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# First Publication to Describe a Protocol for the Liquid Storage of Stallion Spermatozoa for 7 Days

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## 1. Introduction

The ability to store spermatozoa in a synthetic medium for longer than 72 h without cryopreserving will provide a number of benefits including the transport of semen from stallions whose spermatozoa do not tolerate cooling or freezing and reduced biosecurity concerns associated with the use of animal-derived components such as skim milk and eggs. In addition, the extended sperm longevity will permit the use of more relaxed mare synchronisation and insemination regimens and the ability to ship semen without the need for a dry shipper will reduce the costs and logistical constraints associated with the use of frozen semen. The aim of this project was to develop a protocol for the processing and storage of semen storage in a synthetic, chemically defined stallion sperm extender without the need to chill or cryopreserve.

## 2. Materials and Methods

*UoN extender.* The precise formulation of the UoN extender described in this abstract cannot be fully disclosed (patent pending). UoN extender is a protein-free balanced salt and amino acid solution which has been modified to support the unique requirements of stallion spermatozoa [1-4] with further modifications. This medium has been supplemented with 100 U/mL Nystatin, 0.25 mg/mL gentamicin, 50 U/mL penicillin and 50 μg/mL streptomycin to curtail microbial growth.

*Semen processing.* Following collection, semen was extended 1:2 (semen:extender) using EquiPlus with gentamicin (Minitube). The initial extension in a protein-based medium is necessary to

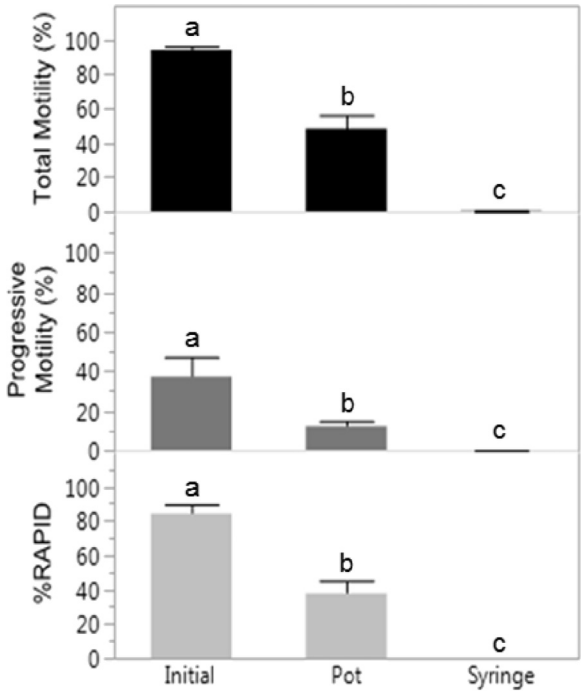


**Figure 1.** Motility parameters of stallion spermatozoa stored in UoN extender in either a 5 mL sterile Sarstedt flat-bottom pot or a B.Braun syringe for 7 days at RT.

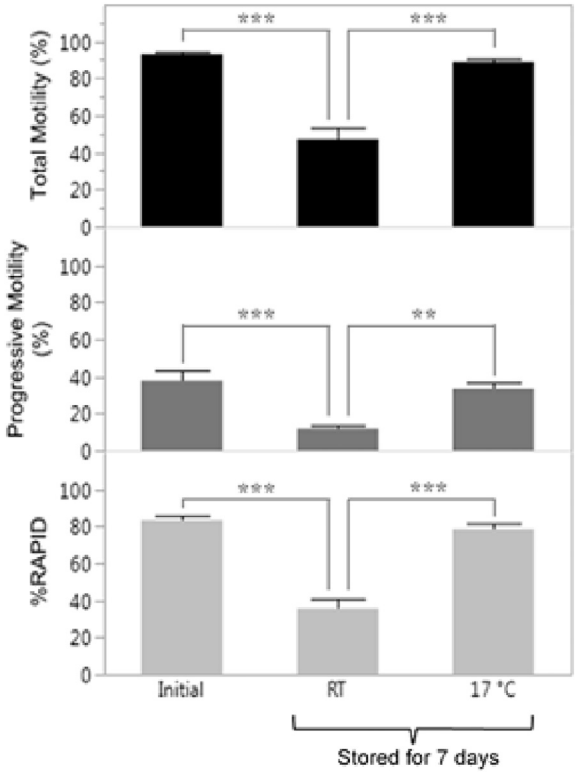


**Figure 2.** Motility parameters of stallion spermatozoa stored in UoN extender at RT or 17 °C.

scavenge toxic seminal plasma proteins and as UoN medium is protein-free this initial step is necessary. Extended semen was either centrifuged directly at 350 x *g* for 15 min ('non-purified') or layered onto an EquiPure gradient and centrifuged at 400 x *g* for 20 min to isolate high density (putative high quality) cells ('purified'). Sperm pellets were then resuspended to 50 x $10^6$ spermatozoa/mL in either UoN extender or EquiPlus with gentamicin as indicated. All samples were stored aerobically.

*Effects of storage container.* The motility of non-purified spermatozoa (N=3) was assessed via computer assisted sperm analysis (CASA; IVOS Hamilton Thorne) and 3 mL aliquots were placed into either a 5 mL Sarstedt flat-bottom sterile specimen pot or a B.Braun Inject latex-free insemination syringe and stored at RT (approx. 22 °C) for 7 days after which CASA was performed. For all proceeding experiments sperm was stored in 3 mL aliquots within 5 mL Sarstedt flat-bottom sterile specimen pots.

*Effect of temperature.* Non-purified spermatozoa (N=6) were stored either at RT or 17 °C for 7 days before CASA. 17 °C was selected as 1) below this stallion spermatozoa lose their ability to regulate intracellular calcium [5] and 2) there are commercially available 17 °C pig semen shippers.

*Effect of purification.* Purified and non-purified spermatozoa (N=12) were stored at 17 °C for 7 days before CASA.

*Comparison between chilled and UoN extender-stored spermatozoa.* EquiPure isolated spermatozoa (N=12) were stored in either EquiPlus (at 4 °C) or UoN extender (at 17 °C) for 7 days. Viability, acrosome integrity and motility were assessed. Spermatozoa were then capacitated for 2 h [6], motility was reassessed, a heterologous (bovine) zona binding assay [6] was performed using 10,000 progressively motile spermatozoa and capacitation was assessed via Western blot using an anti-phospho-tyrosine antibody (Sigma).

*Statistical analyses.* All analyses (ANOVA, Chi Square, REML or Students t-tests) were performed using JMP v13 software (SAS Institute) at  = 0.05.

## 3. Results and Discussion

*Effects of storage container.* There was a decrease in total, progressive and rapid motility over time ($P \leq 0.05$). All motility parameters of spermatozoa stored in the pot were higher than spermatozoa stored in the syringe ($P \leq 0.01$; Fig 1).

*Effect of temperature.* There was an effect of storage temperature on all motility parameters ($P \leq 0.0001$), with no decline in any motility parameter over 7 days when spermatozoa were stored at 17 °C. All motility parameters of spermatozoa stored at RT were significantly lower than both the initial motilities and the motilities of spermatozoa stored at 17 °C (Fig 2).

*Effect of purification.* There was a significant effect of EquiPure treatment on all parameters. Purified spermatozoa had higher total and %RAPID motility than non-purified spermatozoa after 7 days ($P \leq 0.01$; Fig 3).

*Comparison between chilled and UoN extender-stored spermatozoa.* Following storage, spermatozoa stored in UoN medium had higher total ($P \leq 0.01$) and %RAPID ($P \leq 0.001$) motility than chilled spermatozoa (Fig 4). Following capacitation, all motility

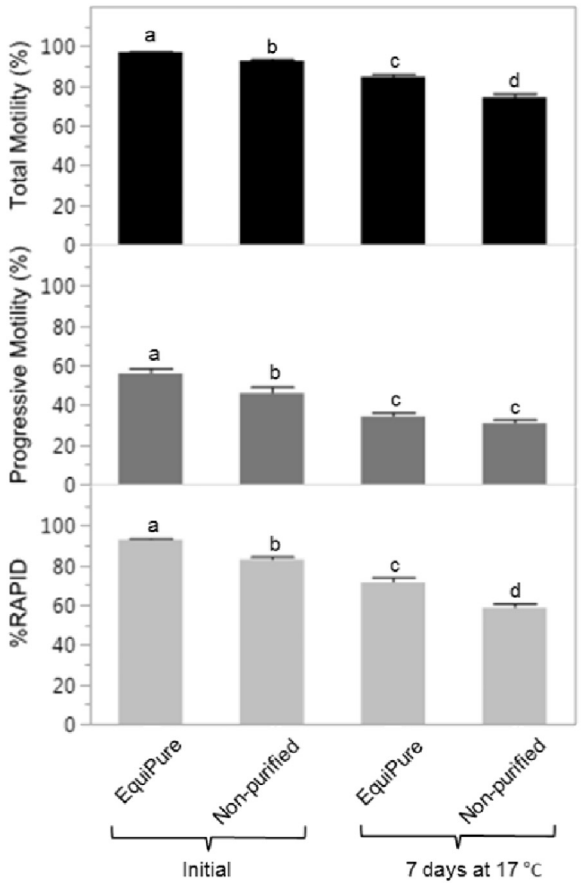


**Figure 3.** Motility parameters of purified and non-purified stallion spermatozoa stored in UoN extender at 17 °C for 7 days.

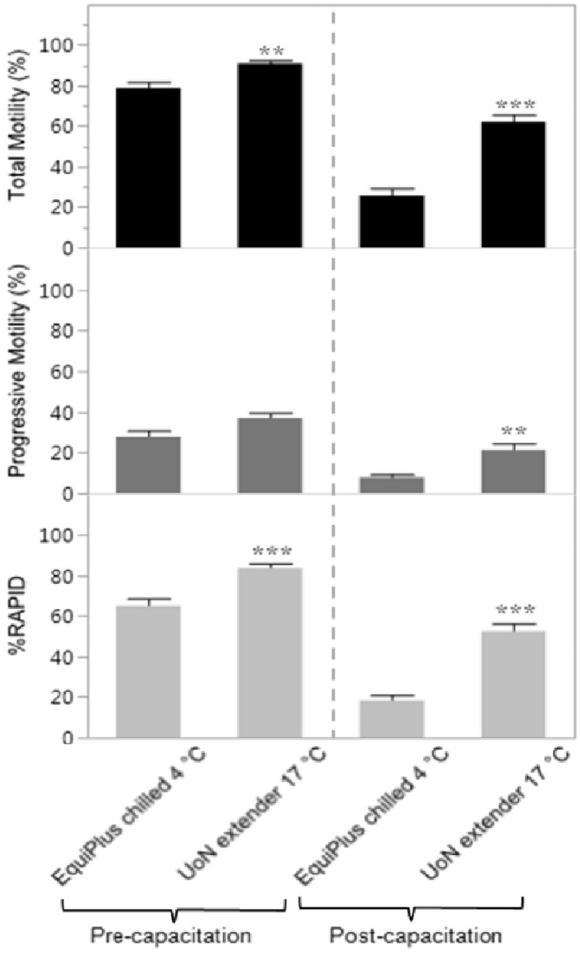


**Figure 4.** Motility of chilled and UoN extender-stored spermatozoa following 7 days storage pre- and post-capacitation.