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Equine seminal plasma on preserving the viability of frozen-thawed ram sperm

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Abstract

Re-suspension of frozen-thawed ram sperm with ovine and bovine whole seminal plasma (SP) is beneficial for its post-thawing viability. However, neither the influence of SP incubation duration nor the re-suspension with equine SP has been tested. In the first experiment, frozen-thawed ram sperm were incubated with SP from bovine (BSP), equine (ESP) or ovine (OSP) or without SP (-SP) for short (5 min) or long (6 h) periods. Viability parameters such as sperm progressive motility, percentage of live cells and membrane functionality were assessed every 2 h for 6 h. All SP treatments showed higher spermatozoa viability than the -SP treatment in most evaluations. Incubation time did not affect sperm viability for BSP treatment, however, ESP and OSP induced a transitory benefic effect in the short incubation period and detrimental effect in the longer period. In the second experiment, frozen-thawed sperm, with or without short incubation with SP, were selected by swim-up, and their DNA fragmentation rate was assessed using comet assay immediately after swim-up completion and after 5 h of incubation. BSP, ESP and OSP protein profiles were determined by SDS-PAGE. Only ESP was associated with sperm DNA stabilization capacity and SP from rams and bulls showed protein profiles different from that of stallions. These experiments indicate that equine or ovine, but not bovine whole SP supplementation to post-thawing incubation medium of frozen-thawed ram sperm affects its viability in a time dependent manner. The beneficial effect of ESP on stabilizing DNA integrity, even after sperm washing with swim-up method and incubation for 5 h, can be determined by SPP or by antioxidant components from SP.

Keywords: heterologous seminal plasma, sperm cryopreservation.

Introduction

The main challenge for cervical artificial insemination (AI) programs in ewes with frozen-thawed sperm is the achievement of pregnancy rates similar to those obtained with laparoscopic intrauterine AI. The inconsistent results of cervical AI with cryopreserved ram sperm may be due to its impaired transport and survival in the female reproductive tract and by its reduced fertility (reviewed by Salamon and Maxwell, 2000). Sperm deterioration during the freezing-thawing process is attributed to several causes; some of them related to the removal of seminal plasma proteins (SPP). This hypothesis is supported by events such as cold-shock reversion (Pérez-Pé *et al*., 2001; Barrios *et al*., 2005); antioxidant defense (Peris *et al*., 2007; Marti *et al*., 2008); better sperm capacitation and motility (Maxwell *et al*., 1999; El-Hajj Ghaoui *et al*., 2007); and improved ability to penetrate cervical mucus and fertility after cervical AI obtained by sperm exposed to ovine (Maxwell *et al*., 1999) or bovine seminal plasma (Kareta *et al*., 1972; Gunay *et al*., 2006). However, contradictory results observed after the addition of homologous seminal plasma (SP) to ram sperm before AI highlight the importance of SP and sperm handling for preservation of spermatozoa viability (El-Hajj Ghaoui *et al*., 2007; O'Meara *et al*., 2007).

Pelleted or in-straw frozen ram sperm are commonly thawed and re-suspended into a glass tube with 20-30% SP before AI takes place (Maxwell *et al*., 1999; El-Hajj Ghaoui *et al*., 2007; O'Meara *et al*., 2007), which is laborious and time consuming. An alternative procedure would be an in-straw media mixture, similar to the method proposed for direct embryo transfer (Leibo, 1984), which would allow a mixture of sperm and SP after thawing under field conditions without additional re-suspension steps. In addition, frozen-thawed ram sperm may be supplemented with SP from other species, minimizing the effect of limited volume of SP and the seasonal variation in protein composition of ram ejaculates (Pérez-Pé *et al*., 2001; Cardozo *et al*., 2006).

Beneficial effects for ram sperm viability were observed following addition of bovine SP before freezing (Gunay *et al*., 2006) and after thawing (Kareta *et al*., 1972; García-López *et al*., 1996). Such an effect can be attributed to homology between bovine and ovine SPP (Bergeron *et al*., 2005; Jobim *et al*., 2005). A similar homology was also observed among bovine and equine SPP (Ménard *et al*., 2003), indicating that SP proteins may play similar biological roles in different mammalian species. Thus, considering the greater SP content in stallions' ejaculates, it appears relevant to test the addition of equine SP to frozen-thawed ram sperm. However, neither the influence of SP incubation duration nor the re-suspension with equine SP has been tested. The objectives of this study were: 1) to determine the influence of incubation length after in-

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straw re-suspension of frozen-thawed ram sperm with heterologous (bovine and equine) or homologous SP on the sperm progressive motility, percentage of live cells, membrane functionality and DNA integrity and 2) to determine the electrophoretic SPP profile of bovine, equine and ovine SP.

Materials and Methods

Chemicals and reagents

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were prepared using water from a Milli-Q Synthesis System (Millipore, Bedford, MA, USA).

Experimental design

The Ethics Committee in Animal Experimentation of the Agro-Veterinary Research Center/Santa Catarina State University approved the procedures herein. The effects of the addition of heterologous versus homologous SP to frozen-thawed ram sperm were compared in two experiments comprising sperm incubation for 6 h. In the first experiment, a short SP incubation period was tested in order to simulate the physiologic removal of the adsorbed SP proteins during sperm transport through female genital secretions. In the second experiment, the spermatozoa DNA fragmentation rate was evaluated by single cell gel electrophoresis test (comet assay) after short incubation with SP and spermatozoa selection through the swim-up procedure. The total SPP concentration of all SP was determined by direct spectrophotometry and the molecular protein profile by SDS-PAGE to detect potential similarities.

Experimental animals and whole SP preparation

All animals were bred on a private farm (27°19'50.68" - 50°36'48.05"). The SP was obtained during summer (January) from single ejaculates collected with an artificial vagina from five bulls (bovine SP - BSP) and five stallions (equine SP - ESP), and from 16 ejaculates collected from 8 rams (ovine SP - OSP). Each ejaculate was centrifuged three times at room temperature (1200 g, 20 min) until a clear supernatant was recovered. The SP samples were homogeneously pooled within the species and filtered through a 0.22 µm pore membrane before frozen storage at -20°C in 1 ml aliquots. SP was thawed at room temperature just before use.

Sperm collection and freezing

Two ejaculates were collected daily per ram with an artificial vagina from the same eight rams used as SP donors. Collections occurred in the summer (February), before the ovine breeding season, in two replicates with a one-week interval. Right after the collections, ejaculates were diluted (1:1, v/v) in the Tris-glucose-citric acid-egg yolk (TY) extender (Evans and Maxwell, 1987) with 8% (v/v) glycerol. Semen samples were cooled (0.3-0.5°C/min) in ice-water bath to 5°C. Ejaculates meeting minimum quality standards (wave motion of 4 or 5 on a 0-5 scale and concentration of$\geq 3 \times 10^9$ spermatozoa/mL, as determined with a Neubauer chamber) were pooled and maintained at 5°C for 2 h, before isothermal loading in French mini straws. The straw loading was comprised of a 0.175 ml sperm-extender column with 250 x 10^6 spermatozoa followed by a 1.0 cm air bubble and 0.045 ml of SP or TY extender column (25% of total dose volume), allocated to the following treatments: BSP; ESP; OSP; and a control treatment without SP (-SP). Straws were sealed with polyvinyl alcohol powder and frozen in nitrogen vapor before immersion in liquid nitrogen (Evans and Maxwell, 1987).

Experiment 1

Sperm thawing and re-suspension

Three straws per treatment were thawed in a 37°C water bath for 30 sec and then agitated to mix the sperm and SP or TY columns. Just after homogenization, straws were emptied in 1.5 ml conical tubes and the content was homogeneously pooled within treatments. After incubation for 5 min at 37°C, the samples were re-suspended to 20 x 10^6 sperm/ml with isothermal TY extender with no SP or enriched with 25% of BSP, ESP or OSP. The samples re-suspended in pure TY comprised the short SP incubation groups (BSPS, ESPS and OSPS) while those re-suspended in TY with SP or without SP comprised the long incubation groups (BSPL, ESPL, OSPL and -SP), respectively.

Sperm quality evaluations

All treatments were incubated at 37°C for 6 h and sperm quality was assessed every 2 h. Sperm progressive motility was assessed (5% increments) in a slide under a coverslip with light microscopy (200X) equipped with heat-stage (at 37°C). The percentage of live cells was assessed by Eosin-Nigrosin staining (Swanson and Bearden, 1951) and the membrane functionality was determined by the hypoosmotic swelling test (Correa and Zavos, 1994), modified by the use of 100 mOsm/l Tris-glucose-citric acid (TGC) extender (Evans and Maxwell, 1987) for dilution of samples (1:100, v/v) and incubation at 37°C for 1 h. After incubation, two hundred spermatozoa per sample were analyzed per slide.

Experiment 2

Sperm DNA fragmentation

Sperm DNA fragmentation was assessed by single cell gel electrophoresis using the neutral comet

assay (Linfor and Meyers, 2002), adapted for ram sperm. Three straws from each treatment were thawed, pooled and incubated for 5 min. To simulate the physiological removal of non-motile spermatozoa and of the SPP adsorbed during spermatozoa transit through the female genital tract, a swim-up sperm selection procedure (60 min, at 37°C) was performed in TGC. To standardize the assay, a frozen-thawed sample from the -SP treatment and a fresh sperm sample extended in TY (1:2, v/v) from a single ram were used to compose three additional treatments, in which samples were diluted but not submitted to swim-up: (1) -SP frozen-thawed sperm diluted in TGC; (2) non-frozen sperm diluted in TGC (NFI), representing spermatozoa with intact DNA, serving as a negative control and; (3) non-frozen sperm incubated with 400 µm H_2O_2 in TGC (NFF), for induction of sperm DNA fragmentation, serving as a positive control. The dilution of -SP, NFI and NFF was done simultaneously with the start of the swim-up for the other treatments.

After completing the swim-up, the first sperm DNA fragmentation evaluation (moment 1 h) was conducted for samples of selected (BSPS, ESPS, OSPS and -SP) and unselected spermatozoa (-SP, NFI and NFF) at a concentration of 20 x 10^6 spermatozoa/ml. The second DNA fragmentation evaluation was done after incubation at 37°C in TGC for an additional 5 h (moment 6 h).

Single cell gel electrophoresis

Prior to slide preparation, two 0.5% agarose solutions were prepared in Tris, boric acid and EDTA buffer (TBE), with normal (solution 1) and low (solution 2) melting points. Initially, 300 µl of solution 1 was scattered on the slide and dried at 40°C. To prepare the second agarose layer, 5 µl of sperm solutions from each treatment were re-suspended in TGC (1 x 10^6 sperm/ml) and homogenized with 95 µl of solution 2. Subsequently, 50 µl of this new solution was poured over the first gel layer, covered with a 24 x 50 mM coverslip and chilled at 5°C for 15 min. After carefully removing the coverslip, a third layer of solution 2 was poured on, covered with a coverslip and chilled at 5°C for 15 min. After the solidification of the third agarose layer and the removal of the coverslip, the slides were incubated for 2 h at 5°C in the first lysing solution (2.5 M NaCl, 100 mM N_4EDTA, 10 mM TRIS base [pH 10], 1% Triton X-100 and 1% lauryl-sarcosine). Subsequently, the slides were incubated for 4 h at 37°C in the second lysing solution (2.5 M NaCl, 5 mM TRIS base [pH 7.4], 0.5% lauryl-sarcosine and 20 µg/ml RNase A). Finally, the slides were incubated for 12 h at 37°C in the third lysing solution (2.5 M NaCl, 5 mM TRIS base [pH 7.4], 40 mM dithiothreitol and 200 mg/ml proteinase K). After three baths in distilled water (20 min each), the slides were immersed in electrophoresis TBE buffer (pH 8.0), for 20 min.

Electrophoresis was performed at 120 mA for 60 min, using the BIO-RAD Mini-Protean 4 Cell System (Bio-rad Laboratories, USA). After electrophoresis, the slides were washed in distilled water and kept in a vertical position for 30 min to dry. Subsequently, they were fixed in 92°GL alcohol for 5 min and dried at room temperature for 60 min. The slides were then stained with 50 ml of ethidium bromide (50 mg/ml), covered with a coverslip and evaluated under ultraviolet light in an epifluorescence microscope. Sperm DNA fragmentation was classified as positive (presence) or negative (absence), based on the tail of fragmented DNA that migrated from the sperm head causing a "comet pattern" (Linfor and Meyers, 2002).

Spectrophotometry and SDS-PAGE of SP proteins

For the BSP, ESP and OSP samples, the total protein concentration was determined by direct spectrophotometry (Bradford, 1976) and the protein profile was determined by the denaturing polyacrylamide gel electrophoresis technique - SDS-PAGE (Lodish *et al*., 2000). The SDS-PAGE procedure was conducted from standardized samples (25 µg/µl protein in water) of the same treatments. After standardization, a buffer solution was added (0.6173M Tris-HCl – [pH 6.8], 2M β-mercaptoethanol, 10% SDS and 0.1% bromophenol blue in water), at a 1:1 ratio for all samples and the protein was denatured by heating for 5 min at 90°C. The polyacrylamide gel was prepared at 17% and electrophoresis was performed on the BIO-RAD Mini-Protean 4 Cell System (Bio-Rad Laboratories, USA), with a constant current of 25 mA for 2 h. The gel was stained with Brilliant Coomassie Blue for 4 h and bleached using a 45% methanol and 10% acetic acid water solution (v/v). The SDS-PAGE Molecular Weight Broad Range (Bio-Rad Laboratories, USA) was used as a molecular weight marker.

Statistical analysis

Data for sperm motility, membrane functionality and percentage of live cells were submitted to arcsine transformation and evaluated by ANOVA with repeated measures, using the PROC MIXED (SAS Institute Inc. Cary, NC, USA). The rate of sperm DNA fragmentation was analyzed by the Chi-square analysis using Minitab® (Minitab Inc., USA). The significance level was set at $P < 0.05$.

Results

In experiment 1, sperm motility and percentage of live cells were higher ($P < 0.05$) for frozen-thawed ram sperm with a short incubation period than with a long incubation period in the ESP treatment (Table 1). However, membrane functionality at 6 h was similar ($P > 0.05$) across incubation periods. When OSP was used, after 4 h of incubation, all evaluated sperm quality parameters were higher after the short incubation period than those observed after the long incubation period ($P < 0.05$).

Table 1. Progressive motility, percentage of live cells and membrane functionality rates of frozen-thawed ram spermatozoa within a 6 h period, after short (5 min) or long (6 h) incubation period without seminal plasma (-SP) or with seminal plasma (SP) from bovine (BSP); equine (ESP) or ovine (OSP).

Treatment	Period	Progressive motility (%)				Live spermatozoa (%)				Membrane functionality (%)			
		0 h	2 h	4 h	6 h	0 h	2 h	4 h	6 h	0 h	2 h	4 h	6 h
-SP	long	45.0 ± 2.0^{a}	41.6 ± 1.6^{a}	25.0 ± 2.8^{bc}	10.0 ± 2.8^{ab}	52.6 ± 1.2^{a}	41.6 ± 1.6^{cd}	30.6 ± 2.3^{c}	21.6 ± 1.6^{d}	41.6 ± 2.0^{a}	28.3 ± 2.0^{a}	16.3 ± 0.8^{bc}	9.3 ± 0.6^{c}
BSP	long	45.0 ± 3.0^{Aa}	43.3 ± 1.6^{Aa}	25.0 ± 7.6^{Bc}	8.3 ± 1.6^{Abc}	51.3 ± 1.6^{Aa}	43.0 ± 1.7^{Abc}	38.0 ± 1.1^{Bb}	31.0 ± 1.5^{Ab}	41.0 ± 1.1^{Aa}	31.6 ± 2.3^{Aa}	19.6 ± 0.8^{Bb}	17.6 ± 1.7^{Aa}
	short	45.0 ± 3.0^{Aa}	41.6 ± 1.6^{Aa}	31.6 ± 1.6^{Aabc}	10.0 ± 2.8^{Aab}	52.0 ± 2.0^{Aa}	46.0 ± 2.6^{Aab}	41.6 ± 1.2^{Aa}	34.3 ± 0.8^{Aab}	41.0 ± 0.5^{Aa}	33.0 ± 1.5^{Aa}	25.0 ± 1.5^{Aa}	19.0 ± 1.5^{Aa}
ESP	long	45.0 ± 2.0^{Aa}	25.0 ± 2.8^{Bb}	10.0 ± 0^{Bd}	8.3 ± 1.6^{Bbc}	51.6 ± 0.8^{Aa}	38.3 ± 2.7^{Bd}	29.3 ± 0.6^{Bc}	26.0 ± 1.0^{Bc}	41.0 ± 1.5^{Aa}	22.6 ± 3.5^{Bb}	19.6 ± 4.9^{Bb}	19.6 ± 2.0^{Aa}
	short	45.0 ± 2.0^{Aa}	41.6 ± 1.6^{Aa}	38.3 ± 1.6^{Aa}	16.6 ± 3.3^{Aa}	50.7 ± 0.3^{Aa}	48.3 ± 0.8^{Aa}	42.3 ± 0.8^{Aa}	36.0 ± 2.0^{Aa}	42.7 ± 1.4^{Aa}	30.6 ± 2.4^{Aa}	27.0 ± 1.0^{Aa}	22.0 ± 2.5^{Aa}
OSP	long	45.0 ± 3.0^{Aa}	43.3 ± 1.6^{Aa}	13.3 ± 3.3^{Bd}	3.3 ± 1.6^{Bc}	51.6 ± 1.2^{Aa}	43.0 ± 0.5^{Abc}	31.3± 0.8^{Bc}	22.6 ± 2.8^{Bd}	42.0 ± 2.0^{Aa}	30.3 ± 1.4^{Aa}	13.3 ± 1.7^{Bc}	13.0 ± 1.1^{Bb}
	short	45.0 ± 3.0^{Aa}	43.3 ± 1.6^{Aa}	35.0 ± 2.8^{Aab}	13.3 ± 3.3^{Aab}	53.0 ± 1.0^{Aa}	43.4 ± 1.0^{Abc}	40.6 ± 0.6^{Aab}	34.6 ± 0.6^{Aa}	43.0 ± 1.0^{Aa}	30.6 ± 2.8^{Aa}	25.6 ± 2.7^{Aa}	18.0 ± 1.1^{Aa}

^{AB}Least square means having distinct capital superscripts in the same column differ among incubation periods ($P < 0.05$). ^{ab}Least square means having distinct superscripts in the same column differ among treatments ($P < 0.05$)

Sperm motility for the ESPL treatment was lower ($P < 0.05$) than the other treatments at 2 h of incubation (Table 1). At 4 h, sperm motility was similar for ESPL and OSPL ($P > 0.05$), but both treatments presented lower motility than that of the other treatments ($P < 0.05$). At 6 h, sperm motility was similar for -SP and BSPS, ESPS and OSPS ($P > 0.05$) and all such values were higher ($P < 0.05$) than those observed with the long incubation periods. The percentage of live cells was similar ($P > 0.05$) among the treatments with short SP incubation, regardless of the source of SP, and this percentage was higher ($P < 0.05$) at 4 h than in all other treatments. Although BSPS, ESPS and OSPS treatments presented similar ($P > 0.05$) sperm membrane functionality at 4 h of incubation, it was higher than that of the other treatments ($P < 0.05$). At 6 h, the -SP group showed the lowest ($P > 0.05$) sperm membrane functionality compared to all other SP treated groups. At this time-point, in SP treatments the sperm membrane functionality was depressed only for the OSPL group ($P < 0.05$).

In the experiment 2, the sperm DNA fragmentation rate was higher ($P < 0.05$) for the NFF treatment (90%) than for the other treatments at moment 1 h (Table 2). At that period, sperm DNA fragmentation rates for NFI (24%) and BSPS (35%) were similar ($P > 0.05$), and only the NFI treatment presented a reduced rate ($P < 0.05$) in comparison with all other treatments. At moment 6 h, sperm DNA fragmentation for the NFF treatment remained at 90% and was higher ($P > 0.05$) than all other treatments (Table 2). At that period, DNA fragmentation rates were similar ($P > 0.05$) for NFI and BSPS, ESPS and OSPS with short incubation. The ESPS was the only treatment with stabilized sperm DNA integrity ($P > 0.05$), presenting rates of 40% and 52% at 1 h and 6 h, respectively (Table 2).

Table 2. DNA fragmentation rates of frozen-thawed and non-frozen ram spermatozoa not-selected or selected by swim-up (1 h in Tris-glucose-citric acid extender).

Treatment	Selected		Not selected	
	1 h	6 h	1 h	6 h
BSPS	35^{bcX}	55^{BCY}	-	-
ESPS	40^{bX}	52^{BCX}	-	-
OSPS	40^{bX}	60^{BCY}	-	-
-SP	40^{bX}	63^{BY}	50^{bX}	70^{BY}
NFI	-	-	24^{cX}	50^{CY}
NFF	-	-	90^{aX}	90^{AX}

^{ab}Least square means having distinct superscripts in the same column differ at 1 h ($P < 0.05$). ^{AB}Least square means having distinct superscripts in the same column differ at 6 h ($P < 0.05$). ^{XY}Least square means having distinct superscripts in the same line differ between incubation periods ($P < 0.05$). BSPS, ESPS and OSPS = frozen-thawed sperm short incubated with bovine, equine or ovine seminal plasma, respectively; -SP = frozen-thawed sperm incubated without seminal plasma; NFI and NFF = non-frozen sperm incubated without seminal plasma with non-induced DNA damage or with induced DNA damage, respectively.

The total protein concentration for BSP, ESP and OSP samples was 25.1, 3.0 and 16.8 µg/µl, respectively. The protein profile was similar for BSP and OSP, especially regarding the 14 kDa band. ESP exhibited a protein profile distinct from that detected for BSP and OSP, displaying a variety of bands within 6 and 50 kDa intervals as showed in Fig. 1.

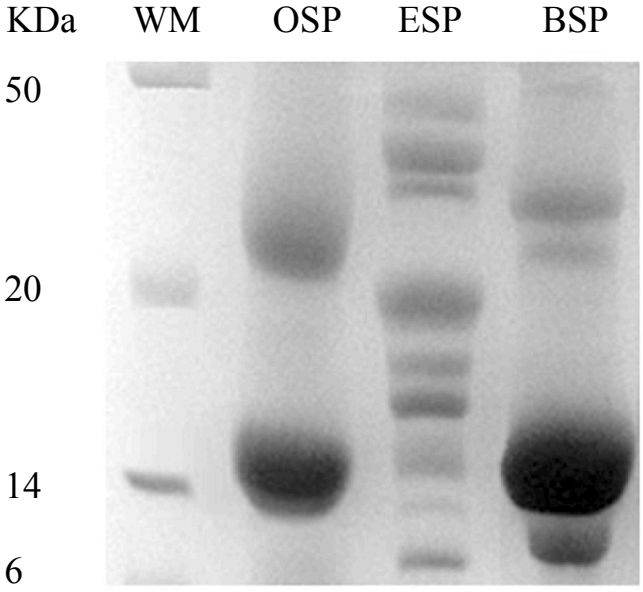

Figure 1. One-dimensional polyacrylamide electrophoresis gel of Ovine (OSP), Equine (ESP) and Bovine (BSP) seminal plasmas. The amount of protein was standardized to 25 µg per lane and the gel was stained with Comassie Blue. Molecular weight marker (WM) is on the left.

Discussion

The main findings of the present study are that the beneficial effects of re-suspension of frozen-thawed ram sperm with SP are time dependent and that equine SP is a viable supplement to restore sperm viability after thawing. Despite the known benefits of the addition of SP from bovine (Kareta *et al*., 1972; Gunay *et al*., 2006) and ovine (Maxwell *et al*., 1999) on improving fertility of ram frozen-thawed sperm, conflicting results (El-Hajj Ghaoui *et al*., 2007; O'Meara *et al*., 2007) suggest that the use of SP in AI programs still needs optimization. These inconsistent results are probably due to a long interval between sperm processing and the AI itself, which has also been linked to inconsistent results obtained with the addition of homologous SP (El-Hajj Ghaoui *et al*., 2007; O'Meara *et al*., 2007). In our study, the proposed straw loading system allowed a fast sperm SP re-suspension after thawing, eliminating extra-straw manipulation. This procedure prevents additional sperm handling before its use, but does not eliminate either volume constraints or seasonal changes in ram ejaculate composition (Cardozo *et al*., 2006) nor the risk of transmission of species-specific diseases. However, the use of bulls and stallions as donors for SP banks would enable the use of SP in large AI programs, avoiding the limitations related to the use of homologous SP.

In the first experiment the sperm viability of the -SP group was slightly compromised in most evaluation periods for all evaluated parameters compared to SP treatments, corroborating previous observations (Maxwell *et al*., 1999; El-Hajj Ghaoui *et al*., 2007). The BSP treatment showed similar results in most evaluation periods for all evaluated parameters, regardless of the incubation period. However, for ESP and especially for OSP treatments the long incubation period was comparable to the -SP group. This result may be attributed to the detrimental effect of long exposure to SPP in the ESPL and OSPL groups rendering a very sensitive sperm membrane modification by efflux of cholesterol and phospholipids (Manjunath *et al*., 2002). The time and concentration of harmful SPP in these treatments potentially supplanted the scavenging capacity of low-density lipoprotein fraction in the egg yolk component of the sperm diluent (Manjunath *et al*., 2002; Bergeron *et al*., 2005), however, for the BSP treatment the benefic and harmful SPP was balanced in both incubation periods.

Despite the difficulty in controlling the allocation of beneficial, harmful or inert SP components on the surface of spermatozoa, especially for ovine and equine SP, our results indicate that the short incubation period allows a satisfactory manifestation of beneficial components on SP, improving ram sperm viability after thawing.

In the second experiment, on the first evaluation (at 1 h) only the BSPS treatment presented a DNA fragmentation rate similar to that observed for the NFI group, but no differences were observed among BSPS and the other treatments at that period. At second evaluation 5 h later (moment 6 h), except for the ESPS treatment, the DNA fragmentation rate for all other treatments showed characteristic DNA fragmentation over time. This slight stabilization in the DNA fragmentation rate observed in the ESPS treatment may be determined by antioxidant concentration in the ESP, since SP antioxidants are DNA stabilizing (Peris *et al*., 2007). However, despite the lower DNA stabilization over time observed for BSPS and OSPS compared to ESPS, both showed DNA fragmentation rates similar to the NFI group at 6 h, reinforcing a protective effect of homologous and heterologous SP which may eventually lead to improved pregnancy rates due to a reduction in spermatozoa DNA damages potentially related to premature embryo losses.

In the present study, the protein profile of ESP differed from that of BSP and OSP (Fig. 1). The greatest number of bands in the ESP protein profile may be determined by different glycosylation levels of the same proteins not well differentiated by 1 D SDS-PAGE used in this study. However, SP protein profiles from the three species showed electrophoretic bands between 14 to 20 kDa molecular weights (Fig. 1.), which are compatible with cold-shock protection conferred to ram sperm (Barrios *et al*., 2005). Considering that the homology between bovine, equine and ovine SP proteins is around 15 to 35% (Druart *et al*., 2013), our results reinforce the observations that beneficial effects of SP may not only be related to the protein coat, but also to relevant effects of enzyme antioxidant activity protecting sperm membrane (Marti *et al*., 2008) and sperm DNA (Peris *et al*., 2007). These results highlight the need to determine the role of SP components, such as antioxidants, protease inhibiting molecules and growth factors on sperm longevity. This may explain the beneficial effects observed in the present study with ESPS treatment.

In conclusion, equine or ovine, but not bovine whole SP supplementation to post-thawing incubation medium of frozen-thawed ram sperm affects its viability in a time dependent manner. The beneficial effect of ESP on stabilizing DNA integrity, even after sperm washing with the swim-up method and additional incubation for 5 h, can be determined by other components other than SP proteins. Further studies must be conducted to evaluate the effects of equine whole SP supplementation of frozen-thawed sperm on pregnancy rates following ewe cervical AI.

Conflict of interest

The authors declare that there is no conflict of interest in this work.

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References

Barrios B, Fernández-Juan M, Muiño-Blanco, Cebrián-Pérez JA. 2005. Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. *J Androl,* 26:539-549

Bergeron A, Villemure M, Lazure C, Manjunath, P. 2005. Isolation and characterization of the major proteins of ram seminal plasma. *Cell Biol Biochem*, 71:461-470.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72:248-254.

Cardozo JA, Fernández-Juan M, Forcada F, Abecia A, Muiño-Blanco T, Cebrián-Pérez JA. 2006. Monthly variations in ovine seminal plasma proteins analyzed by two-dimensional polyacrylamide gel electrophoresis. *Theriogenology*, 66:841-850.

Correa JR, Zavos PM. 1994. The hyposmotic swelling test: Its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. *Theriogenology*, 42:351-360.

Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R, Gibb Z, Crossett B, Tsikis G, Labas V, Harichaux G, Grupen CG, de Graaf SP. 2013. Proteomic characterization and cross species comparison of mammalian seminal plasma. *J Proteomics*, 91:13-22.

El-Hajj Ghaoui R, Thomson PC, Leahy T, Evans G, Maxwell WMC. 2007. Autologous whole ram seminal plasma and its vesicle-free fraction improve motility characteristics and membrane status but not in vivo fertility of frozen-thawed ram spermatozoa. *Reprod Domest Anim*, 42:541-549.

Evans G, Maxwell WMC. 1987. *Salamon's Artificial Insemination of Sheep and Goats*. Sydney: Butterworths. 194 pp.

García-López N, Ollero M, Cebrián-Pérez JA, Muiño-Blanco T. 1996. Reversion of thermic-shock effect on ram spermatozoa by adsorption of seminal plasma proteins revealed by partition in aqueous two-phase systems. *J Chromatogr*, 680:137-143.

Gunay U, Dogan I, Nur Z, Manolov I, Sagirkaya H, Soylu MK, Kaptan C, Akpinar L. 2006. Influence of bull seminal plasma on post-thaw ram semen parameters and fertility. *Bull Vet Inst Pulawy*, 50:503-507.

Jobim MIM, Oberst ER, Salbergo CG, Wald VB, Horn AP, Mattos RC. 2005. BSP A1/A2-like proteins in ram seminal plasma. *Theriogenology*, 63:2053-2062.

Kareta W, Pilch J, Wierzbowski S. 1972. Fertility of frozen ram semen diluted in citrate buffer with or without added bull seminal plasma. *In*: Proceedings of the 7th International Congress on Animal Reproduction and AI, 1972, Munich. Munich: ICAR. v.3, pp. 1479-1484.

Leibo SP. 1984. A one-step method for direct nonsurgical transfer of frozen-thawed bovine embryos. *Theriogenology*, 21:767-790.

Linfor JJ, Meyers S. 2002. Detection of DNA damage in response to cooling injury in equine spermatozoa using single-cell electrophoresis. *J Androl*, 23:107-113.

Lodish H, Berk A, Lawrence Zipursky S, Matsudaira P, Baltimore D, Darnell J. 2000. *Molecular Cell Biology*. 4th ed. New York: W.H. Freeman. 1296 pp.

Manjunath P, Nauc V, Bergeron A, Ménard M. 2002. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. *Biol Reprod*, 67:1250-1258.

Marti E, Mara L, Marti JI, Muiño-Blanco T, Cebrián-Pérez JA. 2008. Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. *J Androl*, 29:459-467.

Maxwell WMC, Evans AG, Mortimer ST, Gillan L, Gellatly ES, McPhie CA. 1999. Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. *Reprod Fertil Dev*, 11:123-126.

Ménard M, Nauc V, Lazure C, Vaillancourt D, Manjunath P. 2003. Plasma phospholipid-binding proteins reveals the presence of a novel member of this family of protein in stallion seminal fluid. *Mol Reprod Devl*, 66:349-357.

O'Meara CM, Donovan A, Hanrahan JP, Duffy P, Fair S, Evans ACO, Lonergan P. 2007. Resuspending ram spermatozoa in seminal plasma after cryopreservation does not improve pregnancy rate in cervically inseminated ewes. *Theriogenology*, 67:1262-1268.

Pérez-Pé R, Cebrián-Pérez JA, Muiño-Blanco T. 2001. Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. *Theriogenology*, 56:425-434.

Peris SI, Bilodeau JF, Dufour M, Bailey J. 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. *Mol Reprod Dev*, 74:878-892.

Salamon S, Maxwell WM. 2000. Storage of ram semen. *Anim Reprod Sci*, 62:77-111.

Swanson, EW, Bearden, HJ. 1951. An eosin-nigrosin stain for differentiating live and dead bovine spermatozoa. *J Anim Sci*, 10:981-987.