Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa

A. Morrier[1], F. Castonguay[1,2], and J. L. Bailey[1,3]

Centre de Recherche en Biologie de la Reproduction, [1]Département des sciences animales, Université Laval, Québec, Canada, G1K 7P4; [2]Dairy and Swine Research and Development Centre, Agriculture and Agri-Food Canada, Lennoxville, Québec, Canada, J1M 1Z3. *Received 18 May 2001, accepted 16 March 2002.*

Morrier, A., Castonguay, F. and Bailey, J. L. 2002. **Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa**. Can. J. Anim. Sci. **82**: 347–356. Fresh extended ram semen has a short fertile lifespan whereas acceptable fertility with cryopreserved semen is achieved only by laparoscopy, which limits widespread artificial insemination in sheep. Although glycerol is considered essential for freezing spermatozoa, it is often included in extenders for short-term storage at above-freezing temperatures. To test the hypothesis that glycerol reduces the function of fresh sperm, ram semen was divided into two aliquots and diluted with commercial extenders that were identical, except that one contained 7% glycerol (n = 6). In a second experiment, ram semen was prepared for cryopreservation by a one-step dilution with a 7% glycerol extender or gradually, with a two-step protocol, to test the hypothesis that the method and time of glycerol addition affects sperm quality after freezing and thawing (n = 7). For both experiments, semen was diluted in a synthetic oviductal fluid (SOF-m) and sperm quality was assessed by computer-assisted motility, viability and chlortetracycline fluorescence (CTC) patterns (an indicator of capacitation status). The presence of glycerol did not affect the quality of fresh sperm ($P > 0.27$). For cryopreserved sperm, the method of glycerol addition also did not affect thawed sperm. However, a decrease in sperm motility and viability, and different distribution of CTC patterns occurred due to the duration of time in extender and in SOF-m ($P \leq 0.0002$). Cryo-capacitation was also observed. In conclusion, the presence of glycerol in the extender did not reduce ram sperm quality during conservation of the semen at 5°C or when it was used to completely and rapidly dilute the semen before cooling for cryopreservation.

Key words: Sheep, Triladyl, Biladyl, chlortetracycline, artificial insemination, spermatozoa.

Morrier, A., Castonguay, F. et Bailey, J. L. 2002. **Additon de cholestérol et conservation de la semence de béliere fraiche et cryoconservée**. Can. J. Anim. Sci. **82**: 347–356. La semence de bélier fraîche (conservée à 5°C) a une durée de vie limitée tandis que la semence cryoconservée ne donne des taux de fertilité acceptables que lorsque la laparoscopie est utilisée, ce qui limite l'expansion de l'insémination artificielle chez l'ovin. Même si le glycérol est essentiel à la congélation des spermatozoïdes, il est souvent inclus dans les diluants destinés à la conservation de courte durée à des températures au-dessus du point de congélation. Afin de tester l'hypothèse selon laquelle le glycérol réduit la fonction des spermatozoïdes frais, la semence de bélier a été divisée en deux aliquotes et diluée avec deux diluants commerciaux identiques, à l'exception du fait qu'un contenait 7% de glycérol (n = 6). Dans une seconde expérience, la semence de bélier était diluée en une seule étape avec un diluant contenant 7% de glycérol ou graduellement, en deux étapes afin de tester l'hypothèse selon laquelle la méthode et le temps d'addition du glycérol affectent la qualité spermatique lors de la congélation-décongélation (n = 7). Dans les deux expériences, la semence était diluée dans un fluide synthétique mimant l'oviducte de la brebis (SOF-m) et la qualité de la semence était évaluée selon la motilité totale et progressive, la viabilité ainsi que les patrons de fluorescence obtenus par la coloration à la chlortétracycline (CTC), patrons indiquant l'état de capacitation du spermatozoïde. Le glycérol n'a pas affecté la qualité de la semence fraîche. Pour la semence cryoconservée, la méthode d'addition du glycérol n'a pas eu d'effet sur la semence décongelée. Cependant, l'incubation dans le SOF a engendré une diminution de la motilité et de la viabilité spermatique ainsi qu'une distribution différente des patrons de CTC ($P < 0.0002$). Un phénomène de cryocapacitation a également été observé. En conclusion, le glycérol ne semble pas endommager la qualité de la semence ovine pendant la conservation à 5°C ou lorsqu'il est ajouté rapidement à la semence avant le refroidissement précédent la cryoconservation.

Mots clés: Mouton, Triladyl, Biladyl, chlortétracycline, insémination artificielle, spermatozoïde

The cryoprotective benefits of glycerol on spermatozoa were discovered by Polge et al. (1949) and are attributed mostly to its water-binding properties [reviewed by Salomon and Maxwell (1995)]. Since then, the use of glycerol to preserve spermatozoa during freezing has become widespread (Hammerstedt et al. 1990; Bailey et al. 2000). Recent studies have demonstrated that glycerol remains the most effective cryoprotective compound for freezing mammalian semen and no enhancement was showed by the addition of other compounds (Molinia et al. 1994).

Therefore, for cryopreservation, glycerol is the most commonly used cryoprotectant for ram semen (Salomon and Maxwell 2000). However, when glycerol should be added during cryopreservation has been investigated, with con-

[3]*Corresponding author (e-mail: Janice.Bailey@CRBR.ulaval.ca).*

Abbreviations: **CTC**, chlortetracycline; **SOF-m**, synthetic oviductal fluid

flicting results. Colas (1975) suggested that glycerol is slightly toxic to ram spermatozoa and that its negative effects could be reduced by its addition at a temperature near 0°C. Contrary to this recommendation, Salomon (1968) [reviewed by Salomon and Maxwell (2000)] reported that a single dilution of semen with an extender containing glycerol was as effective as a two-step dilution. In a recent experiment, it was found that glycerol addition improved the post-thaw motility of stallion semen when performed at 22°C instead of 4°C (Vidament 2000).

Despite its beneficial effects for sperm cryopreservation, Meryman in 1966 (Fahy 1986) demonstrated that glycerol could also have a damaging effect during freezing and thawing of red blood cells. Glycerol alters the membrane bilayers and interacts with bound proteins and glycoproteins. It also increases the bioenergetic requirements of spermatozoa (Hammerstedt et al. 1990). Furthermore, it has been shown that glycerol decreases fertility in sheep when the semen is stored at 5°C (Abdelhakeam et al. 1991) and accelerates the acrosome reaction in ram spermatozoa (Slavik 1987).

With such negative consequences, the use of glycerol is generally recommended only for cryopreservation of semen. However, for convenience, some artificial insemination centres include glycerol in the extender for conservation of fresh ram semen above freezing temperatures (Hackett 1982).

In Canada, the ovine industry is expanding. However, its development is limited by the fact that fresh ovine semen can be conserved only for 8 h (Baril et al. 1993). Furthermore, due to its very poor fertility, artificial insemination with frozen ram semen requires laparoscopy, which considerably increases labour and costs (Maxwell et al. 1996).

Two series of experiments were conducted in this study. To test the hypothesis that glycerol decreases the quality of fresh ram spermatozoa stored at 5°C, two commercial diluents were evaluated, one with and the other without glycerol. In the second experiment, glycerol was added to fresh ram semen immediately after collection or after cooling to 5°C. The semen was then frozen to assess whether the timing of cryoprotectant addition during cryopreservation affects sperm quality. For both experiments, semen was diluted in a solution that mimicked the genital tract of the ewes (SOF-m) and motility, physiological status (assessed by CTC fluorescence) and viability, were used as indicators of sperm quality.

MATERIALS AND METHODS

Chemicals and Animal Care Guidelines

All experiments were conducted as authorized by the university animal care committee and according to the guidelines of the Canadian Council on Animal Care (1993). Unless otherwise specified, all products used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experiment 1: Effect of Glycerol on Fresh Semen

Five mature Dorset-Polled and one Hampshire ram aged from 2 to 7 yr were housed at the *Centre d'Expertise en Production Ovine du Québec* (CEPOQ, La Pocatière, QC, Canada). Rams were housed under a light regime, alternating from long (16 h of light) to short days (8 h of light) each month. For each replicate, semen from these rams was collected twice consecutively (within 10–15 min) with an artificial vagina. These two ejaculates were then pooled for experimentation. Therefore, each male was used once for this experiment ($n = 6$). Semen volume was measured, and sperm morphology and subjective motility were visually assessed by a trained technician using phase contrast microscopy (400×) at room temperature. Samples with less than 75% motile spermatozoa were rejected. Sperm concentration was assessed using a spectrophotometer, previously calibrated by hemocytometry.

Immediately after collection, the semen was divided into two aliquots and each diluted with one of two commercial extenders (Minitube Canada, Woodstock, ON, Canada), Biladyl (0% glycerol; only "solution A") or Triladyl (with 7% glycerol). Both extenders had the same antibiotic cocktail, including Tylosin, Gentamicin, Lincomycin and Spectinomycin and both were supplemented with 20% fresh egg yolk (vol/vol) as per the manufacturer's directions. Commercial eggs were used and kept at 4°C until egg yolk preparation for dilution of semen. Once prepared, the only difference between these diluents was the presence of glycerol. Semen was diluted with the extenders (prepared and prewarmed to 30°C) at room temperature to a final concentration of 1.6×10^9 spermatozoa mL^{-1} (Baril et al. 1993). Diluted semen was placed into 250 µL straws (IMV, l'Aigle, France), sealed with polyvinyl acid, progressively cooled to 5°C over 3 h in a transport box (CIOQ) and maintained at this temperature. This specially designed box contains the appropriate proportion of ice and water at 30°C in order to reach 5°C in 3 h (approximately 0.14°C min^{-1}). The temperature of the box can then be maintained at 5°C for up to 36 h. The same transport box was used for all replicates.

Immediately after preparation for storage in either Biladyl or Triladyl ("Time 0h") and at 8-h intervals (until 24 h), one sealed semen straw of semen was cut open (with clean scissors) and diluted (about 32-fold; to 50×10^6 spermatozoa mL^{-1}) in synthetic oviductal fluid (SOF-m), modified slightly from that described by Pérez et al. (1996a, b), with gentamycin as the antibiotic instead of streptomycin. The SOF-m was composed of 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM glucose, 3.3 mM sodium lactate, 0.33 mM sodium pyruvate, 1 mM glutamine, 20 µM penicillamine, 10 µM hypotaurine and 50 mg L^{-1} gentamycin. This solution was supplemented with 20% heat-inactivated oestrus sheep serum (the same lot for all replicates within each experiment) and adjusted to pH 7.36, 290 mOsm kg^{-1}.

Sperm quality (motility, viability, CTC assay) was assessed immediately following dilution in SOF-m ("Time t0"). The samples diluted in SOF-m were incubated in a humidified atmosphere of 5% CO_2 in air at 39°C. After 6 and 24 h in the SOF-m, sperm quality was re-assessed. Figure 1 shows a schematic description of this protocol.

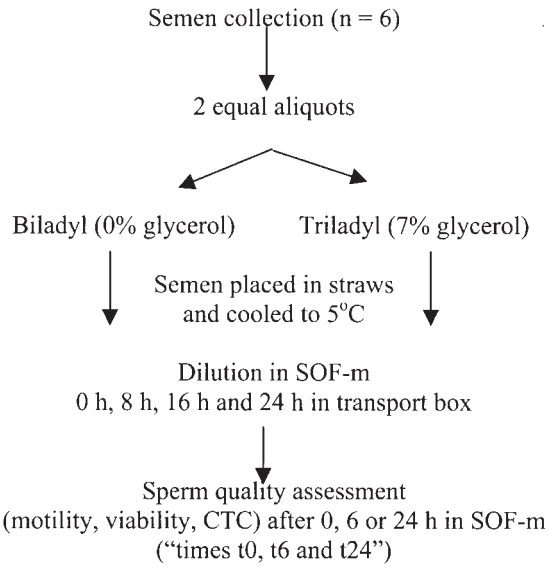

Fig. 1. Outline of the protocol for fresh semen (exp. 1).

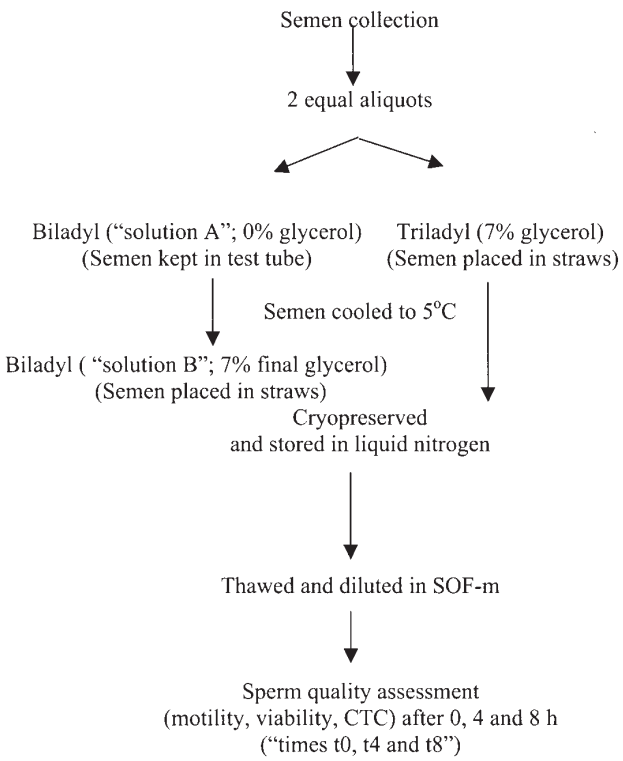

Fig. 2. Outline of the protocol for cryopreserved semen (exp. 2).

Experiment 2: Effect of Preparation Protocol on the Quality of Cryopreserved Ram Semen

Three mature Dorset-polled and one Hampshire rams aged from 2 to 7 yr were collected and semen was assessed as described in exp. 1 (n = 7; each ram was used once or twice). Immediately after collection, the semen was divided into two identical aliquots and extended with Biladyl or Triladyl. For Triladyl (7% glycerol, one-step dilution), semen was diluted to 4×10^8 spermatozoa mL^{-1} with extender supplemented with 20% egg-yolk (prepared as for exp. 1, prewarmed to 30°C). The diluted samples were slowly cooled to 5°C within 3 h in a cold room (approximately 0.14°C min^{-1}), then frozen (according to industry standards) in 250 µL straws. The straws were frozen by plunging them directly in liquid nitrogen at –196°C. Straws were stored in liquid nitrogen for 4 to 8 wk. For Biladyl, the semen was extended at room temperature to 8×10^8 spermatozoa mL^{-1} with warmed "solution A", kept at 30°C supplemented with 20% egg yolk and the antibiotic cocktail (as described in the manufacturer's directions). The diluted samples were slowly cooled to 5°C within 3 h in a cold room, concomitant with the Triladyl aliquots, then "solution B" (containing 14% glycerol) from Biladyl supplemented with 20% egg-yolk (prepared as before) was added at 5°C to reach a sperm concentration of 4×10^8 spermatozoa mL^{-1} and a final glycerol concentration of 7%. Semen was frozen as for Triladyl.

Semen was thawed by plunging straws into a 37°C water bath for 30 s. After thawing, semen was diluted in SOF-m (50×10^6 spermatozoa mL^{-1}). As described for fresh semen (exp. 1), a straw was thawed, then diluted in SOF-m (50×10^6 spermatozoa mL^{-1}). Sperm quality was assessed immediately following dilution in SOF-m ("Time t0"), then 4 and 8 h after dilution ("Times t4 and t8") (39°C, 5% CO_2 in air) as shown in Fig. 2.

Motility Analysis

For the assessment of sperm motility, three 2-µL samples of spermatozoa in SOF-m (~50×10^6 cells mL^{-1}) were deposited on a warmed (37°C) 10-µm Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel), which was held on the motility analyser (Ceros Analyzer, with software version 7,4G; Hamilton Thorn Research, Beverly, MA). At least 200 cells from five random fields were recorded. The settings used were as follows: frames acquired: 20; frame rate: 20 Hz; minimum contrast: 10; minimum size: 5; lo/hi size gates: 0.2 to 2.0; lo/hi intensity gates: 0.2 to 1.8; non-motile head size: 5; non-motile brightness: 63; medium path velocity (VAP) value: 95; low VAP value: 20; slow cells motile: no; threshold (STR): 60. Only progressive and total motilities were determined for this study.

Viability Assay

Equal volumes of sperm suspension and eosin-nigrosin solution (3.3 g eosin Y, 20 g nigrosin, 1.5 g sodium citrate in 300 mL of water, adjusted to pH 7.0) were mixed (Barth and Oko 1989; Cormier et al. 1997). Twenty µL of this mixture were smeared onto a clean microscope slide (prewarmed to 37°C) and allowed to air dry at 27°C. The sample was covered with Permount (Fisher Scientific, Fair Lawn, New Jersey) and sealed with a coverslip. Two hundred spermatozoa were scored per slide using light microscopy (400×). Sperm coloured pink were considered non-viable and unstained (clear) cells were counted as live.

Chlortetracycline Fluorescence Assay

The CTC fluorescence assay used was based on methods previously described by Ward and Storey (1984) and Pérez et al. (1996a), who developed the assay for ram spermatozoa. For the CTC solution, 5 mM cysteine and 750 µM CTC were diluted in a buffer containing 20 mM Tris base and 130 mM NaCl (pH 7.8). Prior to use, this solution was filtered (0.2 µm) then protected from light. For the assay, 15 µL of CTC solution was mixed with 10 µL spermatozoa diluted in SOF-m. Then, 0.5 µL of 12.5% (vol/vol) glutaraldehyde solution prepared in 2.5 M Tris base was added. Ten µL of this uniformly mixed suspension was placed on a clean microscope slide and covered with a coverslip. The prepared slides were kept in a humidified environment at 4°C and evaluated within 24 h using a fluorescent microscope with blue-violet illumination (excitation at 400–440 nm and emission at 470 nm). All slides were prepared in duplicate. A total of 200 spermatozoa per slide were scored (400×). The CTC patterns were scored as described by Gillan et al. (1997) with spermatozoa categorised as follows: "F" or non-capacitated spermatozoa for those with uniform head fluorescence; "B" or capacitated spermatozoa for those with fluorescence only along the acrosome; and "AR" for acrosome-reacted spermatozoa with dull fluorescence over the head.

Statistical Analyses

The normality of the data was confirmed, and then analyses of variance were performed using the General Linear Models procedure of SAS (SAS Institute Inc. 1990). The statistical model used to analyse sperm parameters (% viable, % motile, % progressively motile, and % CTC pattern F, B and AR sperm) for each storage time in extender (exp. 1, fresh semen) or after thawing (exp. 2, cryopreserved semen) included the effect of ram (i.e., repetition), dilution method (i.e., treatment), and duration of incubation in SOF-m. The two-way interactions among these variables were also tested and the residual error was the error term. The model used to assess sperm parameters for each incubation time in SOF-m included the effect of ram (repetition), dilution method (treatment), and storage time in extender (for exp. 1 only). The two-way interactions among these variables were also tested and the residual error was the error term. When dilution method was not significant, a reduced model was used and the two dilution methods were pooled. When the main effects of the model variables were significant, multiple comparisons were conducted using the Student-Newman-Keuls (SNK) test. Significance was judged at the probability level of $P = 0.05$.

RESULTS

Experiment 1: Effect of Glycerol on Fresh Semen

The initial concentrations of the ejaculates and motility were $3.67 \times 10^9 \pm 0.58$ spermatozoa mL^{-1} and 84 ± 3.3%, respectively (means ± SE), with less then 25% abnormal spermatozoa for each ejaculate. For exp. 1, the presence or absence of 7% glycerol in the extender did not affect motility. The mean progressive and total sperm motility (± SE) are shown in Fig. 3. The duration of storage in the treatment extender and the time of incubation in SOF-m affected total motility ($P < 0.0001$). Progressive motility decreased with incubation time in SOF-m ($P < 0.0001$) and was influenced by the duration of storage in the extender (± glycerol) only after 6 h of incubation in SOF-m ($P < 0.0001$).

Sperm viability was reduced with duration in the extender and incubation in SOF-m ($P < 0.0002$ and $P < 0.0001$; Fig. 4). However, the presence of glycerol did not significantly affect sperm viability.

Chlortetracycline fluorescence patterns were influenced by the duration of conservation at 5°C in the extender and by the time of incubation in SOF-m but not by the addition of glycerol to the extender (Fig. 5). The percentages of spermatozoa showing pattern F (non-capacitated) decreased with time in the extender at 5°C and incubation in SOF-m ($P = 0.0001$), while the percentages of pattern AR sperm (acrosome-reacted) increased ($P = 0.0001$). Overall, the percentage of pattern B sperm (capacitated) was lower after 8 h of conservation in the extender (± glycerol; $P = 0.05$), followed by an additional decrease after 24 h ($P = 0.05$). During incubation in SOF-m, the percentage of pattern B sperm increased after 6 h (t6; $P = 0.05$) followed by a decrease at 24 h (t24; $P = 0.05$), regardless of the duration of storage at 5°C.

Experiment 2: Effect of Preparation Protocol on the Quality of Cryopreserved Ram Semen

The method of cryoprotectant addition did not significantly affect thawed sperm quality. However, as in exp. 1, the total and progressive motilities were reduced during incubation in SOF-m at 39°C ($P = 0.0001$; Fig. 6). The viability of thawed ram spermatozoa was also decreased during incubation in SOF-m ($P = 0.0001$; Fig. 6). For the CTC fluorescence, the percentages of sperm displaying patterns F (non-capacitated) and B (capacitated) decreased with time in SOF-m, as the proportion of sperm with the AR pattern increased ($P = 0.05$, Fig. 7).

DISCUSSION

Experiment 1 was conducted to assess the effects of two types of diluent, one without (Biladyl) and the other with glycerol (Triladyl), on the physiology of ram sperm during conservation of liquid semen at 5°C. The main goal of this first experiment was to test whether the absence of glycerol from the diluent would improve the quality of the fresh semen during conservation at 5°C in an egg-yolk diluent, thereby permitting longer storage. Presently, liquid ram semen has a fertile lifespan of only about 8 h when conserved in fresh state. In the second experiment, the effect of glycerol addition at 30°C (one step) or at 5°C (after an initial dilution and cooling) was evaluated on the quality of cryopreserved ram sperm. Several sperm parameters (total and progressive motility, viability and CTC fluorescent patterns) were assessed during storage in the extender and also during incubation in a physiological solution that mimicked the genital tract of the ewe, SOF-m (Pérez et al. 1996a, b; 1997). Therefore, the physiology of the sperm were evaluated in a "synthetic female tract" to better reflect sperm function after insemination. Furthermore, in our experiments,

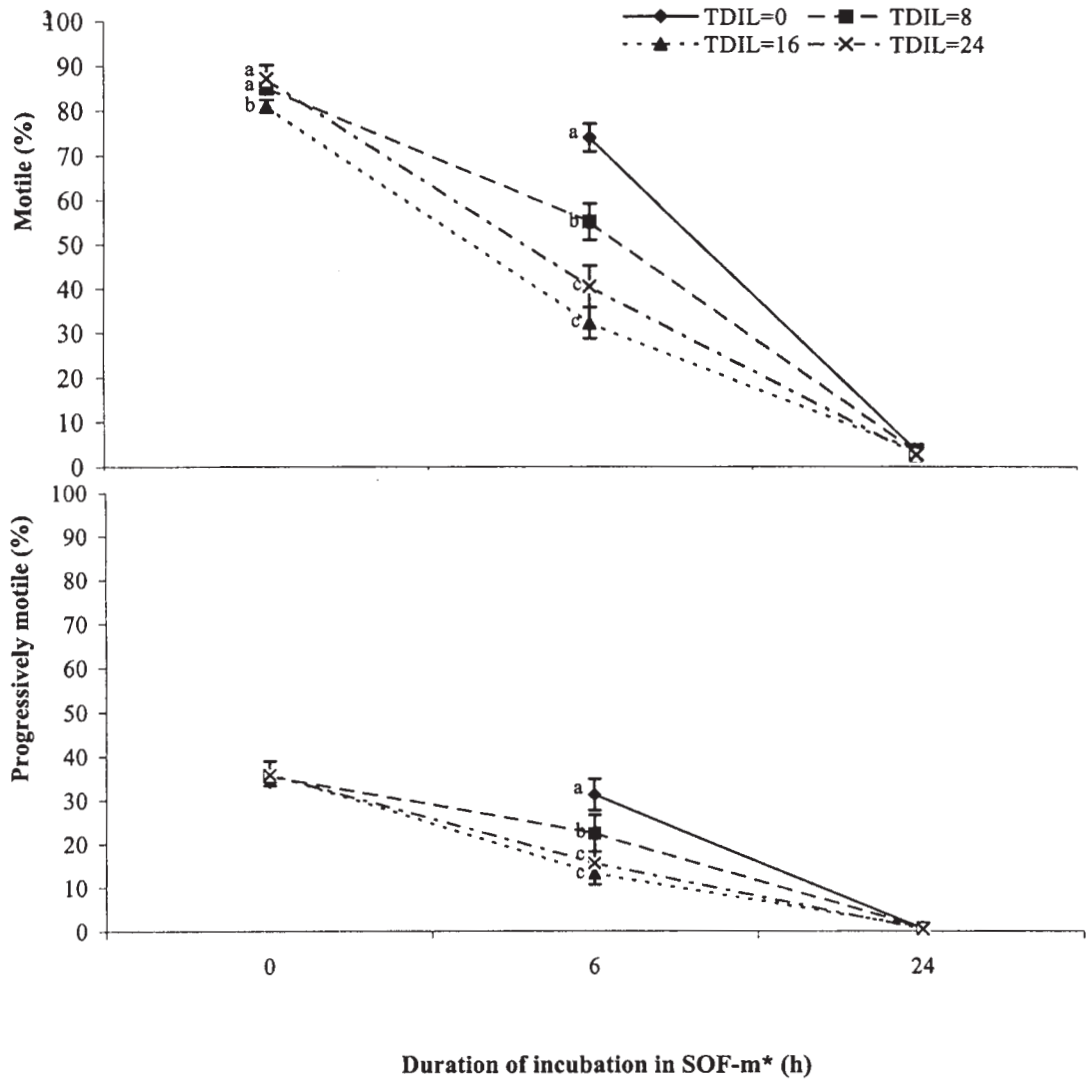

Fig. 3. Effects of conservation at 5°C in the semen extender and incubation in SOF-m on the percentages of total and progressively motile ram spermatozoa. Semen was diluted either in Biladyl (0% glycerol) or Triladyl (7% glycerol) and stored for 0, 8, 16 or 24 h at 5°C (TDIL = 0, 8, 16 and 24, respectively) then diluted in SOF-m at 39°C, 5% CO_2 for 0, 6 or 24 h (t). Shown are means ± SE (n = 6). There was no effect of glycerol ($P > 0.7521$), and therefore data combined for glycerol treatment are shown. For total motility there was a main effect of repetition, time in extender or time in SOF-m ($P < 0.01$). For progressive motility the same effects were observed ($P < 0.01$), except for time in the extender.
* Motility differs within storage time in extender at 5°C (TDIL) due to incubation in SOF-m at 39°C at all times ($P < 0.05$).
a,b,c Motility differs within duration of incubation in SOF-m due to time in the extender (TDIL) at 5°C ($P < 0.05$).

semen was not washed before incubation in SOF-m in order to better mimic insemination in the field (Cormier et al. 1997).

The CTC assay was used to assess and follow the physiological changes of the sperm. Chlortetracycline is a fluorescent antibiotic that binds membrane calcium (Saling and Storey 1979). The distribution of membrane calcium (bound to proteins and/or lipids) is thought to change during capacitation and may be associated with an influx of calcium leading to the acrosome reaction (Gillan et al. 1997). Sperm CTC fluorescence patterns appear to be related to the physiological stages (non-capacitated, capacitated, acrosome reacted) of ram sperm (Pérez et al. 1996a), as with other species (Ward and Storey 1984; Lee et al. 1987; Fraser et al. 1995). In this study, CTC-stained sperm were scored according to three staining categories (F, B and AR) as in the original findings of Saling and Storey (1979) and Ward and Storey (1984) with mouse sperm. Gillan et al. (1997) applied the same nomenclature for ram sperm. However, others have used four CTC-staining patterns for ram sperm (I, II, III, IV; Pérez et al. 1996a). Form II seems to be unique to ram sperm and would represent an earlier stage of capacitation than form III. In our study, forms I and II were both counted as pattern F (non-capacitated).

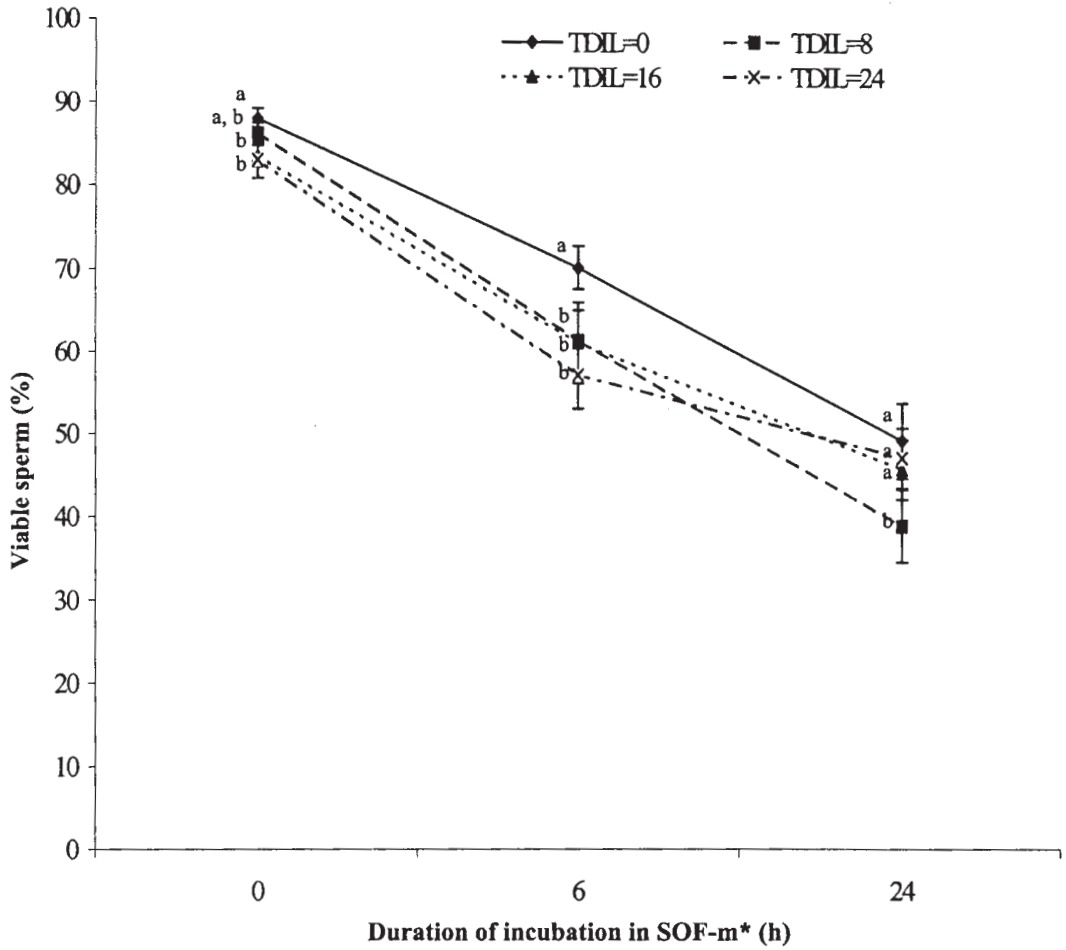

Fig. 4. Effect of conservation at 5°C in the semen extender and incubation in SOF-m on the percentages of viable ram spermatozoa. Semen was diluted either in Biladyl (0% glycerol) or Triladyl (7% glycerol) and stored for 0, 8, 16 or 24 h at 5°C (TDIL = 0, 8, 16 and 24 h, respectively) then diluted in SOF-m at 39°C, 5% CO_2 0, 6 or 24 h. Shown are means ± SE (n = 6). The presence or absence of glycerol did not affect sperm viability at any time point ($P > 0.8021$) and so treatments were combined. There was a main effect of repetition, time in the extender or time in SOF-m ($P < 0.01$).
* Viability differs within storage time in extender at 5°C (TDIL) due to incubation in SOF-m at 39°C at all times ($P < 0.05$).
a,b,c Viability differs within duration of incubation in SOF-m due to time in the extender (TDIL) at 5°C ($P < 0.05$).

Effect of Glycerol on Fresh Semen

In exp. 1, the presence or absence of 7% glycerol in the egg-yolk extender did not significantly affect the motility parameters or the viability of the sperm, either in the extender during storage for 24 h at 5°C or after dilution in SOF-m for 24 h at 39°C. There was an increase in pattern B after 6 h in SOF-m, followed by a decrease after 24 h in SOF-m, suggesting that as sperm undergo spontaneous acrosome-reactions, the proportion of pattern B decreases. Also, no differences in CTC pattern distribution among the sperm were observed due to glycerol, although Slavik (1987) showed that the acrosome reaction of ram sperm is accelerated by glycerol, which was postulated as one of the causes of lower conception rates after insemination with frozen ram sperm. Abdelhakeam et al. (1991) found that the addition of glycerol (3%) decreased lambing rates from 83 to 41%. However, we observed no apparent effect of over twofold higher glycerol concentrations on the function of fresh sperm. The damaging effect of glycerol on sperm is considered to be mostly related to its osmotic impact. Upon glycerol addition, the cells rapidly shrink, which is associated with the release of intracellular water. A slower return to the original volume follows, as the glycerol penetrates into the sperm (Hammerstedt et al. 1990).

However, in vitro tests done on semen are not always related to the fertility of semen in vivo (Colas 1975; Tardif et al. 1999). It is possible that the impact of glycerol is within the female genital tract. In our study, the incubation in SOF-m was conducted in order to mimic the environment in vivo. Many proteins of the female reproductive tract interact with sperm (Abe et al. 1995) and the reproductive tract secretions of the ewes can capacitate ram sperm in vitro (Chavarria and Reys 1996). Thus it is possible that the reduced fertility of ram semen in vivo with glycerol was not reflected in our in vitro conditions, despite efforts to mimic

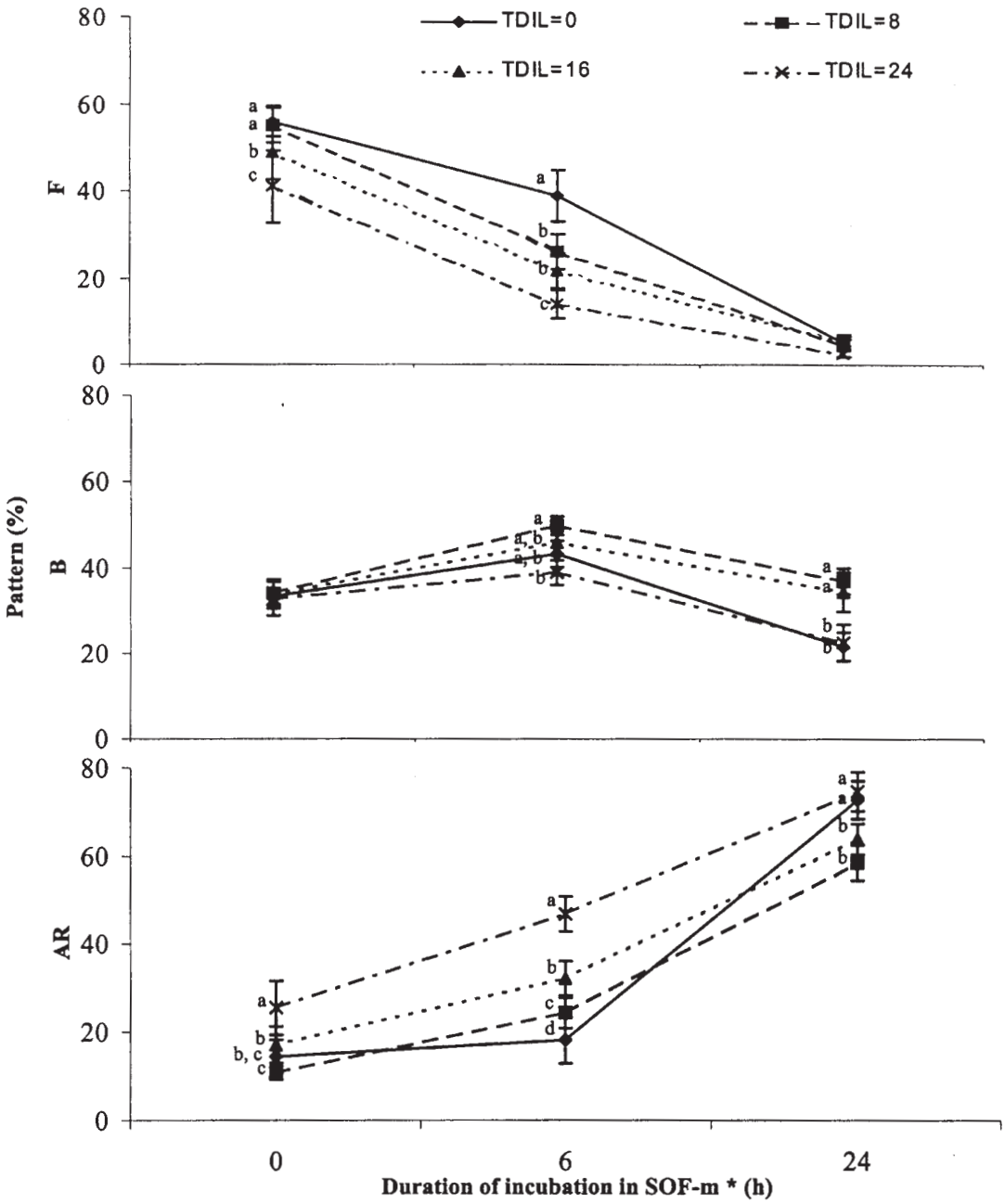

Fig. 5. Effect of conservation at 5°C and subsequent incubation in SOF-m on the distribution of CTC-patterns of fresh spermatozoa (means ± SE; $n = 6$). The presence or absence of glycerol did not affect the distribution of CTC patterns during storage at 5°C or in SOF-m ($P > 0.2797$) and so treatments are pooled together. There was a main effect of repetition, time in the extender or time in SOF-m for all three patterns ($P < 0.01$).

* Patterns differ within storage time in extender at 5°C (TDIL) due to incubation in SOF-m at 39°C at all times ($P < 0.05$) unless indicated[1].

a,b,c CTC pattern differs within duration of incubation in SOF-m due to time in the extender (TDIL) at 5°C ($P < 0.05$).

[1]Pattern B: Duration of incubation in SOF-m at 6 h was different for TDIL = 8 and TDIL = 16 and duration of incubation of 24 h in SOF-m was significantly different for TDIL = 24 ($P < 0.05$).

Pattern AR: Only 24 h of incubation was significantly different for TDIL = 0 and TDIL = 8 ($P < 0.05$)

the in vivo environment. It is also possible that glycerol reduces the fertility of fresh ram semen due to the effects on the ewe, which were not examined in this study. Previous reports have suggested that glycerol irritates the female genital tissues (Tajima et al. 1989; Abdelhakeam et al. 1991; Hammerstedt and Graham 1992), which could reduce fertil-

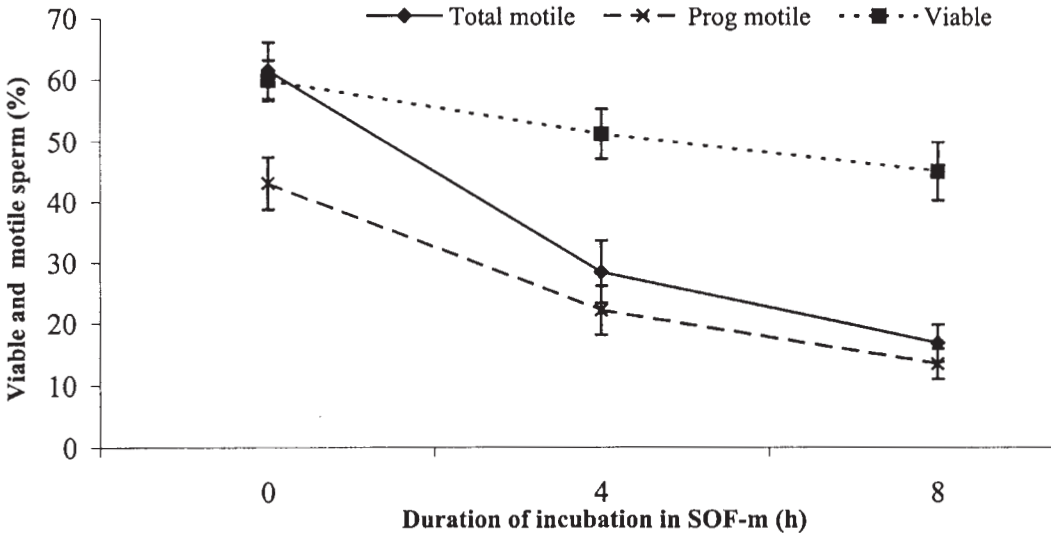

Fig. 6. Effect of a one- or two-step method of dilution on the percentages of viability, total and progressively motile ram spermatozoa after thawing. Semen was cryopreserved (Biladyl, two-step method with glycerol added at 5°C; Triladyl, one step method with glycerol added immediately after collection at 37°C), stored, thawed and then incubated in SOF-m at 39°C (in 5% CO_2 in air). Shown are means ± SE (n = 7). Method of dilution did not affect sperm motility at any time ($P > 0.3351$), and so the treatments are pooled together. Viability, total and progressive motilities changed due to the duration of incubation in SOF-m at 39°C at all times ($P < 0.05$). There was also a main effect of repetition ($P < 0.01$).

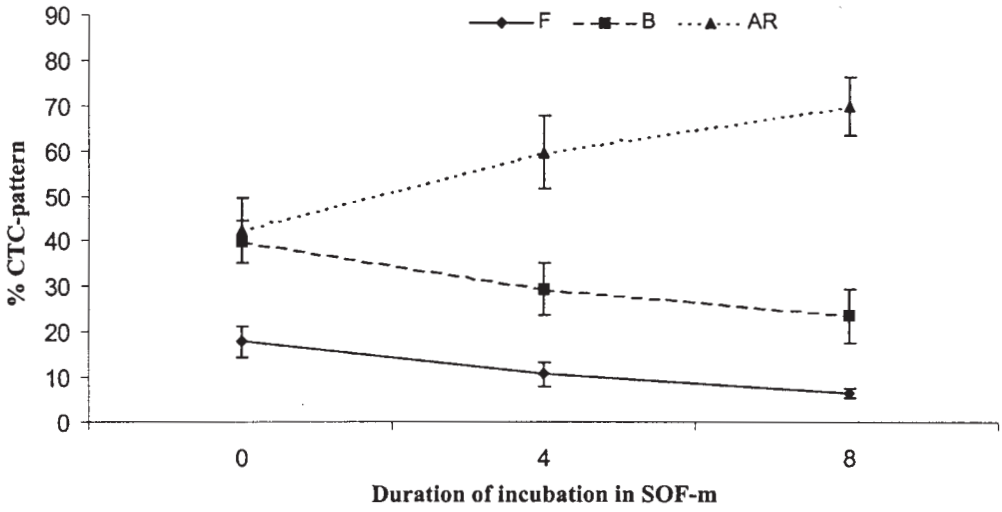

Fig. 7. Effect of a one- or two-step method of dilution on the distribution of CTC-patterns F, B and AR of ram spermatozoa. Semen was cryopreserved (Biladyl, two-step method with glycerol added at 5°C; Triladyl, one-step method with glycerol added immediately after collection at 37°C), stored, thawed and then incubated in SOF-m at 39°C (5% CO_2 in air). Shown are means ± SE (n = 7). Method of dilution did not affect CTC-patterns at each time point ($P > 0.8072$) and so treatments are pooled. The proportion of each CTC-pattern differs due to the duration of incubation in SOF-m at 39°C at all times ($P < 0.05$). There was a main effect of repetition ($P < 0.01$).

ity. However, in our experiment, no putative contraceptive effect was observed on ram semen in vitro.

Effect of Glycerol Addition Protocol during Cryopreservation on Sperm Quality

From the results obtained in the experiment with cryopreserved semen, it is concluded that the addition of glycerol either immediately after collection (one-step) or at 5°C (two-step) does not affect the subsequent quality of frozen-thawed ram sperm assessed by motility, viability and CTC-patterns. Our results agree with those of Branny et al. (1966) [reviewed by Salomon and Maxwell (1995)] and Mattos et al. (1982) [reviewed by Salomon and Maxwell (1995)], who reported no differences when glycerol was added at either

32 or 3°C and 22 or 5°C, respectively. Similarly, in other reports, no differences were observed for bovine sperm glycerated at 5, 10, 20 or 35°C (Salisbury et al. 1978) and the quality of cryopreserved dog sperm was not influenced by a glycerol exposure at either 37 or 4°C (Pena et al. 1998).

However, the results of exp. 2 contradict Blackshaw (1960) and Colas (1975), who found that the addition of glycerol at 5 and 4°C, respectively, was more suitable for cryopreservation of ram sperm than addition of glycerol at 30°C. According to Colas (1975), glycerol toxicity on ram sperm is reduced when added at a temperature near 0°C. Despite those findings, the addition of glycerol in a one-step dilution close to body temperature is practical and is a widely used method of preparing ram semen for freezing (Evans and Maxwell 1987).

It is well known that freezing and thawing of ram sperm lead to severe cellular changes, including altered membrane integrity, reduced motility and fertilising ability [reviewed by Maxwell and Watson (1996)]. Immediately after thawing and diluting in SOF-m, spermatozoa showed more B (capacitated) and AR (acrosome reacted) patterns than in fresh semen (Fig. 8). Therefore, the majority of the sperm were already capacitated after thawing. Watson (1995) suggested that freezing-thawing cause sperm to bypass certain normal processes and resemble capacitated cells. Bailey et al. (2000) hypothesised that cryopreservation actually induces capacitation ("cryo-capacitation"). Our results support these theories and previous data obtained with bulls (Cormier et al. 1997) and rams (Pérez et al. 1996b; Gillan et al. 1997) that showed cryopreservation accelerates or induces capacitation.

CONCLUSION

Glycerol is mainly reserved for cryopreserved semen, which is likely why there are few studies on its effects during fresh semen conservation. Despite previous reports of glycerol's negative effects on sperm function, the present study used several in vitro assays of sperm quality over time in physiological conditions and did not observe any detrimental impact of glycerol. However, for the ovine industry to fully reap the benefits of artificial insemination with both fresh and cryopreserved ram semen, further investigations in vivo should test the effect of the presence and inclusion method (time and temperature) of glycerol.

ACKNOWLEDGEMENTS

We thank the *Centre d'expertise en production ovine du Québec* and *Centre d'insémination ovine du Québec* for providing the semen and Jean Paulin for technical assistance. This work was supported by *le Conseil des recherches en pêche et en agroalimentaire du Québec* (CORPAQ).

Abe, H., Yutaka, S., Takeshi, S. and Hoshi, H. 1995. Bovine oviduct-specific glycoprotein; a potent factor for maintenance of viability and motility of bovine spermatozoa in vitro. Mol. Reprod. Dev. **42**: 226–232.

Abdelhakeam, A. A., Graham, E. F. and Vazquez, I. A. 1991. Studies on the presence and absence of glycerol in unfrozen and frozen ram semen: fertility trials and the effects of dilution methods on freezing ram semen in the absence of glycerol. Cryobiology **28**: 36–42.

Bailey, J. L., Bilodeau J.-F. and Cormier, N. 2000. Semen cryopreservation in domestic animals; a damaging and capaciting phenomenon. J. Androl. **21**: 1–7.

Barth, A. D. and Oko, R. J. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames, Iowa. 285 pp.

Baril, G., Chemineau, P., Cognie, Y., Guérin, Y., Leboeuf, B., Orgeur, P. and Vallet, J.-C. 1993. Manuel de Formation pour l'Insémination Artificielle Chez les Ovins et les Caprins. Station de la physiologie de la reproduction, Institut national de la recherche agronomique (INRA), Nouzilly, France, 231 pp.

Blackshaw, A. W. 1960. The effects of milk diluent on the viability of ram spermatozoa and their reviva1 after freezing. Aust. Vet. J. **36**: 432–435.

Canadian Council on Animal. 1993. Guide to the care and use of experimental animals. Volume 1. E. D. Olfert, B. M. Cross, and A. A. Mc Williams, eds. CCAC, Ottawa, ON.

Chavarria, M. E. and Reys, A. 1996. Secretions of ovine uterus and oviduct induce in vitro capacitation of ram spermatozoa. Arch. Androl. **36**: 17–23.

Colas, G. 1975. Effect of initial freezing temperature, addition of glycerol and dilution of the survival and fertilizing ability of deep-frozen ram semen. J. Reprod. Fertil. **42**: 277–285.

Cormier, N., Sirard, M.-A. and Bailey, J. L. 1997. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. J. Androl. **18**: 461–468.

Evans, G. and Maxwell, W. M. C. 1987. Frozen storage of semen Pages 122–141 *in* Salomon's artificial insemination of sheep and goats. Butterworths, Sydney, Australia.

Fahy, G. M. 1986. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology **23**: 1–13.

Fraser, L. R., Abeydeera, L. R. and Niwa, K. 1995. Ca^{2+}-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol. Reprod. Dev. **40**: 233–241.

Gillan, L., Evans, G. and Maxwell, W. M. C. 1997. Capacitation status and fertility of frozen–thawed ram spermatozoa. Reprod. Fertil. Dev. **9**: 481–487.

Hackett A. J. and Wolynetz, M. S. 1982. Reproductive performance of totally confined sheep bred with semen extended in a lactose-egg yolk-glycerol buffer and stored at 5°C. Can. J. Comp. Med. **46**: 327–333.

Hammerstedt, R. H., Graham, J. K. and Nolan, P. 1990. Cryopreservation of mammalian sperm: What we ask them to survive. J. Androl. **11**: 73–88.

Hammerstedt, R. H. and Graham J. K. 1992. Cryopreservation of poultry sperm: the enigma of glycerol. Cryobiology **29**: 26–38.

Lee, M. A., Trucco, G. S., Bechtol, K. S., Wummer, N., Kopf, G. S., Blasco, L. and Storey, B. T. 1987. Capacitation and acrosome reaction in human spermatozoa monitored by a chlortetracycline fluorescence assay. Fertil. Steril. **48**: 649–658.

Maxwell, W. M. C. and Watson, P. F. 1996. Recent progress in the preservation of ram semen. Anim. Reprod. Sci. **42**: 55–65.

Molinia, F. C., Evans, G. and Maxwell, W. M. C. 1994. Incorporation of penetrating cryoprotectants in diluents for pellet-freezing ram spermatozoa. Theriogenology **42**: 849–858.

Pena, A. I., Barrio, F., Quintela, L. A. and Herradon P. G. 1998. Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. Theriogenology **50**: 163–174.

Pérez L. J., Valcárce, I. A., de las Heras M. A., Moses, D. F. and Baldassarre, H. 1996a. In vitro capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. Theriogenology **45**: 1037–1046.

Pérez, L. J., Valcárce, I. A., de las Heras, M. A., Moses, D. F. and Baldassarre, H. 1996b. Evidence that frozen/thawed spermatozoa show accelerated capacitation in vitro as assessed by chlortetracycline assay. Theriogenology **46**: 131–140.

Pérez, L. J., Valcárce, I. A., de las Heras M. A., Moses, D. F. and Baldassarre, H. 1997. The storage of pure ram semen at room temperature results in capacitation of a subpopulation of spermatozoa. Theriogenology **47**: 549–558.

Saling, P. M. and Storey, B. T. 1979. Mouse gamete interactions during fertilization in vitro. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. J. Cell. Biol. **83**: 544–555.

Salisbury, G. W., Vandemark, N. L. and Lodge, J. R. 1978. Principles and techniques of freezing spermatozoa. Pages 494–554 *in* W. H. Freeman, ed. Physiology of reproduction and artificial insemination of cattle. Freeman and Co., San Francisco, CA.

Salomon, S. and Maxwell, W. M. C. 1995. Frozen storage of ram semen 1. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci. **37**: 185–249.

Salomon, S. and Maxwell, W. M. C. 2000. Storage of ram semen. Anim Reprod. Sci. **62**: 77–111.

SAS Institute, Inc. 1990. SAS/STAT® user's guide: Statistics. Version 6, 4th ed. Vol 2. SAS Institue, Inc., Cary, NC.

Slavik, T. 1987. Effect of glycerol on the penetrating ability of fresh ram spermatozoa with zona-free hamster eggs. J. Reprod. Fertil. **79**: 99–103.

Tajima, A., Graham, E. F. and Hawkins, D. M. 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. J. Reprod. Fertil. **85**: 1–5.

Tardif, S., Laforest, J. P., Cormier, N. and Bailey J. L. 1999. The importance of porcine sperm parameters on fertility in vivo. Theriogenology **52**: 447–459.

Vidament, M., Ecot, P., Noue, P., Bourgeois, C., Magistrini, M. and Palmer, E. 2000. Centrifugation and addition of glycerol at 22°C instead of 4°C improve post-thaw motility and fertility of stallion spermatozoa. Theriogenology **54**: 907–919.

Ward, C. R. and Storey, B. T. 1984. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev. Biol. **104**: 287–296.

Watson, P. F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. **7**: 871–891.