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Seasonal differences in ram seminal plasma revealed by partition in an aqueous two-phase system

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Abstract

Seminal plasma plays an important role in maturation of spermatozoa through hormonal, enzymatic and surface-modifying events. We have previously shown that adsorption of seminal plasma proteins (SPPs) to the sperm cell surface partially restores the functional characteristics of damaged spermatozoa, reproducing those of live cells. In the present report, we investigate the hypothesis that seasonal differences in seminal plasma could affect its ability to recover membrane integrity of cold-shocked sperm. The effect of seminal plasma proteins, obtained in breeding (bsSPPs) and non-breeding (nbsSPPs) season, on cold-shocked ram spermatozoa previously freed from seminal plasma, was analysed by centrifugal counter-current distribution (CCCD) in an aqueous two-phase system as well as membrane integrity determination by fluorescence markers. Cold-shock treatment greatly lowered cell viability in both breeding and non-breeding season spermatozoa. The cold-shocked sperm viability obtained was approximately 20%. The loss of heterogeneity and the decrease in viability revealed by CCCD analysis was reversed by the addition of increasing amounts of bsSPP, which induced restoration of the surface characteristics of viable-like spermatozoa, as well as an increase in the number of recovered viable sperm. However, this restoring effect was much lower when nbsSPPs were added, even in a sixfold higher concentration than used with bsSPPs. Incubation of cold-shocked cells with both kinds of proteins performed in both seasonal periods, showed that the recovering effect was related to the season when the plasma sample was obtained rather than to the semen season. The addition of bsSPPs to cold-shocked sperm accounted for a nearly 50% reversion for both studied breeding seasons. However, the reversion percentages obtained with nbsSPPs were significantly lower ($P<0.05$) than those found with bsSPPs in both studied seasonal periods. This different reversion capacity of bsSPPs and nbsSPPs was related to a different protein composition, as revealed by comparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. The bands of 20, 21, 24, 36 and 67 kDa of the bsSP sample profile decreased in winter–spring SP, and were even less intensely stained in summer SP. Densitometric analysis of the stained gel patterns allows automatic comparison among the separated bands, and revealed an important decrease in the content of several bands. The 21.5 kDa band showed the highest decrease, lowering to 14% in June–August plasma with respect to the value obtained in September–December plasma.

Keywords: Aqueous two-phase systems; Partitioning; Ram sperm

1. Introduction

Seminal plasma (SP) plays an important role in

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maturation of spermatozoa, through hormonal, enzymatic, and surface-modifying events. SP contains a variety of biochemical components, some of which are relatively specific for the regulation of sperm function. However, the effects of SP in maintaining spermatozoa motility and viability still remain controversial, especially in spermatozoa subjected to cold-shock or cryopreservation process. Removal of SP by dialysis or centrifugation, or dilution of ejaculate with cryoprotective medium, are standard methods for the cryopreservation of semen, although the beneficial effect of SP removal on spermatozoa survival is still a matter of speculation. Thus, SP was reported to reduce the deleterious effects of cold-shock in boar [1,2] bull and ram [3], and human [4,5] spermatozoa. Conversely, detrimental effects of SP on sperm survival after freezing–thawing have also been reported in several species [6–10]. These contradictory results could be due to the complex composition of SP, and to variations in composition among sires. Thus, differences in seminal plasma protein profiles from bulls of different fertility [11–14] and the influence of the individual composition of stallion SP on the suitability for semen cryopreservation [15] have been found. Apart from individual differences, seasonal changes in the protein content and composition of SP have recently been found in ram, a species with seasonal reproduction [16].

Interactions of seminal plasma with the sperm surface are supposed to play an important role in the fertilisation process. Sperm–seminal plasma protein (SPP) interactions have been studied by counter-current distribution (CCD) in aqueous two-phase systems [17], which is a useful technique for separating cell populations based on their surface properties. CCD is a chromatography process with one stationary (lower) phase and one mobile (upper) phase. The cell sample is partitioned in one system and the two phases are then, in a systematic way, brought into contact with fresh opposite phases. However, the loss of viability due to dilution and washing during the separation process is always a major technical problem for sperm cells [17]. Thus, the long period of time necessary for phase separation at unit gravity [18] may increase cell death during the separation process. Shorter separation procedures can be carried out by using different equipment in which centrifugation speeds up the phase separation process (CCCD, centrifugal counter-current distribution) [19]. Thus, we have shown [20] that CCCD in a Ficoll–dextran–polyethylene glycol (PEG) two-phase system is a suitable technique for resolving ram sperm heterogeneity, and it has already been used for the separation of sperm cell populations [21–23]. As a consequence of the separation procedure, fractions located further to the left or right of the CCCD profiles will contain cells with higher affinity for the lower dextran- or upper PEG-rich phase, respectively. As previously reported [23], assessment of viability by fluorescent probes showed a different enrichment of live cells in the separated fractions. Dead spermatozoa presented an enhanced affinity for the dextran-rich phase (i.e., preferentially located in the left-hand fractions of the profile), while live cells showed a higher affinity for the PEG-rich phase, being thus preferentially located to the right side of the profile. Moreover, using CCCD, we have already shown that acquisition of SPPs by adsorption to the sperm cell surface modifies the functional characteristics of damaged spermatozoa, reproducing that of live cells [24–26]. More recently, we have proved that this effect is mainly due to one seminal plasma protein of approximately 20 kDa [27].

In the present study, we investigate the hypothesis that seasonal differences in seminal plasma could affect its ability to recover membrane integrity of cold-shocked sperm. The effect of seminal plasma proteins, obtained in breeding and non-breeding season, on cold-shocked ram spermatozoa previously freed from seminal plasma, was analysed by CCCD in an aqueous two-phase system and fluorescence markers. Likewise, comparative sodium dodecyl sulfate–polyacrylamide gel electyrophoresis (SDS–PAGE) analyses of the protein composition of ram seminal plasma from ejaculates obtained in both seasons, were carried out.

2. Experimental

2.1. Reagents

Dextran T-500 (M_r 500 000) and Ficoll 400 (M_r 400 000) were obtained from Pharmacia (Uppsala,

Sweden). PEG (M_r 6000) was purchased from Serva Feinbiochemica (Heidelberg, Germany). All other chemicals used were of analytical-reagent grade.

2.2. Sperm collection

All the experiments were performed using fresh ram spermatozoa. Semen was collected from eight mature *Rasa aragonesa* rams by using an artificial vagina. The rams, which belonged to the National Association of Rasa Aragonesa Breeding (ANGRA), ranged from 2 to 4 years of age, and were kept at the Faculty of Veterinary Medicine under uniform nutritional conditions. Second ejaculates from four rams were pooled and used for each assay to eliminate individual differences. Experiments were carried out over time, maintaining sires with an abstinence period of 2 days on the basis of previous results [28].

2.3. Preparation of cell samples

A seminal plasma-free sperm population obtained by a dextran/swim-up procedure [29] performed using a medium devoid of Ca_2Cl and $NaHCO_3$ was used. For thermal shock, approximately $2 \cdot 10^8$ cells obtained after the swim-up process were incubated for 5 min at 25°C, transferred to 5°C for 10 min, and then replaced at 25°C for a further 5 min. An aliquot of approximately $1 \cdot 10^6$ cells was recovered, diluted to 0.5-ml with the swim-up medium, and then incubated with seminal plasma proteins to assess their effects.

2.4. Evaluation of semen samples

Sperm concentration was calculated in duplicate using a Neubauer's chamber (Marienfeld, Germany).

Cell viability, (membrane integrity) was assessed by fluorescent staining with carboxifluorescein diacetate and propidium iodide [30]. The cells were then examined under a Nikon fluorescence microscope, and the numbers of propidium iodide-negative (membrane-intact) spermatozoa and propidium iodide-positive (membrane-damaged) spermatozoa per 100 cells were estimated and recorded. At least 200 cells were counted in duplicate for each sample. Results are expressed as the percentage of membrane-intact spermatozoa±S.E.M. Means were compared using the Student's *t*-test (Statview 4.0) to determine significant differences between samples.

2.5. Collection of seminal plasma and proteins

Seminal plasma was obtained by spinning 1 ml of semen at 12 000 *g* for 5 min in a microfuge at 4°C. The supernatant was centrifuged again, and seminal plasma was recovered and, after filtering through a 0.45-μm Millipore membrane (Millipore Ibérica, Madrid, Spain), kept at −20°C.

SPPs were obtained by filtering the whole seminal plasma through Microsep (Pall Filtron, Madrid, Spain) microconcentrators of 3 kDa molecular mass cut-off and spinning for 12 h at 3000 *g* at 4°C. The obtained sample concentrate was diluted with two volumes of a medium containing 0.25 *M* sucrose, 4 m*M* sodium phosphate (pH 7.5), 0.1 m*M* EGTA, 10% (v/v) of '10×buffer stock HEPES' (50 m*M* glucose, 100 m*M* HEPES, 20 m*M* KOH) and centrifuged again. The seminal plasma proteins were recovered and stored at −20°C. Protein concentration was assessed by the Bradford method [31].

Proteins obtained from seminal plasmas corresponding to the period from September to January (decreasing photoperiod) were called breeding season seminal plasma proteins (bsSPPs), while the proteins from seminal plasma obtained out of this period were called non-breeding season seminal plasma proteins (nbsSPPs).

2.6. Assessment of seminal plasma protein effect

Different protein amounts were added to $1 \cdot 10^6$ cold-shocked spermatozoa and incubated for 1 h at 20°C in a rotation moving shaker. Results are expressed as the percentage of reversion of cold-shock effect in the sample containing plasma proteins with respect to the control sample (assessing the percentage of membrane-intact spermatozoa, i.e., propidium iodide-negative). The relation between total recovered cells (evaluated as the difference between cell viability after 1 h of incubation with and without proteins) and total theoretically recoverable cells (evaluated as the difference between cell viability in the swim-up obtained sample and the cold-shocked sample after 1 h of incubation) was used to express results, or:

$$[(Vp60 - Vc60)/(Vs - Vc60)] \cdot 100$$

where Vp is the protein added, cold-shocked sample viability; Vc is the cold-shocked sample viability; Vs is the swim-up sample viability; and 60 indicates the incubation time (min) after addition of the proteins.

2.7. Two-phase system

The two-phase system used consisted of 5.5% (w/w) dextran T500, 2% (w/w) PEG 6000, 10.5% (w/w) Ficoll 400, 0.25 *M* sucrose, 0.1 m*M* EGTA, 4 m*M* sodium phosphate, pH 7.5, 10% (v/v) of '10× buffer stock HEPES'.

2.8. Centrifugal counter-current distribution

We designed the counter-current distribution machine on the basis of the invention by Akerlund [19]. The apparatus contains 60 chambers arranged in a circle, which allows transfers of the upper (mobile) phases relative to the lower (stationary) phases. CCD is performed during centrifugation by keeping the denser (bottom) phases in the outer half while the lighter (upper) phases are in the inner half of each chamber. As no elution or pumping of any phase takes place, the overall process consists of a circular multistep transfer of 60 upper over 60 bottom batch phases. Each transfer in this centrifugal-enhanced CCD system includes the following: first, the phases are shaken at unit gravity to mix them thoroughly and they are then separated by centrifugation (1000 *g*). After the phases have separated and while they are still rotating at full speed (1000 *g*), the upper (inner) phases are transferred to the next chambers. After deceleration, a new cycle can be performed. A detailed scheme of the device was already reported by Akerlund [19].

To carry out CCCD experiments, a two-phase system of the above composition was prepared and mixed. In each experiment the volume of the system loaded in chambers was the estimated amount required to maintain the desired volume of the bottom phase (0.7 ml). Two cell samples (each approximately $1 \cdot 10^8$ cells) obtained after the swim-up process and submitted to cold-shock were loaded in chambers 0 and 30, and 29 transfers were carried out. Thus, two samples were analysed at the same time to allow direct comparison between them. To assess SPP effect, a CCCD run was performed by adding SPPs to the sample chamber as well as to the four previous chambers. All operations were carried out at 20°C. After the run, the solutions were transformed into a one-phase system by addition of one volume of a dilution buffer (polymer-free medium). The fractions were then collected and the cells counted under a light microscope.

Partition results are expressed as the percentage of cells counted in each fraction with respect to the value obtained in the chamber containing the maximal amount of cells. For viability assessment, cells from three consecutive chambers throughout the run were pooled and stained as indicated above. Results are expressed as percentage of viable cells in each sample. As a consequence of the separation procedure, sperm cell populations with a marked affinity for the lower dextran-rich phase, (mainly due to a low hydrophobicity), partition in the left part of the profile. Sperm cells which partition almost equally in both phases distribute in the central sector. Finally, sperm populations with a high affinity for the upper PEG-rich phase (mainly due to a high hydrophobicity), partition in the right sector of the profile.

2.9. SDS–PAGE analysis

SDS–PAGE was carried out in a gradient (4–22.5%) of polyacrylamide gel according to the method described by Laemmli [32] using a protean II vertical slab gel electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). The samples containing 75 μg of proteins were diluted 1:1 with the sample buffer (25% glycerol, 2% SDS, 0.027 *M* Tris–HCl, pH 6.8, 5% 2-mercaptoethanol, 0.018% EDTA and 0.1% bromophenol blue) and heated for 5 min at 100°C. Electrophoresis was carried out for 20 h at 110 V at room temperature. A mixture of molecular masses ranging from 6.5 to 205 kDa (Sigma, St. Louis, MO, USA) was used as a standard. Gels were stained with 0.025% Coomassie R (Serva Feinbiochemica). Densitometric analysis of the stained gel was carried out using Gel Doc 1000 with Molecular Analyst software (Bio-Rad).

3. Results

As a first approach towards the understanding of the effect of SPPs on cold-shocked sperm survival, CCCD analysis was performed with and without the addition of SPPs. The study was carried out during the breeding season, using proteins obtained from plasmas of this season (bsSPPs), and during the non-breeding season, using non-breeding season seminal plasma proteins (nbsSPPs).

Following preparation with the dextran/swim up procedure [29], ram spermatozoa were separated from seminal plasma obtaining a highly viable population. The season of semen sample collection did not significantly affect the selection process [29]. Cold-shock treatment caused an important decrease in cell viability in both breeding and non-breeding season, from 72±3.3 to 20±2.4%, and from 73±2.2 to 19.2±1.3% ($n=4$), respectively. After the addition of SPPs, sperm membrane surface changes would be expected if adsorption of proteins were to occur. To prove this hypothesis, CCCD experiments were performed by adding SPPs to the two-phase system. All distribution profiles presented in this study are highly reproducible and are representative of three different experiments with different semen samples. Obtained results indicate that adsorption of SPPs to cold-shocked spermatozoa modified cell surface characteristics. Fig. 1a shows the partition behaviour of breeding season ram spermatozoa freed from seminal plasma by a dextran/swim-up procedure, and subjected to cold-shock. As expected with a low cell viability sample [23], the obtained CCCD profile showed a displacement to the left, with loss of heterogeneity and an important decrease in recovered viability compared to the control sample selected by swim-up, which we have already reported [33]. To test the ability of SPPs to restore the surface properties of cold-shocked cells, the CCCD run was performed in the presence of either bsSPPs or nbsSPPs. The addition of increasing amounts of bsSPPs induced a progressive displacement of the profile to the right, and an increase in the number of recovered viable spermatozoa (Fig. 1b, c and d, 0.18 mg, 0.75 mg and 1.12 mg bsSPPs, respectively). These results indicate the ability of bsSPPs to reverse cold-shock membrane damage, restoring the surface characteristics of viable-like spermatozoa, in accordance with our previous results by scanning electron microscopy and biochemical markers [27].

This restoring effect was much lower when the CCCD run was performed by adding nbsSPPs to cold-shocked cells (Fig. 2). It must be noted that the profile of cold-shocked spermatozoa from ejaculates obtained in non-breeding season (Fig. 2a) was strongly displaced to the left, with an important loss of heterogeneity and viability, compared to the breeding season profile (Fig. 1a). This could be due to a greater susceptibility of non-breeding season cold-shocked sperm to the CCCD process, since the viability of cells loaded in the CCCD sample chamber was similar in both seasons (approximately 20%). However, increasing amounts of nbsSPPs (1.3 mg Fig. 2b, 2.5 mg Fig. 2c), even almost six times

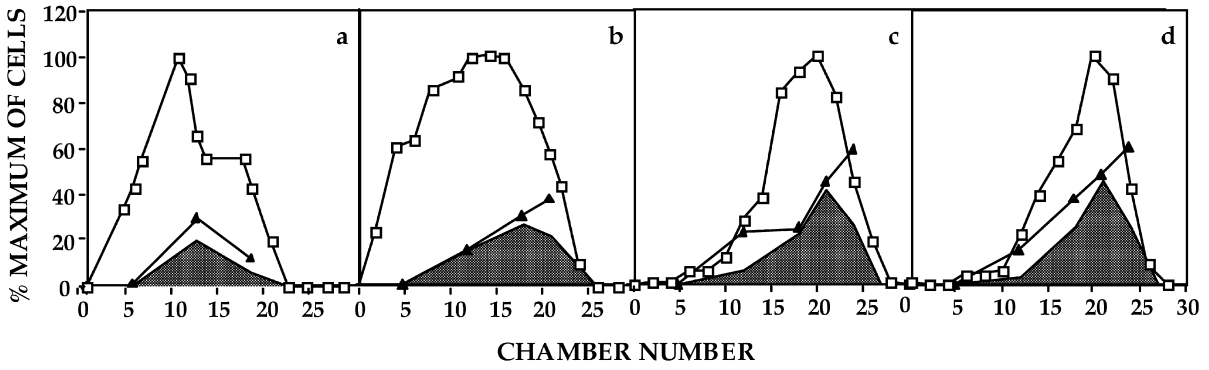

Fig. 1. Effect of the inclusion of breeding season seminal plasma proteins (bsSPPs) on the CCCD profiles of fresh ram spermatozoa subjected to cold-shock. (a) Control, (b) 0.18 mg bsSPPs, (c) 0.75 mg bsSPPs, (d) 1.12 mg bsSPPs. (□) Percentage of maximum of cells; (▲) percentage of viable cells; dark area (■), distribution of total viable cells.

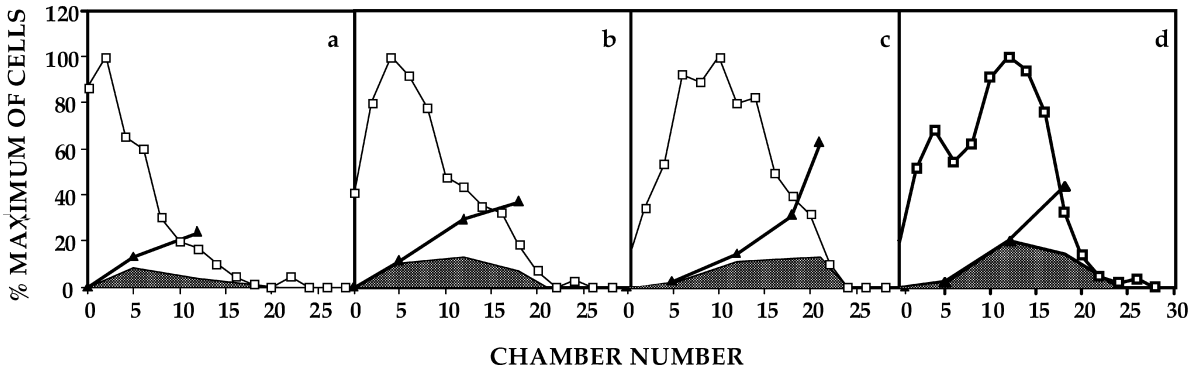

Fig. 2. Effect of the inclusion of non-breeding season seminal plasma proteins (nbsSPPs) on the CCCD profiles of fresh ram spermatozoa subjected to cold-shock. (a) Control, (b) 1.3 mg nbsSPPs, (c) 2.5 mg nbsSPPs, (d) 6.5 mg nbsSPPs. (□) Percentage of maximum of cells; (▲) percentage of viable cells; dark area (■), distribution of total viable cells.

higher than that used in breeding-season experiments (6.5 mg, Fig. 2d) did neither restore the profile to the right, nor increase recovered cell viability.

To further test whether the higher restoring effect of bsSPPs was dependent on the semen season quality or on the seminal plasma samples used, incubation of cold-shocked cells with both kinds of proteins (bsSPPs and nbsSPPs) was performed in both seasonal periods. Obtained results (Table 1) showed that SPP effect was related to the plasma sample collection season rather than to the semen season. The addition of bsSPPs to cold-shocked sperm obtained during the breeding season accounted for a nearly 50% reversion with both protein amounts assayed. These results were not significantly different to those found for bsSPPs with sperm obtained during non-breeding season. However, the reversion percentages obtained with nbsSPPs were significantly lower than values found with bsSPPs in both seasonal periods semen samples (Table 1).

In order to find out whether this different reversion capacity of bsSPPs and nbsSPPs was related to a different protein composition, seminal plasma samples from different seasons were analysed by SDS–PAGE. We classified seminal plasma samples into three seasonal groups. The breeding season seminal plasma (bsSP) corresponded to samples obtained in September, October, November and December, while seminal plasma obtained in the non-breeding season were divided into 2 groups, plasma from January to May (winter–spring SP), and plasma from June to August (summer SP). Comparative SDS–PAGE analysis showed differences in the patterns of different season seminal plasma (Fig. 3, Table 2). There was a detectable loss of five bands in nbsSP samples. The bands of 20, 21, 24, 36 and 67 kDa of

Table 1
Effect of the addition of breeding season seminal plasma proteins (bsSPPs) or non-breeding season seminal plasma proteins (nbsSPPs) on sperm membrane integrity of ejaculates obtained in both seasons

SPP (μg)	% Reversion			
	Breeding season sperm		Non-breeding season sperm	
	bsSPPs	nbsSPPs	bsSPPs	nbsSPPs
700	49.51 ± 8.57^{a} (5)	18.28 ± 6.85^{b} (4)	35.92 ± 3.73^{c} (4)	18.01 ± 2.47^{d} (4)
1400	46.67 ± 9.25^{a} (6)	20.66 ± 3.73^{b} (5)	52.27 ± 15.9^{a} (7)	8.49 ± 2.61^{b} (4)

Percentage of propidium iodide-negative spermatozoa assessed as $[(Vp60-Vc60)/(Vs-Vc60)]\cdot 100$, where Vp is the protein added, cold-shocked sample viability; Vc is the cold-shocked sample viability; Vs is the swim-up sample viability; and 60 indicates the incubation time (min) after addition of the proteins. Data are mean values±S.E.M. of the number of experiments indicated in parentheses. Different superscripts in rows indicate significant differences: a, b: $P<0.05$; c, d: $P<0.01$.

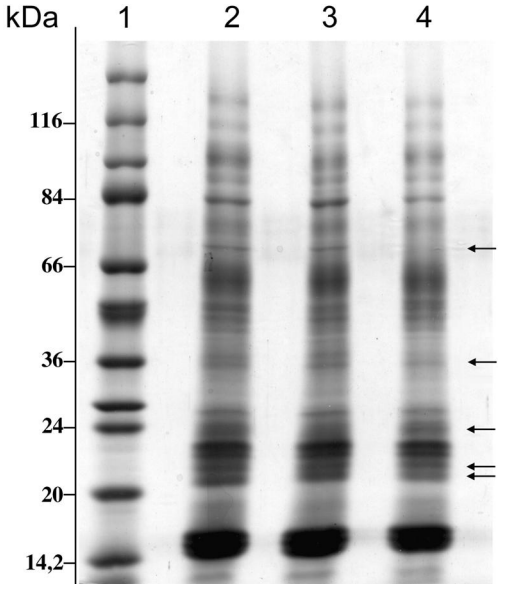

Fig. 3. Coomassie Brilliant Blue stained protein bands in ram seminal plasma separated by SDS–PAGE (75 μg protein per lane). Lanes: 1=molecular mass markers; 2=September–December seminal plasma; 3=January–May seminal plasma; 4=June–August seminal plasma. Arrows indicate bands that have decreased in non-breeding season seminal plasma.

the bsSP sample profile (Fig. 3, lane 2), decreased in winter–spring SP (Fig. 3, lane 3) and were even more less intensely stained in summer SP (Fig. 3, lane 4). Densitometric analysis of the stained gel patterns allows automatic comparison among the separated bands, and revealed an important decrease in the content of several bands. The 21.5 kDa band showed the highest decrease, lowering to 14% in June–August plasma with respect to the value obtained in September–December plasma.

Table 2
Densitometric quantification of relative areas (%) of protein bands in seminal plasma from different seasons

kDa	September–December	January–May	June–August
121.9	0.85	1.1	1.19
100.8	5.3	3.01	6.87
82.3	3.16	4.64	3.37
67.2	0.65	0.71	0.18
42.7	0.46	0.2	0.2
36	4.49	3.5	1.99
32.5	0.27	0.26	0.05
30.75	0.11	nd	nd
26.5	3.72	2.91	1.0
24	8.02	6.34	2.71
22.5	13.24	15.73	9.27
21.5	6.2	5.05	0.9
20.5	5.47	3.33	2.99

nd, Non detected.

4. Discussion

Several studies have provided direct evidence that specific components of seminal plasma, particularly proteins, are adsorbed onto the surface of ejaculated sperm [34–37]. Some of these adsorbed proteins maintain the stability of the membrane until the process of capacitation in the female genital tract [38], when their removal is a prerequisite for fertilisation [36]. Some other sperm-coating components ultimately contribute to membrane loss; in other words, to exocytosis. Thus, it is critically important that capacitation and the acrosome reaction occur in a temporal-spatial framework that is conducive to and facilitative of fertilisation. In addition, the need for some of these adsorbed proteins, not only to acquire the fertilising capacity, but also to maintain cell viability has already been reported [39].

Biochemical studies of cold-shock on mammalian spermatozoa have demonstrated that proteins and lipids are released during the treatment, and morphological damage takes place, especially on the acrosome and plasma membrane [10,40]. Since it has been suggested that post-cooling spermatozoa have the features of premature capacitation [41,42], the reduced longevity and fertilising ability of cryopreserved mammalian spermatozoa has been proposed to be due to their early capacitation state [42–44].

In previous studies we have demonstrated, by using CCCD, that adsorption of SPPs to the sperm cell surface modifies the functional characteristics of damaged spermatozoa, reproducing that of live cells [25,26,29]. More recently, we have verified the effect of seminal plasma proteins by electron microscopy and biochemical markers [27]. However, we have found that the efficacy of SPPs to revert the cold-shock damage was to different extent dependent on the sample. Changes in fertility of frozen semen associated with seasonal changes in ram semen

quality have been reported [45], and related not only to sperm concentration but also to the protein content and composition of SP [16]. With these considerations in mind, we proposed the hypothesis that the different restoring effect of SPPs on damaged spermatozoa was in relation with the season in which the SP samples were obtained. The results presented in this study, both by biochemical markers and by CCCD, confirm this hypothesis. The addition of either bsSPPs or nbsSPPs to cold-shocked sperm, promoted cell surface changes accounting for a displacement of the CCCD profile to the right with an increment in the number of viable cells, as the protein amount increased. However, while the addition of 1.12 mg of bsSPPs (Fig. 1d) resulted in a main peak in the right sector of the profile and a high percentage of recovered viability, the same amount of nbsSPPs (Fig. 2b) resulted not only in a profile displaced to the left but also in a very low recovered viability. Moreover, with an amount of nbsSPPs six times higher (Fig. 2d) than the maximum assayed with bsSPPs (Fig. 1d), the profile still remained in the left hand of the run, with rather low sperm viability. It must be pointed out that the cold-shocked sample viability was similar in both breeding and non-breeding season samples, which rules out the possible influence of sample viability in the obtained results. One explanation could be the major susceptibility to the CCCD process (including filtration to remove the swim-up medium) of cold-shocked sperm during the non-breeding season, which would affect the capability of nbsSPPs to restore the surface properties of cold-shocked cells. However, when damaged spermatozoa from the non-breeding season were directly incubated with either bsSPPs or nbsSPPs, important differences in the reversion capacity of bsSPPs and nbsSPPs were found (Table 1). The fact that the recovering effect of 0.7 mg of bsSPPs was lower in non-breeding season than that obtained in breeding season while no differences were found with 1.4 mg, could be explained on the basis of the requirement for the increased amount of adsorbed proteins as a consequence of the major susceptibility to the cold-shock of sperm during the non-breeding season. Nevertheless, the restoring effect was not found when nbsSPPs were added. This lack of restoring capacity could be due to the absence or decrease of several proteins in the SP during the non-breeding season as revealed by SDS–PAGE analysis (Fig. 3, Table 2). These results are in agreement with those found by Smith et al. [16] which reported the absence of several proteins in non-breeding season ram SP. It is worth noting that we have recently proved that the recovering effect appeared to be mainly due to one seminal plasma protein of approximately 20 kDa [27]. As an important decrease in the 21.5 kDa band (as well as in 20.5 kDa band) was found in the present study during the non-breeding season, this could be the reason for the lower recovering effect of nbsSP. Similarly, the decrease in fertility following the freezing–thawing procedure of bull semen has been related with the loss of a 25-kDa membrane protein [13], and a 26-kDa seminal plasma protein was shown to be prevalent in bulls of high fertility [11]. The inference of other proteins which also decreased in the non-breeding season cannot be ruled out.

Another possible explanation for the different restoring capacity between bsSP and nbsSP could be the presence of a critical factor in seminal plasma that would promote (bsSP) or avoid (nbsSP) SPP adsorption to the sperm plasma membrane, resulting in a significantly different recovery of sperm viability.

The results reported here confirm, once more, the possibility of using phase partitioning to detect spermatozoa surface changes associated with induced experimental conditions, constituting a powerful analytical approach for analysing sperm cell surface physiology. The observation, both in this study and by ultrastructural analysis [27], that seminal plasma proteins are adsorbed onto cold-shocked spermatozoa, indicates that SPPs are able to recover sperm membrane integrity of damaged cells. Moreover, it has been proved that this ability is dependent on the season of SP collection. Experiments to identify the exact components responsible for the sperm membrane integrity recovering effect are currently in progress.

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