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Use of image analysis to assess the plasma membrane integrity of ram spermatozoa in different diluents

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Abstract

Sperm membrane integrity can be assessed by examining a large number of fluorochrome-stained sperm cells over a relative short period of time by flow cytometry or fluorimetry. However, many small laboratories lack a flow-cytometer or fluorimeter for sperm analysis. This study was designed to develop a new image analysis method to evaluate the membrane integrity of ram spermatozoa with the aid of open software, and was divided into three experiments. In the first experiment, the new computer-assisted method was validated by mixing fresh semen samples with different volumes of killed semen in order to know the proportions of damaged spermatozoa in the samples. In the second trial, the new method was compared with the traditional manual counting, and the effect of three extender media on the suitability of the new developed method was evaluated. In the third experiment, the method proposed was tested by comparing the use of milk-, citrate- or TRIS-based diluents for ram semen preservation at 15 °C. In all experiments, semen was assessed for plasma membrane integrity and for percentage of motile and progressive sperm by CASA. In the new computer-assisted method, two images of the sperm cells in a given microscopy field are captured and the number of total- and membrane-damaged cells counted. In the first trial, proportions of damaged sperm cells in each sample determined by the automated procedure agreed closely ($r^2 = 0.98$, $P < 0.001$) with the predicted theoretical values. In experiment 2, the results of membrane integrity obtained using the new method were highly correlated with those provided by the conventional manual counting after PI-CFDA double staining ($r = 0.99$, $P < 0.001$), and also correlated with sperm motility and progressive motility percentages. Viability was significantly higher after dilution with citrate-, than with Tris-based medium, but similar to PBS (70.32 ± 3.93, 55.48 ± 5.76 and 65.38 ± 3.15, respectively), After 0, 24 and 48 h of storage, significantly higher percentages of motile, progressive, and membrane-intact spermatozoa were recorded for the milk than for the Tris extenders. Our results validate the new computer-assisted method for assessing sperm membrane integrity in the sheep, and indicate that the milk extender is less damaging to the sperm of this species than citrate or Tris extenders.
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Keywords: Membrane integrity; Image analysis; Fluorescence microscopy; Sperm diluent

1. Introduction

To improve routine sperm analysis, fast, objective and accessible methods of assessing different aspects of sperm viability need to be developed. The plasma membrane, or plasmalemma, is essential for sperm function. This delicate, unstable layer provides physical

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protection to the cell, acts as a selective barrier for the passage of substances, and plays a pivotal role in interactions with other cells, such as epithelial cells from the female genital tract and oocytes [1].

Many of the methods currently used to assess the status of the plasmalemma are based on the increased permeability of damaged sperm membranes to different substances. These extensively used procedures include measuring membrane permeability to water in a hypo-osmotic solution (hypo-osmotic swelling test, HOST) [2], to stains (eosin–nigrosin method) [3], or to fluorescent probes (Hoescht or propidium-iodide, PI, alone or combined with the permeable fluorochromes CFDA or SYBR) [4–8]. Complementary techniques have been used to increase the precision and feasibility of sperm membrane integrity measurements. Among these, flow cytometry and fluorimetry serve to assess the membrane integrity of large numbers of fluorochrome-stained sperm cells over a relatively short period of time [4,8,9]. A recently developed computer-assisted sperm analyzer (CASA; Sperm Vision, Minitüb, Germany) allows for the automated assessment of sperm membrane integrity in a selected microscopy field [10]. These methods are viewed as important advances in routine sperm quality evaluation. However, whereas not all sperm analysis laboratories have a flow-cytometer, fluorimeter or an adapted CASA system, most will have access to a simple fluorescence microscope. The aim of this study was to develop a novel method of evaluating the membrane integrity of ram spermatozoa in which a fluorescent microscope and a computer with open software are the main pieces of equipment needed. A secondary objective was to use the method proposed to compare milk-, citrate- and Tris-based diluents for ram semen storage at 15 °C.

2. Materials and methods

2.1. Extenders

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Extenders were prepared using Milli-Q water (Millipore Ibérica S.A., Barcelona, Spain).

The three extenders evaluated were: long-life, ultra-heat-treated (UHT) milk (0.7% fat) (MILK); a citrate-based extender (80.6 mM sodium citrate titrated to pH 7.0 using a 1-M citric acid solution, 55.6 mM glucose, and 1% BSA) (CITRATE); and a Tris-based extender (300 mM Tris titrated to pH 7.0 using a 1-M citric acid solution, 55.6 mM glucose, and 1% BSA) (TRIS). Antibiotics (2000 IU/ml penicillin and 0.4 mg/ml streptomycin) were included in all extenders. Osmolarity was adjusted when needed to 290 mosmol/l by adding sodium chloride.

2.2. Collection, dilution and preparation of semen

Semen was collected by artificial vagina from six adult Rasa Aragonesa rams kept at a research center and fed a standard diet. A total of 54 s ejaculates from the six rams were obtained. The undiluted semen was evaluated for motility under a negative-phase contrast microscope at 100× (Olympus BX40, Olympus Optical Co., Ltd., Japan) and sperm concentration calculated by loading 6 μl into a Neubauer chamber in duplicate, and counting under 100× magnification. The semen samples were diluted to 50×10^6 sperm/ml to assess plasma membrane integrity and motility.

2.3. Assessment of stored sperm samples

2.3.1. Assessment of plasma membrane integrity

Sperm viability (membrane integrity) was assessed by fluorescence staining with carboxyfluorescein diacetate and propidium-iodide (CFDA/PI) [5]. Aliquots (1 ml) of diluted samples were pipetted into 1.5 ml Eppendorf centrifuge tubes, and 10 μl of CFDA solution (0.46 mg/ml in dimethyl sulphoxide) and 10 μl of PI solution (0.5 mg/ml in PBS) were added to the samples. Each aliquot was incubated for 8 min in the dark at 30 °C, and spermatozoa were immobilized with formaldehyde. Stained samples (6 μl) were placed on a glass slide, covered and allowed to settle.

Spermatozoa were examined and photographed under an Olympus BX40 fluorescence microscope at 100×. Two pictures where taken of each field under negative-phase contrast and fluorescence microscopy (Olympus BX40, Olympus Optical Co., Ltd., Japan; U-MWIG3 filter block) using a digital camera adapted to the microscope (either a reflex digital camera Canon Eos 400D controlled using the computer through a remote control program, or a compact digital camera Olympus Camedia 30-40). Negative-phase contrast microscopy and fluorescence microscopy were used to determine the number of total and membrane-damaged sperm cells in a given field, respectively. The image was processed using UTHSCSA Image-Tool open software (Version 3.0, available on-line at http://ddsdx.uthscsa.edu/dig/download.html). After transformation of the image to a gray scale, we used the ‘‘Find Objects’’ option of the menu to count the total number of cells per image. A minimum pixel size was selected to avoid counting small particles. The percentage of membrane-damaged spermatozoa was calculated as the number of

propidium-iodide positive cells (in the fluorescence microscopy image) divided by the total number of cells in the same field (phase contrast microscopy image). At least 1000 cells were examined per sample.

For the traditional counting method, numbers of fluorescein-positive (membrane-intact) and propidium-iodide-positive (membrane-damaged) spermatozoa were determined by fluorescence microscopy (Olympus BX40, Olympus Optical Co., Ltd., Japan; U-MNU2 filter block) [5]. At least 200 spermatozoa were examined per sample.

2.3.2. Sperm motility determination by CASA

Computer-assisted sperm analyzer (ISAS®, Version 1.0, PROISER, Valencia, Spain) was used to assess sperm motility. Sample aliquots (5 μl) were placed in a pre-warmed Makler chamber (10 μm depth; Sefi-Medical Instruments, Haifa, Israel) and examined using an Olympus BX40 microscope (Olympus Optical Co., Tokyo, Japan) equipped with a heated stage set at 37 °C, a 10X negative-phase contrast objective (Olympus A10NH), and a Basler A310F digital video camera (Basler Vision Technologies, Ahrensburg, Germany). Two consecutive drops and at least 500 sperm cells (6 fields) were analyzed by CASA for each sample. Established setup parameters were: cell size (min/max), 14/70 μm^2; minimum curvilinear velocity (VCL), 10 μm^{-1}; connectivity, 14; and minimum number of points/trajectory, 5. The semen variables recorded were motility percentage (MS, %), and progressive motility percentage (PS, %).

2.4. Experimental design

2.4.1. Experiment 1

In the first trial, fresh semen samples were mixed with different volumes of killed semen (semen treated with three cycles of freezing to −20 °C and thawing to room temperature), in order to know the proportions of damaged spermatozoa in the samples. Ram spermatozoa from 18 different ejaculates (6 rams, 3 ejaculates/ram) were pre-diluted to 800×10^6 sperm/ml in MILK, mixed with different proportions (0, 25, 50, 75, and 100%) of killed spermatozoa from the same ejaculate, diluted to 50×10^6 sperm/ml in CITRATE and assessed for viability and motility by CASA. The results were compared to the theoretical values predicted on the basis of the estimations made on fresh and frozen samples.

2.4.2. Experiment 2

The second experiment was performed to compare the new computer-assisted method with the traditional manual counting, and to evaluate effect of three extender media on the suitability of the new developed method. Ram spermatozoa from 18 different ejaculates (6 rams, 3 ejaculates/ram) were pre-diluted in the MILK extender to a concentration of 800 million sperm/ml at room temperature, and stored in a refrigerator at 15 °C until analysis. Aliquots from each sample were diluted to 50×10^6 sperm/ml in PBS, CITRATE or TRIS, to assess plasma membrane integrity (manual or computer-assisted) and motility by CASA.

2.4.3. Experiment 3

This experiment was designed to compare three extenders for ram semen preservation at 15 °C using the new developed method. After collection, ram spermatozoa from 18 different ejaculates (6 rams, 3 ejaculates/ram) were split into three equal volumes, which were diluted in the MILK, CITRATE or TRIS extenders to a final concentration of 800 million sperm/ml at room temperature, packed in 0.25 ml straws and stored in a refrigerator at 15 °C. At the time points 0, 24 and 48 h post-dilution, the semen from three straws per group (MILK, CITRATE and TRIS) was pooled, diluted to 50×10^6 sperm/ml in CITRATE and assessed for viability and motility.

2.5. Statistical analysis

The values obtained were expressed as mean ± standard error of the mean (S.E.M.). Statistic analyses were performed using the SPSS package, Version 14.0 (SPSS Inc., Chicago, IL, USA).

Normality distributions and variance homogeneity of the median value score for each set were checked by the Kolmogorov–Smirnov and Levene tests, respectively. For samples that were normally distributed, differences in membrane integrity and motility between extenders were examined through analysis of variance (ANOVA) using generalized linear models. If the F value was significant, a Tukey test was used for a posteriori multiple comparisons. Pearson's correlation coefficients were determined for comparison of the results obtained by the two methods of evaluation sperm membrane integrity (manual and automated counting), and with results of motility determined by CASA. For non-normally distributed populations, the Kruskal–Wallis test was used for comparison of membrane integrity and motility between extenders, followed by the Mann–Whitney a posteriori test. Spearman's correlation coefficient was used to evaluate the correlation between variables of non-normally distributed populations. The relation between theoretical and

actual values of sperm viability in experiment 1 was evaluated by linear regression test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Characteristics of fresh semen

The volume of the 54 s ejaculates was 0.84 ± 0.0148 ml (mean ± S.E.M.), and the sperm concentration measured by Newbauer chamber was $3976.95 \pm 20.95 \times 10^6$ sperm/ml.

3.2. Experiment 1

As shown in Fig. 1, the new computer-assisted method captures two images: one of all the sperm cells and the other of the membrane-damaged sperm cells, in a given microscopy field. The proportions of damaged sperm cells in each sample determined by the automated procedure agreed closely ($r^2 = 0.98$, $P < 0.001$) with the predicted theoretical values (Fig. 2).

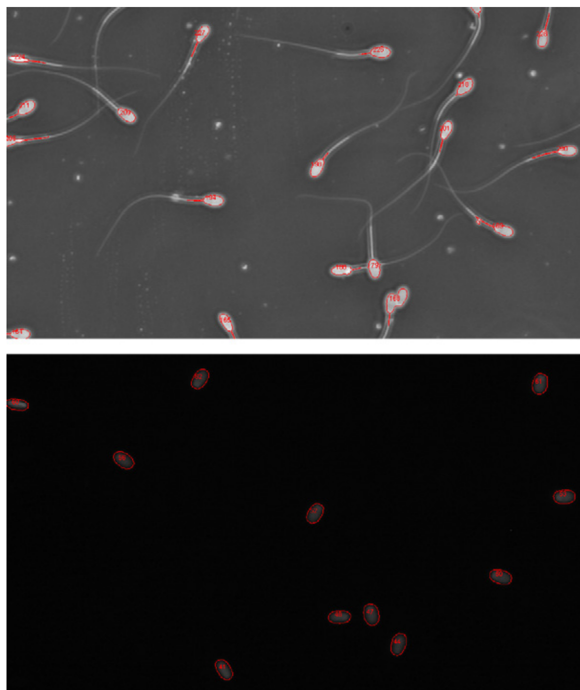

Fig. 1. The total number of sperm cells in a given field was determined in a negative-phase contrast image. Dead spermatozoa were identified and counted using a fluorescence image of the same field. Sperm heads were captured and counted with the aid of Image-Tool software.

Correlation coefficients between different sperm characteristics revealed a positive correlation between sperm viability and each of sperm motility and progressive motility percentages, and between sperm motility and progressive motility percentages ($r = 0.90$, 0.82 and 0.93, respectively, $P < 0.001$).

3.3. Experiment 2

No significant differences in sperm motility (79.07 ± 3.36, 83.29 ± 3.62 and $78.64 \pm 3.84\%$) and progressive motility (47.29 ± 1.87, 47.50 ± 2.18 and $46.79 \pm 2.24\%$) percentages were found between samples diluted in CITRATE, PBS and TRIS, respectively. However, viability was significantly higher after dilution with CITRATE than with TRIS (70.32 ± 3.93 vs. 55.48 ± 5.76, respectively, $P < 0.05$), but no significant differences in membrane integrity were observed between PBS (65.38 ± 3.15) and CITRATE or TRIS.

Correlation coefficients between different sperm characteristics revealed a positive correlation between sperm viability and each of sperm motility and progressive motility percentages, and between sperm motility and progressive motility percentages ($r = 0.73$, 0.43, and 0.72, respectively, $P < 0.001$).

The reliability of the new automated method was compared with the traditional manual CFDA/PI technique. The percentages of spermatozoa with intact plasma membranes recorded using the computer-assisted and manual counting methods were highly correlated ($r = 0.99$, $P < 0.001$).

3.4. Experiment 3

Fig. 3 shows the effects of the different diluents and storage times on the proportions of membrane-intact spermatozoa, observed by using the new method proposed, and on sperm motility. Sperm viability was higher by using MILK than TRIS, at all storage periods ($P < 0.001$ at 0 and 24 h, and $P < 0.05$ at 48 h of storage). At 24 h post-storage, the semen samples diluted in CITRATE showed higher proportions of membrane-intact spermatozoa than the TRIS-diluted samples ($P < 0.05$).

At 0 h of storage, significantly higher percentage of motile sperm were recorded for the MILK compared to the CITRATE or TRIS diluents ($P < 0.01$, Fig. 3). At 24 h, a significant lower percentage of motile sperm was recorded for samples diluted in TRIS than in CITRATE ($P < 0.05$) or MILK ($P < 0.001$) diluent.

Spermatozoa in MILK samples showed a progressive motility higher than in TRIS samples at any time

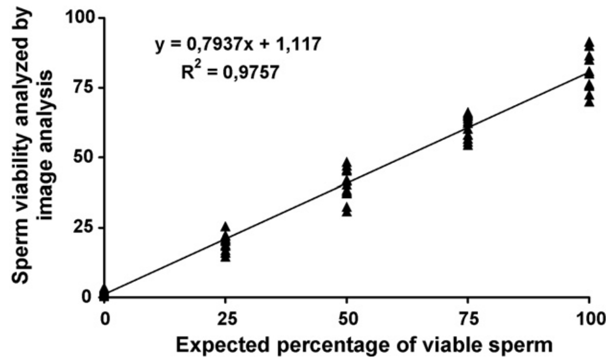

Fig. 2. Regression plot for correlations between percentages of sperm analyzed by the new computer-assisted method and the expected percentages.

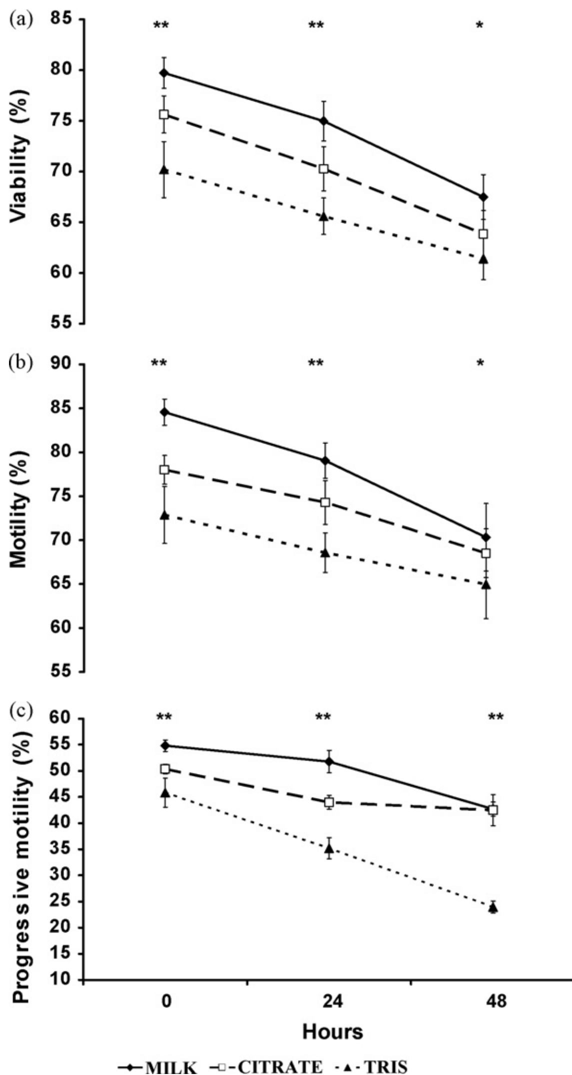

Fig. 3. Mean (±S.E.M.) percentage of sperm with plasma membrane integrity (a), total sperm motility (b) and forward progressive motility (c) of ram spermatozoa at different times of storage at 15 °C after dilution in MILK (◆), CITRATE (□), or TRIS (▲) extenders. Means marked with asterisk are significantly different (*$P < 0.05$, **$P < 0.01$).

during the storage period ($P < 0.001$), and than in CITRATE samples at 0 and 24 h of storage ($P < 0.05$) (Fig. 3). CITRATE diluent significantly improved sperm progressive motility in comparison to TRIS diluent at 24 and 48 h of storage ($P < 0.001$).

As in experiments 1 and 2, correlation coefficients between different sperm characteristics revealed a positive correlation between sperm viability and each of sperm motility and progressive motility percentages, and between sperm motility and progressive motility percentages ($r = 0.9$, 0.77 and 0.74, respectively, $P < 0.001$).

4. Discussion

The plasma membrane integrity (viability) of spermatozoa is considered a valuable measure of sperm quality. However, counting viable cells under the microscope is a time-consuming and subjective method of determining the viability of a few hundred spermatozoa per sample, and is thus not suitable for routinely assessing large numbers of samples [1,9]. The results of the present study suggest that our new method based on image analysis may be an efficient and low-cost alternative to the conventional way of assessing sperm membrane status. The method has been properly validated using known cell population samples by adding different and defined percentages of damaged spermatozoa to fresh semen.

The results of previous studies indicate that an intact sperm cell membrane will more closely reflect semen fertility than sperm motility, but only when large numbers of spermatozoa are examined using flow cytometry or fluorimetry [11–13]. However, the cost of purchasing this equipment is high for many laboratories conducting semen analysis. The method proposed here produces images of 1000–2000 cells in 2–3 min (depending on the sperm concentration of the sample

and the type of digital camera used). The subsequent analysis of the images using the open software package Image-Tool takes an additional 2–3 min. This means that we can analyze some 200–400 sperm/min, increasing its efficiency over the manual counting method by several times. Further advantages of this new method compared to perform manual counts include reduced operator fatigue and the possibility of using low fluorescence intensities (by increasing the camera's exposure time). Further, if desired, a folder with the images captured for each sample can be created such that for high throughput analysis, large numbers of images can be captured and then analyzed at a later stage.

The commercially available computer-assisted sperm analysis system (Visionlab, Minitub) has been recently adapted to assess sperm membrane integrity [10]. The main shortcoming of this analyzer compared to the method proposed here is that it requires the use of high magnifications (400×) to ensure an optimal image and this determines the need to examine at least 15 microscopy fields per sample. In our method, the images are captured by a digital photo camera instead of a video camera. This means that low power magnifications (100×) can be used, reducing the number of fields required. Thus, in this study, three fields were used to efficiently analyze 1000–2000 cells.

The effect of the diluents CITRATE, PBS and TRIS on the suitability of the new developed method has also been evaluated. We chose these diluents because they are easily available and easy to store. PBS and TRIS buffers have been previously used for the assessment of ram sperm viability [14,15]. According to our results, CITRATE allowed a better maintenance of membrane integrity of spermatozoa than TRIS, and is considered as the diluent of choice when using the new developed method in the ram. PBS had intermediate values between CITRATE and TRIS, but differences between them were no significant.

Finally, in experiment 3, we explored the effects of three extenders (milk-, citrate- and Tris-based extenders) on membrane integrity during the storage of ram semen at 15 °C for up to 48 h. Under our conditions, the MILK extender rendered the best motility and viability results at 0 and 24 h of storage, whereas sperm diluted in TRIS showed the lowest *in vitro* survival rate. The literature contains contradictory results concerning the effectiveness of different diluents for ram semen [16], determining that citrate-, Tris-, and milk-based media have been indistinctively recommended for liquid storage of ram semen [17]. Discrepancies may be explained to some extent by differences in the protocols among studies, the use of different sperm concentrations, diluent compositions, times and temperatures of sperm storage, and methods of evaluating sperm quality. Thus, French workers found that the viability and fertility of ram semen diluted in reconstituted skimmed milk was better after storage at 15 °C than at 5 °C for 8–16 h [18,19]. The number of studies that have compared milk, citrate and TRIS diluents for ram semen preservation at 15 °C is limited, but in line with our results, Martí et al. [20] found that a milk-based extender give more satisfactory results than a synthetic Tris-based diluent after semen storage at 15 °C for 6 h.

In summary, our findings validate the effectiveness of the new image analysis method for assessing sperm viability in studies such as this one, designed to find ways of improving the quality of semen used in artificial insemination programs. This rapid, simple procedure has the benefit over other available methods that it only requires a fluorescence microscope and suitable image analysis software as the main pieces of equipment. Although we chose the ram as the experimental animal for this study, we have successfully used this new computer-assisted method for the routine analysis of semen in other species, such as the cow and pig.

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