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Sperm Viability in Ram Semen Diluted and Stored in Three Different Extenders

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López A, Söderquist L, Rodriguez-Martinez H: Sperm viability in ram semen diluted and stored in three different extenders. Acta vet. scand. 1999, 40, 1-9. – Semen was collected with an artificial vagina from 4 one-year-old rams, in order to study the changes in sperm motility and membrane integrity of spermatozoa split-diluted and stored at 5 °C during 7 days in sodium citrate, Tris, and milk-based extenders, respectively. Sperm motility was assessed subjectively and sperm membrane integrity was determined using the fluorescent probes Calcein-AM and Ethidium homodimer. Representative samples were studied using scanning electron microscopy (SEM). The average incidence of sperm motility decreased over time in all the extenders ($p<0.001$). The incidence of spermatozoa showing progressive motility and intact plasma membrane was significantly higher in semen diluted with sodium citrate than in the other 2 extenders following 4 days of dilution until the end of the study. Evaluation with SEM confirmed the findings obtained with the supra vital fluorescent dyes. The results of the present study indicated that there were no differences between sodium citrate-, Tris- or milk-based extenders when ovine liquid semen was stored at 5 °C during a short period (2 days). However, when semen was stored for longer time, spermatozoa in the sodium citrate-based extender sustained its viability better.

liquid; cooled; membrane integrity; motility; storage; sodium citrate; Tris.

Introduction

In vitro evaluation of semen quality is used to obtain information of the fertilizing potential of the semen and of the sire from which it was collected (*Woelders* 1991). This is not only important for research on sperm physiology and semen preservation, but also for artificial insemination programmes.

Freezing and thawing of semen causes serious damage to ram spermatozoa and impairs fertilization (for review see: *Salamon & Maxwell* 1995a, b; *Maxwell & Watson* 1996). The use of fresh diluted and chilled ram semen might be an alternative to frozen semen when used for artificial insemination during a short period after collection. Cooled ram semen (compared with fresh ram semen) suffers from a decrease in motility and morphological integrity, accompanied by a decline in the survival in the female reproductive tract, reduction in fertility and increased embryonic loss (*Maxwell & Salamon* 1993). These damages are, however, less pronounced in diluted and cooled semen than in frozen-thawed ram semen (*Maxwell & Salamon* 1993).

The integrity of the sperm plasma membrane is an essential requirement for general cell function and especially for fertilization (*Woelders* 1991). There are various microscopy tech-

niques that can be used for evaluation of its integrity e.g. phase contrast, differential interference contrast and fluorescence (*Woelders* 1991, *Johnson et al.* 1996). *Gardner et al.* (1986) and *Harrison & Vickers* (1990) described the use of 2 fluorescent probes: carboxyfluorescein diacetate and propidium iodide, to assess the membrane integrity of spermatozoa. The use of fluorochrome probes in combination with UV-light microscopy has been reported to be a useful technique for assessment of the incidence of sperm membrane damage (reviewed by *Rodriguez-Martinez et al.* 1997). In addition, *Januskauskas & Rodriguez-Martinez* (1995) using bull semen, and *Althouse & Hopkins* (1995) working with boar semen, evaluated membrane damage of sperm using the fluorophore probes Calcein AM and Ethidium homodimer (CAM/EthD-1), which has the advantage that it enables estimation of the proportion of spermatozoa with intact plasmalemma as well as the proportion of spermatozoa exhibiting motility (*Rodriguez-Martinez et al.* 1997). Use of scanning electron microscopy (SEM) enables a detailed examination of the cell surface and is therefore an important complement to verify the type and extent of membrane damage (*Eriksson & Rodriguez-Martinez* 1996, *Söderquist et al.* 1997).

The aim of this study was, therefore, to monitor sperm viability (as sperm motility and incidence of plasma membrane damage) in ram semen, diluted in 3 different extenders using a split-sample technique, and stored at 5 °C for up to 7 days.

Materials and methods

Animals, semen collection and experimental design

Four one-year-old rams of Swedish Fine Wool breed were used in this study. The rams were housed at the Department of Obstetrics and Gynaecology of the Swedish University of Agricultural Sciences. Semen samples were assessed before beginning the study, with ejaculate volume, initial sperm concentration, motility and morphology all being within the respective normal ranges for rams according to the standards of the semen laboratory of the Department. Two consecutive ejaculates were collected from each ram (during a period of approximately 5 to 10 min) using an artificial vagina. The consecutive ejaculates were pooled and processed as a single sample. The semen from each ram was divided immediately after collection into 3 equal aliquots and diluted in one of the following extenders: a) "sodium citrate" (2.90% aqueous solution of tri-sodium citrate mixed with 20% of egg yolk); b) "Tris" (Tris buffer 30.28g, fructose 12.50g, citric acid 17.00g per 1000 ml extender plus 20% of egg yolk or c), "skim milk" (The extender was prepared from a basic solution made out of 88g dried non-fatty milk powder in 800 ml distilled water. Two hundred twenty ml of that solution and 12 ml of egg yolk were mixed to complete the extender). Antibiotics (penicillin and streptomycin) were included in all extenders. After dilution, to a final concentration of 1000 million per ml, the semen was cooled to 5 °C (within 1 h) in closed 2 ml plastic vials and maintained at 5 °C in a water bath throughout the 7 day-experiment. Samples taken from each extender and ram were evaluated twice daily. The remaining extended semen was kept for up to 14 days, and samples from each extender and ram were finally evaluated at that time.

Sperm motility

Semen droplets were subjectively assessed for progressive sperm motility (PM) on a phase contrast microscope equipped with a warm stage (37 °C).

Assessment of sperm membrane integrity

Sperm membrane integrity was assessed by in-

cubating semen samples in a staining medium containing the membrane-permeant, cytoplasmic esterase-marker Calcein-AM (CAM) and the membrane-impermeant DNA-marker Ethidium homodimer (EthD-1) (Molecular Probes Inc, Eugene, OR USA) in Phosphate Buffer Solution (PBS), as described by *Januskauskas & Rodriguez-Martinez* (1995). Semen was diluted at a ratio of 1:2 (v/v) in Tris buffer and 30 μl of the dilution were added to equal parts of staining medium and incubated in the dark for 15 min at 35 °C. Random fields were observed under a microscope (600×) equipped with a warm stage (37 °C). One hundred spermatozoa were examined in 5 μl from a stained sample, and the spermatozoa were classified according to *Januskauskas & Rodriguez-Martinez* (1995) into 4 groups: green immotile (GI) with intact plasma lemma, entirely stained green with CAM and immotile; green motile (GM) green stained and motile; green-red (GR) having damaged plasma lemma but intact visible acrosomes (green) when the post acrosomal region stained red with EthD-1, and red (R) having both plasma lemma and acrosomal membrane damaged when stained with EthD-1 and unstained with CAM. Only the GI and GM spermatozoa were considered as having an intact plasma lemma.

On day 7 after semen collection/dilution, semen aliquots were incubated during one hour with caffeine (0.5 and 1 mM, respectively) and progressive motility was assessed before and after incubation.

Following membrane integrity assessment, samples of semen from all the rams and treatments were fixed in a solution of 3% glutaraldehyde in 0.067M cacodylate buffer (500 mOsm, pH 7.2), and representative samples were routinely processed for SEM. The suspensions of spermatozoa were post-fixed in aqueous 1% osmium tetroxide for one h and dehydrated in increasing concentrations of acetone. Spermatozoa were critical-point dried, mounted on stubs, and sputtered with palladium/gold. Samples were examined in a SEM electron microscope (JEOL JSM-6320 SEM) at 5 kV.

Statistical analysis:

Data were examined by repeated-measures ANOVA. The effects of the extender, incubation time and the interaction between extender and incubation time were studied. Rams within extenders were included in the experimental error when extenders were compared. Individual means were compared by the least significant difference test ($p<0.05$) when the main effects were significant. All analyses were done using SAS (*SAS Institute, Inc.* 1993). Results are expressed as mean ± sem.

Results

The average incidence of sperm motility decreased ($p<0.001$) over time in all the extenders (Fig. 1b, c). An interaction between extender and incubation time was found ($p = 0.05$) for progressive motility and percentage of motile green spermatozoa, but not for the percentage of immotile green spermatozoa. A statistically significant difference ($p<0.05$) was found between the sodium citrate-based extender and the other extenders (Tris and milk-based) both for progressive motility and the incidence of sperm membrane integrity (Fig. 1b, c). This difference was detected 4 days after the beginning of the experiment and was maintained until the end (7 days) of the study. Furthermore, differences between the sodium citrate-based and the milk-based extenders were observed 2.5 days after semen collection/dilution (Fig. 1b, c). The percentage of spermatozoa showing progressive motility was less than 10% for the Tris as well as the milk-based extender from day 5 until the end of the study, while the sperm motility

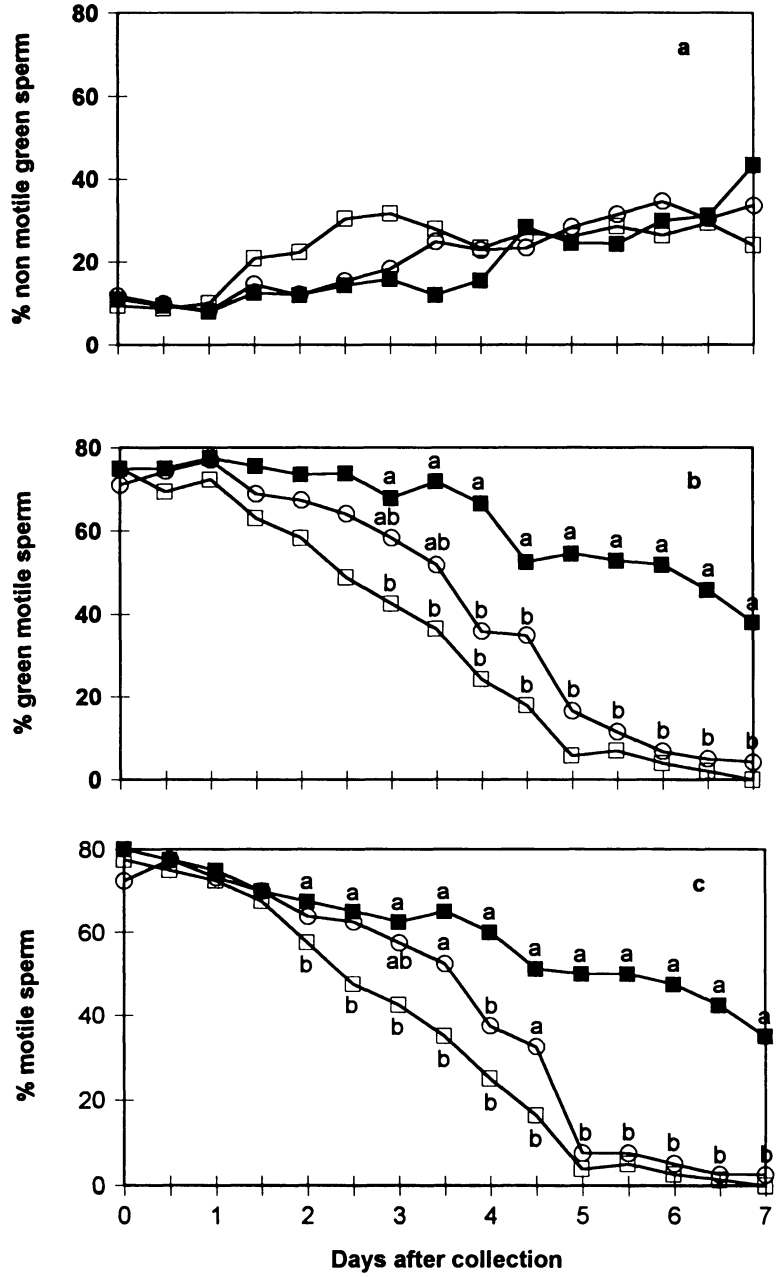

Figure 1. Mean percentage of non motile CAM-green (a), CAM-green motile (b), and motile spermatozoa (c) for milk-based extender (□), sodium citrate (■), and Tris (O) extenders from the time of collection to 7 days thereafter. Different letters at the same sampling occasion indicate statistically significant differences ($p<0.05$).

never decreased below 35% for sodium citrate. The incidence of CAM-green motile spermatozoa was less than 15% for both the Tris and milk-based extenders from 6 days till the end of the study. The incidence of GM spermatozoa never decreased below 40% in the sodium citrate-based extender. No statistically significant differences were found among the extenders for the incidence of GI spermatozoa. Sperm incubation with caffeine appeared unable to increase the rate of progressive motility in the studied samples. Scanning electron microscopy confirmed the findings obtained with the supravital fluorescent dyes; i.e. changes in morphology increased over time and a higher incidence of plasma membrane-deteriorated spermatozoa were observed in Tris as well as in the milk-based extenders compared to the sodium citrate-based extender on days 7 and 14 (Figs. 2 a-d).

Discussion

In the present study, the results indicated a clear advantage of the use of a sodium citrate extender compared to a milk- or Tris-based extender when storing ram semen at 5 °C. Ram semen diluted in sodium citrate-based diluent maintained motility during a longer period of time, and sperm damage was lower after approximately 4 days storage at 5 °C.

The incidence of sperm membrane damage in processed ram spermatozoa is mainly due to changes caused by handling of semen, either at 35-37 °C; during the lowering of temperature from 37 °C to 5 °C (cold shock), and/or during incubation at 5 °C (ageing of spermatozoa). In the present study, the handling procedures at 35-37 °C were quickly finished, and therefore it is unlikely that damage of spermatozoa occurred during this step. When ram spermatozoa are rapidly cooled to near the freezing point, motility and metabolic activity are irreversibly depressed and both acrosome and plasma membranes disrupted (*White* 1993). Cold shock is probably related to the phase transitions of membrane lipids resulting in phase separations and loss of selective permeability (*Watson* 1995). There is an important loss of plasma membrane phospholipids when ram (and other species) spermatozoa are cold shocked. Susceptibility of sperm to cold shock is linked with a high ratio of unsaturated: saturated fatty acids in the phospholipids and a low cholesterol content, which gives a less stable sperm membrane (*White* 1993). Phospholipids in egg yolk might protect the sperm membrane against cold shock in semen diluted in that kind of extenders. In the present study, sperm motility and membrane integrity were assessed immediately after semen samples reached a temperature of 5 °C and they were high (more than 75%). Cold shock changes, even though it might have provoked changes in the spermatozoa, were not pronounced enough to be detected. Thereafter, most of the changes probably occurred during incubation at 5 °C, especially after 48 h when the differences among extenders could be detected. Spermatozoa need easily metabolizable substrates to obtain energy for their functions; and both milk and egg yolk provide substrate for spermatozoa metabolism (*Maxwell & Salamon* 1993). Spermatozoa diluted in both sodium citrate- and Tris-based extenders containing 20% egg yolk maintained progressive motility and suffered less membrane damage during the 3.5 days of storage compared to the milk based-extender containing only 5.2% of egg yolk. It seems, therefore, that the milk extender is better for storage of ram semen at 15 °C than at 5 °C (*Langford & Fiser* 1980). Within an aerobic or even a partially aerobic medium, the production of reactive oxygen is inevitable (*Vishwanath & Shannon* 1997). During the present study, semen samples were stored in closed vials, but the air interphase be-

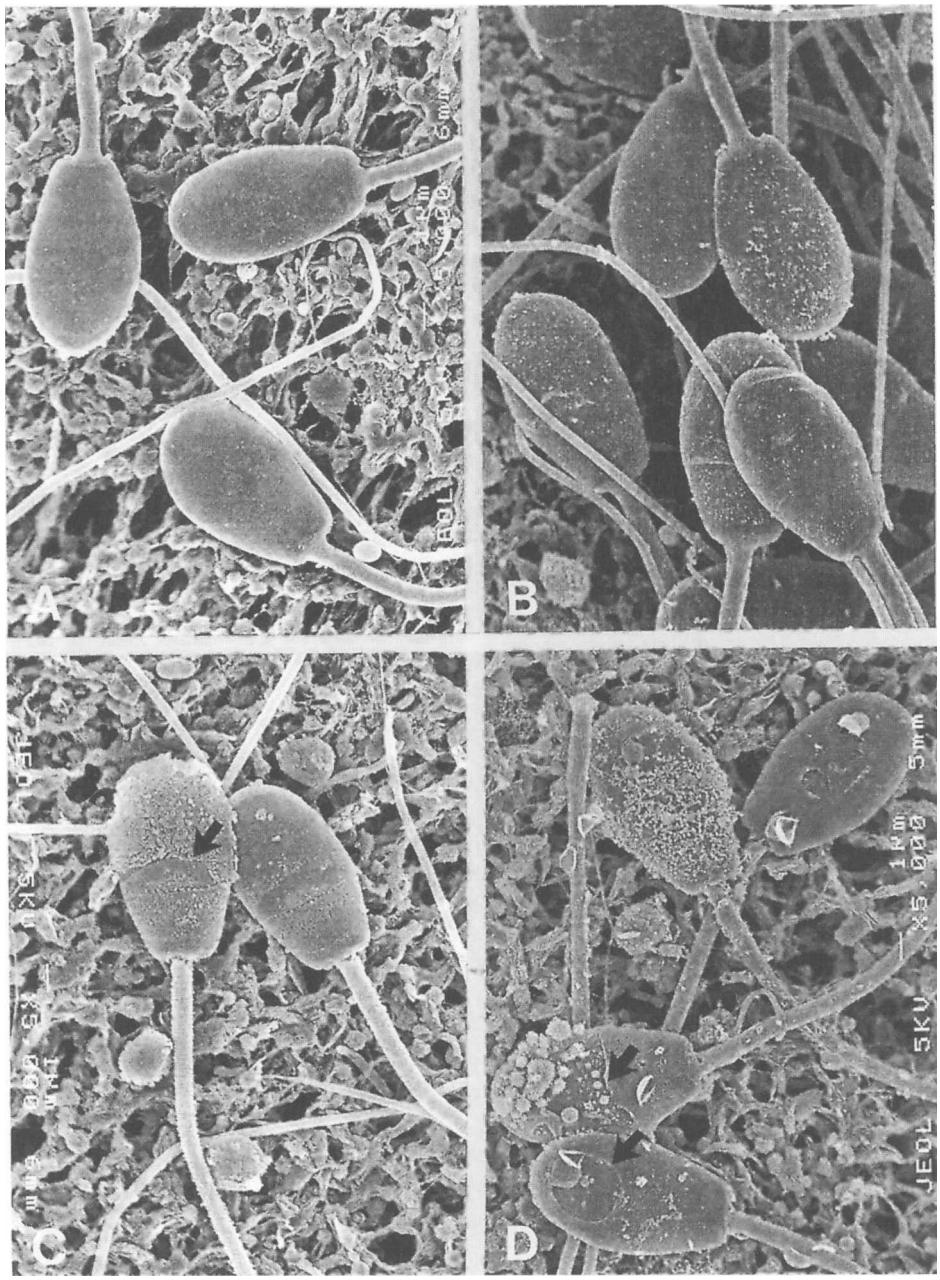

Figure 2. SEM-micrographs of ram spermatozoa after 3 days (A) and at the end of incubation time at 5 °C in sodium citrate- (B), Tris- (C), and milk-based (D) extenders (5000x). Note the damages in the plasmalemmae (arrows) at the sperm head region.

tween the upper part of the tube and the semen surface might have contained enough oxygen to maintain the aerobic metabolism of the spermatozoa between assessments. Storage at 5 °C does not completely arrest spermatozoa metabolism, therefore the accumulation of the toxic products, including free radicals, might be involved in the damage that spermatozoa suffered. The effects of peroxidation on ram sperm include irreversible loss of motility, inhibition of fructolysis and respiration, and structural damage to plasma membrane (*White* 1993). In this respect, it is worth noting that *Maxwell & Stojanov* (1996) were able to improve in vitro fertilization rates with ram semen by adding catalase or superoxide dismutase to the storage medium.

Why sodium citrate extenders appear so beneficial for liquid storage of ram semen is yet unknown. The reports in the literature are to some extent contradictory. *Shindler & Amir* (1961) observed that sodium citrate based-extenders maintained sperm motility longer than milk-based extenders, and *Salamon & Robinson* (1962) obtained greater lambing percentages with semen diluted in sodium citrate than with milk-based extenders. On the other hand, other authors did not find any differences between these extenders (*Blackshaw* 1960, *Salamon* 1962). Sodium citrate, combined with sugars, was used as one of the first extenders when freezing of ram semen was attempted. However, better results obtained with other extenders, especially with Tris- and milk-based extenders, surpassed the use of sodium citrate for freezing of ram semen (*Salamon & Maxwell* 1995a). Extenders containing Tris as the main component have also been used for liquid storage of ram semen with different success (*Salamon et al.* 1979, *Maxwell & Salamon* 1993). In the present study, the sodium citrate-based extender was better than the Tris-based extender for liquid storage of ram semen for more than 4 days. The damage caused to the spermatozoa during longer storage periods at 5 °C might be better prevented using inorganic extenders, like sodium citrate-based extenders, but when the semen is frozen-thawed, organic extenders, like milk or Tris-based extenders, provide a better protection to the sperm membrane. In the present study, more than 20% of the spermatozoa diluted in Tris- or milk-based extenders, did not show any signs of membrane damage according to the fluorescent staining pattern (CAM-green). However, the spermatozoa soon became immotile and remained so for up to 14 days at 5 °C. Other authors have found similar results with bull semen using CAM/Eth-1, (*Januskauskas* 1995), and boar semen evaluated with carboxyfluorescein diacetate and propidium iodide (*Harrison & Vickers* 1990) as well as SYBR-14 (*Johnson et al.* 1996). It was suggested that the lack of motility was due to an inadequate environment, since the incubation of the poorly motile spermatozoa with 3mM dithiothreitol was enough to recuperate motility. Therefore, the immotile cells would indeed have remained undamaged and capable of expressing motility under right environmental conditions (*Harrison & Vickers* 1990). In the present study the motility was unaffected after the incubation of spermatozoa with caffeine, despite caffeine being used at the same concentrations by other authors as a good motility stimulator for ram spermatozoa (*Dacheux & Paquignon* 1980, *Amann et al.* 1982). The lack of stimulation with exogenous caffeine remains therefore unexplained and requires further investigation.

The results of the present study indicated no statistical difference between sodium citrate-, Tris- and milk-based extenders when liquid ram semen was stored at 5 °C during a short period (2 days). However, when semen was cold-stored for longer periods, spermatozoa diluted in the sodium citrate-based extender sustained

better viability, i.e. showed higher incidence of percentage of progressive motility and lower incidence of membrane damage. Whether this long term storage of ram semen influences its fertility after AI is not known and is to be tested in the future.

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Sammanfattning

Spermieviabilitet hos baggsperma spädd i tre olika spädningsvätskor.

Sperma samlades med hjälp av artificiell vagina från fyra stycken ett år gamla baggar i syfte att studera förändringar i spermiemotilitet och membranintegritet hos spermier spädda med (split-sample teknik) natriumcitrat-, Tris- respektive mjölkbaserad spädningsvätska och förvarade vid 5 °C under sammanlagt sju dagar. Spermiemotiliteten bedömdes subjektivt under faskontrast-mikroskop och spermiemembranintegriteten bestämdes med hjälp av de fluorescerande markörerna Calcein-AM och Ethidium homodimer.

Representativa prover studerades i svepelektronmikroskop (SEM). I medeltal sjönk den procentuella spermiemotiliteten i alla spädningsvätskorna med tiden ($p<0.001$). Frekvensen spermier som uppvisade progressiv motilitet och intakta plasmamembraner var signifikant högre i sperma spädd i natriumcitrat lösning jämfört med de två andra spädningsvätskorna efter 4 dagars förvaring och fram till försökets slut. Utvärdering i SEM verifierade de fynd som gjorts med hjälp av de fluoroscerande markörerna. Resultaten från denna studie indikerar att det inte fanns någon skillnad mellan förvaring i natriumcitrat-, Tris- eller mjölkbaserad spädningsvätska när baggsperma förvarades vid 5 °C under en kortare period (2 dagar). När sperma förvarades under längre tid bibehöll emellertid spermier spädda i natriumcitrat lösning sin livsduglighet bättre.

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