Effect of heterologous seminal plasma and semen extenders on motility of frozen–thawed ram spermatozoa

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ABSTRACT
Ram seminal plasma increases the fertility of frozen–thawed ram spermatozoa deposited into the cervix. The aim of the current study was to compare the effect of ram seminal plasma to that of bull seminal plasma, dog prostatic fluid, protein-free TALP, TrilEq (Triladyl with 0.5 m of Equex STM paste added to each 100 m) and heat-treated skim milk on longevity and percentages of progressively motile and aberrantly motile frozen–thawed ram spermatozoa. Three ejaculates from each of 6 rams were extended in TrilEq, pooled and frozen in straws as a single batch per ram. One hundred and eight straws (3 straws from each ram for each fluid) were thawed in random order. Once thawed, a straw was emptied into a tube with 0.85 m of the appropriate fluid at 37 °C and kept at that temperature for 6 h. Motility was assessed at ×200 magnification immediately (time zero) and 2, 4 and 6 h after thawing. Progressive motility decreased from each time to the next (P < 0.05) and was 39.0 % (0 h), 26.0 % (2 h), 19.6 % (4 h) and 12.6 % (6 h); SEM 1.24, n = 108 for each group. Ram seminal plasma resulted in higher progressive motility than bull seminal plasma, lower than milk, and similar to the other fluids. Ram seminal plasma resulted in lower aberrant motility than protein-free TALP and similar aberrant motility to other fluids. The effect of ram seminal plasma and dog prostatic fluid was very similar. The effect of ram seminal plasma on the fertility of frozen–thawed ram spermatozoa deposited into the cervix is not due an exceptionally beneficial effect on the motility of spermatozoa.

Keywords: frozen–thawed semen, ram, seminal plasma sperm.

INTRODUCTION
Fresh ram semen may yield good fertility after cervical insemination, whereas frozen–thawed ram semen has to be deposited into the uterus for acceptable fertility. The need for uterine insemination restricts the use of frozen–thawed ram semen because there is no method that is easy, repeatable, non-invasive and that does not require expensive equipment to achieve intrauterine insemination. The addition of ram seminal plasma to frozen–thawed ram spermatozoa holds promise as it improves the fertility achieved with cervical insemination to acceptable levels11. Ram semen contains relatively little plasma, which makes it an impractical additive where large numbers of ewes need to be inseminated with frozen–thawed semen. There is thus a need to search for another fluid that would also yield acceptable fertility of frozen–thawed ram spermatozoa after cervical insemination. Like frozen–thawed ram spermatozoa, frozen–thawed dog spermatozoa also result in high fertility after uterine insemination but poor fertility after vaginal insemination19. The addition of dog prostatic fluid to frozen-thawed dog spermatozoa improves their fertility after vaginal insemination19. The ejaculate of the domestic dog contains a large amount of seminal plasma, largely derived from the prostate gland12. Adding bovine seminal plasma to frozen-thawed African buffalo (Syncerus caffer) spermatozoa improves their ability to fertilise bovine oocytes in vitro, confirming that heterologous seminal plasma can have an effect on the fertility of spermatozoa21.

The motility of ram spermatozoa is considered essential for their migration through the cervix and from the central region of the cervical canal towards the cervical mucosa where they are stored and released over time as required for fertilisation10. It is therefore possible that seminal plasma promotes the fertility of frozen–thawed ram spermatozoa deposited in the cervix by maintaining or promoting their motility. When searching for a fluid to replace ram seminal plasma as a post-thaw additive to frozen–thawed ram spermatozoa prior to cervical insemination, its effect on the motility of frozen–thawed ram sperm should be compared to that of ram seminal plasma.

Heat-treated skim milk is an effective extender for fresh ram spermatozoa prior to cervical insemination9, resulting in high fertility, presumably due to its protein fraction, which may buffer against changes in pH and partially protect spermatozoa during cooling for storage10. Adding dog prostatic fluid to frozen–thawed dog spermatozoa not only improves their fertility after vaginal insemination12,13, but also their motility and longevity after thawing9. Adding protein-free TALP to frozen–thawed dog spermatozoa, however, maintains their post-thaw motility better than dog prostatic fluid14.

The aim of the current study is to compare the effects of bull seminal plasma, dog prostatic fluid, heat-treated skim milk and protein-free TALP on the motility of frozen–thawed ram spermatozoa to the effect of ram seminal plasma.

MATERIALS AND METHODS
Experimental animals
The semen donors were 2 Dorper, 2 Merino and 2 Dohne Merino rams, aged 2–4 years, kept at Faculty of Veterinary Science of the University of Pretoria in open-roofed pens with cement floors and fed lucerne hay and commercial sheep pellets, with water ad libitum. Semen was collected by means of an artificial vagina, using a non-oestrous ewe tied in a neck clamp as a mount. The ejaculates were collected during May, which coincides with the optimum breeding season for sheep in South Africa.

Experimental design
One batch of semen from each of 2 rams from each of 3 breeds was frozen and...
18 straws from each batch thawed and 3 straws individually allocated to each of 6 fluids that were added after thawing. In this way, the semen from 3 straws of each batch were separately diluted with the same type of fluid. The fluids were ram seminal plasma, bull seminal plasma, dog prostatic fluid, heat-treated skim milk, protein-free TALP and Triladyl with Eqex STM paste (TrilEq). After thawing and dilution the semen was incubated at 37 °C for 6 h and the motility evaluated (t0), 2 (t2), 4 (t4) and 6 (t6) hours after thawing. The response variables were percentage progressively motile spermatozoa, percentage aberrantly motile spermatozoa and percentage immotile spermatozoa.

Collection, preparation and storage of fluid

TrilEq. Triladyl was prepared by adding 1 volume of Triladyl concentrate™ (Minitub, Tiefenbach, Germany), which contains glycerol, tris, citric acid, fructose, tylosin, gentamicin, lincomycin and spectinomycin in unknown concentrations as this is a trade secret, to 3 volumes of deionised water and 1 volume of egg yolk. Finally, to each 100 ml of Triladyl, 0.5 ml of Eqex STM paste (Nova ChemiCalSales, Scituate, MA, USA) was added to give 100.5 ml of TrilEq. TrilEq was used as freezing extender and as 1 of the fluids added to frozen-thawed semen. TrilEq was prepared in advance and stored at −70 °C until shortly before use.

Seminal plasma from rams and bulls and prostatic fluid from dogs. An ejaculate was collected from each of the 6 rams, pooled and centrifuged at 3500 g for 30 minutes and the supernatant filtered through a 0.22 μm filter. This process was repeated and the supernatants pooled until a sufficient quantity was available to yield 20 aliquots of 0.85 ml of the sperm free, filtered seminal plasma that were transferred into Cryo-tubes (NUNC, Denmark) and stored at −70 °C.

One ejaculate from each of 8 bulls (2 Holsteins, 2 Ayshires, 2 Tulis and 2 Brahman) belonging to the Taurus Artificial Insemination Centre was collected by means of an artificial vagina and treated in the same manner as described for rams to yield 20 aliquots of seminal plasma.

The post-sperm fraction of 1 ejaculate from each of 6 dogs was collected by means of digital massage and then processed in the same manner as described for seminal plasma of rams and bulls to yield 20 aliquots of prostatic fluid.

Heat-treated skim milk. Ultra high temperature (UHT) skim milk (Clover South Africa (Pty) Ltd) contained 4.9 g carbohydrates, 3.4 g protein, 0.4 g milk fat and 123 mg calcium per litre.

Protein-free TALP. The composition of Protein-free TALP used was: NaCl, 113.96 mmol/l; KCl, 3.19 mmol/l; NaHCO3, 24.88 mmol/l; NaH2PO4 mono-hydrate, 0.40 mmol/l; MgCl2 hexahydrate, 0.49 mmol/l; CaCl2 dihydrate, 2.00 mmol/l; HEPES, 10.07 mmol/l; Phenol red, 0.01 mmol/l; sodium pyruvate, 0.50 mmol/l; sodium lactate, 15.78 mmol/l; gentamycin, 0.025 g/l

Freezing of semen

One ejaculate was collected from each ram on each of 3 consecutive days and discarded, subsequent to which each ram rested for 3 days before another 3 ejaculates were collected from each, with a 60-minute interval between ejaculates. These last 3 ejaculates from each ram were extended, cooled to 5 °C, pooled and frozen as 1 batch per ram, as described below. Immediately after collection of an ejaculate destined for freezing, a small drop of it was suspended from a glass slide kept at 37 °C and examined under a microscope at ×40 magnification to confirm that the semen had strong wave movement (at least 4 on a scale from 0 to 5). Each ejaculate with adequate wave movement was immediately extended with 2 volumes of TrilEq at 30 °C in a 15-mℓ tissue culture tube that was placed in a 50 ℓ water jacket at 25 °C. This was placed in a beaker with 250 ℓ water at 15 °C, which was transferred to a cool room at 5 °C. The 2nd and 3rd ejaculates from the ram were processed in the same way as the 1st and 150 minutes after the 3rd was placed in the cool room they were pooled in a 50 ℓ tissue culture tube. The concentration of spermatozoa in the pool was determined by means of a haemacytometer and the pool further extended with TrilEq at 5 °C to a final concentration of 200 million per ml. Two hours after the 3 ejaculates were pooled the suspension of spermatozoa was inverted repeatedly to ensure thorough mixing. Fifty 0.25 ml French straws, marked with the name of the ram, were filled and sealed with PVC powder. The straws were frozen by suspending them horizontally 5 mm apart and 4 cm above liquid nitrogen in stable nitrogen in a Styrofoam box for 20 minutes before plunging them into the liquid nitrogen. The straws were then stored in a liquid nitrogen container at −196 °C.

Statistical analysis

The statistical software package NCSS (www.ncss.com, Kaysville, UT USA) was used for all statistical analyses. Breed was initially included in the model, with ram nested in breed, but, because breed had no significant effect (P > 0.05) and breed was of no particular concern with respect to the aim of the study, it was removed prior to the final analysis.

Repeated measures ANOVA was used to test for differences among the post-thaw motility with the rams as subjects and fluid and time as factors causing within-subjects variation. The interaction between fluid and time was included in the model, whereas the subject interactions were combined with error. Bonferroni’s test was used to compare the mean of
each fluid to that of ram seminal plasma, which served as control. For all comparisons the level of significance was set to $P < 0.05$.

**RESULTS**

**Fresh semen quality**

Although there was a large variation in sperm concentration among rams, each ejaculate had good motility (mass motility score 5, percentage progressively motile spermatozoa exceeding 70 % and percentage aberrantly motile spermatozoa of 1 % or lower).

**Motility after thawing and dilution**

Ram, fluid and time significantly affected the percentage progressively motile spermatozoa, the percentage aberrantly motile spermatozoa and the percentage immotile spermatozoa ($P < 0.001$). Table 1 shows the mean percentages of progressively motile, aberrantly motile, and immotile spermatozoa for each fluid at different times of incubation. Ram seminal plasma resulted in a higher percentage progressively motile spermatozoa than bull seminal plasma and a lower percentage than heat-treated skim milk ($P < 0.05$). A similar level during the following 4 hours, while no such rise occurred with the other fluids. The interaction between fluid and time with respect to percentage immotile spermatozoa was mainly because, for bull seminal plasma, the percentage immotile spermatozoa increased more steeply during the 1st 2 hours of incubation than for the other fluids.

**DISCUSSION**

Although ram seminal plasma improves the fertility of frozen–thawed ram spermatozoa deposited into the cervix and heat-treated skim milk does not, ram seminal plasma does not preserve the in vitro motility of such spermatozoa as well as heat-treated skim milk. This observation suggests that ram seminal plasma exerts its beneficial effect on the fertility of frozen–thawed ram spermatozoa deposited into the cervix via a mechanism other than exceptional preservation of progressive motility. Assuming that ram seminal plasma preserves progressive motility of frozen–thawed spermatozoa that were deposited into the cervix sufficiently well to result in acceptable fertility, the results of the current study suggest that dog prostatic fluid and protein-free TALP, both of which preserved progressive motility as well as ram seminal plasma, should also adequately preserve their progressive motility. In the dog, however, vaginally inseminated frozen–thawed spermatozoa.
zoa resulted in higher fertility after the addition of dog prostatic fluid than protein-free TALP. The observation that seminal plasma of the domestic bull improves the ability of African buffalo spermatozoa to fertilise bovine oocytes in vitro suggests that it is reasonable to suspect that effects of seminal plasma on fertility need not be restricted to an autologous effect.

Sheep and dogs share certain characteristics with respect to the utility of frozen–thawed spermatozoa in the 2 species: fertility resulting from deposition of frozen–thawed spermatozoa is good after deposition in the uterus, but poor after deposition at the external cervical opening, and fertility after deposition at the latter site is better if seminal plasma is added to the frozen–thawed spermatozoa prior to insemination.

Further research is required to compare the effects of ram seminal plasma and dog prostatic fluid on the fertility of frozen–thawed ram spermatozoa deposited into the cervix.

REFERENCES
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