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Soy lecithin as a potential alternative to powdered egg yolk for buck sperm cryopreservation does not protect them from mitochondrial damage

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ABSTRACT

The aim of this study was to address whether soy lecithin (SL) was an effective non-penetrating cryoprotectant for buck sperm cryopreservation in the presence of seminal plasma. There was also an attempt to determine the optimal concentration of BHT as an antioxidant in powdered egg yolk (PEY) or in SL based media. Two ejaculates were collected from six bucks and mixed ejaculates were aliquoted into washed, using centrifugation procedures, and unwashed samples. In Experiment 1, washed sperm were re-suspended in PEY (15%) or SL (1%) media, while unwashed semen was only diluted in SL medium. In Experiment 2, washed and unwashed sperm were diluted in PEY and SL media, respectively, with there being different BHT concentrations (0.6, 2.0 and 5.0 mM). In both experiments, after 4 h of refrigeration, there were no differences neither in sperm viability nor plasma membrane functional integrity (HOST) between groups when there were evaluations using eosin-nigrosine staining. After thawing, however, there was a negative effect on motility of washed sperm preserved in SL media. Furthermore, results from cytometry evaluations indicated there was a larger population of thawed sperm with intact plasma (SYBR-14$^+$/PI-) and acrosome (PE-PNA-) membranes, but inactive mitochondria (Mitotracker deep red-) when SL media were used. When there was BHT supplementation, there was only a slight enhancement of motility of spermatozoa preserved in PEY media with 5 mM BHT. In conclusion, when effectiveness and efficiencies are considered, PEY is the non-penetrating cryoprotectant that should be utilized for buck sperm cryopreservation.

1. Introduction

In recent years, soy lecithin (SL) has emerged as a suitable substitute for egg yolk in the conventional extenders in the quest to develop a non-animal-based alternative for commercial semen extenders. Results of numerous studies indicate that with the use of soy-based extenders there were varying outcomes with regard to efficacy of these extenders for sperm preservation, therefore, there continues to be a need for improvements in efficacy when this extender is used (Layek et al., 2016).

With buck semen cryopreservation, the elimination of seminal plasma utilizing centrifugation procedures is recommended because seminal plasma contains lipases from the secretions of the bulbourethral glands that interact with egg yolk producing toxic substances that detrimentally affect sperm cells, reducing the freezing capacity for sperm samples (Leboeuf et al., 2000). Se-

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men washing, however, is a cumbersome process, time-consuming and can lead to sperm loss or damage (Tuli and Holtz, 1994). The SL and egg yolk differ in lipid composition and fatty acid content (Palacios and Wang, 2005; Le Grandois et al., 2009); therefore, the SL interacts differently than egg yolk constituents with the enzymes contained in the seminal plasma.

Furthermore, extender supplementations with antioxidants represent potential opportunities for reducing the detrimental effects on sperm quality caused by freeze-thawing processes. In previous years, the antioxidant butylated hydroxytoluene (BHT) has been widely used. The supplementation of extender with this synthetic analog of vitamin E has positive effects reducing the cold-shock effect on bull (Shoae and Zamiri, 2008), buffalo (Ijaz et al., 2009), ram (Farshad et al., 2010) and buck (Memon et al., 2011) sperm. Antioxidant concentration, however, is an important aspect to be considered when determining cryopreservation media composition because antioxidant concentration may affect media efficacy and antioxidant capacity (Memon et al., 2012). The objectives of this study, therefore, were to assess SL as a viable alternative to be used in buck sperm freezing media in the presence of seminal plasma, as well as to determine the optimal BHT antioxidant concentration for freezing media.

2. Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated. The PEY was obtained from NIVE (Nunspeet Holland Eiproducten). Animal procedures were performed using procedures consistent with Spanish laws for animal welfare and experimentation.

2.1. Freezing extenders preparation

The basic extender used in this study was Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) solution defined by Salamon and Maxwell (2000), consisting of Tris (0.3 M), citric acid anhydrous (94.7 mM), and D (+)-glucose (27.75 mM). Glycerol (5% v/v, final concentration) and antibiotics (1000 IU/mL sodium penicillin and 1.0 mg/mL streptomycin sulfate) were subsequently added. The PEY or SL were also subsequently added to a final concentration of 15% (v/v) and 1% (w/v), respectively.

Preparation of the extender based on PEY was performed as described by Marco-Jiménez et al. (2004). Briefly, PEY was diluted (1:1.25) with Milli Q water and stirred for 20 min, while the SL extender was prepared by the addition of 1% (w/v) of lecithin (Sigma 11145) to the basic extender, as described by Forouzanfar et al. (2010) in ram sperm. Different concentrations of butylated hydroxytoluene (BHT) were used as antioxidant agents (0.6, 2.0 and 5.0 mM dissolved in DMSO). The final concentration of DMSO in the sperm preservation medium was 0.25% (v/v), as reported by Khalifa et al. (2008).

2.2. Semen collection and freezing protocol

Two different consecutive experiments were performed. In each experiment bucks of the *Blanca de Rasquera* breed that were approximately 2 years of age were used to conduct the study. Semen was collected twice weekly with two ejaculates per collection being collected from each male using an artificial vagina in *Caldes de Montbui*, Spain, where semen donors were housed.

In Experiment 1, there was a total of six semen collections conducted ($n = 6$). At the time of semen collections, two ejaculates were obtained from a total of six *Blanca de Rasquera* bucks. The twelve ejaculates collected in every replicate were immediately pooled and divided in two samples. One sample was washed with utilization of dilution (1:5) procedures in TCG and centrifuged at $600 \times g$ for 10 min. After the supernatant was removed, the sediment was diluted and centrifuged again using the same procedures as utilized for the previous centrifugation. The supernatant was carefully removed and the sediment was divided into two aliquots and re-suspended in an extender (1:4) containing a PEY concentration of 15% (v/v) or 1% (w/v) SL. The other sample (unwashed) was directly diluted (1:4) in the medium with 1% SL, resulting in three experimental groups: i) washed sperm diluted in PEY medium (control); ii) washed sperm diluted in SL medium; and iii) unwashed sperm diluted in SL medium. Sperm concentration of the samples was subsequently evaluated using a Neubauer hemacytometer and there were dilutions to a final concentration of 400×10^6 sperm/mL. All samples were chilled for 4 h at 5 °C before freezing. Sperm were subsequently packaged in 0.25-mL straws (IMV Technologies, L'Aigle, Cedex, France) and sealed utilizing polyvinyl alcohol. Straws were frozen in liquid nitrogen vapor for 10 min., 5 cm above the surface of the liquid nitrogen and later plunged into the liquid nitrogen for storage.

In Experiment 2, there was a total of six semen collections conducted ($n = 6$) using the procedures described for Experiment 1. During each collection replicate, two ejaculates were obtained from a total of six bucks. The twelve ejaculates collected were pooled and aliquoted into two equal samples. One sample was washed twice using procedures described for Experiment 1 and the resulting sediment was divided into four equal aliquots and re-suspended in the different extenders containing egg yolk (15%) utilizing different BHT concentrations (0.0, 0.6, 2.0 and 5.0 mM). The other sample (unwashed) was divided into four equal aliquots and immediately diluted in a medium with SL (1%), containing different BHT concentrations. With utilization of this experimental design, there were eight different groups (four groups of washed sperm diluted in PEY media and four groups of unwashed sperm diluted in SL media with there being BHT concentrations of 0.0, 0.6, 2.0 and 5.0 mM for both washed and unwashed samples). All samples were subsequently chilled for 4 h at 5 °C before freezing utilizing procedures described for Experiment 1.

2.3. Sperm evaluation

In both experiments, sperm evaluation techniques were performed after 4 h of refrigeration at 5 °C and immediately after thawing sperm samples. After cooling, there was motion variable analyses of the refrigerated sperm samples performed. In addition, structural sperm membrane integrity was estimated using eosin/nigrosine vital staining procedures (Hancock, 1951), while

the analysis of the functionality of the plasma membrane was assessed using the hypoosmotic swelling test (HOST), which consisted of incubating 20 μL of sperm suspension in 180 μL of hypotonic solution (100 mOsm) at 37 °C (Forouzanfar et al., 2010) and determining the hypoosmotic shock resistance of spermatozoa. With both procedures, immediately after cooling and after 30 min. incubation in the hypoosmotic solution, eosin/nigrosine vital staining was performed by placing 10 μL of a sperm sample and 10 μL of eosin/nigrosine by smearing the sample on a microscope slide and observing the smear contents using an optical microscope at 1000×, assessing 200 sperm/smear and two smears/sample.

After cryopreservation, two straws from each experimental group and replicate ($n = 6$) were thawed by immersion in a water bath at 37 °C for 30 seconds and the content was poured into a dry tube maintained at the same temperature for motion variable and flow cytometry analyses.

2.3.1. Motion variable analyses

Sperm motility variables were assessed using a computer-assisted sperm analysis (CASA) system ISAS® (PROISER S.L., Valencia). Aliquots of refrigerated and frozen-thawed sperm samples were diluted (1:100) in PBS, and a 10 μL drop of sperm suspension was placed on a slide and covered with a coverslip (24 x 24 mm). Sperm motility was assessed at 38 °C at 200× using a phase contrast microscope (Olympus BH-2, Japan). For each sample, more than three fields per drop were analyzed and a minimum of 200 sperm evaluated. Percentages of total motile (%) and progressively motile sperm cells (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average pathway velocity (VAP, μm/s), linearity coefficient (LIN = [VSL/VCL]x100,%), straightness coefficient (STR = [VSL/VAP]x100,%), amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz) were evaluated. The settings used for the sperm image analyses were as follows: number of images (25/s), optical (Ph-), scale (20xOlympus), particle area (>3 a < 70 micras^2), slow sperm (10–45 micras/s), average sperm (45–75 micras/s), rapid sperm (>75 micras/s), progressive (80% STR) (Cox et al., 2006; Berlinguer et al., 2009).

2.3.2. Flow cytometric analysis

Plasma and acrosome membrane integrity as well as mitochondrial function of thawed sperm were evaluated using flow cytometry procedures utilizing quadruple-staining, as described by Tabarez et al. (2017). The following fluorescent probes were used: LIVE/DEAD® sperm viability kit (SYBR-14 and Propidium Iodide (PI); L-7011, Invitrogen S.A.) for plasma membrane integrity (viability), PE-PNA (GTX01509, Antibody Bcn, S.L.) for acrosome integrity and Mitotracker deep red (M22426, Invitrogen S.A.) for the detection of mitochondrial activity.

The analysis was performed using a final concentration of 1 nM of SYBR-14 (diluted in DMSO), 1.5 μM of PI, 2.5 μg/mL PE-PNA (1 mg/mL of stock solution in a buffer composed of 3.0 M ammonium sulfate, 50 mM sodium phosphate and 0.05% sodium acid, pH 7.0 containing 1 mM [Ca^{2+}] and [Mn^{2+}] ions) and 1.5 nM of Mitotracker deep red (diluted in DMSO) with 1 mL of diluted semen in PBS to a final sperm concentration of 1×10^6/mL. Samples were mixed and incubated at 37 °C for 10 min. and then remixed immediately prior to conducting the analyses. Stained sperm suspensions were subsequently run through a flow cytometer. Fluorescent probes SYBR-14, PE-PNA and PI were excited in the flow cytometer using a 488 nm blue solid-state laser while the Mitotracker deep red was excited using a 633 nm He/Ne excitation laser. The equipment used was the BD FACSCanto flow cytometer (BD Biosciences, CA) and samples were analyzed using BD FACSDiva (BD Biosciences, CA).

Dead cells became PI-positive, and their red fluorescent signal was detected using filter detector 679 L P (detection of emitted photons with a wavelength >670 nm). Live cells were SYBR-14 positive, and the green fluorescent signal was detected using filter detector 530/30BP (detection of emitted photons with a wavelength of 515–545 nm). Acrosome-damage cells were stained positively for PE-PNA, and the orange fluorescent signal was detected using the filter detector 585/42BP (detection of emitted photons with a wavelength of 564–650 nm). Sperm mitochondrial function was assessed with Mitotracker deep red using the filter detector 660/20BP. This dye is sequestered in mitochondria and emits red fluorescence in functional mitochondria (high mitochondrial membrane potential). This staining with four simultaneous fluorescences had minimal emission overlap, and therefore, compensation was adjusted for emission detectors used with the respective compensating controls. Non-sperm structures were gated out for conducting the analysis, based on scatter properties as observed in the forward-scatter (FSC) and side-scatter light (SSC) (scatter-gated sperm analysis). In addition, events with scatter characteristics similar to sperm cells but without DNA content (very little SYBR-14 or PI staining) were also gated out (doubly-gated sperm analysis). The flow cytometer was calibrated each day with calibration beads Rainbown 8 peaks (Sphero^{TM}) and used at the average flow rate (60 μL/min). The recording of scatter and fluorescent properties of all events stopped when 20,000 double-gated events were recorded. The sperm populations were divided by regions and quadrants.

After evaluation, spermatozoa were separated into eight populations: 1) viable cells with intact acrosome and active mitochondria (SYBR14+/PI-/PE-PNA-/Mitotracker+); 2) viable cells with a damaged acrosome and a functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker+); 3) viable cells with an intact acrosome and non-functional mitochondria (SYBR14+/PI-/ PE-PNA-/Mitotracker-); 4) viable cells with a damaged acrosome and a non-functional mitochondria (SYBR14+/PI-/PE-PNA+/Mitotracker-); 5) non-viable cells with an intact acrosome and functional mitochondria (SYBR14-/PI+/PE-PNA-/Mitotracker+); 6) non-viable cells with a damaged acrosome and functional mitochondria (SYBR14-/PI+/PE-PNA+/Mitotracker+); 7) non-viable cells with an intact acrosome and non-functional mitochondria (SYBR14-/PI+/PE-PNA-/Mitotracker-); and 8) non-viable cells with damaged acrosome and non-functional mitochondria (SYBR14-/PI+/PE-PNA+/Mitotracker-).

2.4. Statistical analyses

Statistical analyses were conducted using the statistical package SPSS 20 for Windows (IBM Corp. Released, 2011). Results are presented as means and standard error of the mean (± S.E.M.). Data for all sperm variables were subjected to tests of normality and homoscedasticity, Shapiro-Wilk and Levene, respectively. When necessary, data were arc-sin transformed. Data for all variables were analyzed using an analysis of variance utilizing the GLM univariate except for the sperm motility quality variables evaluated using the CASA system, which were analyzed using multivariate GLM. Differences between means were analyzed using the Duncan test. For all statistical analyses, there were mean differences considered to exist when there was a $P < 0.05$.

3. Results

3.1. Experiment 1

After refrigeration, there were no differences ($P > 0.05$) in sperm viability between SL based treatment groups, regardless of the presence of seminal plasma. Similarly, there were no differences when values for both SL based treatment groups were compared with the control treatment group based on inclusion of PEY medium without seminal plasma. Furthermore, percentages of spermatozoa with an intact and functional plasma membrane when evaluated using the HOS Test in PEY medium did not differ from those observed for washed and unwashed sperm preserved in SL based media. Seminal plasma removal resulted in a decrease in total and progressive motility in washed sperm preserved in SL media and there were differences in mean values for all kinetic variables except the amplitude of lateral head displacement (ALH), when compared to the mean values for other treatment groups (Table 1).

After sperm thawing, there was a similar negative effect ($P < 0.05$) on total and progressive motility in washed sperm preserved in SL based medium, with there being markedly lesser values than those for sperm frozen using the control protocol or in SL medium in the presence of seminal plasma. There were also differences in mean values for some variables of quality of movement between treatment groups. The mean values for washed sperm preserved in SL based medium were less ($P < 0.05$) for VSL, LIN, STR and BCF than those for washed sperm preserved in PEY media and unwashed sperm preserved in SL. There were no differences in mean values among treatment groups for VCL, VAP and ALH variables (Table 1).

When thawed sperm samples were evaluated using flow cytometer procedures, the mean percentage values for washed sperm cryopreserved in PEY medium were greater ($P < 0.05$) for sperm with an intact plasma membrane (SYBR14+/PI- sperm population) than the mean percentage values for SL-preserved sperm, although there were no differences in mean percentages among these groups for plasma membrane functionality (Table 1). Furthermore, within these sperm populations with an intact plasma membrane, the proportion of viable sperm with functional mitochondria (SYBR14+/PI-/PE-PNA-/Mitotracker+) was greater in the samples in which there were washed sperm and preserved in PEY while the population with intact plasma and acrosome membranes and non-functional mitochondria (SYBR14+/PI-/ PE-PNA-/Mitotracker-) was less compared with the percentages of both populations for both the washed and unwashed sperm samples preserved in SL-media (Table 2). It is also noteworthy that from the eight expected subpopulations based on the cytometry staining utilized, only six populations were detected because there was not detection of sperm with an intact plasma membrane that had a damaged acrosome with or without functional mitochondria.

Table 1
Effects of replacing powdered egg yolk (PEY) with soy lecithin (SL) in preservation medium with or without seminal plasma separation from sperm on viability, plasma membrane functionality when the results from the HOS test and motility variables were evaluated in refrigerated and frozen-thawed buck sperm (mean ± SEM)

	After 4 h refrigeration			After thawing		
Variable	Control	SL Washing	SL	Control	SL Washing	SL
Viability (%) *	88.7 ± 2.0	82.5 ± 4.4	87.1 ± 3.2	41.0 ± 3.8 [a]	25.3 ± 1.8 [b]	26.0 ± 2.1 [b]
HOST (%) *	80.3 ± 3.9	71.6 ± 5.4	68.5 ± 4.5	29.3 ± 3.4	28.5 ± 1.9	25.9 ± 1.3
MT (%)	89.8 ± 1.6 [a]	49.1 ± 12.8 [b]	81.1 ± 6.3 [a]	46.8 ± 9.1 [a]	8.3 ± 2.6 [b]	34.3 ± 4.8 [a]
MP (%)	37.8 ± 3.3 [a]	5.7 ± 1.9 [b]	35.5 ± 4.9 [a]	18.7 ± 4.0 [a]	1.4 ± 0.4 [b]	15.3 ± 2.1 [a]
VCL (μm/s)	124.7 ± 5.0 [a]	96.0 ± 6.8 [b]	117.3 ± 2.7 [a]	123.9 ± 4.3	108.1 ± 8.2	110.5 ± 4.9
VSL (μm/s)	64.9 ± 2.6 [a]	26.5 ± 5.7 [b]	56.2 ± 4.3 [a]	48.5 ± 4.4 [a]	29.5 ± 6.9 [b]	50.1 ± 5.5 [a]
VAP (μm/s)	81.3 ± 2.2 [a]	48.1 ± 7.4 [b]	72.6 ± 3.5 [a]	66.6 ± 5.2	50.0 ± 8.0	64.6 ± 4.9
LIN (%)	52.3 ± 3.2 [a]	28.6 ± 4.3 [b]	48.5 ± 3.9 [a]	39.7 ± 2.3 [a]	26.1 ± 4.8 [b]	44.7 ± 4.7 [a]
STR (%)	77.2 ± 1.5 [a]	50.3 ± 4.3 [b]	73.9 ± 2.6 [a]	70.6 ± 2.2 [a]	51.3 ± 3.8 [b]	70.8 ± 3.8 [a]
ALH (μm)	4.6 ± 0.2	4.3 ± 0.2	4.4 ± 0.2	4.9 ± 0.1	4.8 ± 0.3	4.2 ± 0.2
BCF (Hz)	8.8 ± 0.4 [a]	6.3 ± 0.9 [b]	9.9 ± 0.5 [a]	9.6 ± 0.7 [a]	5.1 ± 0.6 [b]	9.4 ± 0.3 [a]

Different letters in rows indicate differences ($n = 6$, $P < 0.05$); Control: PEY and washing. TM: total motility, PM: progressive motility, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average cell-path velocity, LIN: Linearity index, STR: straightness index, ALH: amplitude of lateral head displacement, BCF: beat-cross frequency; (*): The techniques used to determine the percentages of viability (plasma membrane integrity) and HOST (plasma membrane functionality by hypoosmotic swelling test) variables were different after refrigeration (utilizing Eosine/Nigrosin Staining for optical microscope observation) and after thawing (utilizing Quadruple Staining for cytometer analyses for evaluating SYBR14+/PI- sperm populations)

Table 2
Effect of replacing powdered egg yolk (PEY) with soy lecithin (SL) in preservation medium in with or without seminal plasma separation from sperm on membrane integrity and mitochondrial function of post-thawed buck spermatozoa (mean ± SEM).

(%)	Control	SL Washing	SL
Viable sperm with intact acrosome and active mitochondria	36.3 ± 4.2 [a]	12.0 ± 2.3 [b]	14.2 ± 3.0 [b]
Viable sperm with intact acrosome and inactive mitochondria	4.7 ± 1.6 [a]	13.3 ± 1.2 [b]	12.2 ± 2.4 [b]
Total Acrosome Damage	29.9 ± 2.1	27.6 ± 3.5	20.8 ± 4.3

Different letters in rows indicate differences ($n = 6$, $P < 0.05$); Control: PEY and washing

There was no difference ($P > 0.05$) in acrosome damage (PE-PNA+) in sperm cryopreserved in SL media when there was or was not seminal plasma present compared to sperm preserved in the PEY medium without seminal plasma (Table 2).

3.2. Experiment 2

After 4 h of refrigeration, there were no differences when there was supplementation of extender with BHT as an antioxidant in different sperm cryopreservation media at any concentration ($P > 0.05$) on sperm viability, plasma membrane functionality when there was assessment using the HOS test and for a majority of the mean values for kinetic variables. There were only differences in mean values for STR, ALH and BCF among treatment groups (Table 3). There were lesser mean values for straightness coefficient (STR) for sperm preserved in SL-based medium with 0.6 mM BHT compared with the mean values for sperm preserved in the PEY-based media. Furthermore, the mean values for ALH were similar among treatment groups, except for sperm preserved in PEY-based medium with 5 mM BHT and for those preserved in SL-based medium with 2 mM BHT, with the least and greatest ALH values ($P < 0.05$), respectively. In addition, the BCF values for sperm preserved in SL-based media were less as the BHT concentration in the extender increased, with there being lesser values when there were greater concentrations of BHT (2 and 5 mM) compared with those when there was no supplementation of media with BHT. For the sperm preserved in PEY-based media the mean values for this variable increased as the antioxidant concentration increased, being greater in the extender with the largest BHT concentration (5 mM) than in the samples from the other treatment groups, except for the treatment with 2 mM BHT, for which there were no differences (Table 3).

After the analysis of post-thaw sperm motility, the lack of effect on mean values for kinetic variables when there was the inclusion of BHT as an antioxidant was even more remarkable than for the analysis after refrigeration. There was only the maximal total motility for sperm preserved in PEY where there was supplementation with the largest concentration of BHT (5 mM) with there being different sperm motility in samples preserved in PEY media without supplementation of the antioxidant or with supplementation of 2 mM of BHT (Table 4).

In addition, there were no differences ($P > 0.05$) among treatment groups when post-thaw sperm viability was evaluated (SYBR14+/PI- sperm population) utilizing flow cytometry. The mean values for the sperm population with intact plasma and acrosome membranes and non-functional mitochondria differed among treatment groups with there being greater percentages of sperm in this population when there was preservation utilizing the SL media regardless of whether there was supplementation with antioxidant at any of the concentrations evaluated. Similar to the results from Experiment 1, there was a similar trend in the proportion of viable sperm with a functional mitochondria, with the proportion of these sperm being greater when there was use of the PEY extenders regardless of whether there was supplementation with BHT (Table 5). Furthermore, there were no sperm cells de-

Table 3
Effect of the addition of BHT at different concentrations in soy lecithin (SL) or powdered egg yolk (PEY) based media on sperm viability, plasma membrane functionality using the HOS test and evaluation of motility variables for buck sperm after 4 h of refrigeration.

Variable	W-PEY 0 BHT	W-PEY 0.6 BHT	W-PEY 2 BHT	W-PEY 5 BHT	SL 0 BHT	SL 0.6 BHT	SL 2 BHT	SL 5 BHT
Viability (%)	82.3 ± 3.6	81.0 ± 3.5	78.5 ± 6.1	80.2 ± 4.8	81.2 ± 3.8	81.7 ± 3.4	82.0 ± 3.9	77.0 ± 7.6
HOST (%)	69.8 ± 4.7	71.6 ± 4.1	71.8 ± 3.9	71.8 ± 3.3	65.6 ± 2.0	63.9 ± 6.2	56.5 ± 4.8	63.5 ± 5.8
TM (%)	78.8 ± 4.7	80.2 ± 4.2	77.1 ± 5.2	80.7 ± 3.8	75.1 ± 4.2	82.8 ± 1.2	80.9 ± 2.7	76.8 ± 4.6
PM (%)	31.6 ± 4.0	30.6 ± 3.3	31.5 ± 2.3	31.4 ± 2.8	26.4 ± 4.4	27.6 ± 5.3	30.0 ± 2.5	26.7 ± 1.8
VCL(μm/s)	116.5 ± 5.7	116.3 ± 6.7	116.6 ± 5.2	112.3 ± 5.3	121.4 ± 5.7	114.1 ± 4.9	110.6 ± 5.6	105.6 ± 7.9
VSL(μm/s)	52.7 ± 5.4	51.9 ± 4.6	53.6 ± 4.2	52.6 ± 5.3	48.9 ± 4.1	46.5 ± 5.0	50.7 ± 4.6	45.5 ± 4.0
VAP(μm/s)	68.7 ± 5.6	68.0 ± 5.6	69.7 ± 5.1	68.0 ± 5.7	69.8 ± 4.6	66.4 ± 4.6	69.6 ± 5.3	63.5 ± 5.7
LIN (%)	45.4 ± 3.1	45.2 ± 2.8	46.4 ± 2.4	47.0 ± 3.1	40.6 ± 3.0	40.9 ± 4.1	45.0 ± 2.3	42.4 ± 2.5
STR (%)	74.0 ± 2.6 [a]	73.5 ± 2.4 [a]	74.6 ± 1.9 [a]	74.3 ± 2.0 [a]	66.9 ± 2.7 [ab]	65.4 ± 4.2 [b]	68.7 ± 2.3 [ab]	67.0 ± 0.8 [ab]
ALH (μm)	4.7 ± 0.2 [b]	4.4 ± 0.1 [b]	4.5 ± 0.1 [b]	3.7 ± 0.1 [a]	4.3 ± 0.2 [b]	4.6 ± 0.1 [b]	5.3 ± 0.1 [c]	4.7 ± 0.4 [b]
BCF (Hz)	8.4 ± 0.6 [ab]	8.4 ± 0.2 [ab]	9.3 ± 0.2 [bc]	10.2 ± 0.3 [c]	8.8 ± 0.4 [b]	8.4 ± 0.1 [ab]	7.6 ± 0.3 [a]	7.6 ± 0.1 [a]

Different letters in rows indicate differences ($n = 6$, $P < 0.05$); W: Washing; 0, 0.6, 2, 5: mM concentration of butylated hydroxytoluene (BHT); TM: Total motility, PM: Progressive motility, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average cell-path velocity, LIN: Linearity index, STR: straightness index, ALH: amplitude of lateral head displacement, BCF: beat-cross frequency; The technique used to determine the percentage viability (plasma membrane integrity) and HOST (plasma membrane functionality using the hypoosmotic swelling test) variables using Eosine/Nigrosin Staining for optical microscope observation

Table 4
Effects of the addition of different concentrations of BHT to soy lecithin (SL) or powdered egg yolk (PEY)-based media on motility variables for buck sperm after thawing.

Variable	W-PEY 0 BHT	W- PEY 0.6 BHT	W- PEY 2 BHT	W- PEY 5 BHT	SL 0 BHT	SL 0.6 BHT	SL 2 BHT	SL 5 BHT
TM (%)	21.6 ± 2.4 [a]	31.8 ± 3.7 [ab]	20.8 ± 3.0 [a]	37.2 ± 5.2 [b]	22.9 ± 2.7 [ab]	32.6 ± 2.5 [ab]	31.9 ± 2.5 [ab]	31.5 ± 4.3 [ab]
PM (%)	4.9 ± 0.8	7.7 ± 2.0	4.5 ± 0.6	12.6 ± 3.1	3.5 ± 1.4	6.3 ± 2.3	8.2 ± 2.4	7.7 ± 2.4
VCL(μm/s)	100.2 ± 3.3	101.7 ± 5.5	99.3 ± 4.8	97.9 ± 4.5	95.7 ± 3.5	93.1 ± 5.5	89.3 ± 4.7	92.9 ± 5.2
VSL(μm/s)	28.8 ± 2.1	33.6 ± 4.4	29.3 ± 1.0	38.3 ± 4.2	25.9 ± 4.1	28.2 ± 5.0	30.8 ± 4.1	30.3 ± 4.3
VAP(μm/s)	49.7 ± 1.6	53.1 ± 3.4	49.1 ± 2.5	55.2 ± 3.7	49.6 ± 2.6	50.8 ± 2.4	50.6 ± 3.5	51.1 ± 3.3
LIN (%)	31.2 ± 2.1	34.7 ± 3.7	32.0 ± 1.7	39.6 ± 3.3	28.4 ± 3.0	30.7 ± 5.4	33.9 ± 3.3	31.9 ± 3.2
STR (%)	57.7 ± 3.0	61.0 ± 4.6	59.3 ± 3.2	65.4 ± 3.5	50.8 ± 4.3	51.5 ± 6.7	56.1 ± 4.0	54.7 ± 4.4
ALH (μm)	4.6 ± 0.2	4.5 ± 0.2	4.6 ± 0.3	4.2 ± 0.2	4.4 ± 0.2	4.1 ± 0.3	3.9 ± 0.2	4.0 ± 0.2
BCF (Hz)	6.9 ± 0.6	7.0 ± 0.6	6.2 ± 0.5	7.7 ± 0.3	5.9 ± 0.6	6.4 ± 0.8	6.5 ± 0.5	6.9 ± 0.4

Different letters in rows indicate difference ($n = 6$, $P < 0.05$); W: Washing; 0, 0.6, 2, 5: mM concentration of butylated hydroxytoluene (BHT); TM: Total Motility, PM: Progressive Motility, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average cell-path velocity, LIN: Linearity index, STR: straightness index, ALH: amplitude of lateral head displacement, BCF: beat-cross frequency

Table 5
Effects of non-penetrating cryoprotectant and inclusion of BHT for cryopreservation medium on membrane integrity and mitochondrial function of post-thawed buck spermatozoa.

Sperm (%)	W-PEY 0 BHT	W- PEY 0.6 BHT	W- PEY 2 BHT	W- PEY 5 BHT	SL 0 BHT	SL 0.6 BHT	SL 2 BHT	SL 5 BHT
Viability	30.5 ± 4.9	40.1 ± 2.1	35.9 ± 5.6	41.9 ± 3.6	32.1 ± 3.1	32.4 ± 2.2	31.8 ± 4.5	32.6 ± 2.0
Viable, intact acrosome and active mitochondria	28.9 ± 4.5 [ab]	39.2 ± 1.9 [a]	33.3 ± 5.2 [a]	40.6 ± 3.4 [a]	14.3 ± 1.7 [bc]	12.2 ± 4.2 [c]	9.9 ± 3.0 [c]	10.2 ± 3.2 [c]
Viable, intact acrosome, and inactive mitochondria	1.6 ± 0.8 [a]	0.8 ± 0.4 [a]	2.5 ± 1.6 [a]	1.3 ± 0.5 [a]	17.8 ± 4.6 [b]	20.1 ± 5.8 [b]	21.9 ± 5.1 [b]	22.4 ± 4.0 [b]
Total Acrosome Damage	39.2 ± 4.9 [a]	38.9 ± 3.2 [a]	39.2 ± 4.5 [a]	35.1 ± 3.0 [ab]	30.0 ± 2.1 [ab]	28.3 ± 2.1 [b]	25.5 ± 2.7 [b]	25.5 ± 2.9 [b]

Different letters for each variable indicate differences ($n = 6$, $P < 0.05$). PEY: powdered egg yolk; SL: Soy lecithin; W: Washing; 0, 0.6, 2, 5: mM concentration of butylated hydroxytoluene (BHT); Viable: sperm with intact plasma membrane.

tected which had an acrosome reaction, based on number of sperm with an intact plasma membrane and reacted acrosome. There, however, was a negative effect of centrifugation (washing) on sperm acrosome integrity because there was a marked amount of acrosome damage based on the acrosome integrity assessments for washed sperm cryopreserved in PEY compared with unwashed sperm preserved in SL, except for unwashed sperm preserved in SL without supplementation with BHT and washed sperm preserved in PEY with the supplementation of 5 mM of BHT which did not differ of any of the different cryopreservation treatments (Table 5).

4. Discussion

In the present study, replacing PEY with SL in sperm preservation medium was found to maintain the functional and structural integrity of sperm plasma membrane after refrigeration, without the need of eliminating seminal plasma by centrifugation. Furthermore, values for kinetic variables of unwashed sperm preserved in SL were greater than those for spermatozoa preserved in the same medium but without seminal plasma, after refrigeration. These values were similar to those when washed sperm were preserved in PEY media as well as for a majority of the motility values analyzed after thawing. After thawing, however, the integrity of sperm plasma membrane was greater when PEY was used as a cryoprotectant in Experiment 1 indicating SL has lower capacity to prevent damage caused by cryopreservation. Furthermore, this finding was supported by the results of the present study where there were greater percentages of sperm cells with intact plasma and acrosome membranes and non-functional mitochondria when sperm were preserved in the SL media, regardless of whether semen was or not washed.

In numerous studies where there was utilization of soy-based extenders for buck sperm cryopreservation, there has been similar post-thaw motility, and plasma as well as acrosome membrane integrity as compared to when Tris-egg yolk semen extenders were used for cryopreservation (Sarıözkan et al., 2010; Roof et al., 2012; Salmani et al., 2013). In none of these studies, however, was there an assessment of the capacity of SL to protect sperm from mitochondrial membrane damage during the freeze-thawing process. In a study with rams, Najafi et al. (2013) found there were lesser proportions of viable spermatozoa, lesser mitochondrial activity, and lesser progressive motility in sperm preserved with 1% of SL in the medium after thawing, which is similar to the results of Del Valle et al. (2012), where the SL actively induced specific types of mitochondrial damage. The reason why egg yolk can provide greater protection to the mitochondria could be related to its composition, because not only does it contain phosphatidylcholine but it also contains low-density lipoproteins, which may have a greater capacity to protect mitochondria (Le Grandois et al., 2009). Further studies, therefore, are needed before SL can be a useful substitute for conventional extenders. The PEY appears to

be, at present, the most effective non-penetrating cryoprotectant for sperm preservation in goats, even though seminal plasma removal has to occur if PEY is used as a cryoprotectant.

Nevertheless, it is noteworthy that when buck sperm is preserved in a SL medium, seminal plasma interacts differently as compared with when egg yolk is used, which makes semen centrifugation unnecessary for semen processing (Roof et al., 2012) and if centrifugation is conducted this practice can be detrimental when the motility data are evaluated after refrigeration and thawing. Results of the present study indicate SL may be a very practical option as an extender for buck semen because sperm are not subjected to the centrifugation process when SL is used for buck semen preservation. Centrifugation can result in sperm loss or damage (Tuli and Holtz, 1994), as well as loss of functions of important proteins contained in the seminal plasma, known as Goat Seminal Plasma (GSP) proteins (Villemure et al., 2003), which are very important for adequate sperm function (Cardozo et al., 2006). There, however, may be damage to sperm mitochondria during refrigeration similar to that detected in thawed spermatozoa in the present study, therefore, there is no assurance of the suitability of SL-based media for sperm preservation at 5 °C.

There were no beneficial effects of the inclusion of BHT in the extender in the present study. After refrigeration, sperm characteristics were similar in both media (PEY and SL) with there being only slight differences ($P < 0.05$) in the STR, ALH and BCF, thus, there were not any marked beneficial effects of the cryoprotectant and/or of the BHT concentration evaluated in the present study. Furthermore, these small differences in mean values for kinetic variables were not detected after thawing, which is consistent with the previous results of the study of Naijian et al. (2013) in which there were no differences in the motility of frozen-thawed buck spermatozoa at different BHT concentrations (0.5, 1, 2 and 4 mM) when used to supplement egg yolk-based medium. Nevertheless, in the present study, there was the greatest total motility after thawing in spermatozoa preserved in the PEY-based medium supplemented with 5 mM BHT, although there was only different when washed sperm were preserved in PEY with 2 mM BHT or without BHT supplementation of the media.

Furthermore, there were no differences in sperm membrane integrity when supplementing the extender with BHT at the various concentrations. There, however, was an increase in the number of spermatozoa with intact plasma and acrosomal membranes but without mitochondrial function when the SL-based media was used as the extender, regardless of the concentration of the BHT supplementation to the extender. With the use of SL, there are marked alterations in the mitochondrial membrane, which are not always indicated by modifications in sperm motility, and the supplementation of BHT to the extender did not counteract these detrimental effects, regardless of the BHT concentration used for supplementation. This damage on the mitochondria could be due to a reduction in the cardiolipin content because the surface area of the mitochondria was less when there was the use of SL as extender. Cardiolipin is the primary phospholipid of the mitochondrial inner membrane (Petit et al., 1994), having a structural and functional effect on the many multimeric complexes associated to this membrane, and therefore, on the organization of the components of the mitochondrial respiratory chain which is the primary function of cardiolipin (Paradies et al., 2014).

Furthermore, there tends to be a negative effect on acrosome integrity as a result of sperm washing (centrifugation) as Tuli and Holtz (1994) also described. In the present study, the addition of BHT (5 mM) to the PEY media resulted in a reduction in the acrosome damage to washed sperm with there being no differences in acrosome integrity when there was imposing of the other treatments, regardless of whether the sperm were or not washed.

The components used in the thawing diluents could also interact with buck sperm, affecting the behaviour in the different media or even the survival and fertilizing capacity in the female tract depending on the preservation regimen used as previously suggested by Ramirez-Vasquez et al. (2019). Unfortunately, because of the design of the present study there could not be evaluations of the potential interactions between the cryopreservation treatments and the thawing medium used. More studies focused in this area could be of great interest and value to the scientific community because there are frequently inconsistent results from studies in this area of investigation, therefore, making repetitive studies very important for improvements in the approaches for sperm preservation..

5. Conclusions

In conclusion, the results of this study indicate PEY is the most suitable non-penetrating cryoprotectant for buck sperm cryopreservation, whereas SL could be a viable alternative for egg yolk based media when there are 5 °C conditions used for preservation, especially because the use does not involve the removal of seminal plasma. There should, however, not be discounting of the alterations in mitochondrial function that result from the use of SL. Because of the aims of the present study, it was beyond the scope to ascertain the sperm fertilizing capacity with use of the preservation approaches that were evaluated; therefore, fertility studies, either *in vitro* or *in vivo*, are necessary to assess the possible adverse effects of mitochondrial alterations that are associated with the use of SL as the base extender. There was a minor effect of the antioxidant BHT that was used in the sperm preservation media in the present study at different concentrations. Whether adding BHT to preservation media improves fertility requires further research.

Author Statement

The corresponding author is responsible for ensuring that the descriptions are accurate and agreed by all authors, as well MJ Palomo is the responsible for funding acquisition and for designing the experimental work.

All the authors contribute obtaining the data. A. Tabarez is responsible for most of the sperm analyses, statistical analyses and Spanish writing. MJ Palomo, as her supervisor, is responsible for the revision of the work.

Declaration of Competing Interest

None of the authors have any conflict of interest to declare.

Uncited references

Sarıözkan et al. (2010).

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