Conservation of fresh ram spermatozoa at 5°C in the presence of seminal plasma

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Morrier, A., Castonguay, F. and Bailey, J. L. 2003. **Conservation of fresh ram spermatozoa at 5°C in the presence of seminal plasma**. Can. J. Anim. Sci. **83**: 221–227. Seminal plasma aids sperm transport and contains factors beneficial for sperm function. In artificial insemination, however, diluting the semen reduces the concentration of seminal plasma. To test the hypothesis that supplemental seminal plasma in extended ram semen improves conservation at 5°C, we added various concentrations of seminal plasma to semen during storage, and investigated subsequent sperm function in vitro. Semen was divided into three aliquots, extended in a commercial diluent (Triladyl) supplemented with 0, 10 or 25% (vol:vol) ovine seminal plasma and cooled to 5°C. After 8 and 24 h at 5°C, sperm were suspended in a modified synthetic oviduct fluid (SOF-m) at 39°C to mimic the female genital tract at insemination. Sperm aliquots were assessed for motility and chlortetracycline fluorescence after 0, 4 and 8 h in the SOF-m. No significant differences were observed due to seminal plasma supplementation during conservation at 5°C or incubation in SOF-m at 39°C. However, decreased sperm motility and fewer non-capacitated sperm were observed concomitant with an augmentation of capacitated and acrosome-reacted cells during incubation in SOF-m. Therefore, the hypothesis that diluent supplementation with homologous seminal plasma improves ram sperm conservation or subsequent sperm function was not supported.

Key words: Ovine, ram, sperm, motility, viability, chlortetracycline fluorescence, artificial insemination, SOF

Morrier, A., Castonguay, F. et Bailey, J. L. 2003. **Conservation des spermatozoïdes frais de bélier à 5 °C en présence de plasma séminal**. Can. J. Anim. Sci. **83**: 221–227. Le plasma séminal facilite le transport du sperme et renferme des facteurs qui lui sont bénéfiques. Lors de l'insémination artificielle cependant, en diluant la semence, on réduit aussi la concentration du plasma. Pour vérifier l'hypothèse qu'un supplément de plasma séminal améliorerait la conservation du sperme de bélier allongé à 5 °C, les auteurs ont ajouté à ce dernier une quantité variable de plasma séminal pendant le stockage et examiné subséquemment la motilité des spermatozoïdes *in vitro*. Le sperme a été divisé en trois parties, allongé avec un diluant commercial (Triladyl) auquel on a ajouté 0, 10 ou 25 % (v:v) de plasma séminal d'ovin puis a été refroidi à 5 °C. Après avoir passé 8 ou 24 heures à cette température, le sperme a été mis en suspension dans un fluide synthétique modifié imitant celui des trompes de Fallope (SOF-m) à 39 °C, de manière à reproduire les conditions dans le tractus génital de la femelle à l'insémination. Les auteurs ont évalué la motilité des spermatozoïdes et la fluorescence à la chlortétracycline des aliquotes de sperme laissés 0, 4 et 8 heures dans le SOF-m. Aucune variation sensible n'a été relevée à la suite de l'addition de plasma séminal lors de la conservation du sperme à 5 °C ou son incubation dans le SOF-m à 39 °C. Néanmoins, la motilité réduite des spermatozoïdes et le plus petit nombre de spermatozoïdes non capacités sont cohérents avec la hausse du nombre de cellules capacitées et de cellules avec réaction acrosomique pendant l'incubation dans le SOF-m. On en déduit que la dilution du sperme de bélier avec du plasma séminal homologue n'améliore pas sa conservation ni le fonctionnement subséquent des spermatozoïdes.

Mots clés: Ovins, bélier, sperme, motilité, viabilité, fluorescence à la chlortétracycline, insémination artificielle, fluide synthétique modifié des trompes de Fallope

Seminal plasma facilitates sperm transport in the female genital tract [reviewed by Yanagimachi (1994)] and may regulate capacitation by contributing decapacitation factors to the sperm (Maxwell and Johnson 1999). However, artificial insemination implies the dilution of semen, which removes certain proteins adsorbed to the sperm surface, and reduces the concentration of natural antioxidants and other compounds present in seminal plasma. In fact, it has been suggested that better diluents for freezing and storage of semen would be formulated following the systematic study of epididymal compounds (Setchell et al. 1993) or seminal plasma proteins (Barrios et al. 2000).

The specific effects of seminal plasma on sperm during semen conservation are controversial, but many recent reports indicate a positive influence of seminal plasma components on sperm quality. The inclusion of seminal plasma

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Abbreviations: **AR**, for acrosome-reacted sperm with dull fluorescence over the head; **B**, capacitated sperm for those with fluorescence only along the acrosome; **CTC**, chlortetracycline; **F**, non-capacitated sperm for those with uniform head fluorescence; **SOF-m**, synthetic oviduct fluid; **VAP**, medium path velocity

in the media improved the viability and membrane integrity of boar (Maxwell et al. 1998) and ram sperm (Maxwell et al. 1997) subjected to flow cytometric sorting. Seminal plasma can suppress premature capacitation and acrosome reactions (Cross 1993), due to the presence of membrane-stabilizing decapacitation factors (Maxwell and Johnson 1999). Furthermore, its proteins are thought to prevent or restore the membrane integrity of cold-shocked ram sperm (Barrios et al. 2000; Pérez-Pé et al. 2001). Although removal of seminal plasma before freezing increased the viability of ram sperm, plasma membrane integrity, motility and heterogeneity were enhanced when either whole seminal plasma or a specific fraction were added to the semen diluent (Ollero et al. 1997a,b, 1998). Moreover, Pérez-Pé et al. (2002) reported that seminal plasma prevented the appearance of a capacitation-associated tyrosine phosphoprotein in ram sperm during cold-shock, reinforcing the theory that seminal plasma components stabilize the sperm plasma membrane.

Maxwell et al. (1999) demonstrated that the addition of seminal plasma to frozen-thawed semen prior to artificial insemination improved sperm motility, with more uncapacitated and fewer acrosome-reacted sperm compared to unsupplemented controls. Furthermore, it was found that the addition of seminal plasma increased pregnancy rates of ewes after cervical insemination with frozen-thawed ram sperm. Therefore, the hypothesis of the present study was that the addition of homologous seminal plasma to a commercial semen diluent improves ram sperm quality during conservation at 5°C. Motility and physiological status assessed by the chlortetracycline (CTC) fluorescence assay 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 were conducted in accordance with the guidelines of the Canadian Council on Animal Care (1993). All products used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Seminal Plasma Preparation

One ejaculate from each of 10 rams (two Charollais, two Polled-Dorset, one Romanov, three Hampshire, and two Texel) was pooled and centrifuged (12 000 × *g*, 15 min, 22°C). Ejaculates with less than 75% sperm motility were rejected. The supernatants were collected and held at –20°C until use (Catt et al. 1997).

Semen Collection

Six mature Polled-Dorset, one Hampshire, one Texel, one Charollais, one Arcott Canadian, and two Arcott Rideau rams aged from 2 to 7 yr were housed at the Centre d'Insémination Ovine du Québec (CIOQ, La Pocatière, QC). Rams were under light regime, alternating from long (16 h of light) to short days (8 h of light) monthly. Semen was collected twice consecutively with an artificial vagina and the two ejaculates were then pooled for experimentation. Therefore, each male was used once for this experiment ($n = 12$). Volume was determined and morphology and subjective motility were visually assessed by a trained andrologist, using phase contrast microscopy (400×) at room temperature. Samples with less than 75% motile sperm were rejected. Sperm concentration was assessed with a calibrated spectrophotometer (Spectronic 20D, Bausch and Lomb, Markham, ON).

Semen Preparation and Conservation

Immediately after collection, the semen was divided into three aliquots. Each aliquot was diluted with Triladyl (Minitube Canada, Woodstock, ON), a commercial diluent prepared with 20% egg yolk (from commercial eggs kept at 4°C until use), as per manufacturer's directions. Triladyl is composed of Tris [Tris(hydroxymethyl)aminomethane], citric acid, fructose and 7% glycerol containing tylosin, gentamycin, lincomycin and spectinomycin as antibiotics. Each aliquot was supplemented with either 0, 10 or 25% (vol:vol) seminal plasma. Semen was extended at room temperature with Triladyl ± seminal plasma (prewarmed to 30°C) to reach a final concentration of 1.6×10^9 sperm mL^{-1} (Baril et al. 1993) as normally conducted at CIOQ. Diluted semen was placed into straws of 250 µL (IMV, l'Aigle, France), sealed with polyvinyl acid, progressively cooled to 5°C over 3 h in a transport box (CIOQ), and subsequently maintained at this temperature. This specially designed box contains the exact proportion of ice and water at 30°C in order to reach 5°C in 3 h (approximately 0.14°C min^{-1}); thereafter, the temperature can be maintained at 5°C for 36 h. The same transport box was used for all replicates.

At 8 and 24 h post-collection, one sealed straw of semen was cut open (with clean scissors) and diluted (about 32-fold; to 50×10^6 sperm mL^{-1}) in synthetic oviductal fluid (SOF-m; Morrier et al. 2002). 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 estrus sheep serum (the same lot for all replicates) and adjusted to pH 7.36, 290 mOsm kg^{-1}.

Sperm quality (motility and 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 4 and 8 h in the SOF-m, sperm quality was re-assessed. Figure 1 shows a schematic description of this protocol.

Sperm Motility Analysis

For the assessment of sperm motility, three 2-µL samples of sperm in SOF-m (~50×10^6 cells mL^{-1}) were deposited into a 10-µm Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) placed on the motility analyser (Hamilton Thorn Research, Beverly, MA) as described by Morrier et al. (2002). At least 200 cells from five random fields were recorded. The settings used were the following:

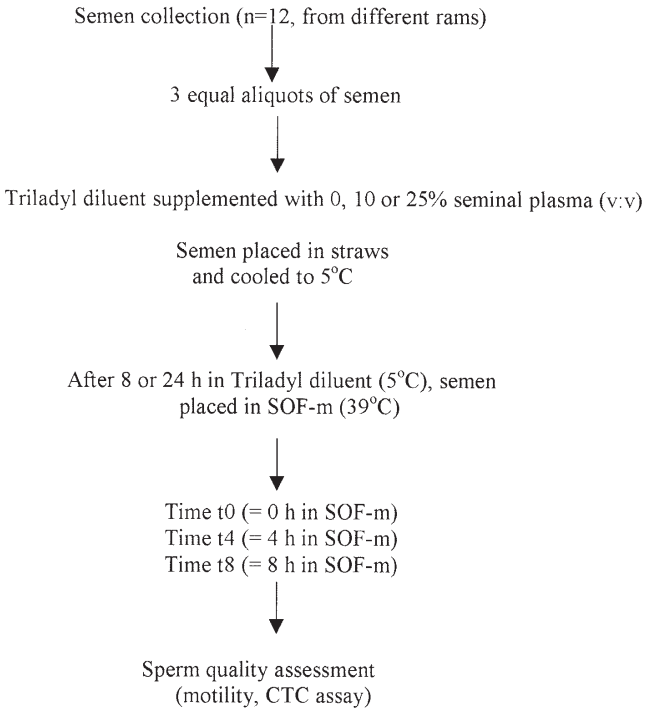

Fig. 1. Outline of experimental protocol.

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; and threshold (STR), 60. Only progressive (proportion of cells moving with a certain straightness) and total motilities (or motile sperm; proportion of cells moving at or above a minimum speed) were determined for this study.

Chlortetracycline Fluorescence Assay

The CTC fluorescence assay used was conducted as described previously (Morrier et al. 2002). In brief, the CTC solution was 5 mM cysteine and 750 μM CTC diluted in a buffer of 20 mM Tris base and 130 mM NaCl (pH 7.8). For the assay, 15 μL CTC solutions were mixed with 10 μL sperm diluted in SOF-m, and fixed with 0.5 μL 12.5% (vol:vol) glutaraldehyde solution prepared in 2.5 M Tris base. Ten μL of this suspension were placed on a microscope slide, covered with a coverslip and evaluated by fluorescent microscopy. Slides were prepared in duplicate and 200 sperm slide^{-1} were scored at 400× magnification. The CTC patterns were scored as described by Gillan et al. (1997) as follows: "F" or non-capacitated sperm for those with uniform head fluorescence; "B" or capacitated sperm for those with fluorescence only along the acrosome; and "AR" for acrosome-reacted sperm with dull fluorescence over the head.

Statistical Analyses

The normality of the data was confirmed. Analysis of variance was performed using the General linear models procedure, followed by the Fisher's protected least significant differences (LSD) test when there were significant variable effects. The statistical model used for sperm parameters (percentage motile, percentage progressively motile, and percentage CTC pattern F, B and AR sperm) included the effect of ram, the volume of seminal plasma added (treatment), duration of conservation in the diluent at 5°C, and time of incubation in SOF-m at 39°C. The two-way interactions among these variables were also tested and the residual error was the error term. Significance was judged at the probability level of $P < 0.05$. SAS Institute, Inc. (1990) software was used for the analyses.

RESULTS

The initial concentrations of the ejaculates and total motility were $3.67 \times 10^9 \pm 1.31$ sperm mL^{-1} and 88 ± 4% (means ± SD), respectively, with less than 25% morphologically abnormal sperm for each ejaculate. The presence of seminal plasma in the Triladyl diluent did not affect the percentage of total motile sperm ($P = 0.5321$; Fig. 2) at any of the time points studied. However, the percentage of total motile sperm increased very slightly during conservation in diluent at 5°C (83% after 8 h versus 86% after 24 h, with a least significant difference of 2.4%; $P < 0.05$). The percentage of total motile sperm decreased during subsequent incubation in SOF-m at 39°C ($P < 0.05$) at all times (0 to 4 to 8 h).

The percentage of progressively motile sperm was not affected by the seminal plasma treatments at any of the time points studied ($P = 0.8932$; Fig. 3). However, the conservation in Triladyl diluent at 5°C increased the percentage of progressively motile sperm (41% after 8 h versus 44% after 24 h when the data of all SOF time points are combined, with a least significant difference of 1.7%; $P < 0.05$). The percentage of progressively motile sperm was lower during incubation in SOF-m at 39°C ($P < 0.05$) after 8 h of conservation at 5°C. The percentage of progressively motile sperm actually increased after 4 h of incubation in SOF-m at 39°C, then decreased again when the semen was conserved for 24 h in Triladyl (46, 49 and 36% for times 0, 4 and 8 h in SOF-m, respectively; all times were significantly different with a least significant difference of 2.3%).

The percentage of sperm displaying the non-capacitated CTC pattern (pattern F) was not affected by the addition of seminal plasma to the Triladyl diluent at any of the time points studied ($P = 0.5701$, Fig. 4). However, the percentages of F pattern sperm decreased during conservation in Triladyl diluent at 5°C and incubation in SOF-m at 39°C ($P < 0.05$). The inclusion of seminal plasma did not affect the frequency of AR-pattern sperm ($P = 0.6027$) either. Contrary to the F pattern, however, the percentage of AR pattern sperm increased with time in either the Triladyl at 5°C or SOF-m at 39°C. The percentage of B pattern sperm was increased due to conservation Triladyl at 5°C ($P < 0.05$) and duration of incubation in SOF-m ($P < 0.05$).

DISCUSSION

In the light of reports by Maxwell et al. (1997, 1999) on the benefits of seminal plasma on sperm function, our study utilized a different, more practical approach: the addition of

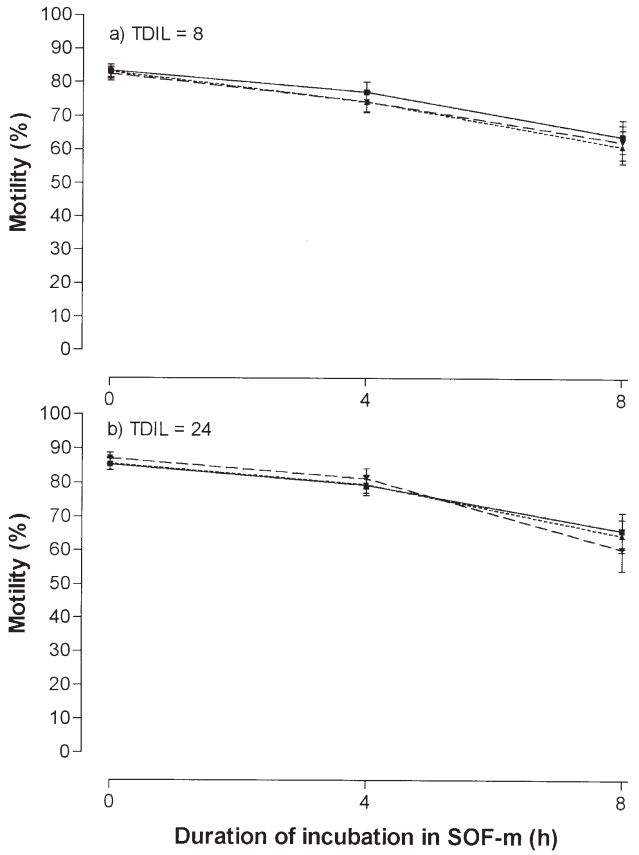

Fig. 2. Percentages of motile sperm (means ± SE) from ram semen conserved in a liquid state at 5°C for (a) 8 or (b) 24 h in Triladyl diluent (TDIL = 8 and TDIL = 24, respectively) containing 0, 10 or 25% (vol:vol) seminal plasma, and after incubation in SOF-m at 39°C, 5% CO_2 for 0, 4 and 8 h (n = 12). Seminal plasma did not affect the percentage of motile sperm (P = 0.5321). Sperm motility increased very slightly during 8 and 24 h of storage in Triladyl diluent at 5°C ($P < 0.05$). Within time of conservation in Triladyl (TDIL = 8 or TDIL = 24), motility declined ($P < 0.05$) with each successive duration (0, 4 and 8 h) of incubation in SOF-m at 39°C.

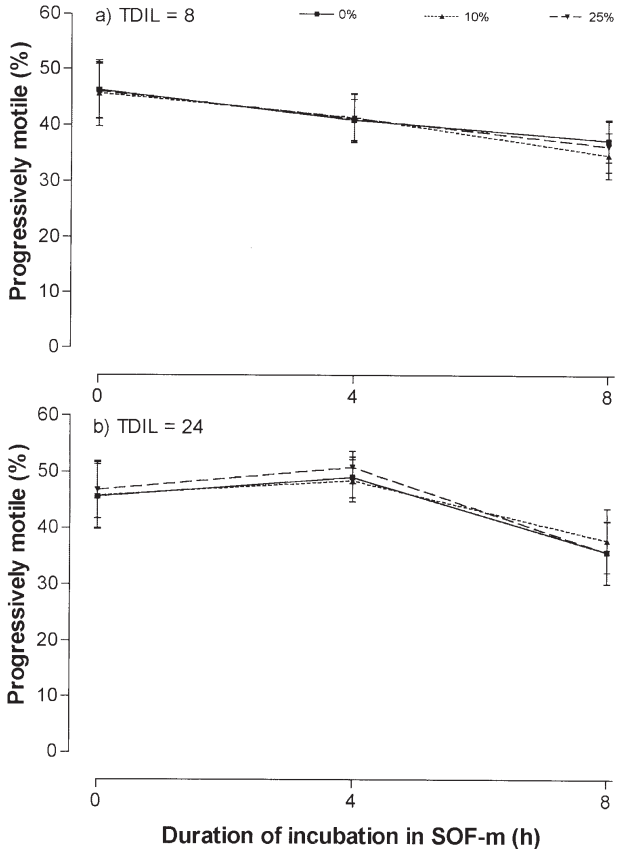

Fig. 3. Percentages of progressively motile ram sperm (means ± SE) conserved in liquid state at 5°C for (a) 8 or (b) 24 h in Triladyl diluent (TDIL = 8 and TDIL = 24, respectively) containing 0, 10% or 25% (vol:vol) seminal plasma and after dilution in SOF-m at 39°C, 5% CO_2 for 0, 4 and 8 h (n = 12). Seminal plasma did not affect the percentage of progressively motile sperm (P = 0.8932). The percentage of progressively motile sperm differed due to conservation in the Triladyl diluent at 5°C only at 4 h of incubation in SOF-m ($P < 0.05$) (TDIL = 8 compared to TDIL = 24). Within time of conservation in Triladyl (TDIL = 8 or TDIL = 24), motility declined ($P < 0.05$) with each successive duration (0, 4 and 8 h) of incubation in SOF-m at 39°C.

seminal plasma during dilution for artificial insemination, avoiding semen manipulation post-conservation. We tested the hypothesis that supplementation of ram semen with homologous seminal plasma during chilled conservation would protect sperm function. It is commonly recommended to use cooled ram semen for insemination within 8 h of collection (Baril et al. 1993), due to a marked decline in sperm fertilizing capacity within 24 h (Evans and Maxwell 1987; Maxwell and Salamon 1993). Therefore, we evaluated sperm parameters after 8 h (which is within the recommended time frame for artificial insemination) and after 24 h (when the first marked drop in sperm function is evident).

Contrary to our hypothesis that seminal plasma improves ram sperm conservation at 5°C, sperm motility, viability and distribution of CTC-patterns were not improved with the inclusion of 10 or 25% seminal plasma. Although several studies have provided observations that seminal plasma added to semen would be helpful to sperm (Barrios et al. 2000; Graham 1994), others have demonstrated that seminal plasma is detrimental to sperm motility (de Lamirande and Gagnon 1984) and viability (García and Graham 1987). Ollero et al. (1997b) suggested that those controversial results could be explained by the presence of two components in ram seminal plasma. A higher molecular weight compound, present in the >10 kDa seminal plasma fraction (Ollero et al. 1997a) would protect ram sperm; however, a low molecular weight factor would prevent the adsorption of beneficial seminal plasma proteins to the sperm after ejaculation.

Several studies have reported a beneficial effect of seminal plasma on sperm undergoing flow-cytometric processing (Ashworth 1994; Catt et al. 1997; Maxwell et al. 1997). However, these studies were performed at high dilution rates ($< 1 \times 10^7$ sperm mL^{-1}). At such high dilution levels, the cells die rapidly, a phenomenon called the "dilution effect" [Mann and Lutwak-Mann (1981) in Maxwell and Johnson (1999)], which particularly affects ram sperm

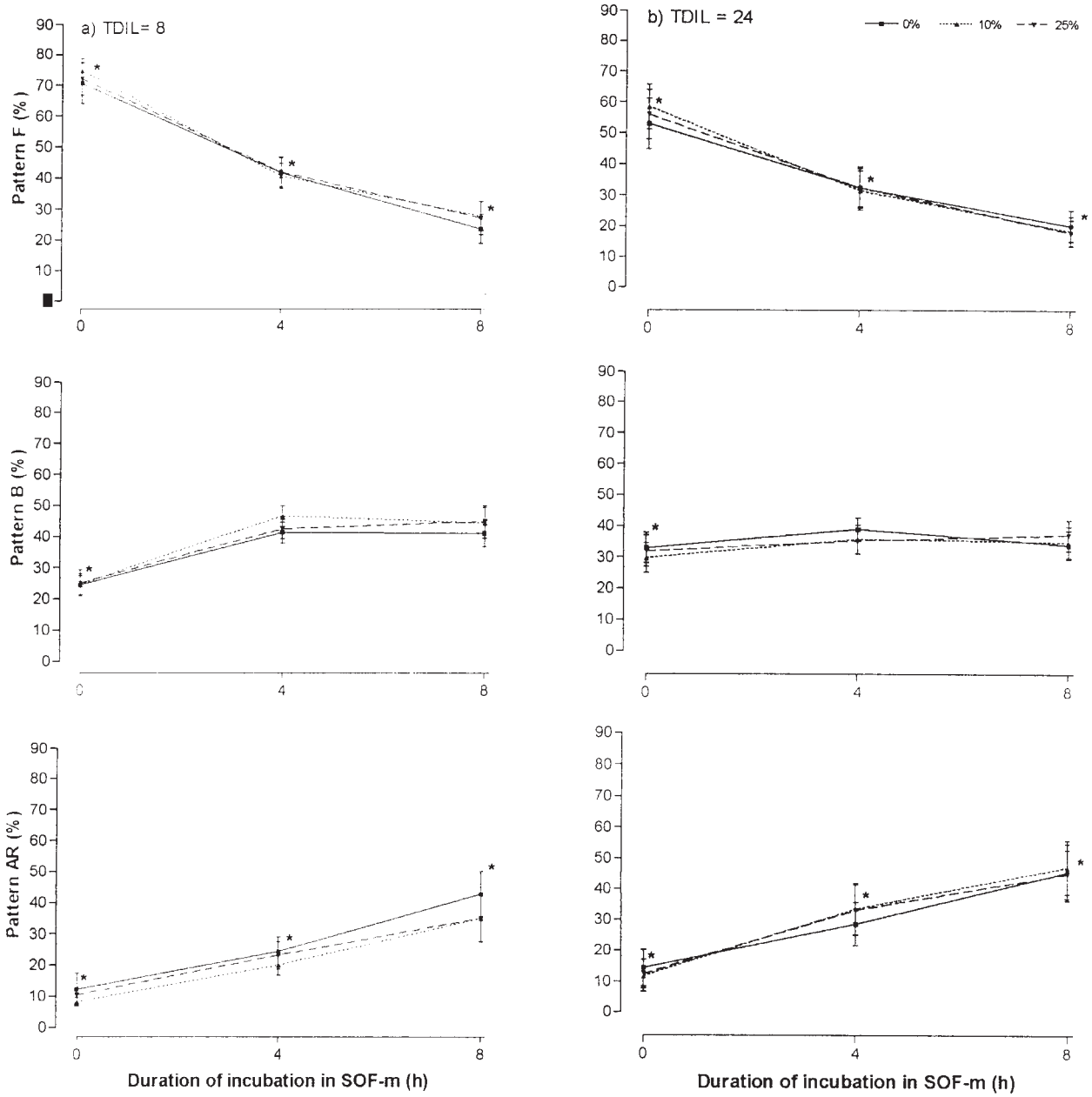

Fig. 4. Percentages of sperm (means ± SE) displaying CTC patterns F, B and AR during conservation in a liquid state at 5°C for (a) 8 or (b) 24 h in Triladyl diluent (TDIL = 8 and TDIL = 24, respectively) containing 0, 10% or 25% (vol:vol) seminal plasma, and after dilution in SOF-m at 39°C, 5% CO_2 for 0, 4 and 8 h (n = 12). The presence of seminal plasma did not affect CTC pattern distribution ($P > 0.05$). The overall CTC pattern distribution differed within time of incubation in SOF-m at 39°C, due to duration of conservation in Triladyl diluent at 5°C (TDIL = 8 versus TDIL = 24; $P < 0.05$). *For each CTC pattern, the proportion of sperm differs within time of conservation in Triladyl diluent at 5°C (TDIL = 8 or TDIL = 24), due to incubation in SOF-m at 39°C at all times (0, 4 and 8 h; $P < 0.05$).

[Mann (1984) in Maxwell and Johnson (1999)]. However, in our study, 1.6×10^9 sperm mL^{-1} were used (a comparatively low dilution rate). Furthermore, Harrison et al. (1982) suggested that using a protein-depleted medium results in two factors that can explain the dilution effect: (1) poor stimulation of motility and (2) the tendency of live sperm to adhere to container surfaces. According to Harrison et al. (1982), serum albumin protects against the dilution effect by stimulating sperm motility and preventing sperm adhesion. In our study, sperm were diluted in a medium rich in proteins (Triladyl is prepared with 20% egg-yolk). The egg yolk (contained in the diluent) and the estrous ewe serum (present in the SOF-m) probably play the same role as serum albumin, thus possibly explaining why the seminal plasma addition did not improve the physiology of ram sperm.

Maxwell et al. (1999) reported an improvement in fertility rates and sperm motility, an increase in the percentage of uncapacitated sperm, and a concomitant decrease in capaci-

tated and acrosome-reacted sperm when seminal plasma was added to frozen-thawed ram sperm washed of the semen extender prior to cervical insemination of ewes (compared to a control without seminal plasma). Although that study was interesting, their protocol is impractical for large-scale application under field conditions. As well, the beneficial effect of seminal plasma was observed relative to sperm extended only in Dulbecco's phosphate-buffered solution (DPBS). This a protein-free medium; a decrease in the proportion of agglutinated cells when seminal plasma was added to the diluent can also explain the beneficial effect observed. Furthermore, it was not established whether the beneficial results observed were specific to seminal plasma proteins, since no other protein sources were tested. In the present experiment, egg yolk (which is rich in proteins), could have masked the effect of seminal plasma. Bedford et al. (1995) proposed a possible negative interaction between egg yolk and seminal plasma; the mechanism is unknown, but may involve lipid peroxidation and release of free radicals, which would harm sperm and reduce their function (Bedford et al. 1995). In our experiment, it is possible that the beneficial effects of seminal plasma were hidden by the presence of egg yolk. Perhaps a positive effect of seminal plasma would be evident using a less-protein-rich semen diluent, such as a simpler Tris-based extender. Alternatively, pre-incubating freshly collected semen with the supplemental seminal plasma prior to extension might reduce competition between seminal plasma proteins and diluent proteins for the sperm plasma membrane, thereby avoiding any interference by the proteins in the egg yolk-based Triladyl.

In conclusion, the effects of seminal plasma on sperm function and fertility remain controversial. Seminal plasma seems to act in duality: some seminal plasma proteins are beneficial to sperm membranes, while others are detrimental. Further investigation is necessary to understand this complex mixture. In theory, a better understanding of seminal plasma proteins could improve the formulation of better diluents for artificial insemination. Specific protein fractions or even individual proteins from seminal plasma could be screened for positive (or detrimental) effects on sperm function and semen preservation. However, the present work does not indicate that supplementing fresh ram semen (diluted in egg yolk) with additional seminal plasma improves sperm conservation at 5°C.

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