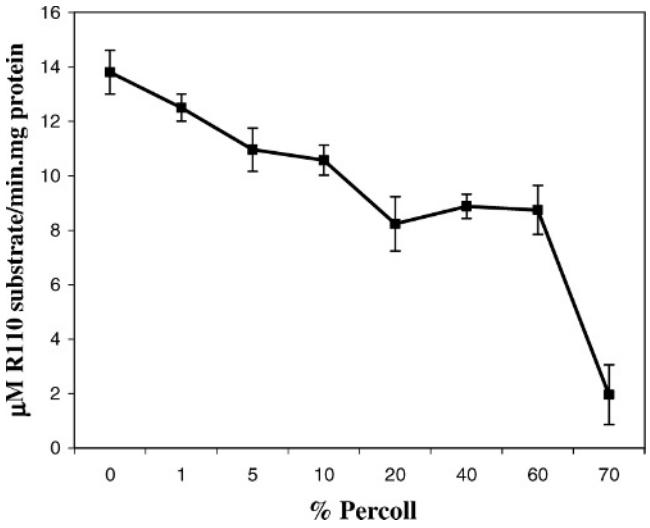
Table 3. *Carboxyfluorescein diacetate/annexin V sperm subtypes in fresh semen and semen after 4 sperm preparative techniques, and decrease in apoptotic sperm relative to fresh semen*

Sperm samples	Sperm subtypes (%)			Decrease in apoptotic sperm (%)
	Intact	Apoptotic	Dead	
Fresh	9.0 ± 1.9†	82.8 ± 2.3†	8.2 ± 2.4†	...
Swim-up	29.0 ± 3.4‡	67.0 ± 3.7‡	4.0 ± 0.6	19.1 ± 5.1*
Percoll gradient	26.3 ± 4.0‡	72.0 ± 4.1	1.7 ± 0.3‡	13.0 ± 3.7
Sucrose washing	14.5 ± 1.9†	80.5 ± 1.7†	5.0 ± 0.4	2.8 ± 2.2
Filtration	13.2 ± 1.5†	82.3 ± 1.8†	4.5 ± 0.8	0.6 ± 1.0

* $P < .05$; †,‡ indicate significant differences ($P < .05$); results are expressed as percentages and values are means M± SEM (n = 8).

motility and viability. The accurate measurement of the semen fertilizing potential is of great importance in determining the acceptability of processed semen for breeding purposes. As the low fertility rate obtained with frozen-thawed semen is possibly due to premature capacitation-like changes (Thomas et al, 2006), assessment of the sperm capacitation state as well as other sperm characteristics such as apoptotic markers, could give substantial information about the sperm fertilizing capacity.

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, 2004), others to a phenomenon of abortive testicular apoptosis (Sakkas et al, 2004), and some to pathologic causes (Oehninger et al, 2003). 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, 2006) and bulls (Anzar et al, 2002).



Caspase activity, expressed as nM R110 substrate/min·mg protein, of a commercial caspase-3 (0.025 µg/mL) mixed with increasing amounts of Percoll. Mean values ± SEM (n = 2).

Thus, it appears that the selection of nonapoptotic spermatozoa is one of the prerequisites for achieving good results after assisted reproduction (Paasch et al, 2005). Therefore, efforts must be focused on the selection of a sperm preparative technique able to discard apoptotic spermatozoa.

In the present study, we compared 4 preparative methods taking into account not only the percentage of viable recovered sperm but also the capacitation state and apoptotic markers. Apart from the inherent interest of this study, it is essential to mention that, to our knowledge, this is the first report about apoptosis in ram sperm.

Although there are numerous studies comparing swim-up and Percoll gradient methods, the obtained results are contradictory. Some investigators have found no differences between samples obtained by either method (Smith et al, 1995), some found swim-up more advantageous (Palomo et al, 1999), while others have demonstrated that the use of the Percoll gradient resulted in better sperm quality (Ding et al, 2000; Somfai et al, 2002). Both the classical swim-up method (Berger et al, 1985) and the Percoll gradient (Arcidiacono et al, 1983) involve centrifugation steps, which have detrimental effects on ejaculated spermatozoa (Aitken and Clarkson, 1988; Alvarez et al, 1993; Mortimer, 1994). It is worth pointing out that the dextran/swim-up technique that we previously developed (García-López et al, 1996) and used in this study does not include centrifugation, thereby avoiding sperm viability loss.

Our comparison of the dextran/swim-up method, Percoll density gradient centrifugation, sucrose washing, and filtration procedure revealed that the swim-up technique resulted in the best quality spermatozoa. The dextran/swim-up sample showed the highest percentages of viable and noncapacitated spermatozoa, the lowest degree of PS translocation, and a caspase activity value lower than fresh semen. We already described the enrichment in viable sperm in the dextran/swim-up sample (García-López et al, 1996) and the sperm capacitation state (Pérez-Pé et al, 2002); however, the

apoptosis status in this selected sample was not evaluated until this current study.

The higher level of PS translocation in Percoll-selected sperm compared with swim-up samples was not correlated with a higher caspase activity, despite the fact that PS translocation has been related to caspase activity (Martin et al, 1996). This suggests that the almost null caspase activity in the Percoll sample is due to an artifact more than a lack of activity. The additional experiment that we carried out with Percoll and a commercial caspase suggests that Percoll interferes with the enzyme determination as has been documented by others (Andersson and Hjorth, 1984; Awasthi and Misra, 2001).

The study of the capacitation state showed that Percoll density gradient yielded a higher percentage of capacitated spermatozoa than the swim-up technique, even greater than the proportion in the fresh sample. This observation is in accordance with that of Moohan and Lindsay (Moohan and Lindsay, 1995), who found that Percoll-selected spermatozoa showed an increased hyperactivation and an ability to undergo capacitation more readily than swim-up samples. This could be due to the fact that the Percoll method selects spermatozoa with different surface membrane properties than those selected by swim-up. Particularly, the Percoll-separated spermatozoa did not possess coating envelopes or a high level of surface fucose residues in comparison with the swim-up spermatozoa, and this may facilitate capacitation (Tanphaichitr et al, 1988). This fact, which could be desirable in sperm preparation techniques for human-assisted reproduction, could be a disadvantage in animal artificial reproduction when timing between ovulation and insemination is not always completely synchronized or the time interval between sperm preparation and insemination could be extended.

The other 2 methods studied, sucrose washing and filtration, yielded a similar sperm viability value, both lower than the fresh sample. Furthermore, the percentage of acrosome-reacted spermatozoa increased after both treatments compared with the control, swim-up, and Percoll samples. In addition, although caspase activity was lower after sucrose washing, it was not accompanied by a significant decrease in spermatozoa with exposed PS.

These results indicate once more that methods involving mechanical stress, such as centrifugation, are harmful to ram spermatozoa. In Percoll density gradient or sucrose washing, 1 or more centrifugation steps are carried out. Although the first one is more effective than the second in selecting nonapoptotic spermatozoa, both exert damage to the cell membranes that results in a decrease in viable and noncapacitated sperm. Furthermore, Percoll, which is a PVP-coated silica, has deleterious effects on sperm membranes (Strehler et al, 1998).

Filtration also exerts mechanical damage on sperm membranes as revealed by the decrease in viability. This could be due to the effect of the vacuum and/or the recurrent pippetting to which sperm are subjected during the process. In addition, the inefficient elimination by filtration of some factors in the seminal plasma with negative effects on spermatozoa cannot be ruled out (Ollero et al, 1997). Also, in the filtration process only seminal plasma is removed, while debris and all dead cells are kept and could affect live spermatozoa probably by production of high levels of reactive oxygen species, which have been reported as inductors of apoptosis (Wang et al, 2003; Grunewald et al, 2005).

In conclusion, results of this study confirm that the dextran/swim-up is a suitable method for selecting high-quality sperm. This is in accordance with our previous observation that ram sperm selection by this simple procedure increases fertilization rates following intrauterine insemination in superovulated ewes (Grasa et al, 2004). The high sperm concentration in ram ejaculates is not a drawback for the yield of the technique. The high percentage of noncapacitated spermatozoa and the low levels of apoptotic markers in the dextran/swim-up sample could, together with the high motility and viability values, contribute to the increased fertility rate of the selected sample compared with the control one. This is the first report on apoptotic markers in ram sperm, and it also confirms the higher capacity of the dextran/swim-up procedure to select nonapoptotic, viable, and noncapacitated sperm, compared with the other 3 sperm preparation methods. This should be considered to enhance the results of AI techniques in ovine reproduction.

Acknowledgments

The authors thank ANGRA for supplying the sires and S. Morales for the collection of semen samples.

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