

Fertility of ram spermatozoa pellet-frozen in zwitterion-buffered diluents

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Summary — The fertility (positive ultrasound scanning 60–75 days after insemination) of ram spermatozoa frozen in Tris- or zwitterion/glucose/egg yolk/glycerol-based diluents was examined during the natural breeding season (autumn). In *Experiment 1* Tris-citrate, high glucose and Hepes-NaOH diluents were used and ewes ($n = 266$) were inseminated (induced oestrus) with 20 (uterus) or 180 (cervix) million motile spermatozoa. There was no difference in fertility (range: 10.9–71.1%) between diluents, despite the superior post-thawing motility and progressive velocity of spermatozoa in Hepes-NaOH compared with the other diluents ($P < 0.001$). In *Experiment 2*, there was no difference in fertility (range: 4.1–50.0%) between Tris-citrate and Hepes-NaOH diluents after insemination (induced oestrus) of ewes ($n = 290$) with 10 (uterus) or 100 (cervix) million motile spermatozoa, respectively. In *Experiment 3* Tris-citrate, Hepes-NaOH and Pipes-NaOH diluents were used and ewes ($n = 492$) were inseminated (natural oestrus) with 8 or 24 (uterus), or 80 or 240 (cervix) million motile spermatozoa. There were no differences in fertility (range: 35.4–52.9%) between insemination doses or diluents despite the superior motility and acrosome integrity in the zwitterion compared with the Tris-citrate diluents ($P < 0.001$). In all experiments, the fertility of frozen spermatozoa was higher for intrauterine than cervical insemination (51.9 vs 22.1%, $n = 1\ 048$; $P < 0.01$). It was concluded that post-thawing in vitro quality was superior and the fertility comparable after freeze-thawing spermatozoa in zwitterion compared with Tris-citrate diluents.

fertility / zwitterion diluent / frozen-thawed ram spermatozoa

Résumé — **Fertilité après congélation en pastilles de spermatozoïdes de bélier dans divers dilueurs tamponnés.** Nous avons mesuré le pouvoir fécondant des spermatozoïdes de bélier après congélation dans des dilueurs à base de tris, ou à base de jaune d'œuf-glycérol-tampon amphotère. Dans un premier essai, 271 brebis ont été inséminées (20 millions (utérus) ou 180 millions (cervix) de spermatozoïdes mobiles) avec du sperme congelé dans des milieux à base de Tris-citrate, de glucose ou d'Hépes-NaOH. En dépit d'une meilleure motilité et d'une plus grande vitesse de déplacement des spermatozoïdes après congélation dans la suspension d'Hépes-NaOH ($p < 0,001$), aucune différence de fertilité n'a été observée entre les trois dilueurs. Une deuxième comparaison (Tris-citrate

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versus Hépes-NaOH) réalisée sur 290 animaux avec 10 millions (utérus) ou 100 millions (cervix) de spermatozoïdes mobiles n'a pas non plus permis de dégager de différence de fertilité entre les deux milieux utilisés. Enfin, dans une troisième série d'essai ($n = 491$), nous avons comparé les solutions à base de Tris-citrate, d'Hépes-NaOH ou de Piper-NaOH en utilisant 8 et 24 (utérus) ou 80 ou 240 (cervix) millions de spermatozoïdes mobiles. Aucune différence de résultat n'a pu être mise en évidence entre les doses ou entre les dilueurs employés et ce malgré une motilité et une intégrité de l'acrosome des spermatozoïdes meilleures dans les milieux tampon-amphotère que dans le milieu Tris-citrate. Dans l'ensemble, les taux de fertilité sont plus élevés après insémination intra-utérine qu'après insémination cervicale ($p < 0,001$). Ils sont comparables pour les trois dilueurs mis à l'essai bien que la qualité *in vitro* des spermatozoïdes soit meilleure dans les solutions à base de tampon amphotère que dans celles contenant du tris et du citrate.

fertilité / zwitterion / sperme congelé-décongelé de bélier

INTRODUCTION

Zwitterion-buffered diluents have been developed that are superior to Tris-citrate-based diluents for freezing the semen of boars (Crabo et al, 1972), bulls (Graham et al, 1972; Parrish and Foote, 1980; Garcia and Graham, 1989), humans (Prins and Weidel, 1986; Weidel and Prins, 1987) and turkeys (Brown et al, 1972). Recently, we reported better post-thawing motility and acrosome integrity of ram spermatozoa frozen in Tes-, Hepes- and Pipes-based diluents titrated to pH 7.0 with NaOH or Tris than for those frozen in Tris-citrate diluents (Molinia et al, 1994a). Improvements in post-thawing sperm quality may enhance the sperm transport in the female tract and the eventual fertilisation.

The most unequivocal test of a diluent for freezing spermatozoa is a fertility trial (Evans, 1988). The purpose of this study was to determine whether the *in vitro* improvements in sperm quality after freezing in Hepes-NaOH or Pipes-NaOH compared with Tris-citrate-based diluents results in improved fertility *in vivo*. The fertility of ram spermatozoa frozen-thawed in these diluents and artificially inseminated into the cervix or uterus was determined in three experiments. Different insemination doses of motile frozen-thawed spermatozoa were used to see if a possible difference in fertility between the diluents tested in these

experiments could be attributed to the number of spermatozoa inseminated.

MATERIALS AND METHODS

Experimental animals and locations

Mature Merino rams of mixed ages were housed at Hay NSW (*Experiment 1*); Katanning, WA (*Experiment 2*) and Sydney, NSW (*Experiment 3*) and were maintained on dry, natural pasture or in animal houses. They were supplemented with lupin grains/pellets *ad libitum*. Inseminations were performed in autumn on mature Merino ewes at Hay, NSW (*Experiment 1*), Mt Barker, WA (*Experiment 2*) and Jugiong, NSW (*Experiment 3*). All ewes were maintained on dry, natural pasture and were supplemented with lupin grains *ad libitum*.

Semen collection, dilution and freezing

Semen was collected during autumn by artificial vagina. Only ejaculates with good wave motion were used (score 4 or 5 on a 0–5 scale; Evans and Maxwell, 1987). Aliquots of semen were pooled and diluted threefold at 30 °C. In all experiments, a standard Tris-citrate diluent (control) and Hepes-NaOH diluent were used. The former contained 360 mM Tris, 114 mM citric acid, 33 mM glucose, 18.0% egg yolk and 6.0% glycerol (Salamon and Visser, 1972) and the latter contained 236 mM Hepes titrated to pH 7.0 with 1 M NaOH and 13.5% egg yolk, which was centrifuged prior to the addition of 85 mM glucose

and 6.0% glycerol (Molinia et al, 1994a). In addition, a high glucose diluent (similar to Tris-citrate but containing 300 mM Tris, 95 mM citric acid and 30 mM glucose) was used in *Experiment 1* and a Pipes-NaOH diluent (similar to Hepes-NaOH but containing 119 mM Pipes titrated to pH 7.0 with 1 M NaOH) was used in *Experiment 3*. For each experiment several batches of semen were used, each batch was collected over a 30 min interval and consisted of pooled semen ejaculates from each of four rams. The diluted semen was cooled to 5 °C in 1.5–2 h and then frozen in pellet form on dry ice (0.2 mL/pellet; Evans and Maxwell, 1987). Frozen pellets were transferred into liquid nitrogen and stored for several weeks before thawing. The fresh semen used as the cervical insemination control in *Experiment 1* was diluted in Tris-citrate diluent (Salamon and Visser, 1972) so that the final concentration of the diluent components was the same as that for the frozen-thawed semen (240 mM Tris, 76 mM citric acid, 22 mM glucose, 12% egg yolk and 4% glycerol).

Synchronisation of oestrus

The ewes were treated with intravaginal progestagen sponges (Repromap, Upjohn, Rydalmere NSW) for 12–14 days (*Experiments 1 and 3*) or Chronogest sponges (Intervet, Australia Pty Ltd) for 13–15 days (*Experiment 2*). In *Experiments 1 and 2*, the ewes received an intramuscular injection of 400 and 350 iu Pregnant mare serum gonadotrophin (PMSG) (Pregnecol, Horizon Animal Reproduction, North Ryde, NSW), respectively, and were joined with 10% vasectomised rams at sponge removal (SR) to increase the degree of oestrus synchrony, but the ewes were not marked. In *Experiment 3*, PMSG was not administered at SR and the ewes were inseminated at the second oestrus after synchronisation. In this case, the ewes were run with testosterone-treated wethers (4%) fitted with Sire-sine[®] harnesses and marking crayons from day 15 after SR to identify oestrus females. Marked ewes were removed twice daily at 0700 and 1800 h and yarded separately prior to insemination.

Intrauterine and cervical insemination

Frozen-thawed semen (all experiments) and diluted fresh semen (*Experiment 1*) was held at 30 °C in a waterbath and used for insemination

within 30 min. Approximately equal numbers of ewes were inseminated per diluent and per batch for each of the inseminating methods. For intrauterine insemination, the semen was loaded into plastic or glass pipettes fitted with a 5 mm 24 gauge hypodermic needle, using a 1 mL syringe. The semen was deposited into both uterine horns by laparoscopy while the ewes were under local anaesthesia (Evans and Maxwell, 1987). In *Experiments 1 and 2*, the ewes were inseminated 50–60 or 59–63 h after SR, respectively, while in *Experiment 3*, the ewes were inseminated 8–12 h after detection of oestrus. For cervical insemination, semen was loaded into plastic single-shot disposable pipettes using a 1 mL syringe and deposited into the cervix with the aid of a speculum and headlamp (Evans and Maxwell, 1987). In *Experiments 1 and 2*, the ewes were inseminated 50–60 or 53–57 h after SR, respectively, while in *Experiment 3*, the ewes were inseminated twice, 8–12 and 20–24 h after detection of oestrus. Pregnancy was determined by ultrasound scanning 61–66 (*Experiment 3*), 65 (*Experiment 1*) or 75 days (*Experiment 2*) after insemination.

Assessment of post-thawing motility

Successful fertilisation of oocytes is dependent on the presence of viable spermatozoa in the female tract at the appropriate time, which in turn depends on the viable life of spermatozoa. Post-thawing motility of spermatozoa during incubation was used in all experiments as an estimate of the viable life of the spermatozoa. Pellets from *Experiments 1 and 3* were thawed in dry 10 mL test-tubes in a waterbath at 37 °C for 5 min then diluted 20-fold with Dulbecco's phosphate-buffered saline (PBS). In *Experiment 1*, the percentage progressive motility and progressive velocity were objectively assessed using a Hamilton Thorn Motility Analyser (HT-M2000 Version 7.2, Daintree Industries, Victoria) after 0, 3 and 6 h incubation at 37 °C as described previously (Molinia et al, 1994b). In *Experiment 3*, the percentage motile spermatozoa was subjectively assessed under a coverslip on a warm stage (37 °C) using phase contrast microscopy (100 x) after 1, 2, 4 and 6 h incubation at 37 °C. Three (*Experiment 1*) or four (*Experiment 3*) separate assessments (replicates) of pellets from each batch of diluents were made. Samples were coded and presented by an assistant to the assessor in random order, so that the

assessor did not know the identity of the individual samples.

Assessment of acrosome integrity

Changes in the spermatozoa acrosome play an important role in the fertilisation process. Spermatozoa require an intact (undamaged) acrosome before they can participate in this process (Evans and Maxwell, 1987). Thus, frozen-thawed spermatozoa were assessed for acrosome integrity after 0, 3 and 6 h incubation at 37 °C in *Experiment 1*, or within 30 min of thawing and dilution in *Experiment 3*. A drop of diluted semen was smeared on a slide and allowed to air dry. Smears were stained as for rabbit spermatozoa using a modified staining procedure (in naphthol yellow S and erythrosin B for 14 min; Bryan and Akruk, 1977). Slides were examined by phase contrast microscopy (1 000 x) using an oil immersion objective and white light. Three (*Experiment 1*) or 4 (*Experiment 3*) separate assessments (replicates) of pellets from each batch of diluents were made. Spermatozoa ($n = 200$ per slide) were assessed for the percentage of intact acrosomes.

Experiment 1

In this experiment 266 ewes were inseminated in the uterus or cervix with 20 or 180 million motile frozen-thawed spermatozoa per ewe, respectively. Tris-citrate, high glucose and Hepes-NaOH diluents were used. Three batches of frozen semen pellets of each of the three diluents were made prior to the experiment. As a cervical insemination control, an additional 50 ewes were inseminated in the cervix with fresh spermatozoa. The fresh semen was collected within 30 min prior to insemination and diluted with Tris-citrate diluent (as described above) to a dose of 180 million motile spermatozoa in 0.2 mL.

Experiment 2

In this experiment 290 ewes were inseminated in the uterus or cervix with 10 or 100 million motile frozen-thawed spermatozoa per ewe, respectively. Tris-citrate and Hepes-NaOH diluents were used and seven batches of frozen semen pellets of

each of the two diluents were made prior to the experiment.

Experiment 3

In this experiment 492 ewes were inseminated with frozen-thawed spermatozoa in the uterus (8 or 24 million motile per ewe) or cervix (80 or 240 million motile per ewe). Tris-citrate, Hepes-NaOH and Pipes-NaOH diluents were used. Ten batches of frozen semen pellets of each of the three diluents were made prior to the experiment.

Statistics

Motility and acrosome integrity data for *Experiments 1* and *3* were subjected to angular transformation and analysis of variance for a split-plot design, with the different diluents as the main plots and post-thawing incubation time as the subplot. All analyses were performed using GENSTAT V (Mark 4.03, 1980, Lawes Agricultural Trust, Rothamsted Experimental Station, UK) and $P < 0.05$ was considered to be statistically significant.

Motility results are presented as means over the three or four observation times \pm pooled standard error of means (sem) calculated from the standard error of differences of means in the ANOVA program. When there were interactions involving incubation time, data were converted to the percentage loss of motile spermatozoa during incubation. (Percentage loss of motile spermatozoa during incubation = (% of motile sperm at 0 or 1 h (*Experiments 1* or *3*) minus % of motile sperm at 6 h \times 100%) / % of motile sperm at 1 h.) Only interactions showing differences in the percentage loss of motile spermatozoa during incubation are presented. Acrosome integrity results are presented as means of readings conducted at one or three observation times \pm pooled standard error of means (sem) calculated from the standard error of differences of means in the ANOVA program.

Significant differences between individual treatment means were determined by the least significant difference procedure where appropriate. Where there were significant interactions, the same error mean square term was used to test the relevant main effects as these were fixed

components and no random components were involved in the interactions.

Fertility data for each experiment were assessed by χ^2 analysis of contingency tables.

RESULTS

Experiment 1

There was an effect of freezing diluent on the percentage progressive motility and progressive velocity of spermatozoa ($P < 0.001$), the best diluent being Hepes-NaOH, but this did not effect the percentage of intact acrosomes. There was an interaction between diluent type and semen batch on the percentage progressive motility. In batches 2 and 3 higher motility was observed in Tris-citrate than in the high glucose diluents (25.9 vs 19.7% mean motile spermatozoa; $P < 0.01$), while the converse was true for batch 1 (21.0 vs 30.0% mean

motile spermatozoa; $P < 0.001$). However, the overall percentage progressive motility and velocity was superior in the Hepes-NaOH-based diluent than in the other two diluents for all batches ($P < 0.001$; table I).

There was also an interaction between diluent type and incubation time on the percentage progressive motility ($P < 0.05$). In diluents containing Hepes-NaOH, the percentage loss of motile spermatozoa during incubation (ie, the relative proportionate loss of motility over time) was lower than in diluents containing Tris-citrate or high glucose (53 vs 72%).

Fertility was higher after intrauterine than cervical insemination (62.2 vs 16.5%; $P < 0.01$) and, after cervical AI, fresh spermatozoa gave higher fertility results than the frozen-thawed spermatozoa (42.0 vs 16.5%; $P < 0.01$, table I). There were no differences in fertility between diluents, despite superior percentage progressive motility and progressive velocity in Hepes-NaOH than in the other diluents.

Table I. Effect of freezing diluent on the mean post-thawing motility, velocity, acrosome integrity ^a and fertility at induced oestrus of frozen-thawed ram spermatozoa from *Experiment 1*.

	Type of diluent			Fresh
	Tris-citrate	High glucose	Hepes-NaOH	
Progressive motility (%)	24.3	23.1	32.5*	
Progressive velocity $\mu\text{M/s}$	59.6	59.6	77.7*	
Intact acrosomes (%)	44.1	39.7	38.8	
<i>Fertility: ewes pregnant/inseminated (%)</i>				
Intrauterine ^b	28/42 (67)	19/40 (48)	32/45 (71)	
Cervical ^c	9/43 (21)	9/50 (18)	5/46 (11)	21/50* (42)

* Different from other treatments in each row $P < 0.001$. ^a Mean post-thawing percentage progressive motility, progressive velocity and acrosome integrity values are averaged for three batches of pooled semen, three replicates and three incubation times for each of the diluents ($n = 27$). ^b Inseminated in the uterus with 20 million motile frozen-thawed spermatozoa per ewe. ^c Inseminated in the cervix with 180 million motile fresh or frozen-thawed spermatozoa per ewe.

Table II. Effect of freezing diluent on the fertility at induced oestrus of frozen-thawed ram spermatozoa ^a from *Experiment 2*.

Fertility: ewes pregnant/inseminated (%)	Type of diluent	
	Tris-citrate	Hepes-NaOH
Intrauterine ^b	33/71 (46)	37/74 (50)
Cervical ^c	3/73 (4)	8/72 (11)

^a Seven batches of pooled semen were used. ^b Inseminated in the uterus with 10 million motile frozen-thawed spermatozoa per ewe. ^c Inseminated in the cervix with 100 million motile frozen-thawed spermatozoa per ewe.

Experiment 2

Fertility data are presented in table II. Fertility was higher after intrauterine than cervical insemination (48.3 vs 7.6%; $P < 0.001$), but there were no differences in fertility between Tris-citrate and Hepes-NaOH diluents.

Experiment 3

There was an effect of freezing diluent ($P < 0.001$) and of semen batch on both the post-thawing percentage motility and acrosome integrity. Higher motility and acrosome integrity were observed in both Hepes-NaOH and Pipes-NaOH than in the Tris-citrate diluents ($P < 0.001$; table III).

There was an interaction between diluent type and incubation time on the percentage motility ($P < 0.01$). In diluents containing zwitterion buffers, the percentage loss of motile spermatozoa during incubation (ie, the relative proportionate loss of motility over time) was lower than in diluents containing Tris-citrate (21 vs 32%).

Fertility was higher after intrauterine compared with cervical insemination (48.0 vs 32.1%; $P < 0.001$), but for each of these insemination methods, there was no effect of insemination dose (intrauterine: 49.5 vs

46.5%; cervical: 31.5 vs 32.6%; table III). There were no differences in fertility between diluents, despite superior motility and acrosome integrity in the zwitterion as compared with the Tris-citrate diluents ($P < 0.001$, table III).

DISCUSSION

Cryopreservation of ram semen greatly reduces sperm quality, compared with fresh semen. Accordingly, poor sperm transport in the female tract has generally resulted in poor fertility of frozen-thawed spermatozoa following cervical insemination (Lightfoot and Salamon, 1970). Laparoscopic intrauterine insemination (Killeen and Caffery, 1982) and transcervical insemination procedures (Halbert et al, 1990) overcome this problem, as they permit deposition of frozen-thawed semen closer to the site of fertilisation. However, these techniques are either relatively expensive, time-consuming and require a high level of expertise (laparoscopy) or have proved unreliable in practice (transcervical insemination). An alternative to better insemination techniques is to improve the quality of the frozen-thawed spermatozoa. A possible hypothesis is that improvements in sperm velocity, viability and motility may facilitate the passage of

Table III. Effect of freezing diluent on the mean post-thawing motility ^a, acrosome integrity ^b and of insemination dose ^c on the fertility at natural oestrus of frozen-thawed ram spermatozoa from *Experiment 3*.

	Type of diluent			
	Tris-citrate	Hepes-NaOH	Pipes-NaOH	
Motility (%)	50.8	55.3*	55.1*	
Intact acrosomes (%)	54.8	59.1*	59.4*	
<i>Fertility: ewes pregnant/inseminated (%)</i>				
Intrauterine dose (x 10 ⁶)				
8	15/35 (43)	18/34 (53)	17/32 (53)	
24	13/35 (37)	18/34 (53)	16/32 (50)	
Total	28/70 (40)	36/68 (53)	33/64 (52)	97/202 (48)
Cervical dose (x 10 ⁶)				
80	20/49 (41)	12/49 (24)	14/48 (29)	
240	17/48 (35)	17/48 (35)	13/48 (27)	
Total	37/97 (38)	29/97 (30)	27/96 (28)	93/290 (32)

* Different from Tris-citrate $P < 0.001$. ^a Mean post-thawing motility values are averaged for ten batches of pooled semen, four replicates and quatre incubation times for each of the diluents ($n = 160$). ^b Mean post-thawing acrosome integrity values are averaged for ten batches of pooled semen and four replicates for each of the diluents. ^c Insemination doses are total numbers of motile frozen-thawed spermatozoa per ewe.

greater numbers of sperm through the female tract to the site of fertilisation, and that improvements in the percentage of sperm with intact acrosomes would ensure that a larger proportion of sperm that had reached this site would be able to fertilise ova successfully *in vivo*. The purpose of the current study was to observe whether improvements in the *in vitro* quality of frozen-thawed spermatozoa in zwitterion-compared with Tris-citrate-based diluents would be reflected in improved fertility.

Improvements in post-thawing percentage motility and progressive velocity (tables I and III) and acrosome integrity (table III only) were observed in zwitterion-compared with Tris-citrate-based diluents, which confirmed our previous findings (Molinia et al, 1994a). However, no additional information

was obtained from the objective assessments of post-thawing motility in *Experiment 1* compared with the subjective assessments in *Experiment 3* as reported previously (Molinia et al, 1994b). Batch effects on post-thawing motility and/or acrosome integrity values in *Experiments 1* and *3* were due to differences in frozen-thawed semen quality between batches, as each batch consisted of different pooled ejaculates collected at different times from each of the animals. As a comparative measure of the ability of diluents to maintain the viability of spermatozoa after thawing, results were presented as the percentage loss of motile spermatozoa in *Experiments 1* and *3* where there were interactions involving incubation time. When the overall mean motility was high the percentage loss of motile sperma-

tozoa during incubation was low and vice versa. Thus it was observed that spermatozoa with superior motility (in zwitterion diluents) were likely to sustain the motile cell population longer, while those with lower motility (in Tris-citrate and high glucose diluents) were likely to have a higher incidence of cell mortality.

In the three field experiments presented in this study, there was no demonstrable effect of diluent type on the fertility of frozen-thawed ram spermatozoa following cervical or laparoscopic AI. This demonstrates that the *in vitro* measures of motility and acrosome integrity were poorly correlated with the *in vivo* fertility results as reported previously in the ram (Eppleston et al, 1986). The poor results from the cervical inseminations in this study imply that the *in vitro* improvements in the quality of frozen-thawed spermatozoa were insufficient to render a significant improvement in fertility. For the intrauterine inseminations, the numbers of spermatozoa used for insemination were probably too high to detect possible differences between the diluents.

It was apparent from the cervical insemination data that much greater improvement in the post-thawing quality of ram spermatozoa is necessary to improve sperm transport through the genital tract of the ewe. There was evidence of this in *Experiment 1* where the fertility of frozen-thawed spermatozoa was approximately 40% that of fresh spermatozoa even though the same numbers of motile spermatozoa were used in the insemination dose. Pregnancy rates above 30% were achieved in *Experiment 3* with an insemination dose of frozen semen half that used in *Experiment 1*, probably because the former animals were inseminated at a natural oestrus, while a similar dose in *Experiment 2* yielded very poor fertility (7.6%). However, there was no difference in fertility (31.5 vs 32.6%) when the insemination dose was tripled in *Experiment 3*. In contrast, Salamon (1977) found that

increasing the insemination dose fourfold (from 90 to 360 million motile frozen-thawed spermatozoa) in ewes cervically inseminated at a natural oestrus, increased the fertility from approximately 30 to 55%, although this was based on lambing data, and only a Tris-citrate diluent was used. It is apparent that the dose of semen required for acceptable fertility varies between flocks of sheep (in different areas), seasons, synchronisation treatments, times and methods of insemination and the freezing diluents used.

Fertility following intrauterine insemination was far superior to cervical insemination (51.9 vs 22.1%), as the former technique circumvented the problem of sperm transport through the cervix (Lightfoot and Salamon, 1970). Doses of 8–24 million motile frozen-thawed spermatozoa yielded acceptable fertility results in all experiments, the best being obtained with 20 million spermatozoa (*Experiment 1*). This was not surprising as doses as low as 5 million motile frozen-thawed spermatozoa have yielded acceptable fertility (Eppleston et al, 1986). Thus it is possible that no differences were seen between the diluents, as the ewes were inseminated in the uterus with greater than minimum doses of semen required for optimum fertility. However, in *Experiment 3*, there was a small but non-significant increase in fertility between zwitterion- and Tris-citrate-based diluents (52.3 vs 40.0%). It may be possible to demonstrate a significant effect between the diluents if large numbers of ewes are inseminated and if the inseminations are performed with less than minimum doses of spermatozoa, but they would then increase the risk of lower fertility.

It is concluded from this study that the post-thawing motility was superior, acrosome integrity comparable if not better, and fertility similar, after freezing spermatozoa in zwitterion than in Tris-citrate diluents. The improvements in sperm quality in the zwitterion diluents *in vitro* were still insufficient to

improve sperm transport through the cervix. However, a small but non-significant improvement in fertility with these diluents following laparoscopic intrauterine insemination was reported in this study. A larger number of animals should be inseminated to demonstrate the possible beneficial effect of these diluents.

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