

Reprod Dom Anim 42, 541–549 (2007); doi: 10.1111/j.1439-0531.2006.00819.x
ISSN 0936-6768

Autologous Whole Ram Seminal Plasma and its Vesicle-free Fraction Improve Motility Characteristics and Membrane Status but not *In Vivo* Fertility of Frozen–Thawed Ram Spermatozoa

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Contents

Motility characteristics (assessed subjectively and with computer-assisted semen analysis) and membrane status (after staining with chlortetracycline) of washed and non-washed frozen–thawed ram spermatozoa were evaluated after incubation in buffer and buffer containing autologous whole seminal plasma or one of its two fractions: the pellet of membrane vesicles obtained by ultracentrifugation (and used at three times normal protein concentration) or the vesicle-free supernatant fraction. Whole seminal plasma and supernatant, but not membrane vesicles, improved the motility characteristics of spermatozoa after 3 and 6 h of post-thaw incubation compared with the control buffer. Resuspension and incubation with whole seminal plasma, supernatant or membrane vesicles lowered the proportion of acrosome-reacted frozen–thawed spermatozoa compared with the control buffer. Unwashed frozen–thawed semen from three rams, incubated with autologous whole seminal plasma or its fractions and inseminated using cervical or intrauterine artificial insemination, had no effect on pregnancy rates of ewes in synchronized oestrus. However, fertility was higher after laparoscopic than cervical insemination (44.9 vs 12.3%, $p < 0.001$). In conclusion, resuspension and incubation of frozen–thawed ram spermatozoa in autologous whole seminal plasma or its vesicle-free supernatant fraction improved their motility characteristics and, with membrane vesicles, membrane status, but these benefits were not reflected in improved fertility after cervical or intrauterine insemination.

Introduction

Ejaculated spermatozoa must undergo a number of modifications before becoming able to fertilize the oocytes. The interaction of spermatozoa with other semen components, particularly proteins, has been shown to influence the function and fertilizing capacity of spermatozoa (Maxwell et al. 2007). Maxwell et al. (1999) demonstrated a beneficial effect of whole seminal plasma on the function and fertility after cervical insemination of frozen–thawed ram spermatozoa. It has been suggested that plasma membrane vesicles, a fraction isolated from ram seminal plasma, may have a role in modifying sperm function in relation to their motility and capacitation status (Breitbart et al. 1983). However, we were unable to find any effect of these vesicles on motility characteristics or *in vitro* fertility of frozen–thawed ram spermatozoa at their normal concentration in seminal plasma (El-Hajj Ghaoui et al. 2007). Moreover, the vesicle-free supernatant fraction of seminal plasma, rather than the seminal plasma membrane vesicles, was shown to improve the function and *in vitro* fertility of frozen–thawed ram spermatozoa

when added to the post-thaw medium (El-Hajj Ghaoui et al. 2007).

The objective of the present study was to determine the effect of whole ram seminal plasma and its two fractions, supernatant and pellet of membrane vesicles (separated after ultracentrifugation and used at 3× their normal protein concentration), on sperm motility and membrane status when added to washed and non-washed ram spermatozoa after they had been frozen and thawed. We further tested the *in vivo* fertility of frozen–thawed ram spermatozoa, treated with seminal plasma fractions post-thaw, deposited by cervical and laparoscopic insemination into the reproductive tracts of ewes in synchronized oestrus.

Materials and Methods

Chemicals and reagents

Tris, glucose, citric acid, glycerol, streptomycin, penicillin and chlortetracycline (CTC) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Experimental animals, housing and locations

Procedures described herein were approved by The University of Sydney's Animal Ethics Committee. Semen was collected from four, three and another three Merino rams for Expts 1, 2 and 3, respectively. The rams were maintained on a fully prepared chaff-based ration (oaten : lucerne chaff, 1 : 1) with supplementary lupin grain in an animal house at the Faculty of Veterinary Science, University of Sydney, Camperdown, NSW.

The fertility tests involved 132 and 346 mature Merino ewes for Expts 2 and 3, respectively. The ewes were maintained at The University of Sydney Arthurleigh Farm at Marulan, NSW, grazing on native pastures and supplemented with barley and/or lupin grain. The fertility tests were conducted during the breeding season.

Semen collection and freezing

For Expt 1, semen was collected from the four rams twice a week by artificial vagina. The ejaculates (six from each ram) were split equally into two parts. The first part was washed twice with Salamon's buffer [Tris (hydroxymethyl) aminomethane (300 mM), glucose (30 mM) and citric acid (monohydrate) (94.7 mM)] and

centrifuged each time ($600 \times g$, 30°C , 10 min) to concentrate the spermatozoa and eliminate seminal plasma. The concentrated spermatozoa were diluted (1 + 1; sperm pellet + diluent) with Salamon's diluent [(Tris (hydroxymethyl) aminomethane (300 mM), glucose (30 mM), citric acid (94.7 mM), egg yolk (15% v/v), glycerol (5% v/v), penicillin (100 000 IU) and streptomycin (73500 IU)] and frozen in pellet form before storage in liquid nitrogen (Evans and Maxwell 1987). The second part was diluted (1 + 1; semen + diluent) immediately after collection with Salamon's diluent and cryopreserved using the same method described for washed semen.

For Expts 2 and 3, semen was collected from three rams once a week by artificial vagina, and either (Expt 2) washed and frozen using the same method described in Expt 1 for washed semen, or (Expt 3) the raw ejaculates were frozen using the same method described in Expt 1 for non-washed semen. Flock fertility control ejaculates from the rams in Expt 2 were frozen as raw ejaculates using the same method described in Expt 1 for non-washed semen.

Fractionation of seminal plasma

Twenty to 25 ejaculates collected from each ram were used for seminal plasma separation. Semen was centrifuged ($2000 \times g$, 4°C) for 20 min on a Spintron GT-15FR centrifuge (Spintron Pty Ltd, Newport Beach, NSW, Sydney). Pellets of spermatozoa were discarded and supernatants were centrifuged again ($2500 \times g$, 4°C , 30 min) to eliminate any remaining spermatozoa and cell debris. Supernatants were measured using a 1 ml measuring disposable pipette (Interpath Services Pty. Ltd, Sydney, NSW, Australia) and stored in Eppendorf tubes in a (-20°C) freezer until use. For Expt 1, aliquots of $650 \mu\text{l}$ seminal plasma were centrifuged ($100\ 000 \times g$, 4°C , 80 min) on a Beckman ultracentrifuge (Beckman, Chatswood, Sydney, NSW, Australia) using special adaptors for such small volumes in a 40.1Ti swing head rotor. For Expts 2 and 3, 5 ml seminal plasma was centrifuged ($100\ 000 \times g$, 4°C , 80 min) on a Beckman ultracentrifuge in a 70Ti fixed rotor. Pellets were washed twice with Salamon's buffer and then resuspended to $650 \mu\text{l}$ (Expt 1) or 5 ml (Expts 2 and 3) with the same buffer. Protein concentrations were determined for whole seminal plasma and the two fractions using a BIO-RAD reagent for protein concentration (Bio-Rad, Regents Park, NSW, Australia) and they were re-frozen for storage in a (-20°C) freezer. The mean concentra-

tion of protein in the seminal plasma for all the rams is presented in Table 1.

Experiment 1

Addition of seminal plasma fractions to frozen-thawed semen

Frozen-thawed spermatozoa were placed in four tubes for resuspension with aliquots of control buffer, whole seminal plasma, supernatant and pellet of vesicles at three times their normal protein concentration. This treatment was included because it was found to have marginally improved both motility characteristics and *in vitro* fertility of frozen-thawed ram spermatozoa in our previous study (El-Hajj Ghaoui et al. 2007). The volume of each aliquot of whole seminal plasma and supernatant added to the tubes was adjusted to provide the same concentration of protein in each tube. This concentration was the equivalent of 20% whole seminal plasma in the final volume of resuspended frozen-thawed spermatozoa. For the pellet of vesicles, the volume added provided the equivalent of 60% ($3 \times 20\%$) whole seminal plasma in the final volume of resuspended frozen-thawed spermatozoa. The final volumes in each tube were then adjusted to $500 \mu\text{l}$ with Salamon's buffer (minimum volume for incubation of spermatozoa) to provide a concentration of 20 million spermatozoa per millilitre. This sperm concentration is the minimum for accurate assessment of sperm motility characteristics by computer-assisted sperm analysis (CASA). The tubes were incubated in a water bath at 37°C for 6 h.

Assessment of motility characteristics of spermatozoa

The motility of frozen-thawed spermatozoa was assessed subjectively (SUBJ) and by CASA at 0, 3 and 6 h after thawing and resuspension. Subjective assessment for forward progressive motility of spermatozoa was determined visually by placing $10 \mu\text{l}$ of semen on a clean, warm (37°C) slide held on a warm stage and under $200\times$ magnification as described by Evans and Maxwell (1987).

Spermatozoa were assessed by CASA (HTM-IVOS v. 12; Hamilton Thorn, Cummings Centre, Beverly, MA, USA) for total motility (TOT), progressive motility (PROG), average path velocity (VAP), average straight-line velocity (VSL), average track speed (VCL), amplitude of lateral head displacement (AHL), beat cross frequency (BCF), straightness (STR), linearity (LIN) and elongation (ELONG). Resuspended sperm samples

	Experiment 1	Experiment 2	Experiment 3
Sperm parameters			
Motility of spermatozoa (%)	90.0 ± 1.89	88.4 ± 0.60	88.7 ± 0.59
Concentration of spermatozoa ($10^6/\text{ml}$)	3299 ± 287	2657 ± 124	3161 ± 203
Protein concentration			
Whole seminal plasma ^a	24.4 ± 7.46	21.6 ± 3.74	15.9 ± 1.21
Supernatant ^a	23.8 ± 7.55	21.3 ± 3.69	15.3 ± 8.98
Vesicles ^a	7.9 ± 3.15	3.6 ± 2.00	5.0 ± 1.70

Table 1. Ejaculate characteristics for the semen collected and used in the three experiments

^aMean of total protein concentration in frozen-thawed seminal plasma and the two fractions supernatant and vesicles measured in mg/ml.

Values shown are mean \pm SEM pooled for rams and ejaculates within rams.

(5.5 μ l) were placed on slides (Cell Vu, Millennium Sciences Corp., New York, USA; pre-warmed to 37°C) and enclosed using a 22 \times 22 mm coverslip before immediate transfer to the CASA. Motility characteristics were determined by assessment of at least three randomly selected microscopic fields (>300 spermatozoa per sample) utilizing factory CASA settings (ram) at an image sampling frequency of 60 Hz.

Assessment of membrane status of spermatozoa

The membrane status of frozen-thawed spermatozoa was also assessed at 0, 3 and 6 h after thawing and resuspension, using the chlortetracycline (CTC) staining method as described by Gillan et al. (1997). Briefly, 45 μ l of resuspended spermatozoa was added to 45 μ l of CTC solution and mixed well. Ten microlitres of glutaraldehyde solution was added with mixing and centrifuged for 30 s to precipitate spermatozoa. A 5- μ l drop of DABCO was placed on a microscope slide and mixed with 5 μ l of the fixed spermatozoa to retard fading of fluorescence. A cover slip was placed on the suspension, excess fluid removed by compression and the edges of the cover slip sealed with colorless nail varnish. Two hundred spermatozoa were classified according to CTC patterns using the nomenclature described by Gillan et al. (1997). The three patterns were: F (characteristic of uncapacitated, acrosome-intact cells), B (characteristic of capacitated, acrosome-intact cells) and AR (characteristic of capacitated, acrosome-reacted cells).

Expts 2 and 3 (*in vivo* fertilization)

Experimental design

Experiment 2 included 3 rams \times 2 treatments (thawed spermatozoa resuspended in buffer or supernatant) with 22 ewes per treatment per ram (N = 132 ewes). All experimental ewes were inseminated cervically but a flock fertility control group of ewes was also inseminated by laparoscopy with 50×10^6 spermatozoa (N = 70 ewes).

Experiment 3 included 3 rams \times 2 treatments (thawed spermatozoa resuspended in buffer or supernatant) for cervical insemination; and 3 rams \times 3 treatments (thawed spermatozoa resuspended in buffer, supernatant or vesicles at 3 \times their normal concentration for intrauterine insemination by laparoscopy). For cervical insemination, one insemination dose (200×10^6 total spermatozoa) was used and 20–23 ewes were randomly allocated to each treatment/ram (N = 127 ewes). Three insemination doses were used for laparoscopic insemination (20, 40 or 60×10^6 total spermatozoa), so eight to nine ewes were allocated to each dose per treatment per ram (N = 218 ewes).

Preparation of ewes

The time of oestrus was controlled in mature Merino ewes by intra-vaginal progestagen-impregnated sponges (40 mg Chronogest; Bioniche Australasia, Armidale, Australia) inserted for 12 days. At sponge removal, each ewe received an intramuscular injection of 400 IU

pregnant mare serum gonadotrophin (PMSG, Pregnecol; Bioniche Australasia, Armidale, Australia) and androgenized wethers were introduced at a rate of three per 100 ewes. Each wether was administered 1 ml of Durateston i.m. (Intervet Australia, Bendigo, Australia) just before joining with the ewes.

Semen preparation

Washed frozen semen from three rams was used in Expt 2 and non-washed frozen semen from three other rams was used in Expt 3. Semen pellets were thawed quickly in dry test tubes (1 pellet per tube for 1 min) in a 37°C water bath and incubated with Salamon's buffer as control or with the supernatant for Expt 2; and with Salamon's buffer as control or with the supernatant or the pellet of vesicles at three times their normal concentration for Expt 3. All the tubes were prepared at the time of insemination, adjusted to the same protein concentration as for Expt 1 and held at 30°C in preparation for insemination (15 min). The motility of spermatozoa was assessed subjectively for each treatment before insemination. Samples containing 50–70% total motile spermatozoa were used in the two experiments.

Insemination

The field experiments were conducted in April 2005 using cervical insemination (Expt 2) and January 2006 using cervical and intrauterine artificial insemination (Expt 3). All ewes were made to fast for 24 h prior to insemination. For Expt 2, cervical insemination with the aid of a headlamp and speculum (Evans and Maxwell 1987) was carried out 57 h after withdrawal of progestagen sponges. Each ewe received a 200 μ l inseminate dose containing 200×10^6 total frozen-thawed and resuspended spermatozoa by means of a graduated insemination pipette.

In Expt 3, ewes were inseminated either cervically or by laparoscopy. For cervical insemination, the ewes received two inseminations, each of 100 μ l containing 100×10^6 total frozen-thawed and resuspended spermatozoa, the first at 54 h and the second at 60 h after sponge removal. Intrauterine inseminations were performed by standard laparoscopy procedures (Evans and Maxwell 1987) 15 min after i.m. sedation (1 ml Ketamine; Ilium, Troy Laboratories, Smithfield, NSW, Australia; 2 ml diazepam; Pamlin, Parnell Laboratories, Alexandria, NSW, Australia) and local anaesthetic (4 ml lignocaine, Troy Laboratories). Ewes were inseminated by laparoscopy once at 57 h after sponge removal with 20, 40, or 60×10^6 total spermatozoa.

Pregnancy diagnosis

For Expts 2 and 3, pregnancy was assessed on day 54 of gestation in all inseminated ewes using real-time cutaneous ultrasound for detection of presence of foetuses and their number. For Expt 2, pregnancy status was also initially determined on day 18 post-insemination by analysis of progesterone concentration (Spectra; Orion Diagnostics, Helsinki, Finland) following collection of

jugular blood samples. All samples were assayed in a single batch and the intra-assay CV was < 10%. Ewes with progesterone concentrations above 1.2 ng/ml were considered pregnant (Robertson and Sarda 1971). Pregnancy rate was calculated as the number of ewes pregnant on day 18 or day 54/number of ewes inseminated $\times 100$.

Statistical analysis

Expt 1: ejaculate characteristics

A one-way ANOVA was performed to compare the motility of spermatozoa and their concentration in the ejaculates used in each of the three experiments as well as the protein concentration of the whole seminal plasma and the two fractions (supernatant and membrane vesicles). Mean values of these data are presented in Table 1.

Expt 1: motility characteristics of spermatozoa

Motility characteristics of spermatozoa were analyzed to compare the four treatments over the three post-thaw incubation times (0, 3 and 6 h) as well as washed and unwashed semen samples using a REML procedure in GenStat (Release 8; Ceanet, Brisbane, Australia).

The model fitted to each of the sperm motility measures was

$$Y_{ijk} = \mu + \text{Tube}_i + \text{Time}_j + \text{Semen}_k + (\text{Tube} \cdot \text{Time})_{ij} \\ + (\text{Tube} \cdot \text{Semen})_{ik} + (\text{Time} \cdot \text{Semen})_{jk} + \text{Ram}_l \\ + (\text{Ram} \cdot \text{Semen})_{kl} + (\text{Ram} \cdot \text{Semen} \cdot \text{Tube})_{ikl} + \varepsilon_{ijkl},$$

where Y is the measure being modelled (e.g. Subjective), Tube is the particular treatment, and Semen refers to 'washed' vs 'non-washed'. Note that 'Tube', 'Time' and 'Semen' have been designated fixed effects, while Ram, Ram·Semen and Ram·Semen·Tube are random effects, i.e.

$$\text{Ram}_l \sim N(0, \sigma^2), (\text{Ram} \cdot \text{Semen})_{kl} \sim N(0, \sigma_{\text{Ram.Semen}}^2),$$

and

$$(\text{Ram} \cdot \text{Semen} \cdot \text{Tube})_{ikl} \sim N(0, \sigma_{\text{Ram.Semen.Tube}}^2)$$

with the residual random errors, $\varepsilon_{ijkl} \sim N(0, \sigma_e^2)$, incorporating the serial correlation structure as needed. The random effect terms, Ram_l , $(\text{Ram} \cdot \text{Semen})_{kl}$, and $(\text{Ram} \cdot \text{Semen} \cdot \text{Tube})_{ikl}$, correspond to the partitioning up of the semen samples from each of the four rams. In addition, a three-way interaction term, $(\text{Tube} \cdot \text{Time} \cdot \text{Semen})_{ijk}$, was also added to each model, but was found to be non-significant in all cases (all $p > 0.5$), so was subsequently discarded.

Expt 1: Membrane status of spermatozoa assessed by CTC staining

For the membrane status data (CTC type = F, B or AR), there were relatively few type F cells and consequently this category was discarded from the analysis. As only two outcome categories were involved, (binary)

logistic regression was used to model the probability of type B, as opposed to type AR. A generalized linear mixed model (GLMM) was fitted to the data of the form

$$\log\left(\frac{\pi_{ijkl}}{1 - \pi_{ijkl}}\right) = \text{const} + \text{Tube}_i + \text{Time}_j + \text{Semen}_k \\ + (\text{Tube} \cdot \text{Time})_{ij} + (\text{Tube} \cdot \text{Semen})_{ik} \\ + (\text{Time} \cdot \text{Semen})_{jk} + \text{Ram}_l \\ + (\text{Ram} \cdot \text{Semen})_{kl} + (\text{Ram} \cdot \text{Semen} \cdot \text{Tube})_{ikl}$$

where π_{ijkl} is the probability of a type B spermatozoon, with fixed effects and random effects terms as specified in the previous model. A three-factor interaction term, $(\text{Tube} \cdot \text{Time} \cdot \text{Semen})_{ijk}$, was also tested, but was found to be non-significant ($p = 0.6$) and subsequently discarded.

Experiment 2

As a result of the very low fertility rate obtained in this experiment, a loglinear model (Poisson regression) was used to assess the effect of the treatment (control vs supernatant) on the pregnancy rate as well as the number of foetuses (prolificacy). Because of the overriding importance of the peripheral plasma progesterone concentration on day 18 post-insemination as a predictor of fertility, which would otherwise obscure any treatment effect, progesterone was also included in the model. These models took the form

$$\log_e\left(\frac{\pi_{ijk}}{1 - \pi_{ijk}}\right) = \text{constant} + \text{Treatment}_i \\ + \beta \times \log_e(\text{Progesterone}_j) + \text{Ram}_k$$

for pregnancy rate, where π_{ijk} is the probability of a successful fertilization, and

$$\log_e \mu_{ijk} = \text{constant} + \text{Treatment}_i \\ + \beta \times \log_e(\text{Progesterone}_j) + \text{Ram}_k$$

for prolificacy, where μ_{ijk} is the mean number of foetuses for the Treatment and progesterone concentration.

Experiment 3

For the analysis of the fertility data (pregnancy success), a logistic regression GLMM was fitted to the data. Because of the different sperm concentrations and range of treatments for the two insemination methods, it was specified that the Treatment, Dose and Treatment \times Dose effects were nested within the Insemination method. The full model fitted was

$$\log_e\left(\frac{\pi_{ijkl}}{1 - \pi_{ijkl}}\right) = \text{constant} + \text{Insem}_i \\ + \text{Insem} \cdot \text{Treatment}_{ij} + \text{Insem} \cdot \text{Dose}_{ik} \\ + \text{Insem} \cdot \text{Treatment} \cdot \text{Dose}_{ijk} + \text{Ram}_l$$

where π_{ijkl} is the probability of a successful fertilization, and where Ram was specified as a random effect. The number of foetuses observed per insemination (prolificacy) was analysed using a similar GLMM, namely

$$\log_e \mu_{ijkl} = \text{constant} + \text{Insem}_i + \text{Insem} \cdot \text{Treatment}_{ij} \\ + \text{Insem} \cdot \text{Dose}_{ik} + \text{Insem} \cdot \text{Treatment} \cdot \text{Dose}_{ijk} \\ + \text{Ram}_l,$$

where μ_{ijkl} is the mean number of foetuses per ewe insemination.

Results

Ejaculate characteristics, Expts 1, 2 and 3

Data on semen characteristics and protein concentration for the three seminal plasma fractions (whole, supernatant and pellet of vesicles) from the ejaculates used in the three experiments, pooled for rams, are presented in Table 1. There was no difference in the motility of spermatozoa between rams within experiment ($p = 0.10, 0.78$ and 0.75 for Expt 1, 2 and 3, respectively). The concentration of spermatozoa was significantly different between rams for Expt 1 ($p = 0.011$) and 3 ($p < 0.001$) but not for Expt 2 ($p = 0.34$). The mean protein concentration for whole seminal plasma and for supernatant was significantly higher than for the pellet of vesicles in the samples used in the three experiments ($p < 0.001, p = 0.004$ and $p < 0.001$ respectively) but no significant difference was detected between the former two fractions ($p > 0.5$).

Experiment 1

Motility characteristics of spermatozoa

The effects of control buffer, whole seminal plasma, supernatant and pellet of vesicles in the post-thaw resuspension medium on the motility characteristics of washed (seminal plasma-free) and non-washed frozen-thawed ram spermatozoa are presented in Fig. 1. Almost universally, whole seminal plasma and supernatant means were significantly higher than the control means for all measures at 3 and 6 h post-thaw incubation (mostly $p < 0.05$), but the three-times vesicle treatment mean values were not significantly different from the control mean values (mostly $p > 0.05$). However, immediately post-thaw (0 h), almost none of the treatment mean values differed significantly for any of the motility characteristics (mostly $p > 0.05$).

For most motility characteristics of spermatozoa, values were higher for non-washed than for washed spermatozoa (Table 2), although the only measures for which the difference reached (marginal) statistical significance were SUBJ ($p = 0.032$) and VSL ($p = 0.072$).

Membrane status of spermatozoa

Overall, 1.0%, 46.8% and 52.2% of spermatozoa displayed the F, B and AR CTC patterns, respectively. There were no detectable treatment effects for F pattern spermatozoa at any time, largely because these occurred at a low frequency, and the response for AR pattern spermatozoa was the complement of that for the B pattern spermatozoa, so only the results for spermatozoa exhibiting the B CTC pattern are presented in Fig. 2. A higher proportion of spermatozoa exhibited

the B pattern, and a lower proportion the AR pattern, when treated with whole seminal plasma, supernatant and pellets of vesicles than for those resuspended in the control buffer alone ($p < 0.05$) at all times of incubation. There was no difference between whole seminal plasma, supernatant or vesicles at any time (all $p > 0.05$).

There was a significant interaction between semen treatment and time ($p = 0.003$) because of a slightly higher proportion of pattern B spermatozoa in the non-washed (74%) than washed (65%) samples at 0 h, but this differential was not present at 3 h (52% and 53% for washed and non-washed, respectively) or 6 h (36% for both washed and non-washed) post-thawing. Overall, the proportion of type B declined and the proportion of AR-pattern spermatozoa increased over time ($p < 0.001$).

Experiment 2

In this experiment, the ewes were cervically inseminated and two treatments, control buffer and supernatant, were applied to the washed frozen-thawed spermatozoa before insemination. Unfortunately, a very low fertility rate (overall 5% of ewes were pregnant) was obtained with only three of 66 and two of 66 ewes pregnant after AI with spermatozoa incubated with control buffer and supernatant, respectively. Of the 70 flock fertility control ewes inseminated by laparoscopy with 50×10^6 frozen-thawed spermatozoa, 44 (62.9%) and 38 (54.3%; with 38 foetuses) were pregnant at days 18 and 54, respectively.

Nevertheless, there was an indication that the fertility rates were higher for the supernatant treatment than the control ($p = 0.09$) after adjusting for progesterone levels (0.63 vs 0.65 ng/ml for control vs supernatant, $p = 0.76$), based on the number of foetuses observed, although no effect was detectable when analysing pregnancy rate ($p = 0.63$). As expected, progesterone was highly predictive of the number of foetuses and pregnancy rate (both $p < 0.001$), despite the small number observed (four and three foetuses for control and supernatant, respectively). Mean progesterone levels were 6.5 ng/ml (range 4.5–10.6) where foetuses were present, and 0.5 ng/ml (range 0.3–1.2) when absent.

Experiment 3

Data on pregnancy at day 54 post-insemination are presented in Table 3. In this experiment, cervical and intrauterine insemination (three sperm doses) were compared, and control buffer, supernatant or pellet of vesicles fractions of seminal plasma were incubated with the non-washed frozen-thawed spermatozoa before insemination.

The pregnancy rate (percentage of inseminations resulting in a pregnancy) was not influenced by the treatment ($p = 0.44$) nor by sperm dose ($p = 0.61$). However, the pregnancy rate after intrauterine insemination by laparoscopy was higher than for cervical insemination ($p < 0.001$, model-based estimates were 44.9% and 12.3% pregnancy for laparoscopic and

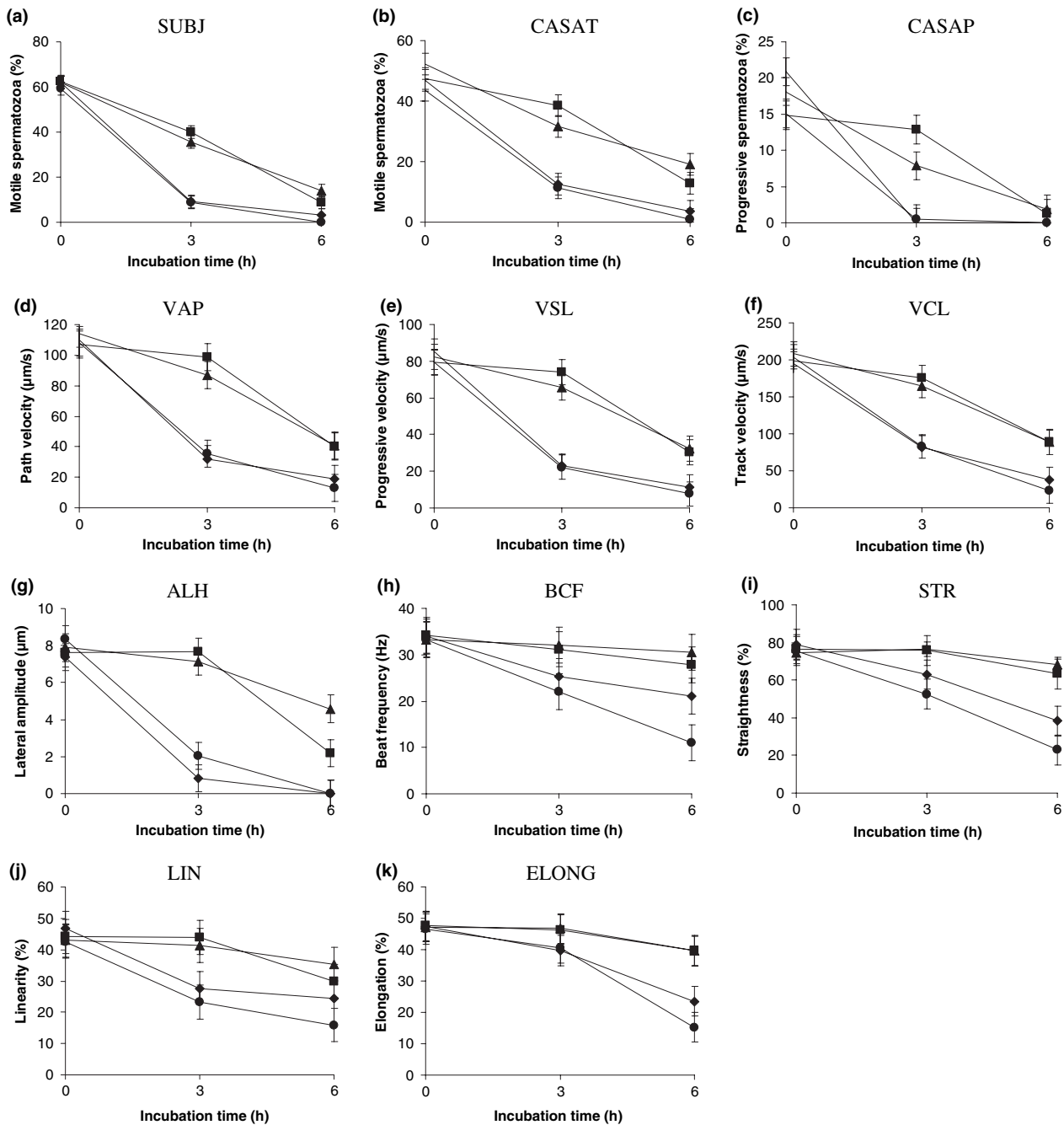


Fig. 1. Motility characteristics of frozen-thawed ram spermatozoa assessed subjectively or by CASA for washed and non-washed semen after incubation with control buffer (●), whole seminal plasma (■), supernatant (▲) and membrane vesicles at 3× their concentration (◆). Data are mean ± SEM for each post-thaw incubation assessment time (0, 3 and 6 h) and were pooled within ram for washed and non-washed semen

cervical insemination, respectively). Similar findings were obtained for prolificacy (number of foetuses). Namely, there was no detectable effect of treatment ($p = 0.48$) nor sperm dose ($p = 0.17$), but laparoscopy resulted in more foetuses per insemination (0.67) than cervical insemination (0.18) ($p < 0.001$).

Discussion

In our previous study (El-Hajj Ghaoui et al. 2007) we demonstrated that whole seminal plasma and its supernatant fraction after ultracentrifugation displayed sim-

ilar protein profiles, but different from the pellet of vesicles fraction, as assessed by one-dimensional gel electrophoresis. Autologous whole seminal plasma or supernatant, when included in the post-thaw buffer, improved the motility characteristics and membrane integrity of washed frozen-thawed ram spermatozoa, and increased their ability to fertilize *in vitro*-matured oocytes, compared with the pellet of vesicles at its normal protein concentration or control buffer without the seminal plasma fractions. Vesicles at three times their normal protein concentration marginally improved both motility characteristics and *in vitro* fertility,

Table 2. Difference of means and *P*-values of spermatozoa characteristics for washed versus non-washed semen. The difference is expressed as the overall mean for non-washed minus the overall mean for washed semen

Spermatozoa characteristic	Difference of mean values (non-washed – washed)	<i>p</i> -value
SUBJ	3.35	0.032
CASAT	2.52	0.235
CASAP	1.604	0.134
VAP	4.81	0.197
VSL	5.33	0.072
VCL	7.1	0.350
ALH	0.281	0.409
BCF	-1.13	0.597
STR	5.27	0.250
LIN	3.29	0.292
ELONG	1.86	0.477

possibly due to contamination from supernatant proteins (El-Hajj Ghaoui et al. 2007).

In the first experiment of the present study we examined the motility characteristics and membrane status

of washed and non-washed frozen–thawed ram spermatozoa during 6 h of incubation in control buffer and buffer containing autologous whole seminal plasma and its two fractions: supernatant and pellet of membrane vesicles, the latter at three times the normal protein concentration. Seminal plasma and its fractions were added to spermatozoa at a protein concentration calculated to provide the equivalent of 20% whole seminal plasma (60% in the case of pellet of vesicles) in the same volume of the resuspension medium, based on the mean protein concentration of the seminal plasma of all rams, as for our previous study (El-Hajj Ghaoui et al. 2007). Non-washed semen was included in this study to see if the elimination of the pre-freezing centrifugation step would improve the post-thaw quality of spermatozoa. This was confirmed in Expt 1, as nearly all motility characteristics and the proportion of B-compared with AR-pattern spermatozoa, were improved for non-washed compared with washed semen. By washing the semen we eliminated the majority of seminal plasma before adding the extender and freezing the spermatozoa. This had a negative effect on motility

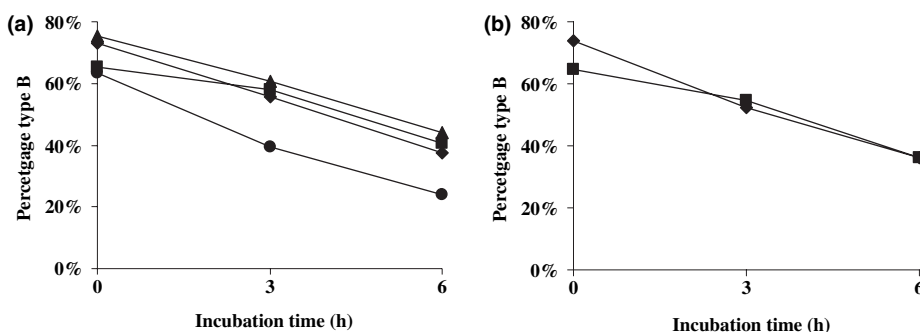


Fig. 2. (a) Percentages of spermatozoa with chlortetracycline pattern B after incubation with control buffer (◆), whole seminal plasma (■), supernatant (▲) and membrane vesicles at 3× normal concentration (●) [data pooled for washed and non-washed spermatozoa]. (b) Percentages of spermatozoa with chlortetracycline pattern B during incubation of washed (◆) or non-washed spermatozoa (■) [data pooled for post-thaw resuspension treatments]. The response for AR-patterned spermatozoa is the complement of that for the B patterned spermatozoa. Data are means ± SEM for each post-thaw incubation assessment time (0, 3 and 6 h) and were pooled within ram

Table 3. Pregnancy and number of foetuses observed on day 54 after cervical or laparoscopic intra-uterine insemination of ewes in synchronized oestrus with non-washed frozen–thawed semen.

Insemination	Treatment ^a	Sperm dose (×10 ⁶)	No. ewes inseminated	No. ewes pregnant (%) ^b	No. of foetuses (%)	
Cervical	Control	200	57	6 (10.5)	8 (14.0)	
	Supernatant	200	61	9 (14.8)	14 (23.0)	
	Total		118	15 (12.7)	22 (18.6)	
Laparoscopy	Control	20	23	14 (60.9)	22 (95.7)	
		40	24	9 (37.5)	10 (41.7)	
		60	24	14 (58.3)	23 (95.8)	
	Total		71	37 (52.1)	55 (78.6)	
		Supernatant	20	24	8 (33.3)	13 (54.2)
			40	23	10 (43.5)	13 (56.5)
	60		23	11 (47.8)	17 (73.9)	
	Total		70	29 (41.4)	43 (61.4)	
		Vesicles	20	24	8 (33.3)	13 (54.2)
			40	25	12 (48.0)	17 (68.0)
	60		23	10 (43.5)	18 (78.3)	
	Total		72	30 (41.7)	47 (65.3)	

^aFrozen–thawed spermatozoa were resuspended with control buffer, or whole seminal plasma, supernatant or pellet of vesicles. The volume of the latter three fractions was adjusted to provide the same concentration of protein, equivalent of 20% whole seminal plasma in the final volume of resuspended spermatozoa. The volumes were then adjusted to 500 μl with buffer (20 million spermatozoa per ml) and incubated at 30°C in preparation for 15 min before insemination.

^bDetermined by real-time cutaneous ultrasound.

characteristics and membrane status, and non-washed spermatozoa also responded better than washed spermatozoa to the post-thaw addition of seminal plasma or supernatant. These differences were probably because of reduced stress from the washing and centrifugation, together with the retention of beneficial seminal plasma proteins, adhering to the sperm membrane during processing, freezing and thawing in the case of non-washed semen.

Whole seminal plasma and supernatant improved the motility of spermatozoa after 3 and 6 h of post-thaw incubation compared with the control buffer (Expt 1). However, membrane vesicles had no effect on motility characteristics, even though included at a higher concentration than in the seminal plasma. Resuspension and incubation with membrane vesicles, whole seminal plasma or supernatant lowered the proportion of AR-pattern frozen-thawed spermatozoa (characteristic of acrosome-reacted cells) after 3 and 6 h incubation compared with the control buffer, suggesting that 'decapacitation factors' in the seminal plasma may have delayed the progression of sperm membranes to the acrosome reacted state, maintaining more capacitated (B-pattern) cells over time available for fertilization. The benefits observed for the concentrated vesicle fraction in this case may have been due to contamination from beneficial seminal plasma proteins or to a direct effect of the vesicle proteins.

In the first field fertility test (Expt 2), cervical insemination with washed frozen-thawed semen resulted in low fertility after spermatozoa were incubated with either supernatant or control buffer, so it was difficult to detect a strong treatment effect. Nevertheless, there was an indication of improved fertility from the supernatant compared with the control group, as detected by the model including plasma progesterone concentration in ewes at day 18 after insemination, and the increased number of foetuses in ewes inseminated with supernatant-treated spermatozoa.

Freezing and thawing of ram semen is known to cause damage to a significant proportion of the spermatozoa (Salamon and Maxwell 1995) and the cells that survive show alterations, particularly in the plasma membrane. The occurrence of capacitation-like changes in frozen-thawed spermatozoa when compared with their initial status after ejaculation (Watson 1995) results in a sperm population with a shorter fertilizing lifespan (Gillan et al. 1997). However, these changes may be ameliorated *in vivo* and *in vitro* when the semen is incubated with the seminal plasma or its fractions (Maxwell et al. 1999; El-Hajj Ghaoui et al. 2007). There was an indication of a beneficial effect from incubating frozen-thawed spermatozoa with the 'decapacitating' proteins in whole seminal plasma or its supernatant fraction but the fertility after cervical insemination was very low (5% of 132 ewes) compared with the flock fertility control group inseminated by laparoscopy (62.9% of 70 ewes), and this was not improved by seminal plasma fractions. While fertility in the flock control group had decreased to 54.3% by day 54 of pregnancy, the early embryonic loss of 13.6% (6/44) was well within the normal range for Merino sheep (Edey 1976). Fertility after cervical insemination of synchronized Merino ewes with fro-

zen-thawed semen is low and highly variable under Australian conditions, and the results obtained in the present study are at the lower end of the reported range (17.8–57.5% in Australia, 3.7–47.2% in New Zealand; Salamon and Maxwell 1995). It was concluded that the low fertility obtained in Expt 1 was most probably caused by the stress of centrifugation and washing spermatozoa as part of the pre-freeze processing procedure, exacerbated by the insemination of these compromised spermatozoa, with a reduced fertilizable lifespan, at a single fixed time.

For these reasons, and based on the improved viability of non-washed compared with washed spermatozoa in Expt 1, another fertility test (Expt 3) was conducted in which the washing procedure was eliminated and only non-washed frozen-thawed semen was used for both cervical and intrauterine insemination. Furthermore, the cervical insemination dose (200×10^6 spermatozoa) was split between two insemination times, in an attempt to improve the chances of viable spermatozoa being present in the oviduct at the time of ovulation. Intrauterine insemination, using three inseminate doses of spermatozoa, was used to increase overall fertility and determine whether seminal plasma fractions would have any effect on fertility after placement of the spermatozoa closer to the site of fertilization. The inseminate doses were designed to be below (20×10^6 spermatozoa), equivalent to (40×10^6 spermatozoa) and higher than (60×10^6 spermatozoa) the recommended dose for commercial laparoscopic insemination of ewes (Evans and Maxwell 1987).

Unfortunately, neither treatment with seminal plasma fractions nor dose of spermatozoa influenced pregnancy rates of ewes after cervical or intrauterine insemination (Table 3), despite the relatively high proportion of spermatozoa remaining motile after thawing (50–70%). The overall pregnancy rate (45% of 213 ewes) obtained after laparoscopic insemination was lower than that normally achieved by commercial artificial insemination programmes in Australia (72%; Hill et al. 1998) and the pregnancy rate after cervical insemination (12.7% of 118 ewes) was still low compared with rates of 20–45% reported using similar sperm concentrations in recent reports from Europe (Gil et al. 2003; O'Meara et al. 2005) but was at the lower end of the reported range of results reported for Australian conditions (Salamon and Maxwell 1995). It is possible that the long-term drought conditions experienced during the conduct of the fertility tests may have contributed to these results, although ewes were kept in good body condition throughout by supplementary feeding.

Other studies have used incubated frozen-thawed ram semen for cervical or intrauterine insemination (Maxwell et al. 1993; Sanchez-Partida et al. 1999; Gil et al. 2002) and fertility after cervical insemination has been improved by addition of seminal plasma to frozen-thawed spermatozoa immediately before insemination (Maxwell et al. 1999). In the present study, the spermatozoa were incubated with the seminal plasma fractions for up to 15 min before insemination but this failed to improve fertility results. Moreover, the effect of seminal plasma *in vivo* was not consistent with the benefits demonstrated *in vitro* (Expt 1). It has been demonstrated

that the proteins present in seminal plasma have a beneficial effect on the motility, membrane integrity (this study) and *in vitro* fertility of spermatozoa (El-Hajj Ghaoui et al. 2007). However, under the conditions of the present study these improvements were not reflected in *in vivo* fertility differences after cervical or laparoscopic insemination.

In conclusion, autologous whole seminal plasma and its fractions, supernatant and membrane vesicles, added to the post-thaw incubation medium of non-washed frozen-thawed ram spermatozoa improved their motility characteristics and membrane status but had no effect on *in vivo* fertilization after cervical or intrauterine artificial insemination. Further work is needed to determine and purify the components of ram seminal plasma, especially proteins, and investigate their beneficial effects if any on *in vivo* fertilization, with the aim of improving fertility after cervical insemination of ewes with frozen-thawed semen.

Acknowledgements

The authors thank Andrew Souter for semen collection and technical assistance, and Simon de Graff for the preparation and synchronization of the ewes for the field trials. Mr Steven Burgun and the staff at Arthursleigh are thanked for their assistance during field experiments.

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Submitted: 05.09.2006

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