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Motility of liquid stored ram spermatozoa is altered by dilution rate independent of seminal plasma concentration

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ABSTRACT

The fertility after use of liquid stored ram semen following cervical AI rapidly decreases if semen is stored beyond 12 h. The dilution of seminal plasma is often cited as a key contributor to the diminished motility and fertility of ram spermatozoa subjected to liquid preservation. Two experiments were conducted to assess the effect of spermatozoa concentration (*i.e.* dilution rate) and percentage of seminal plasma on the motility and viability of liquid stored ram spermatozoa. In Experiment 1, semen was diluted to one of seven concentrations ranging from 0.2 to 1.4×10^9 spermatozoa/ml with milk and assessed for motility after 3 or 24 h of storage at 15 °C. In Experiment 2, semen was collected and washed to remove seminal plasma before re-dilution to 0.2–1.4×10^9 spermatozoa/ml with milk containing 0%, 20% or 40% (final v/v ratio) seminal plasma and assessed for viability and motility after 3 or 24 h of storage at 15 °C. Whereas motility was not affected by spermatozoa concentration after 3 h of storage, the proportion of progressive spermatozoa decreased after 24 h of storage when spermatozoa concentration was greater than 1.0×10^9 spermatozoa/ml. The duration of preservation and the spermatozoa concentration affected spermatozoa motility but had no impact on spermatozoa viability. This negative effect of greater spermatozoa concentrations on motility was independent of the presence and the concentration of seminal plasma. The seminal plasma at both concentrations (20% and 40%) had a protective effect on spermatozoa motility after 24 h of storage. These findings have the potential to improve the efficiency of cervical AI with liquid stored ram semen.

1. Introduction

The preservation of semen for the artificial insemination (AI) of sheep has progressed markedly since the first reports of success in the early decades of the 20th century (Ivanov, 1907, 1912). Over the years, empirical studies have established the appropriate concentrations of sugars,

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pH buffers, proteins and other substances required to confer metabolic support and protection to spermatozoa during dilution for liquid or frozen storage prior to AI. Fertility resulting after use of liquid or frozen stored ram semen remains compromised. The fertility of liquid stored ram semen following cervical AI rapidly decreases if stored beyond 12–24 h (Maxwell and Salamon, 1993), while acceptable fertility with frozen-thawed ram semen can only be achieved by the use of intrauterine AI (facilitated commercially by laparoscopy; Killeen and Caffery, 1982; Maxwell et al., 1984). Despite the added technical challenge, most ewes bred artificially are laparoscopically inseminated with frozen-thawed semen due to the obvious logistical benefits of indefinite storage. However, many European countries continue to AI large numbers of ewes with liquid stored semen due to the low cost and ethical benefits of a non-surgical delivery technique (para-cervical or intra-cervical). Despite geographically localised flocks and excellent transport infrastructure, the 12–24 h time period for optimum fertility places significant strain on artificial breeding enterprises in countries that use liquid stored ram semen. Therefore, the ability to extend the period of maximum fertility when liquid stored ram semen is used would be of great benefit.

The method used to extend and chill ram semen varies somewhat between countries, but the most common system involves dilution with UHT milk or tris–citrate–fructose–egg yolk media prior to storage at 15 °C or 5 °C. The dilution rate used for extension is usually minimal (in the order of 3–4 fold) so as to limit insemination volume and minimise the dilution of beneficial antioxidants and proteins contained within seminal plasma. The deleterious effect of dilution of seminal plasma on the quality of ram spermatozoa (commonly referred to as the "dilution effect"; Ashworth et al., 1994) is often cited as a reason for the diminished motility and fertility following liquid or frozen storage (Maxwell et al., 2007; Leahy and de Graaf, 2012). However, recent research has cast doubt on the need to minimise dilution rate as frozen-thawed ram spermatozoa were found to have superior function when frozen at quite low concentrations (D'Alessandro et al., 2001; Leahy et al., 2010b; Alvarez et al., 2011). As such, the present study was conducted to investigate the effect of dilution rate on the motility of liquid stored ram spermatozoa and whether this was linked to dilution of seminal plasma.

2. Materials and methods

2.1. Experimental design

Two experiments were conducted to assess the effect of spermatozoa concentration (*i.e.* dilution rate) and percentage of seminal plasma on the motility of liquid stored ram spermatozoa. Semen was collected from six adult Lacaune rams and pooled. Each experiment was replicated seven times with the collection of the same six rams. In Experiment 1, semen was diluted to one of seven concentrations ranging from 0.2 to 1.4×10^9 spermatozoa/ml with milk and assessed for motility after 3 or 24 h of storage at 15 °C. In Experiment 2, semen was collected and washed to remove seminal plasma before re-dilution to 0.2–1.4×10^9 spermatozoa/ml with milk containing 0%, 20% or 40% (final v/v ratio) seminal plasma and assessed for viability and motility after 3 or 24 h of storage at 15 °C. In both experiments, 3 and 24 h spermatozoa analyses were conducted following further extension of a sub-sample of each treatment in phosphate buffer saline supplemented with bovine serum albumin (1 mg/ml) to a concentration of 20×10^6 spermatozoa/ml and incubation for 2 h at 37 °C.

2.2. Preparation of seminal plasma

After collection by artificial vagina, semen was pooled from each ram ($n = 6$ Lacaune rams), three ejaculates/ram and centrifuged at $10{,}000 \times g$ for 10 min at 4 °C. The resultant supernatant was aspirated and further centrifuged ($10{,}000 \times g$ for 10 min at 4 °C) to remove any remaining spermatozoa or cellular debris. This supernatant was aspirated, aliquoted into tubes (100 μl) and stored at −80 °C until use was required for insemination.

2.3. Collection, dilution and storage of semen

Ejaculates were collected from Lacaune rams ($n = 6$) using an artificial vagina. Only those ejaculates with an initial wave motion score of 4 or above were processed for liquid storage. In Experiment 1, semen was diluted to 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 or 1.4×10^9 spermatozoa/ml with milk (reconstituted with 11.1 g of skim milk powder in 100 ml of distilled water) supplemented with gentamycin (50 μg/ml). Diluted semen (1 ml) was transferred into 1.5 ml Eppendorf tubes, placed in an incubator and cooled to 15 °C over the course of 3 h and stored at this temperature for 24 h.

In Experiment 2, 1 ml of semen was diluted to 10 ml in phosphate buffered saline and washed by centrifugation ($800 \times g$, 10 min) to remove seminal plasma. The resultant pellet was re-suspended with milk supplemented with 0%, 20% or 40% seminal plasma (v/v percentage of final solution) to the same seven sperm concentrations tested in Experiment 1. Diluted semen was transferred into 1.5 ml Eppendorf tubes, cooled and stored as per Experiment 1.

2.4. Assessment of spermatozoa motility characteristics

The percentage of motile spermatozoa was assessed objectively using computer assisted spermatozoa analysis (CASA; HTM-IVOS v. 12; Hamilton–Thorne, Beverly MA, USA). At each applicable time point, sub samples of each treatment were further diluted to a standard 20×10^6 spermatozoa/ml with phosphate buffered saline supplemented with bovine serum albumin (1 mg/ml) and incubated for 2 h at 37 °C. After this incubation period, semen samples (2 replicates of 10 μl, 20×10^6 spermatozoa/ml) were subsequently placed on slides and enclosed using a 22 mm × 22 mm coverslip before immediate transfer to the CASA. Motility characteristics were determined by assessment of at least 12 randomly selected microscopic fields (>300 spermatozoa/sample) utilising factory CASA settings (ram) at an image sampling frequency of 60 Hz. Spermatozoa tracks