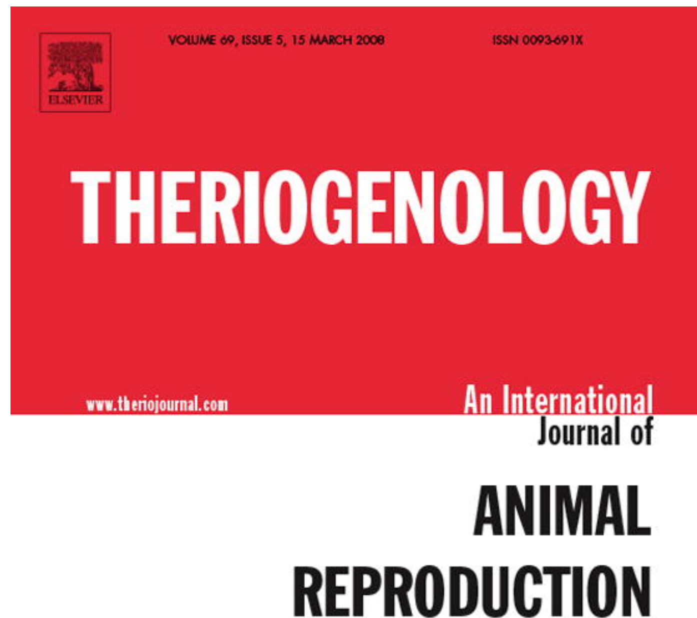
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author's institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

ELSEVIER

Available online at www.sciencedirect.com

Theriogenology 69 (2008) 564–573

Theriogenology

www.theriojournal.com

Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm

M.P. Domínguez [a,1], A. Falcinelli [a,b,1], F. Hozbor [a], E. Sánchez [a], A. Cesari [b,*], R.H. Alberio [a]

[a] *Laboratorio de Biotecnología de la Reproducción, INTA, Ruta 226 Km. 73.5, 7620 Balcarce, Provincia de Buenos Aires, Argentina*
[b] *Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, UNMdP, CC:1245, 7600 Mar del Plata, Buenos Aires, Argentina*

Received 10 April 2007; received in revised form 2 August 2007; accepted 5 November 2007

Abstract

It has been proposed that seminal plasma (SP) in the extender or in post-thaw media can prevent and revert cold-shock damage in cryopreserved ram sperm; however, this was dependent on season. We evaluated sperm parameters from Frisian ram semen incubated for various intervals with SP from all seasons and stored at −18 or −196 °C. At both temperatures, SP from autumn or winter increased ($P < 0.05$) sperm motility, whereas no SP, or SP from spring or summer, had no effect. However, neither viability nor membrane or acrosomal status were modified by SP. Thirteen SP proteins were bound to the sperm surface (16.1, 16.7, 17.4, 23.3, 25.2, 27.5, 35.0, 40.0, 49.0, 53.5, 55.5, 61.0, and 86.0 kDa). The SP proteins that bound to sperm were affected by season, but not by conservation temperature. Sperm incubated with SP from autumn had increased concentrations of five proteins; two were identified (with specific antibodies) as RSVP14 and RSVP20. In conclusion, SP from autumn and winter improved sperm motility of frozen-thawed ram sperm, and storage of ram SP at −18 or −196 °C did not affect protein composition. The SP proteins that bound to the sperm surface may be responsible for sperm membrane stabilization and should be further investigated.
© 2007 Elsevier Inc. All rights reserved.

Keywords: Ram sperm; Cryopreservation; Seminal plasma; Membrane status; Sheep

1. Introduction

Cryopreservation of sperm caused ultrastructural, biochemical, and functional changes that mainly affected the sperm plasma membrane, similar to the physiological changes related to sperm capacitation [1–3]. Consequently, post-thaw sperm viability and therfore fertility was affected, [4–7], as the sperm were prematurely ready to fertilize the ovum. Ram sperm were more sensitive to freezing than gametes from other mammalian species, producing very low pregnancy rates to cervical AI with frozen-thawed semen [8]. Because of the reduced performance of the frozen-thawed ram sperm and the anatomical characteristics of the ewe's cervix, the source of sperm used for cervical insemination is restricted to fresh semen, whereas frozen-thawed semen can be used solely for intrauterine insemination. The latter is used for breeding animal production due to its high price [8,9]. That there were seasonal variations in the quality of ram sperm [10], and that cryotolerance was related to sperm quality, semen collection was limited to the breeding season (autumn, in temperate regions).

* Corresponding author. Tel.: +54 223 4753030; fax: +54 223 4753150.
E-mail address: acesari@mdp.edu.ar (A. Cesari).
[1] These authors contributed equally to this work.

0093-691X/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2007.11.010

Author's personal copy

Seminal plasma (SP) is a mixed secretion from several glands of the male reproductive tract. Similar to many other body fluids, it has a high concentration of certain proteins, making it difficult to identify proteins present in low concentrations [11]. Several SP proteins are adsorbed to the ejaculated sperm surface [12–14]; they maintain the stability of the sperm plasma membrane until capacitation begins in the female reproductive tract [15], where their removal is a prerequisite for fertilization [13]. Several SP components prevented and reverted cold-shock damage on the sperm membrane and improved the viability and fertility of frozen-thawed sperm [16–21].

In bovine SP, a group of proteins called BSP (bovine seminal plasma proteins) associated with the sperm surface and modulated sperm function [22]. Homologous SP proteins were reported in the horse [23,24], pig [25,26], goat [27], bison [28], and sheep [29,30]. There were seasonal variations in quantity and quality of SP proteins [5,31–33], like due to differential protective effects of SP (against cold-shock). This protective effect may be related to antioxidant enzyme activity [34]. Some authors demonstrated the protective activity to two major proteins expressed in the seminal vesicles, called RSVP14 and RSVP20 [19,20], showing that several SP proteins may interact with the sperm surface. The transference of these findings to ram semen cryopreservation make necessary to preserve the SP and to know if it could be collected along the year.

The aims of this work were to determine if (1) the ability of SP to improve sperm parameters (motility, viability, plasma membrane integrity, and capacitation status) was influenced by the conservation temperatures and the season of collection; (2) SP protein composition was affected by season and temperature at which it was stored; and (3) the ability of SP proteins to bind to the sperm surface membrane was affected by storage temperature or season.

2. Materials and methods

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.1. Collection and freezing of semen

Semen was collected (artificial vagina) from three mature *Ost-Friesisches Milchschaf* (Frisian) rams during the autumn (May 2005). Ejaculates were maintained at 32 °C, and those with good wave motion (≥4; range 0–5), 80% progressively motile sperm, and >2.5×10^9 sperm/mL were pooled, maintained at 32 °C, and diluted (1×10^9 cells/mL) in an extender containing 300 mM Tris, 37.7 mM citric acid, 94.7 mM glucose, 20% (v/v) egg yolk, 5% (v/v) glycerol, sodium G penicillin (1×10^6 IU/L) and streptomycin sulfate (1 g/L). The diluted semen was cooled to 5 °C over 2 h, and then held at that temperature for another 2 h. Drops of 150 μL of diluted semen were dispensed in concavities of dry ice. After 10 min, the frozen pellets were plunged in liquid N_2 and stored until thawing.

2.2. Thawing and suspension of sperm

Pellets were held in glass tubes containing 300 μL of extender without egg yolk and glycerol in a water bath at 37 °C. To obtain a viable sperm population, the frozen-thawed semen was centrifuged (700 × *g*, 10 min) at room temperature (RT) in a Percoll® gradient with physiological solution (0.15 M ClNa) in 90–60–30% dilutions. The pellet (200 μL) was collected and suspended in various media (according to each treatment) for 45 or 90 min in a water bath at 37 °C.

2.3. Seminal plasma collection, freezing and thawing

The SP was separated from the semen of the same rams used for sperm cryopreservation. The dates of collection were autumn: 30 March 2006, 1 April 2006, 3 April 2006, 11 April 2006; winter: 18 August 2005, 30 August 2005; spring: 27 September 2005, 30 September 2005; 28 October 2005, 1 November 2005; and summer: 19 January 2006, 23 January 2006, 25 January 2006, 28 January 2006. Semen was centrifuged (2000 × *g*, 15 min, 4 °C) and the supernatants recovered were centrifuged again for SP collection. Finally, the supernatants were pooled (same proportion from each ram) and the SP pools were filtered through a 0.22 μm nitrocellulose acetate membrane (Microclar®, Tigre, BA, Argentina) and divided in two cryovials. Protein concentration of both fractions of the filtrate indicated that no protein was lost during filtration. Each aliquot was stored at either −18 or −196 °C (SP-18 and SP-196, respectively) and subsequently thawed at room temperature.

2.4. Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoresed in 15% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS-PAGE) at RT and 20 mA/gel in a Hoefer

Author's personal copy

mini VE device (Amersham GE, Buckinghamshire, UK), according to Laemmli [35]. Samples were mixed with 5× Laemmli sample buffer conditions and boiled. The molecular weight was estimated using protein low molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were stained with Coomassie brilliant blue R-250.

2.5. Western blotting

After electrophoresis proteins were transferred to PVDF membranes, blocked and incubated with polyclonal antibodies against RSVP14 and RSVP20 (1:2000 and 1:500, respectively) and then incubated with Cy^{IM}5-conjugated goat anti-rabbit IgG (1:5000, Amersham). After extensive washing, the membranes were scanned in a Storm 840 scanner (Amersham) and visualized by excitation at 649 nm and emission at 670 nm. Results were analysed by ImageQuant TL software (Amersham). Polyclonal antibodies against RSVP14 and RSVP20 were generously provided by Dr. Muiño-Blanco [19].

2.6. Protein concentration

Protein concentration of both SP and sperm were assessed by the Bradford method [36].

2.7. Statistical analyses

All data were assessed for normality of distribution using the Shapiro–Wilk test (PROC UNIVARIATE, [37]) and homogeneity of variance using PROC MIXED [37]. Models with homogeneous residual variance or separate residual variance within each group were compared using Akaike criteria to decide whether heterogeneous variance was necessary. Percentage of sperm motility, viability, sperm with a swollen tail, and CTC-patterns were analysed using PROC MIXED [37]. This procedure was used to perform repeated analysis of covariance to test the effect of incubation time (0, 45, and 90 min), treatment (SP-18, SP-196 and control), and their interaction. Data are presented as least square means and the Tukey–Kramer test was used for comparison. The level of significance was set at $P < 0.05$, unless otherwise stated.

2.8. Experimental design

2.8.1. Assessment of SP effects on frozen-thawed-washed sperm

The concentration of sperm remained constant (5×10^6 sperm/mL). An aliquot (100 μL) of the sperm suspension was incubated (37 °C) with the following solutions (treatments) in 200 μL of final volume: control: PBS; SP-18: 20% (v/v) SP-18 in PBS; and SP-196: 20% (v/v) SP-196 in PBS. At 0, 45, and 90 min of culture, the following sperm parameters (%) were assessed:

- Total sperm motility and progressive individual motility were subjectively assessed (on a warm slide, 37 °C), viewed with an optical microscope (400×). Approximately five fields per sample were examined and motility was estimated in increments of 5%.
- Viability was determined by examining 200 sperm stained with eosin–nigrosin [38].
- HOS+ (positive hypo-osmotic swelling test) was assessed as described by García Artiga [39]. A volume of 10 μL of frozen-thawed sperm was added to 1 mL of the hypo-osmotic solution (100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate) and incubated at 37 °C for 30 min. After incubation, one drop of semen was placed on glass slide, covered with coverslip and evaluated under an optical microscope (400×). At least, 200 sperm were counted and the proportion of sperm with a swollen tail sperm was recorded (HOS+).
- Capacitation status was assessed with chlortetracycline (CTC) staining as described by Perez et al. [40], with slight modifications [41]. A CTC-working solution (750 μM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM DL-cysteine, pH 7.8. An aliquot (5 μL) of semen was mixed with 20 μL of CTC-working solution. After 20 s, the reaction was stopped by the addition of 5 μL of 1% (v/v) glutaraldehyde in 1 M Tris–HCl pH 7.8. Smears (5 μL) were prepared on a clean microscope slide, covered with a coverslip, sealed with nail varnish, and kept in the dark at 5 °C. Samples were examined under an epifluorescent inverted Eclipse TE-300 microscope (Nikon, Japan) within 12 h after staining, using green filters (380 nm excitation and 420 nm emission). At least 200 stained sperm were classified into three categories, according to their CTC patterns [42], as follows: F-pattern (uniform fluorescent head; non-capacitated sperm with an intact acrosome); B-pattern (post-acrosomal region without fluorescence; capacitated sperm with an intact acrosome); and AR-pattern (fluorescent-free head or a thin fluorescent band on the equatorial segment; sperm that had undergone an acrosome reaction).

This procedure was repeated five times each season. Statistical analysis was performed as described above.

Author's personal copy

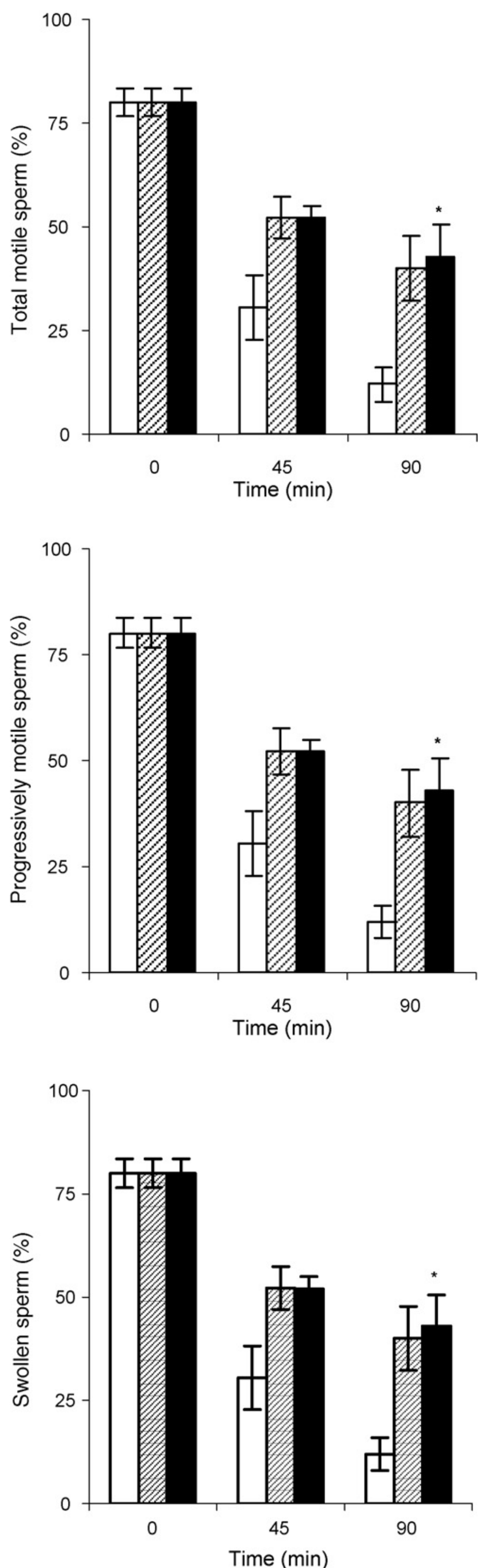


Fig. 1. Mean (±S.E.M.; $n = 5$) percentages of motile and progressively motile sperm, and swollen sperm (HOS test), of frozen-thawed sperm suspended in either SP-18 (cross-hatch bars), SP-196 (dark bars) from autumn or PBS (open bars) immediately after centrifugation (0 min) and after 45 and 90 min incubation at 37 °C, respectively. Within each cluster of three bars, those without a common superscript differed ($P < 0.05$).

2.8.2. Protein analysis

To evaluate the effect of the storage temperature (−20 and −196 °C) and season over the protein composition, total protein concentration was measured and protein separation was performed by SDS-PAGE. The protein pattern of frozen versus fresh spermatozoa were also compared by SDS-PAGE. Three replicates were performed for each assay and a representative picture is shown.

2.8.3. Sperm–SP protein interaction assay

Thawed sperm were washed twice with PBS (300 × g, 10 min) and cell concentration was assessed. Sperm (15 × 10^6) were incubated for 20 min at room temperature with 50 μg of SP proteins and PBS (total volume 150 μL) with constant agitation. Then, the samples were centrifuged (300 × g, 10 min), yielding cells with SP proteins bound to their surface in the pellet and free SP proteins in the supernatant. An aliquot of the supernatant was conserved for SDS-PAGE, and the remainder was incubated with another 15 × 10^6 sperm, allowing a new interaction between the remnant SP proteins in the supernatant and the sperm. Four rounds of interaction were performed until a consistent pattern of SP proteins was detected in the supernatant.

Frozen semen already contains SP; therefore, no new proteins were expected. To determine the increase in SP proteins that were already bound onto sperm, pellets were treated as follows: extraction buffer (1% Tritón-100, 50 mM Tris–HCl pH 9, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF and 2 mM DTT) was added for 1 h at 4 °C. After centrifugation (14,000 × g, 20 min, 4 °C) proteins were recovered from the supernatant and analysed by SDS-PAGE. The differences between incubated and control sperm were analysed by densitometry. The identity of two known retained proteins was assessed by Western blotting.

In the assays, the protein pattern of SP proteins in the fourth supernatant and the proteins extracted from sperm incubated at the first round with SP were analysed. All rounds were analysed by SDS-PAGE at the setup of the assay (Fig. 3b). Three replicates were performed for each assay and a representative picture is shown.

Author's personal copy

3. Results

3.1. Effect of SP on sperm parameters

When frozen-thawed sperm was supplemented with SP from autumn, there was an interaction ($P < 0.05$) between treatments and incubation times on the percentage of total sperm motility, progressive motility, and HOS+ (Fig. 1). The addition of SP tended to improve ($P = 0.07$) total sperm motility at 45 min, even though it was higher ($P < 0.05$) at 90 min, with no significant differences between SP-18 and SP-196 (Fig. 1). The SP-18 improved ($P < 0.05$) the progressive motility at 45 min, whereas SP-196 only showed a tendency ($P = 0.06$) in the same direction. At 90 min, SP improved ($P < 0.05$) progressive motility, with no differences ($P > 0.05$) between SP-18 and SP-196 (Fig. 1b). The SP collected during winter improved ($P < 0.05$) total sperm motility and progressive motility, with no significant differences between SP-18 and SP-196 (Fig. 2). Conversely, when frozen-thawed sperm were incubated with SP obtained in spring and summer, the percentage of total sperm motility, progressive motility were not significantly affected (data not shown).

The addition of SP (regardless of season of collection) to frozen-thawed semen had minimal effects on viability (eosin–nigrosin) nor membrane (hypo-osmotic test) (data not shown) or acrosome status (CTC-pattern, Table 1). The positive effect of autumn SP-196 on the percentage of swollen sperm was different ($P < 0.05$) from SP-18 at 45 min, but there was only a tendency ($P = 0.07$) for a difference between SP-18 and SP-196 at 90 min (Fig. 1). Neither the presence of SP obtained in autumn, nor its absence significantly affected viability or CTC-patterns (Table 1). When frozen-thawed sperm were incubated with SP obtained in spring and summer, HOS+, viability (data not shown) and CTC-patterns (Table 1) were not affected ($P > 0.05$). One exception was the F-pattern; it was increased ($P < 0.05$) by the addition of SP-18 obtained in spring ($9.0 \pm 1.3\%$) compared to the control ($4.1 \pm 0.8\%$), with no difference between SP-18 and SP-196 ($7.5 \pm 1.1\%$) after 90 min of incubation.

3.2. Variations in seminal plasma protein composition and sperm–SP protein interactions

3.2.1. Conservation temperature

The influence of the conservation temperature on SP protein composition and on the ability of SP proteins to interact with the sperm surface was evaluated. There were no differences in the protein profile of the SP stored at different temperatures (Fig. 3a). Frozen-thawed sperm were incubated with SP and the proteins retained after washing were obtained, together with the sperm proteins in a detergent protein extraction; the electrophoretic profile was compared against nonincubated sperm (Fig. 3b and c). To determine if all SP proteins had similar affinity for the sperm surface, the supernatant of SP incubated with sperm was re-incubated several times with new sperm, and remaining proteins were analysed (Fig. 3b). Not all SP proteins bound to the sperm surface, since several bands were still detected in the supernatant after four rounds of interaction (Fig. 3b; lanes 3, 5 and 7). Sperm incubated with SP did not gain new proteins, but instead, there were increases in 13 bands with Mw 16.1, 16.7, 17.4, 23.3,

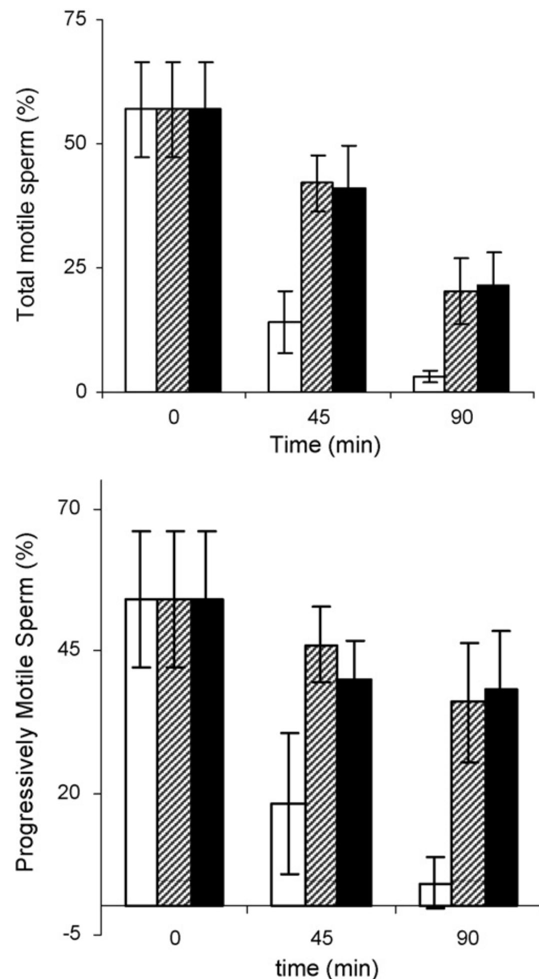


Fig. 2. Mean (±S.E.M.; $n = 5$) percentages of motile and progressively motility sperm, of frozen-thawed sperm suspended in either SP-18 (cross-hatch bars), SP-196 (dark bars) from winter or PBS (open bars), immediately after centrifugation (0 min) and after 45 and 90 min incubation at 37 °C, respectively. Within each cluster of three bars, those without a common superscript differed ($P < 0.05$).

Author's personal copy

Table 1
Mean (±S.E.M.) effect of the addition of SP stored at −18 or −196 °C and duration of incubation on frozen-thawed sperm acrosomal status (evaluated by chlortetracycline)

Season	CTC-pattern (%)	Treatment			SE	Incubation time (min)			SE
		Control	PS-18	PS-196		0	45	90	
Autumn	F-CTC	16.6	19.3	20.2	3.0	26.6 a	14.5 b	15.0 b	2.1
	B-CTC	9.3	10.3	11.2	2.3	7.1 a	10.0 ab	13.4 b	1.6
	AR-CTC	73.3	69.0	67.5	4.5	63.2 a	75.2 b	71.5 b	3.1
Winter	F-CTC	27.9	30.0	30.1	5.3	30.5	29.9	27.5	3.5
	B-CTC	23.0	21.0	19.2	1.7	20.5	19	23.7	1.7
	AR-CTC	49.0	51.0	50.6	6.0	48.9	53.0	48.7	4.3
Spring	F-CTC	4.0 a	8.1 b	7.5 b	1.0	6.0	7.4	6.2	0.8
	B-CTC	12.2	14.1	13.6	1.6	12.2	14.4	13.3	1.6
	AR-CTC	82.2	75.5	77.4	2.6	77.3	78.1	79.7	2.4
Summer	F-CTC	23.9	23.7	21.2	2.0	26.8 a	23.6 a	18.4 b	1.6
	B-CTC	22.8	21.6	22.2	4.2	22.1	22.4	22.1	2.7
	AR-CTC	53.5	54.5	56.2	6.8	51.0 a	53.8 ab	59.3 b	4.3

Within treatment and within duration of incubation, the S.E.M. are consistent among the three columns. Within a row (and within treatment or duration of incubation), means without a common letter differed ($P < 0.05$).

25.2, 27.5, 35, 40, 49, 53.5, 55.5, 61, and 86 kDa (Fig. 3c; lanes 1, 2 and 4), producing a new pattern that differed from fresh ram sperm (Fig. 3d). However, there was no effect of storage temperature (Fig. 3c).

3.2.2. *Effects of season*

The electrophoretic pattern of SP proteins from our experimental animals, obtained during different seasons was compared (Fig. 4), and its ability to bind to the sperm surface, was evaluated in a similar assay to the above described (Fig. 5). Both SP protein concentration and composition varied with season, with a higher protein concentration in the autumn (31 mg/mL) compared to winter, spring and summer (21, 10.5, and 21.2 mg/mL respectively) and a differential protein profile (Fig. 4). Accordingly, the proteins that bound to the sperm surface differed mostly for autumn versus the other three seasons, with minor differences among the other three seasons (Fig. 5). Five proteins (16.1, 17.4, 23.3, 40, and 55.5 kDa) were increased in autumn, whereas four proteins (16.7, 25.2, 27.5, and 35 kDa) were reduced. The remainder of the proteins that bound to sperm did not have seasonal differences. As a control of previously described SP proteins that bind to sperm, the presence of RSVP14 and RSVP20 in the retained fraction of SP from autumn was evaluated by Western blot with their specific antibodies [19]. The bands corresponding to 17.4 kDa and a double band at 23.3 and 25.2 kDa, were recognized, respectively by anti-RSVP14 and RSVP-20 (Fig. 5b).

4. Discussion

In the present study, ram SP was obtained throughout the year, stored at two temperatures, and used to supplement the post-thawing medium of ram sperm. Sperm motility was the main end-point affected by SP incubation. Neither viability nor membrane integrity (hypo-osmotic test) or acrosome status (CTC) were substantially modified by SP.

The addition of SP collected during autumn or winter significantly improved frozen-thawed sperm both total and progressive motility, consistent with previous reports [21]. However, SP from spring and summer did not significantly affect sperm motility. It was noteworthy that storage temperature did not significantly alter the protective effects of SP. These post-thawing sperm motility results were in agreement with those of Maxwell et al. [8] and Mortimer and Maxwell [43], who used cryopreserved SP at −20 °C. For this reason, although sperm viability and membrane status [44] were not modified by the addition of SP in the present study, the improved motility after SP treatment were expected to increase pregnancy rates in ewes after cervical AI, as demonstrated by McPhie et al. [9] and Maxwell et al. [8]. Perhaps improvements in the pregnancy rate described by other authors was due to better sperm motility, and not to prevention of premature capacitation. In that regard, Maxwell and co-workers developed a predictive model for in vitro fertilization success using motility measures in frozen-thawed ram sperm [45]. Conversely, Morrier et al. [46] reported no effect of SP on sperm motility when it was

Author's personal copy

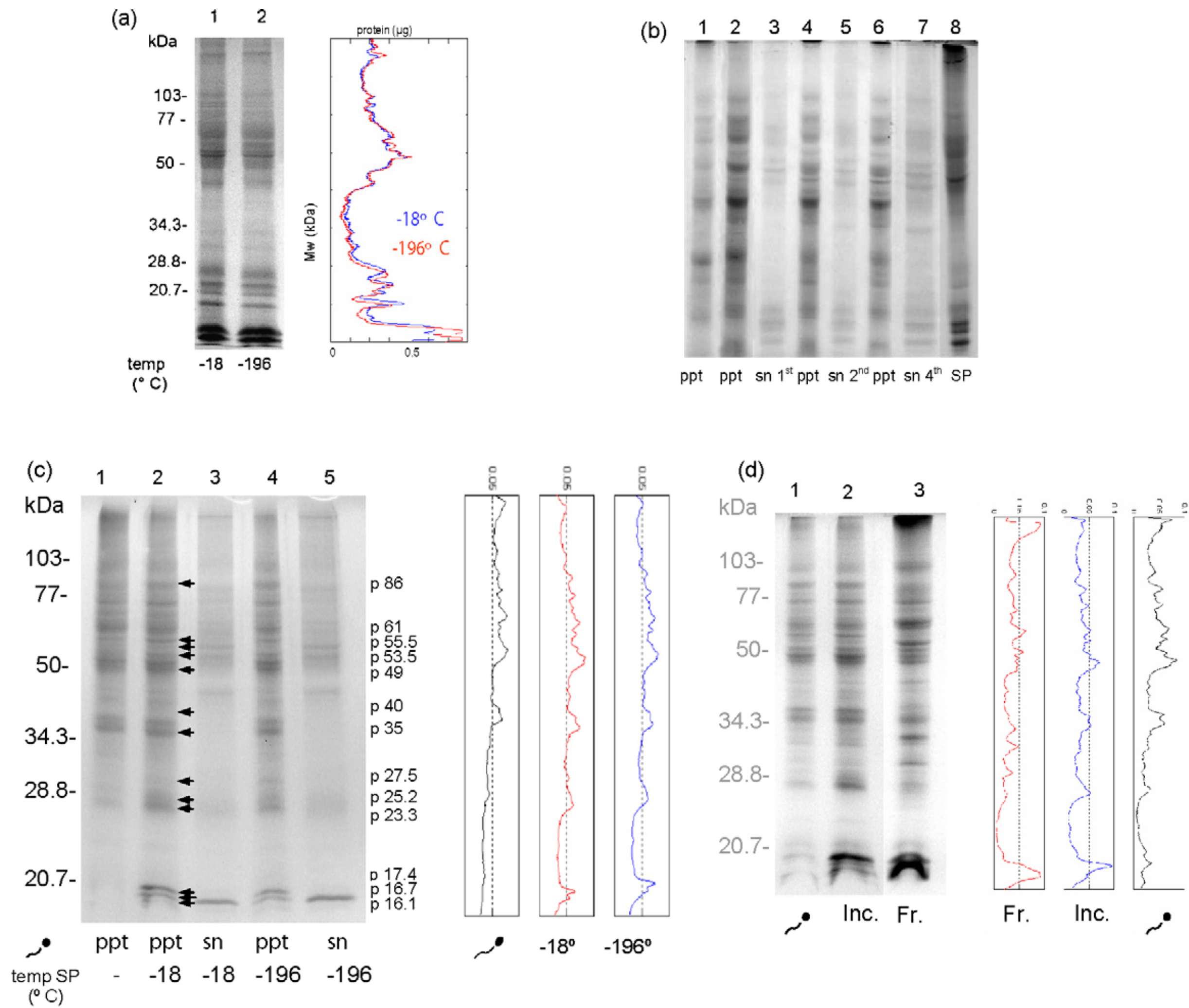


Fig. 3. Interaction of SP proteins with the sperm surface: effect of SP storage temperature. (a) Electrophoresis in polyacrylamide gels (15%) showing protein profile (left) of the SP stored at −18 °C (SP-18; lane 1) and −196 °C (SP-196; lane 2), and scanning densitometry (right). (b) Sperm–SP protein sequential rounds of interaction, (lane 1) frozen-thawed sperm protein extract; (lane 2) protein extract of sperm incubated with SP; (lane 3) SP proteins not retained by the sperm (first round); (lane 4) protein extract of sperm incubated with remnant SP; (lane 5) SP proteins not retained by the sperm (second round); (lane 6) protein extract of sperm incubated with remnant SP; (lane 7) SP proteins not retained by the sperm (fourth round); (lane 8) SP protein pattern. (c) Sperm–SP protein interaction assay with SP conserved at two temperatures (right) and densitometry (left), (lane 1) frozen-thawed sperm protein extract; (lane 2) protein extract of sperm incubated with SP-18; (lane 3) SP-18 proteins not retained by the sperm; (lane 4) protein extract of sperm incubated with SP-196; (lane 5) SP-196 proteins not retained by the sperm. (d) Comparison between the protein profile of frozen-thawed sperm (lane 1), sperm incubated with SP (lane 2) and fresh sperm (lane 3; left). Scanning densitometry (right). References: (sperm symbol) sperm proteins; (ppt) sperm proteins with bound SP proteins; sn: SP proteins not-bound to sperm; (Inc.) sperm incubated with SP; (Fr.) fresh ram sperm. Arrows point differential bands. Mw references are indicated at the left side of the gel.

added to ram-conserved sperm at 5 °C; they suggested that egg yolk proteins could have masked the effect of SP proteins. In the present study, we obtained a live sperm population through a Percoll® gradient. We infer that this procedure may have eliminated those components and allowed the direct effect of SP or some of the SP proteins.

Capacitation is a membrane-associated event that involves intracellular ionic modifications regulated by the transient association of molecules to the sperm surface [47,48]. We found that cryopreservation caused the loss of proteins bound to the sperm surface and that some of these proteins were recovered after sperm–SP incubation; however, the protein pattern of fresh sperm was not reestablished. Some proteins from SP bind to the sperm surface and regulate the cholesterol efflux and protein rearrangement [22,49]. In rams, SP soluble protein fraction depleted from vesicles (tiny organelles of unknown function that originated from testis or epididymis) was responsible for enhancing frozen-thawed ram sperm function and fertility when added to the post-thaw medium [45]. A lipid binding protein

Author's personal copy

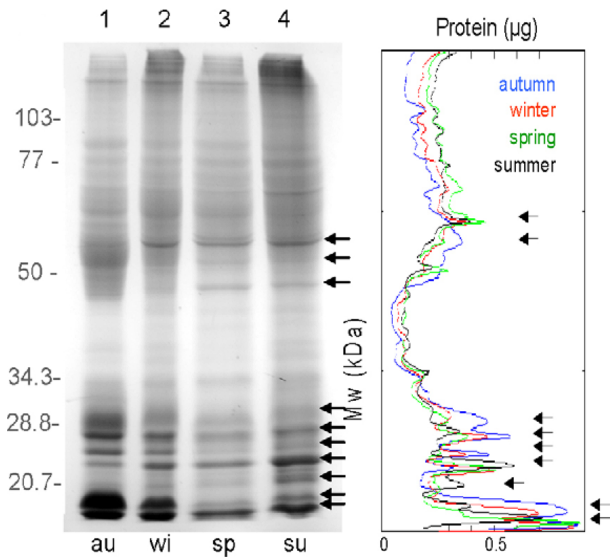


Fig. 4. Effect of season of SP collection. Electrophoresis in polyacrylamide gels (15%) showing protein profile (left) of SP collected in autumns (lane 1); winter (lane 2); spring (lane 3); summer (lane 4); and scanning densitometry (right).

family (BSP) was reported in bovine SP [50]; homologous proteins were detected in stallion (HSP) [23–25], goat (GSP) [27], boar (pBI) [25,26] and ram [30,51]. The BSP-like antigens are also present in rat, mouse, hamster, and human seminal vesicle fluid or SP [52]. Barrios et al. reported that two SP proteins with seminal vesicle origin (RSVP14 and RSVP20), responsible for protecting sperm from cold-shock, were attached to the sperm surface in fresh ejaculated ram sperm [19].

Five proteins (16.1, 16.7, 17.4, 23.3, and 25.2 kDa) had the highest capacity to bind to the sperm surface. Based on their molecular weight, we inferred that these were the same five major ram SP proteins previously isolated and characterized by Bergeron et al. [30]. The 16.7 kDa in the present study may correspond to the 15.5 kDa protein identified as a Spermadhesin [30]; similarly, the 16.1, 17.4, 23.3, and 25.2 kDa proteins may correspond to the 15, 16, 22, and 24 kDa proteins in the previous study [30]. The first and second proteins cross-reacted with antibodies against BSP proteins [30]; these proteins bound to the sperm membrane and modulated sperm function (reviewed in [22]). Protein bands of 17.4, 23.3, and 25.2 kDa cross-reacted with antibodies against RSVP14 and RSVP20; since the latter antibody cross-reacted with a band that was increased in autumn (23.3 kDa) and another one that was reduced in the same season (25.2 kDa), this antibody may not be highly specific.

Specific receptors on the sperm surface might be capable to bind SP proteins that trigger or regulate

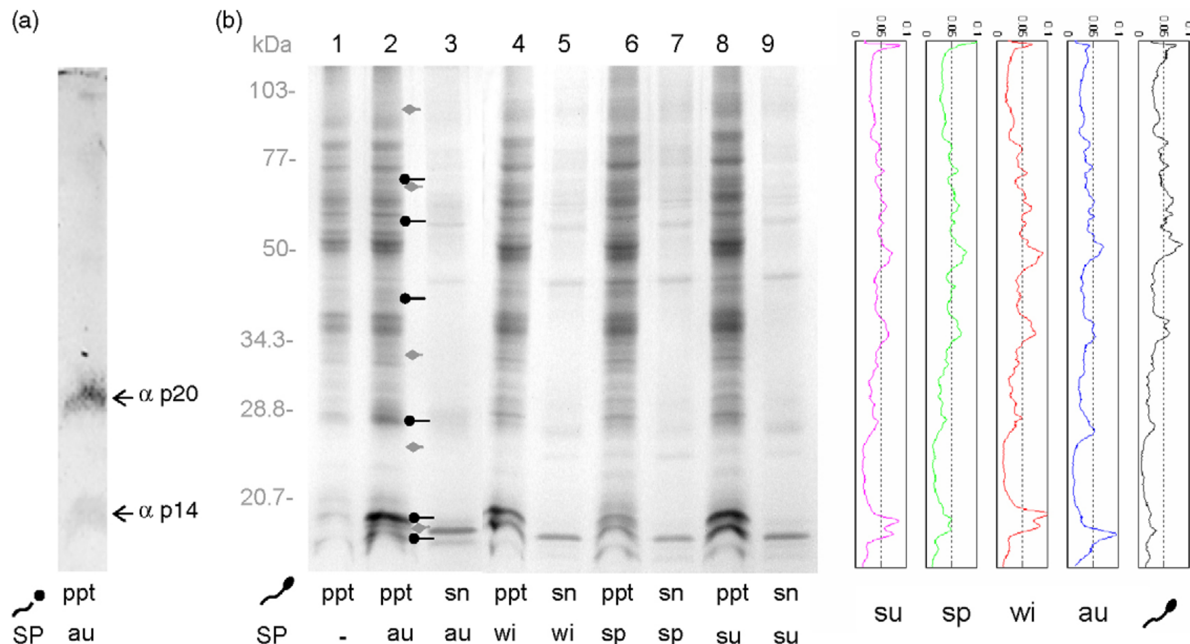


Fig. 5. Interaction of SP proteins with the sperm surface. (a) Sperm–SP proteins interaction assay with SP from different seasons: gel (left) and densitometry (right). (lane 1) Sperm protein extract; (lanes 2, 4, 6, and 8) protein extract of sperm incubated with SP from autumn, winter, spring and summer, respectively; (lanes 3, 5, 7, and 9) SP proteins not retained by the sperm. (⇠ ⬩) reduced bands; (●—) increased bands, comparing autumn with the other seasons. (b) Western blot revealed with antibody anti-RSVP14 and anti-RSVP20 of protein extract from sperm incubated with SP from autumn. References: (au) autumn; (wi) winter; (sp) spring; (su) summer; (sperm symbol) sperm proteins; ppt: sperm proteins with bound SPP; sn: SP proteins not-bound to sperm. Dark arrows point differential bands comparing the autumn with the other seasons. αp14 and αp20: antibodies anti RSVP14 and anti-RSVP20 [19]. Mw references are indicated at the left side of the gel, and immunoreactive bands are indicated on the right (←).

Author's personal copy

signaling events related to capacitation and AR. According to the evidence that SP reverts cold-shock damage on plasma membrane avoiding premature capacitation, we expected a variation in the percentage of CTC-patterns after incubation with SP, consistent with acquisition of SP proteins. However, as mentioned before, it was not significantly affected by the presence of SP obtained throughout the year, except for an increase in the F-pattern proportion after treatment with SP from spring conserved at 18 °C, which could not be explained. In this regard, the explanation for the beneficial effect of SP from autumn, may be either the higher protein concentration itself or the distinctive SP proteins that bind to sperm which may not be involved in sperm capacitation prevention. Conversely, it cannot be excluded that molecules other than proteins may modulate sperm function after sperm–SP interaction. This hypothesis should be further investigated.

In conclusion, the primary findings from this study were that: (1) seminal plasma from autumn and winter improved sperm motility of frozen-thawed sperm; and (2) ram seminal plasma could be stored at −18 or −196 °C without affecting protein composition nor the ability of SP proteins to bind onto the sperm surface.

Acknowledgements

We thank Ing. Gustavo Arenas for the development of the program for quantitative protein analysis and Claudia Faverin for the statistical analysis. We also acknowledge Drs. Muiño-Blanco and Cebrián-Perez for providing their antibodies against RSVP14 and RSVP20. This work was supported by grants from the Universidad Nacional de Mar del Plata, ANPCyT-PICTO-UNMdP 2004 (Project No. 8-375).

References

[1] Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA, et al. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Dev Biol 1999;214:429–43.

[2] Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl 2000;21:1–7.

[3] Chatterjee S, de Lamirande E, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol Reprod Dev 2001;60:498–506.

[4] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:871–91.

[5] Perez-Pe R, Barrios B, Muino-Blanco T, Cebrian-Perez JA. Seasonal differences in ram seminal plasma revealed by partition in an aqueous two-phase system. J Chromatogr B Biomed Sci Appl 2001;760:113–21.

[6] Holt WV. Basic aspects of frozen storage of semen. Anim Reprod Sci 2000;62:3–22.

[7] Holt WV. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 2000;53:47–58.

[8] Maxwell WM, Evans G, Mortimer ST, Gillan L, Gellatly ES, McPhie CA. Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. Reprod Fertil Dev 1999;11:123–6.

[9] McPhie C, Evans G, Maxwell WM. Effect of supplementation of fresh and frozen-thawed semen with seminal plasma on fertility of ewes after cervical and intrauterine insemination. In: 14th international congress on animal reproduction, vol. 2; 1999.

[10] Kafi M, Safdarian M, Hashemi M. Seasonal variation in semen characteristics, scrotal circumference and libido of Persian Karakul rams. Small Rumin Res 2004;53:133–9.

[11] Pilch B, Mann M. Large-scale and high-confidence proteomic analysis of human seminal plasma. Genome Biol 2006;7:R40.

[12] Metz KW, Berger T, Clegg ED. Adsorption of seminal plasma proteins by boar spermatozoa. Theriogenology 1990;34:691–700.

[13] Desnoyers L, Manjunath P. Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. J Biol Chem 1992;267:10149–55.

[14] Amann RP, Hammerstedt RH, Shabanowitz RB. Exposure of human, boar, or bull sperm to a synthetic peptide increases binding to an egg-membrane substrate. J Androl 1999;20:34–41.

[15] Cross NL. Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. Biol Reprod 1996;54:138–45.

[16] Catt SL, Sakkas D, Bizzaro D, Bianchi PG, Maxwell WM, Evans G. Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human spermatozoa. Mol Hum Reprod 1997;3:821–5.

[17] Gillan L, Maxwell WM. The functional integrity and fate of cryopreserved ram spermatozoa in the female tract. J Reprod Fertil Suppl 1999;54:271–83.

[18] Evans JP. Getting sperm and egg together: things conserved and things diverged. Biol Reprod 2000;63:355–60.

[19] Barrios B, Fernandez-Juan M, Muino-Blanco T, Cebrian-Perez JA. Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. J Androl 2005;26:539–49.

[20] Fernandez-Juan M, Gallego M, Barrios B, Osada J, Cebrian-Perez JA, Muino-Blanco T. Immunohistochemical localization of sperm preserving proteins in the ram reproductive tract. J Androl 2006;27:588–95.

[21] Barrios B, Perez-Pe R, Gallego M, Tato A, Osada J, Muino-Blanco T, et al. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod 2000;63:1531–7.

[22] Manjunath P, Therien I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 2002;53:109–19.

[23] Calvete JJ, Mann K, Schafer W, Sanz L, Reinert M, Nessau S, et al. Amino acid sequence of HSP-1, a major protein of stallion seminal plasma: effect of glycosylation on its heparin- and gelatin-binding capabilities. Biochem J 1995;310(Pt 2):615–22.

[24] Menard M, Nauc V, Lazure C, Vaillancourt D, Manjunath P. Novel purification method for mammalian seminal plasma phospholipid-binding proteins reveals the presence of a novel

Author's personal copy

member of this family of protein in stallion seminal fluid. Mol Reprod Dev 2003;66:349–57.
[25] Calvete JJ, Ensslin M, Mburu J, Iborra A, Martinez P, Adermann K, et al. Monoclonal antibodies against boar sperm zona pellucida-binding protein AWN-1. Characterization of a continuous antigenic determinant and immunolocalization of AWN epitopes in inseminated sows. Biol Reprod 1997;57:735–42.
[26] Sanz L, Calvete JJ, Mann K, Gabius HJ, Topfer-Petersen E. Isolation and biochemical characterization of heparin-binding proteins from boar seminal plasma: a dual role for spermadhesins in fertilization. Mol Reprod Dev 1993;35:37–43.
[27] Villemure M, Lazure C, Manjunath P. Isolation and characterization of gelatin-binding proteins from goat seminal plasma. Reprod Biol Endocrinol 2003;1:39.
[28] Boisvert M, Bergeron A, Lazure C, Manjunath P. Isolation and characterization of gelatin-binding bison seminal vesicle secretory proteins. Biol Reprod 2004;70:656–61.
[29] Jobim MI, Oberst ER, Salbego CG, Wald VB, Horn AP, Mattos RC. BSP A1/A2-like proteins in ram seminal plasma. Theriogenology 2005;63:2053–62.
[30] Bergeron A, Villemure M, Lazure C, Manjunath P. Isolation and characterization of the major proteins of ram seminal plasma. Mol Reprod Dev 2005;71:461–70.
[31] La Falci VS, Tortorella H, Rodrigues JL, Brandelli A. Seasonal variation of goat seminal plasma proteins. Theriogenology 2002;57:1035–48.
[32] Cardozo JA, Fernandez-Juan M, Forcada F, Abecia A, Muino-Blanco T, Cebrian-Perez JA. Monthly variations in ovine seminal plasma proteins analyzed by two-dimensional polyacrylamide gel electrophoresis. Theriogenology 2006.
[33] Smith I, Parr J, Murray G, Mcdonald R, Lee R-F. Seasonal changes in the protein content and composition of ram seminal plasma. In: Proceedings of the New Zealand Society of Animal Production; 1999.
[34] Marti E, Mara L, Marti JI, Muino-Blanco T, Cebrian-Perez JA. Seasonal variations in antioxidant enzyme activity in ram seminal plasma. Theriogenology 2007;67:1446–54.
[35] Laemmli UK, Beguin F, Gujer-Kellenberger G. A factor preventing the major head protein of bacteriophage T4 from random aggregation. J Mol Biol 1970;47:69–85.
[36] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[37] SAS II. SAS SAS/STAT user's guide. Cary, NC, USA: SAS; 1999.
[38] Mortimer D. Sperm recovery techniques to maximize fertilizing capacity. Reprod Fertil Dev 1994;6:25–31.
[39] Garcia Artiga C. Test de endosmosis en ovino. In: Jornadas internacionales de reproduccion animal. Murcia, España; 1994
[40] Perez LJ, Valcarcel A, de Las Heras MA, Moses DF, Baldassarre H. In vitro capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. Theriogenology 1996;45:1037–46.
[41] Gil J, Lundeheim N, Soderquist L, Rodriiuez-Martinez H. Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology 2003;59:1241–55.
[42] Fraser LR, Abeydeera LR, Niwa K. Ca(2+)-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol Reprod Dev 1995;40:233–41.
[43] Mortimer ST, Maxwell WM. Effect of medium on the kinematics of frozen-thawed ram spermatozoa. Reproduction 2004;127: 285–91.
[44] Adeoya-Osiguwa SA, Dudley RK, Hosseini R, Fraser LR. FPP modulates mammalian sperm function via TCP-11 and the adenylyl cyclase/cAMP pathway. Mol Reprod Dev 1998;51: 468–76.
[45] Ghaoui E, Gillan L, Thompson P, Evans G, Maxwell W. Effect of seminal plasma fractions from entire and vasectomized rams on the motility characteristics, membrane status, and in vitro fertility of ram spermatozoa. J Androl 2007;28:109–22.
[46] Morrier A, Castonguay F, Bailey J. Conservation of fresh ram spermatozoa at 5 °C in the presence of seminal plasma. Can J Anim Sci 2003;83.
[47] Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? Theriogenology 2002;57:327–44.
[48] Fraser LR, Adeoya-Osiguwa SA, Baxendale RW, Gibbons R. Regulation of mammalian sperm capacitation by endogenous molecules. Front Biosci 2006;11:1636–45.
[49] Moreau R, Manjunath P. Characteristics of the cholesterol efflux induced by novel seminal phospholipid-binding proteins. Biochim Biophys Acta 2000;1487:24–32.
[50] Manjunath P, Sairam MR, Uma J. Purification of four gelatin-binding proteins from bovine seminal plasma by affinity chromatography. Biosci Rep 1987;7:231–8.
[51] Jobim MI, Bustamante Filho IC, Trein C, Wald VB, Gregory RM, Mattos RC. Equine seminal plasma proteins related with fertility. Anim Reprod Sci 2005;89:305–8.
[52] Leblond E, Desnoyers L, Manjunath P. Phosphorylcholine-binding proteins from the seminal fluids of different species share antigenic determinants with the major proteins of bovine seminal plasma. Mol Reprod Dev 1993;34:443–9.