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Effect of diluent composition on the dynamics of sperm DNA fragmentation and other sperm quality parameters in ram during incubation at 37 °C

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ABSTRACT

This study was conducted to assess the effect of different semen extenders on the dynamics of sperm DNA fragmentation (SDF) and other quality sperm parameters in ram. To this end, second ejaculates from 6 adult males were collected using an artificial vagina and diluted in either INRA-96®, milk, MOPS, TRIS, TES, HEPES, citrate or phosphate-based extenders. Semen samples were incubated at 37 °C and the SDF, motility and membrane integrity were assessed after 0, 3, 6 and 24 h intervals. Significantly higher SDF indexes were obtained at 6 and 24 h of incubation in the milk-based than in all the buffer-based extenders. At 24 h, sperm diluted in INRA also reached a higher SDF than MOPS, TRIS, citrate, and phosphate-based extenders. Diluent composition also affected sperm motility and membrane integrity parameters. The diluent had a clear impact on the pH of the samples, with a significant drop at 6 h of incubation in the samples diluted in INRA, milk and citrate-based diluents, but only at the end of the incubation period (24 h) in the other diluents. A significant negative correlation was observed between the pH of the media and SDF (−0.67). It was concluded that the results of the dynamics of sperm DNA fragmentation are highly dependent on the diluent employed during incubation at 37 °C, and this should be taken into account in studies evaluating this parameter. The use of some diluents would reduce, or even prevent, sperm DNA fragmentation during incubation, so that possible differences in susceptibility between males would be masked.

1. Introduction

Semen evaluation *in vitro* (spermiogram) is an essential tool in the selection of males/ejaculates, in the improvement of semen diluents and semen processing protocols and in studies of reproductive biology and toxicology. Given that the usual spermiogram parameters have low prognostic values for *in vivo* fertilising capacity, semen analyses have become increasingly detailed (Rodriguez-Martinez, 2006). The establishment of a viable pregnancy requires that spermatozoa reach the site of fertilisation, but they must also penetrate and fertilise the oocyte and produce a viable embryo. Spermatozoa containing damaged DNA may potentially compete with spermatozoa having normal DNA, decreasing the success rates of fertilisation. High levels of sperm DNA fragmentation (SDF) have been associated with decreased oocyte fecundation, embryo quality and pregnancy rate (Tesarik et al., 2004; Virro et al., 2004). SDF assessment of semen used for assisted reproduction purposes is therefore of interest (Gosálvez et al., 2011).

SDF may be evaluated punctually (SDF static or basal) or, preferably, through the assessment of DNA fragmentation kinetics (SDF dynamics) (López-Fernández et al., 2010; Pérez-Llano et al., 2010). To assess the dynamics of SDF, semen samples are incubated for a varying period at 37 °C, with the aim of emulating the biological conditions experienced by the spermatozoa after insemination. Assessment of SDF dynamics by *in vitro* incubation provides key information about the changes of DNA integrity in the mature spermatozoa that are typically not revealed in basal assessment of SDF (López-Fernández et al., 2008, 2010). As differences in DNA

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quality have been related to the fertilising capacity of the ejaculate (Evenson, 1999), the kinetics of DNA fragmentation must further help predict the fertility capacity of a given sample.

The composition of the media to dilute semen samples may have an impact on the extent of DNA damage and this aspect must be standardised for a given species to obtain reliable results. In this paper, we examine the effect of different diluents on the SDF dynamics and other sperm quality parameters in rams after a period of controlled sperm stress during *in vitro* incubation at 37 °C for 24 h.

2. Materials and methods

2.1. Semen diluent composition

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

Eight semen extenders were evaluated in this study, as previously described (Yániz et al., 2005, 2011): (i) a milk-based extender (long-life UHT skimmed milk, MILK); (ii) the commercial INRA-96® extender (INRA); (iii) a citrate-based extender (80.6 mM sodium citrate adjusted to a pH of 7.0, with a 1 M citric acid solution, 55.6 mM Glucose, 0.8% BSA, and 0.1% polyvinyl alcohol, PVA) (CITRATE); (iv) a TRIS-based extender (250 mM TRIS adjusted to a pH of 7.0, with a 1 M citric acid solution, 55.6 mM Glucose, 0.8% BSA, and 0.1% PVA) (TRIS); (v) a TES-based extender (80 mM TES adjusted to a pH of 7.0, with a 1 M NaOH solution, 55.6 mM Glucose, 0.8% BSA, and 0.1% PVA) (TES); (vi) a HEPES-based extender (125 mM HEPES adjusted to a pH of 7.0, with a 1 M NaOH solution, 55.6 mM Glucose, 0.8% BSA, and 0.1% PVA) (HEPES); (vii) a MOPS-based extender (125 mM MOPS adjusted to a pH of 7.0, with a 1 M NaOH solution, 55.6 mM Glucose, 0.8% BSA, and 0.1% PVA) (MOPS); (viii) a phosphate-based extender (80 mM $Na_2HPO_4{\cdot}2H_2O$ adjusted to a pH of 7.0, with a 220 mM $NaH_2PO_4{\cdot}H_2O$ solution, 55.6 mM Glucose, 0.8% BSA, and 0.1% PVA) (PHOSPHATE). Antibiotics (2000 IU/ml penicillin and 0.4 mg/ml streptomycin) were included in all semen diluents, except in the INRA-96®, and the osmolarity was adjusted to 290 mM, where needed, by the addition of sodium chloride.

2.2. Semen collection, dilution and preparation

All animal procedures were performed in accordance with the Spanish Animal Protection Regulation RD223/1988, which conforms to European Union Regulation 86/609. Semen was collected with the aid of an artificial vagina from 6 adult rams of Rasa Aragonesa breed in a period of less than 30 min prior to analysis. All the animals were fed using a standard diet and kept at the production centre. A total of 24 s ejaculates (the first ejaculate was discarded) were obtained from the 6 rams. Four replicas per ram with a period of abstinence of 3 days were obtained and individually processed (Table 1). The semen was diluted with the CITRATE extender (2% semen + 98% extender) and evaluated for individual sperm motility under a negative-phase contrast microscope (×100 magnification) (Olympus BX40, Olympus Optical Co., Ltd., Japan). The minimum criterion for acceptability was 70% visual sperm motility. The sperm concentration was measured using a computer-assisted sperm analyser (CASA) (ISAS®, Version 1.0, PROISER, Valencia, Spain), after placing the diluted semen sample in a Neubauer chamber in duplicate. The semen of each ejaculate was diluted with either MILK, INRA, CITRATE, HEPES, MOPS, PHOSPHATE, TES or TRIS extenders, respectively, to a final concentration of 800×10^6 sperm/ml. The diluted semen samples were stored in glass test-tubes and incubated 37 °C in a water bath. At 0, 3, 6 and 24 h after dilution, the semen samples were carefully mixed and diluted to 50×10^6 sperm/ml in the same medium used in previous semen dilution to evaluate the sperm motility, and in a citrate-fructose medium to assess the sperm plasma membrane integrity (Yániz et al., 2013a,b) or diluted to 20×10^6 sperm/ml in PBS to assess sperm DNA fragmentation (López-Fernández et al., 2008).

2.3. Assessment of stored semen samples

2.3.1. Assessment of sperm motility

Computer-assisted sperm analyzer (ISAS®, Version 1.0, PROISER,Valencia, Spain) was used to assess sperm motility (Yániz et al., 2008). Briefly, semen sample aliquots (5 μl) were placed in a prewarmed 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 10× 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 were analysed 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, %).

2.3.2. Assessment of sperm cell plasma membrane integrity

Sperm viability (membrane integrity, SV) was determined using acridine orange (AO) and propidium iodide (PI) (Yániz et al., 2013a,b). Aliquots (0.2 ml, 50×10^6 sperm/ml) of diluted samples were pipetted into 1.5 ml Eppendorf centrifuge tubes and fluorochrome solutions were added, so the final concentrations were 5 μl/ml of the acridine orange solution from the DUO-VITAL kit (Halotech, Madrid, Spain) combined with 15 μM PI. Spermatozoa were immobilised with formaldehyde at a final concentration of 0.005%. Labelled samples (4 μl) were placed on a glass slide, covered and allowed to settle. This procedure results in selective labelling of dead and live cells, visualised in red and green colours, respectively.

Digital images of the fluorescence-labelled sperm were obtained using an epifluorescence microscope (DM4500B Leica, Wetzlar, Germany) under a 10× magnification objective equipped with a double bandpass filter (FITC/TxRed dualband filter cube, with a 479/585 nm excitation filter, 505/606 nm dichroic beam splitter and 524/628 nm barrier filter). A JenOptik ProgRes CF CCD (JenOptik AG, Jena, Germany) coupled with Jenoptik Progress Capture Pro image acquisition software was used. The CCD was pre-adjusted with a fixed triggering time of 501 ms. The number of total sperm and the percentages of membrane-intact and membrane-damaged sperm were determined using IMAGE J processing open software (version 1.45e, available on-line at http://rsbweb.nih.gov/ij/download.html), with two plug-ins created for this purpose (Yániz et al., 2013a). At least 300 sperm cells were examined per sample.

2.3.3. Assessment of sperm DNA fragmentation (SDF)

Each semen sample was analysed after adjusting the concentration to 20×10^6 sperm/ml in PBS. For all samples, SDF was analysed using the Sperm-Halomax kit (Halotech DNA, Madrid, Spain). The Sperm-Halomax methodology is based on the sperm chromatin dispersion test (SCD; López-Fernández et al., 2008).

Each experiment was conducted using 15 μl of each diluted aliquot of semen. This volume was mixed with 30 μl of low-melting-point agarose. Two microlitres of the mixture was extended on pre-treated slides (Sperm-Halomax kit, Halotech DNA, Madrid, Spain), covered with a 24 × 24 mm coverslip (Thomas Scientific) and placed on a cold metallic plate in the refrigerator (4 °C) for 5 min. Afterwards, the coverslip was removed and each slide was set up horizontally in 10 ml of lysing solution (Sperm-Halomax kit) for 5 min, and the slide was washed in distilled H_2O for 5 min at room temperature. The nucleoids resulting from the lysing process were dehydrated in a 2 min series of ethanol baths (70, 90 and 100%). Once dried, the slides were stained using a 1:1 mixture of GelRed (10×; Biotium, Hayward, CA, USA) in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA) for DNA staining. This staining method permits manual scoring by allowing for the distinction of sperm containing either fragmented DNA (large and spotty halos of chromatin dispersion) or unfragmented DNA (small and compact halo of chromatin dispersion). Samples were viewed and captured using a Leica DM4500B microscope (Leica Microsystems, Wetzlar, Germany). Sperm were counted and divided into 2 groups: fragmented and non-fragmented, and a percentage was calculated based on measuring 300 cells.

2.3.4. Assessment of pH

All pH measurements were made directly on samples with the aid of a Crison pH-metre (Crison. Instruments S.A., Alella, Spain) at 0, 3, 6 and 24 h of incubation at 37 °C.

2.4. Statistical analyses

Normality of the distributions was checked using the Kolmogorov–Smirnov tests. As all data were normally distributed, parametric tests were used throughout. The effects of time of incubation (0, 3, 6 and 24 h), diluent (MILK, INRA, TRIS, CITRATE, PHOSPHATE, MOPS, TES and HEPES), ram and their interactions on the SDF, CASA sperm motility, sperm viability and the pH of the media were analysed by general linear models (GLM) repeated measures analysis of variance, using the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA). The differences in the sperm quality parameters between extenders within a time of incubation and between times of incubation within extender were tested using the estimated marginal means provided by the SPSS, by creating contrast statements. Pearson's correlation coefficients were determined to identify significant relationships between the results of different parameters of sperm quality (SDF, CASA sperm motility and SV) and the pH of the media during incubation. Values are expressed as the mean ± standard error of the mean (SEM). The statistical level of significance was set at $P < 0.05$.

3. Results

GLM repeated measures of analysis of variance revealed that the SDF, MS, PS and SV were significantly different ($P < 0.001$) between extenders, rams and incubation time periods (Tables 2 and 3). A significant extender × time of incubation interaction for SDF, MS, PS and SV ($P < 0.001$ in all cases) was also recorded. Ram effect was due to the fact that one of the six rams used in the present study obtained higher SDF, and lower MS, PS and SV values throughout