Seminal plasma proteins interacting with sperm surface revert capacitation indicators in frozen-thawed ram sperm

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Abstract

This study was conducted to evaluate the effects of interacting seminal plasma proteins (iSPP) obtained by AV or EE on frozen-thawed ram sperm in order to test the hypothesis whether this fraction could be sufficient to emulate the effect of complete seminal plasma (SP). Additionally, we evaluated whether this proteins has a differential effect between spermatozoa from high and low fertility rams and between breeding and non-breeding seasons. We assessed sperm motility, quality parameters (intracellular reactive oxygen species, membrane fluidity, plasma membrane permeability and mitochondrial activity) and capacitation status. The main findings from this work were: i) iSPP had no effect on sperm motility, whereas SP (AV or EE) addition produced the highest values of total motility (74.13±2.99 and 72.27±2.99 for AV and EE, respectively) and progressive motility (64.97±2.64 and 63.73±2.64 for AV and EE, respectively); ii) iSPP had no effect on sperm quality parameters ($p>0.05$), but whole SP improved all parameters evaluated. Moreover, SP collected by AV yielded significantly higher viability (44.60±2.87) and sperm with stable plasma membrane (44.56±2.49) comparing with the addition of SP collected by EE (35.80±2.47 and 36.67±1.71, respectively); iii) iSPP and SP collected by EE, but not by AV, reverted molecular signals of capacitation as protein tyrosine phosphorylation caused by freezing temperatures; iv) there were no effects of fertility or season in sperm quality parameters evaluated. This study demonstrated that, although the iSPP have a clear decapacitating effect, including the ability to revert cryo-capacitation indicators, they are not sufficient to emulate the effects of complete SP regarding sperm functional parameters.

Keywords: Capacitation; Post-thaw sperm quality; Seminal plasma.

Introduction

The cryopreservation process, involving cooling, freezing and thawing produce serious detrimental changes in ram sperm function (Ari et al., 2011). Sperm cryoinjury includes the reduction of viability, motility and mitochondrial membrane potential, chromatin damage, increase in the production of reactive oxygen species, activation of apoptosis (Said et al., 2010) and perhaps the most important cryoinjury, the premature induction of a capacitation-like status (Bailey et al., 2003). All of these alterations result in reduced longevity of the cryopreserved spermatozoa within the female reproductive tract, decreasing the likelihood of successfully interact with the oviduct or fertilize the ovum.

Seminal plasma (SP) is a mixed secretion from several glands of the male reproductive tract. Supplementation of frozen/thawed ram sperm with SP has been noted to improve sperm characteristics including motility, viability (Ollero et al., 1997; Domínguez-Rebolledo et al., 2007; Maxwell et al., 2007; Domínguez et al., 2008) and the ability to penetrate the cervical mucus (Graham, 1994; Maxwell et al., 1999; El-hajj Ghaoui et al., 2007; Leahy et al., 2010). Moreover, whole ram SP addition improved cryopreservation of goat semen (Ari and Daskin, 2010). Dott et al. (1979) observed that the effects of SP on sperm cells lasted even after the removal of SP.

The beneficial impact of SP on sperm has been attributed to its proteic components, since seminal plasma proteins (SPP) are able to increase sperm resistance against cold shock (Barrios et al., 2000; Pérez-Pé et al., 2001; Colás et al., 2009) and stimulate sperm function and fertilising ability (Maxwell et al., 2007). It has already been shown that SPP support survival of ram spermatozoa acting not only at the plasma membrane

level but also by inhibiting capacitation (Desnoyers and Manjunath 1992; Barrios et al., 2005) and apoptosis-like changes (Mendoza et al., 2013).

Bernardini et al. (2011) demonstrated that a fraction of SPP with affinity by the sperm membrane was able to repair ultrastructural damage and improve motility of frozen/thawed ram sperm. This fraction, enriched in RSVP14 and RSVP22 proteins, was called interacting SP proteins (iSPP). Previous work has demonstrated that these proteins were partially able to protect and repair ram sperm membrane against cold-shock damages and detrimental effects of cryopreservation process (Barrios et al., 2000; Pérez-Pé et al., 2001; Barrios et al., 2005; Ari and Daskin 2010) as well as to maintain fresh sperm in a decapacitated state (Mendoza et al., 2013). Domínguez et al. (2008) observed that the composition and protein concentration of SP varies according to season. Moreover, in a recent work we noted that ram iSPP varies according to the collection method applied and that the iSPP collected by electroejaculation (EE) has a greater concentration of low molecular weight proteins, such as those considerate as crioprotectans, than the same fraction obtained by artificial vagina (AV) (Ledesma et al., 2014).

With this background, in this work we evaluated the effect of addition of iSPP obtained by AV or EE to thawed spermatozoa from ram with low or high fertility and tested the hypothesis that it could be sufficient to emulate the effect of complete SP. Additionally, we evaluated whether the iSPP had a differential effect on sperm quality between breeding and non-breading season.

Materials and Methods

Reagents

Flow cytometry consumables (including the sheath fluid) were purchased from BD Biosciences (San Jose, CA, USA). The rest of the chemicals were acquired from Sigma. Chemicals were of the highest grade available. PBS was prepared in milli-Q water: 136.9 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 7 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, 1.5 mM KH_2PO_4 and 1 g/l PVA (290 mOsm/kg; pH 7.2).

Frozen semen

All experiments were performed using frozen spermatozoa from 10 adult Assaf rams divided in two groups of five rams each, accordingly to their fertility in low (35.3±3.0%) and high (60.4±1.5%) fertility. Frozen semen was supplied by OVIGEN (Centro de Selección y Mejora Genética de Ovino y Caprino de Castilla y León, Toro, Spain).

Animals

All animal procedures were in accordance with the Spanish Animal Protection Regulation RD 1201/2005, according to European Union Regulation 2003/65. Eight mature Assaf rams were used for obtaining SP and iSPP. Two experiments were carried out, one in autumn (breeding) and the other in spring (non-breeding season).

Seminal plasma and interacting seminal plasma proteins: collection and processing

SP and iSPP were obtained from all males by AV and EE according to Marco-Jiménez et al. (2005) separately by two days between methods. We performed one seminal collection during autumn (breeding season) and one seminal collection during spring (non-breeding season). Ejaculates with total motility ≥80% were pooled by collection method and then split in two parts. One of them was used to obtain SP and the other one was used to obtain the iSPP. Briefly, for obtaining SP, half of the pooled semen was

centrifuged twice (2000 x *g* for 15 min at 4 °C). The clear supernatant (SP) was recovered, filtered (0.22 µm) and kept at -80 °C until use. The other half of the semen pool was used to recover iSPP. Ejaculates were washed 10 times with PBS to remove excess of unbound SP (800 x *g*, 10 min). Sperm free from SP were incubated with 200 µl buffer 10 mM Glycine-HCl pH 3 (20 min, room temperature, with agitation). Finally, sperm were centrifuged and the supernatant was conserved and neutralized with 5 µl Tris-HCL 2 M pH 9.5. Protein concentration of SP and iSPP were assessed according to the method described by Bradford (1976), using BSA for the standard curve. The concentration of proteins eluted with low pH buffer from the sperm surface coming from a known volume of SP was calculated as mg/ml.

Experimental design

Three straws of males of low or high fertility were randomly thawed by immersion in a water bath (37 °C, 20 s), layered over 1 mL Androcoll-O^{TM} colloid and centrifuged, to remove dead cells and SP (800 x *g* by 5 minutes and then 1200 x *g* by 10 minutes). The supernatant was removed and the remaining pellet was suspended in 250 µl of PBS and centrifuged again (800 x *g*, 5 minutes). The resulting sperm pellet was resuspended in PBS and the concentration and motility patterns were calculated. We performed four experimental replicates with frozen sperm of rams with low fertility and four experimental replicates with frozen sperm of rams with high fertility during spring. The second experiment was performed in the same way in autumn. An aliquot (160 µl) of sperm suspension (10 x 10^6 spermatozoa) was supplemented with 40 µL of five different solutions (200 µl of final volume) and incubated at 37 °C during one hour. The supplementation media were:

- 40 µL (20% v/v) of SP collected by AV (40 µl= 0.6 mg protein for spring SP or 1.4 mg for autumn SP)
- 40 µL (20% v/v) of SP collected by EE (40 µl= 0.48 mg protein for spring SP or 1.2 mg for autumn SP)
- 40 µL of iSPP from AV collection (7 µg for spring collection or 23 µg for autumn collection) in PBS 0.5% fructose.
- 40 µL of iSPP from EE collection (3.2 µg for spring collection or 29 µg for autumn collection) in PBS 0.5% fructose.
- Negative control: 40 µL of PBS 0.5% fructose.

The amount of iSPP was selected on the basis of the proportional amount of proteins provided by an equivalent volume of complete SP. The concentration of proteins in SP was 35.0 mg/ml and 30.0 mg/ml for AV and EE, respectively, in autumn and 15.0 mg/ml and 12.0 mg/ml for AV and EE, respectively, in spring. The concentration of proteins in iSPP in autumn was 7.8 mg/ml and 5.8 mg/ml for AV and EE, respectively, and in spring it was 0.9 mg/ml and 0.8 mg/ml for AV and EE, respectively.

Fructose concentration was chosen based on the concentration previously measured by us in SP (unpublished data).

Sperm analyses

After thawing, the following analyses were made: motility parameters with CASA system, quality parameters by flow cytometry and capacitation status (tyrosine phosphorylation) by Western blotting. After one hour of incubation at 37 °C, those parameters were evaluated in treatments and negative control.

Motility parameters

Motility parameters were estimated by computer-assisted sperm motility analysis using a CASA system (ISAS 1.0.4; Proiser SL, Valencia, Spain). A Makler counting chamber (10 μm depth; Haifa Instruments, Israel), warmed up to 37 °C, was filled with 5 μL of sample and examined with a phase contrast microscope (Nikon E400; negative contrast optics). At least four fields and 200 cells were recorded at 10× using a video camera (Basler A312f, Basler Vision Components, Exton, PA, USA), at 53 frames/s and an acquisition time of 1 s. The kinematic parameters recorded for each spermatozoon were: total motility (TM; %), progressive motility (PM; %), velocity path average (VAP; μm/s), straight-line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), linearity (LIN; %), straightness (STR; %), wobble (WOB; %), amplitude of the lateral movement of the head (ALH; μm) and beat-cross frequency (BCF; Hz)..

Quality parameters

Stock probes were as follows: Hoechst 33342 (Sigma B2261) 8.9 mM in Milli-Q water; Propidium iodide (PI) (Sigma P4170) 1.5 mM in Milli-Q water; Mitotracker Deep Red 633 (Invitrogen M22426) 100 μM in DMSO; Dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Invitrogen C6827) 1 mM in DMSO; YO-PRO-1 iodide (491/509) (Invitrogen Y3603) 1 mM in DMSO; Merocyanine 540 (M540; Sigma) 1 mM in DMSO. All solutions were kept in the dark at -20 °C until needed.

Flow cytometer configuration

Flow cytometric analyses were carried out on a Cyan Adp flow cytometer (Beckman Coulter, Inc., Brea, USA). The three lasers of the cytometer were used to excite the different fluorochromes. A 325-nm helium-cadmium UV laser was used for exciting the Hoechst 33342, a 488-nm argon-ion laser was used for exciting YO-PRO-1, CM-

H_2DCFDA, M540 and PI and a 633-nm helium-neon laser was used for exciting MitoTracker Deep Red. Forward-scatter light (FSC) and side-scatter light (SSC) signals plus the fluorescence light of each fluorochrome were acquired. A 530/28 filter was used for the fluorescence from YO-PRO-1 and CM-H_2DCFDA, a 575/25 for M540, a 670LP filter for PI , a 424/44 for Hoechst 33342 and 620/20 for MitoTracker deep red. The acquisition was controlled using the Summit V4.3.02 software. All the parameters were read using logarithmic amplification. An acquisition template was set up in the software in order to discriminate spermatozoa from debris within the events acquired. FSC/SSC and Hoechst 33342 vs. PI dot-plots were used to discard debris. A total of 5.000 spermatozoa were acquired per sample, with a flow rate of 200 cells/s (Martínez-Pastor et al., 2010). The analysis of the flow cytometry data was carried out using Weasel v. 3.2 (WEHI, Melbourne, Australia).

Plasma membrane permeability and mitochondrial activity

Variations of plasma membrane permeability were assessed using the YO-PRO-1/PI combination (PI identifies YO-PRO-1+ spermatozoa with extensive membrane damage) and sperm mitochondrial function was assessed using MitoTracker deep red, this dye is readily sequestered in mitochondria and emits red fluorescence in active mitochondria (high mitochondrial membrane potential) Samples were prepared at 10^6 mL^{-1} in PBS with 100 nM YO-PRO-1, 3 µM PI and 100 nM MitoTracker deep red. After 15 min at 37 °C in the dark, samples were analysed. Considering membrane permeability, spermatozoa were classified into: viable, normal membrane permeability (YO-PRO-1-/PI-); viable, increased membrane permeability or apoptotic (YO-PRO-1+/PI-); and dead (YO-PRO-1+/PI+). MitoTracker+/YO-PRO-1− were considered as viable sperm with high mitochondrial membrane potential (normal); MitoTracker-/YO-PRO-1− were considered as viable cells with decreased mitochondrial membrane potential and YO-

PRO-1+ were considered as apoptotic/dead cells (Fernández-Gago et al., 2013). Data were expressed as the percentage of spermatozoa in each category.

Intracellular reactive oxygen species (ROS)

CM-H_2DCFDA was used for the detection of intracellular ROS. CM-H_2DCFDA is oxidized to dichlorofluorescein (DCF), which emits fluorescence at 530 nm in response to the 488 nm excitation. This fluorescent probe was combined with PI for removing the non-viable population from the analysis (Dominguez-Rebolledo et al., 2011). Samples were prepared at 10^6 mL^{-1} in PBS with 1 µM CM-H_2DCFDA and 2.5 µg/ml PI. After 15 min at 37 °C in the dark, samples were analysed. The median of the green fluorescence intensity was used for analysis, after excluding PI+ events (dead spermatozoa).

Membrane fluidity

M540 binds preferentially to membranes with loosely packed lipids, whereas YO-PRO-1 stains the nuclei of cells with increased plasma membrane permeability ("apoptotic" or dead). Samples were prepared at 10^6 mL^{-1} in PBS with 2.7 µM M540 and 0.1 µM YO-PRO-1. After 15 min at 37 °C in the dark, samples were analysed. Spermatozoa belonged to one of three populations: high YO-PRO-1 fluorescence (dead or increased membrane permeability); low M540 fluorescence and low YO-PRO-1 fluorescence (lower membrane fluidity); high M540 fluorescence and low YO-PRO-1 fluorescence (higher membrane fluidity) (Fernández-Gago et al., 2013).

Assessment of capacitation status by Western blot (tyrosine phosphorylation)

Aliquots containing $5x10^6$ cells of each treatment and negative control were centrifuged at 7500 x *g* for 5 min at 4 °C, and the supernatant was discarded. Samples were mixed with 5x Laemmli sample buffer under reducing conditions (100 mM DTT) and boiled.

Finally, extracts were incubated at 100 °C for 5 min. Solubilised proteins were separated by electrophoresis on 10% (w/v) acrylamide SDS–PAGE (Laemmli, 1970) and electrotransferred onto Immun-Blot PVDF membranes (BIO-RAD). Non-specific binding sites on membranes were blocked with 5% skimmed milk (w/v) in TBS-T (10 mM Tris-HCl, pH 8.0, 120 mM NaCl and 0.05% Tween 20) for 1 h at room temperature. Phosphorylated tyrosines were immunodetected by incubating with the primary antibody (Antiphosphotyrosine clone 4G10 monoclonal mouse, Millipore; 1:2000 in blocking solution) overnight at 4°C under agitation. After several washes in TBS-T, the membranes were incubated for 1 h at room temperature with a secondary goat anti-mouse IgG-HRP (1:5000 in blocking solution; GE, NA9311). Thereafter, the membranes were developed with a chemiluminescence reagent (ECL commercial kit, GE Amersham Biosciences) and detected on autoradiography films (Hyperfilm™ Amersham Biosciences). Digitalized Western blot images were analysed using ImageJ 1.43 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015). The phosphorylation signal was evaluated as volume (area/intensity). Thereafter, blots were stripped during 20 min at 60 °C in stripping buffer (62.5 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol, pH 6.5), re-blocked and incubated sequentially overnight with a mouse monoclonal anti-tubulin antibody (1:5000) (Sigma) as a loading control. Secondary antibody incubation and development were performed as described above. Data normalization was performed by dividing the mean densities of phosphorylated proteins by the corresponding tubulin densities and expressed as a percentage.

Statistical analysis

Sperm parameters were compared by ANOVA using the MIXED procedure of the Statistical Analysis System version 8.2 (SAS Institute INC, Cary, NC). Data of thawed sperm evaluation were used as a covariable. The statistical model includes the effect of SP or iSPP, collection method, season and their interactions. Numeric results are expressed as least square means (LSM) ± standard error of the means (SEM). Data were considered statistically significant when $p<0.05$.

Results

Effect of seminal plasma and interacting seminal plasma proteins on sperm motility

The highest percentages of TM, PM and WOB were observed when spermatozoa were incubated with SP obtained by AV or EE (Table 1), whereas incubation with iSPPs had no effects on these parameters. Addition of iSPP caused a decrease in VCL compared with control samples. However, when SP was added this decline was more pronounced.

Regarding to VSL, incubation of spermatozoa with iSPP collected by AV had no effect in this parameter, in opposite to incubation with iSPP collected by EE or SP that caused a big drop in its values. Adding iSPP, regardless of the collection method, had no effect on STR, ALH and BCF, compared to the addition of SP which caused a decrease in those motility measures. Addition of any of the supplemented media had no effect on LIN. Contrary to what happened with VAP, where all media caused its decline. There were no differences between fertility and collection season in any of the motility variables evaluated ($p\geq0.05$).

Effect of seminal plasma and interacting seminal plasma proteins on quality parameters evaluated by flow cytometry

The analysis of physiological parameters using flow cytometry showed that all quality parameters were affected by treatment ($p<0.0001$) (Table 3). We did not find effect of season or fertility on any of the variables studied ($p>0.05$). Addition of SP collected by AV yielded significantly higher viability showed by an enhanced PI-/YO-PRO-1- subpopulation comparing with the addition of SP collected by EE or any of the iSPP ($p<0.001$). Viable sperm with stable plasma membrane (YO-PRO-1-/M540-) was higher in treatments composed by SP, being significantly higher when SP was collected by AV ($p=0.014$). The mitochondrial activity was affected by treatment, reaching the highest values of the sperm subpopulation YP-/MT+ when treatment was composed by any of the SPs. The presence of intracellular ROS was affected by treatment ($p<.0001$) (PI-/H_2DCFDA-) and was lower when SP was added reaching the lower values when SP was collected by AV. iSPP did not exert significant effects comparing to the control on the variables obtained from the flow cytometry analyses.

Effect of seminal plasma and interacting seminal plasma proteins on tyrosine phosphorylation

In order to evaluate the ability of iSPP obtained by AV and EE to revert molecular signals of capacitation caused by freezing temperatures, sperm protein tyrosine phosphorylation was analysed. Two protein bands of approximately 45 and 40 kDa were detected. Densitometry quantification revealed that the phosphotyrosine signal induced by freezing and thawing was reverted after 1 h incubation with SP and iSPP proteins. We observed a significant decrease in the 45 kDa and 40 kDa bands in all

treatments compared with control samples (Fig. 1). Season and fertility showed neither effects nor interaction with treatment.

Discussion

The present study reports that after thawing addition of iSPP had no effects in the majority of the sperm motility parameters evaluated by CASA system. However, it caused a decrease in curvilinear velocity and moreover, iSPP collected by EE caused a big drop in straight line velocity values. iSPP incubation neither exerts significant effects on sperm variables evaluated by flow cytometry. The evaluation of capacitation status through the quantification of phosphotyrosine signal revealed that the signal induced by freezing/thawing was reverted after incubation with SP and with the single addition of iSPP.

Gillan et al. (1997) demonstrated that the cryopreservation process causes changes in ram sperm membrane functionality equivalent to capacitation. Moreover, Pérez-Pé et al. (2002) proved that ram sperm capacitation is associated to protein tyrosine phosphorylation and that exposure of sperm cells to SP proteins prevents this capacitation, evidenced by the decrease of phosphotyrosine signal of protein bands of 45, 40 and 30 kDa. In the present work, we observed that addition of complete SP collected by AV and EE, and more interestingly, that only the fraction compose by their iSPP were able to decrease the phosphotyrosine signal of 45 and 40 kDa bands in sperm cells. This is consistent with our previous observation related to the content of RSVP14 and RSVP20 in these samples (Ledesma et al., 2014b). Since, Barrios et al. (2005) hypothesized a possible decapacitating effect of these plasma proteins on sperm cells. This finding suggest that iSPP could be sufficient to reverse molecular signals of

capacitation caused by freezing, perhaps acting through the inhibition of the signal transduction pathways of capacitation.

Only fully capacitated sperm are competent to undergo the acrosome reaction and fertilize an oocyte. Nevertheless, the life span of capacitated sperm is shorter than that of freshly ejaculated sperm (Holt and Medrano, 1997; Bailey et al., 2000). Thus, it is critically important that capacitation and ovulation occur in a temporal-spatial framework. Cryo-capacitated spermatozoa are less able to interact with the oviductal epithelium, exhibiting elevated metabolic rates and increased membrane fluidity and permeability. They undergo spontaneous acrosome reaction due to an uncontrolled influx of Ca^{2+} and lose their fertilizing ability before reaching the ampulla of the oviduct. Cryo-capacitation would then produce a sperm subpopulation with a shortened life span, effectively reducing the fertilization efficiency of the population as a whole. Likewise, it is very important that these decapacitating proteins can be removed from the sperm membrane to successfully fertilize an oocyte. From the results of the present work we can not confirm how long capacitation is delayed after iSPP addition and whether iSPP effect is further reversed. In order to propose a biotechnological use for the iSPP fraction it should be desirable that capacitation is delayed until sperm reaches the oviduct and interact with secretions of female genital tract.

iSPP had little effect on sperm motility evaluated by CASA, in contrast to previous findings of our group (Bernardini et al., 2011) and others (Rodrigues et al., 2013). These discrepancies could be attributed to the differences of the method used to assess motility and the incubation time evaluated. Since, the determination of motility in the present work was conducted by CASA which is a more objective and accurate tool than optical microscopy. In the present work, total and progressive motility were improved only by the addition of complete SP and these effects were regardless of the collection method

of SP. Our previous results indicated that motility was improved by SP obtained in the breeding season compared to the non-breeding season (Dominguez et al., 2008). Moreover, Cardozo et al (2006) found a correlation between changes in SP protein composition and motility according to the season, which could not be confirmed in this work. However, having found that the control samples had higher values of VSL and VCL compared to treatments incubated with ISPP might confirm that these proteins have a decapacitating effect in sperm cells, since capacitated cells frequently presents a vigorous and non-progressive or nonlinear movement in association with the hyperactivation process, phenomenon seen during capacitation. On the other hand, Mortimer and Maxwell (1999) exposed that hypeactivated ram sperm cells also must show other parameters affected like linearity less than 30% and a maximum value of 9.0 µm in lateral head displacement and that was not observed in this work.

iSPP had no effect on sperm quality parameters evaluated by flow cytometry. Meanwhile, the post-thawing addition of complete SP caused the best values in the sperm quality parameters studied. For the viable and normal membrane permeability sperm population (YO-PRO-1-/PI-), we also detected a greater effect of SP collected by AV than SP collected by EE, in contrast to our initial hypothesis.

In quality parameters and sperm motility, addition of complete SP had better effects than addition of iSSP, in opposite of Bernardini et al., (2011) who demonstrated that sperm membrane ultrastructure was improved in sperm cells treated for 15 min with iSPP obtained by AV.

On the other hand, we didn't observe differences between collection seasons, in opposition of reported by Cardozo et al. (2006) and our group (Dominguez et al. 2008) and

Taken all the results together we can conclude that, although the iSPP have a clear decapacitating effect, including the ability to revert tyrosine phosphorylation due to cryo-capacitation, they are not sufficient to emulate the effects of complete SP regarding sperm functional parameters. The protein concentration or the time of incubation might need adjustment, in order to allow a good interaction of the proteins with the spermatozoa. The results of this work may help to shed light on the effects of iSPP as a first step to the development of strategies for improving the quality and the fertilizing ability of cryopreserved ovine semen.

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Tables and figures

Fig. 1: Capacitation status by tyrosine phosphorylation in ram frozen/thawed sperm samples. Solubilized sperm protein from each treatment (5 x 10^6 cells) were loaded and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS±PAGE) and immunoblotting of sperm protein extract with antiphosphotyrosine antibodies (Py); PVDF membranes were stripped, reblocked and incubated sequentially with an anti-tubulin antibody as a loading control (Tubulin). The experiment was performed four times on each season and a representative experiment is shown. Treatments were as follows: lane 1, seminal plasma (SP) obtained by artificial vagina (AV); Lane 2, seminal plasma obtained by electroejaculation (EE); Lane 3, interacting seminal plasma proteins (iSPP) obtained by artificial vagina; Lane 4, interacting seminal plasma proteins obtained by electroejaculation; Lane 5, control.

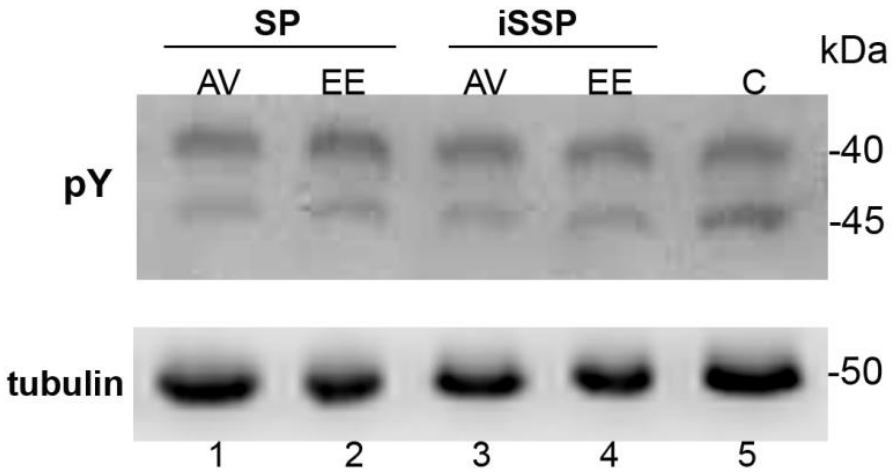

Table 1. Effect of addition of seminal plasma and interacting seminal plasma proteins collected by artificial vagina or electroejaculation on sperm motility parameters (mean ± SEM). No interactions were observed only treatment effect is considered.

Parameter (unit)	SP AV	SP EE	iSPP collected by AV	iSPP collected by EE	Control
TM (%)	74.13 ± 2.99^{a}	72.27 ± 2.99^{a}	60.07 ± 2.99^{b}	53.92 ± 2.99^{b}	54.30 ± 3.01^{b}
PM (%)	64.97 ± 2.64^{a}	63.73 ± 2.64^{a}	53.27 ± 2.64^{b}	46.18 ± 2.64^{b}	48.28 ± 2.78^{b}
VCL (μm/s)	110.19 ± 6.23^{c}	111.41 ± 6.23^{c}	148.92 ± 6.23^{ab}	136.76 ± 6.23^{b}	159.67 ± 6.26^{a}
VSL (μm/s)	80.01± 6.22^{b}	84.27 ± 6.22^{b}	108.22 ± 6.22^{a}	92.55 ± 6.22^{b}	117.40 ± 6.47^{a}
VAP (μm/s)	95.47 ± 5.61^{b}	98.14 ± 5.61^{b}	119.44 ± 5.61^{b}	105.50 ± 5.61^{b}	130.82 ± 5.84^{a}
LIN (%)	76.66 ± 2.76^{a}	78.08 ± 2.76^{a}	73.71 ± 2.76^{ab}	69.10 ± 2.76^{b}	75.71 ± 2.82^{a}
STR (%)	87.95 ± 1.10^{c}	89.28± 1.10^{bc}	92.83 ± 1.10^{a}	91.74 ± 1.10^{ab}	93.06 ± 1.12^{a}
WOB (%)	87.79 ± 1.83^{a}	88.39 ± 1.83^{a}	80.08 ± 1.83^{b}	76.66 ± 1.83^{c}	82.89 ± 1.83^{b}
ALH (μm)	1.50 ± 0.10^{b}	1.48 ± 0.10^{b}	2.10 ± 0.10^{a}	2.12 ± 0.10^{a}	2.07 ± 0.11^{a}
BCF (Hz)	22.58 ± 0.79^{bc}	21.58 ± 0.79^{c}	25.72 ± 0.79^{a}	24.60 ± 0.79^{ab}	25.79 ± 0.84^{a}

SP: seminal plasma; AV: artificial vagina; EE: electroejaculation; TM: total motility percentage; PM: progressive motility percentage; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: lateral head displacement; BCF: beat cross frequency. Values in the same row with different superscripts (abc) are statistically different ($p < 0.01$).

Table 2. Effect of addition of seminal plasma and interacting seminal plasma proteins collected by artificial vagina or electroejaculation on sperm quality parameters (mean ± SEM). No interactions were observed between season and treatment.

Parameter (%)	SP AV	SP EE	iSPP collected by AV	iSPP collected by EE	Control
PI-/ YO-PRO-1-	44.60 ± 2.87^{a}	35.80 ± 2.47^{b}	27.02 ± 2.70^{c}	26.25 ± 2.64^{c}	25.20 ± 2.10^{c}
YO-PRO-1-/ M540-	44.56 ± 2.49^{a}	36.67 ± 1.71^{b}	27.23 ± 2.24^{c}	27.09 ± 2.154^{c}	25.32 ± 2.14^{c}
YO-PRO-1-/ MT+	41.87 ± 4.89^{a}	37.40 ± 2.45^{a}	27.06 ± 2.35^{b}	28.27 ± 2.66^{bc}	26.17 ± 1.57^{b}
PI-/CM-H_2DCFDA-	23.52 ± 4.63^{b}	32.66 ± 4.12^{a}	24.39 ± 4.81^{b}	22.72 ± 3.99^{b}	20.92 ± 4.20^{b}

AV: artificial vagina; EE: electroejaculation; PI-/CM-H_2DCFDA: live sperm with formation of oxygen free radicals; YO-PRO-1-/M540-: live sperm with low membrane fluidity; YO-PRO-1-/MT+: viable sperm with high mitochondrial membrane potential; PI-/YO-PRO-1- viable, normal membrane permeability. Values in the same row with different superscripts (abc) are statistically different ($p < 0.05$).

Table 3. Effect of addition of seminal plasma and interacting seminal plasma proteins collected by artificial vagina or electroejaculation on densitometry quantification of the phosphorylation signal normalized with antitubulin antibody (mean ± SEM).

Protein band (kDa)	SP AV	SP EE	iSPP collected by AV	iSPP collected by EE	Control
45	0.17 ± 0.03^{c}	0.13 ± 0.03^{c}	0.21 ± 0.035^{b}	0.36 ± 0.15^{b}	0.65 ± 0.21^{a}
40	0.37 ± 0.14^{c}	0.38 ± 0.14^{c}	0.38 ± 0.14^{c}	0.53 ± 0.14^{b}	0.77 ± 0.14^{a}

AV: artificial vagina; EE: electroejaculation. Values in the same row with different superscripts (abc) are statistically different ($p < 0.05$).