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Original Research

Potential use of soy lecithin or butylated hydroxytoluene as an alternative to powdered egg yolk for ram semen cryopreservation

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ABSTRACT

The aim of this study was to assess the effect of replacing powdered egg yolk (PEY) with soybean lecithin (SL) or butylated hydroxytoluene (BHT) on ram sperm cryopreservation. Two ejaculates/male were collected via artificial vagina from 8 rams during breeding season. Ejaculates were pooled and washed twice by centrifugation. The pellet was divided into three aliquots, diluted in a Tris-based media with 5% glycerol containing PEY (15%), SL (1%) or BHT (0.6 mM) and cooled for 4 h at 5 °C before freezing. Sperm motility, plasma and acrosome membrane integrity and mitochondria activity as lipid peroxidation were assessed immediately after thawing and after 4 h of resilience incubation in a modified PBS at 38 ºC. After thawing, sperm extended in BHT showed the poorest quality compared to sperm extended in PEY and SL. Similar total and progressive motility were observed in sperm preserved in PEY and SL media. Plasma membrane integrity, however, was significantly higher in sperm extended in SL, although most of them with non-functional mitochondria. Acrosome damage was significant lower in SL sperm samples compared to PEY samples. Highest level of lipid peroxidation was found in sperm preserved in PEY. Resilience test had a negative effect ($P < 0.05$) on plasma and acrosome membrane integrity in all samples, and on progressive motility only in sperm preserved in PEY. In conclusion, soy lecithin could be a potential alternative to PEY for ram cryopreservation, although its adverse effect on sperm mitochondria function has to be strongly considered.

Keywords: Butylated Hydroxytoluene; Cryopreservation; Powdered Egg Yolk; Ram; Soy Lecithin; Sperm

INTRODUCTION

With the new interest towards the characterization and conservation of endangered Catalonia *Aranesa* and *Xisqueta* ram breeds by constituting *ex situ* programs such as semen cryopreservation and development of sperm bank, knowledge of their sperm physiological characteristics regarding frozen-thawed sperm quality is of utmost importance. More so, sperm cryopreservation towards its conclusive use for artificial insemination (AI) is arguably the most important achievement to increasing breeding proficiency, especially for endangered breeds. These two techniques (cryopreservation and artificial insemination) have enabled the worldwide preservation, distribution and use of desired genetic lines at a reasonable cost (Andrabi & Maxwell, 2007), and in rams, the most important challenge is still the availability of high cryopreserved semen quality. During the last decades, the most commonly used extender component in preserving ram sperm has been the egg yolk (EY) (Manjunath, 2012). Its beneficial effect has been as a stabilizer to sperm membranes, protecting against cold shock (Salamon and Maxwell, 2000). Nevertheless, reports on EY in semen extenders poses some challenges such as cryoprotective antagonists, risk of microbial contamination and many more (Aboagla and Terada, 2004; Forouzanfar *et al*., 2010; Emamverdi *et al*., 2013).

In spite of the above mentioned, a previous study from our laboratory concluded that conventional fresh egg yolk (EY) can be replaced with powdered egg yolk (PEY) for freezing ram semen (Garcia *et al*., 2017). This positive result may be due to the homogeneous composition and pasteurization processes of PEY which may have eliminated bacterial contamination or harmful variables affecting semen quality

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(Ansari *et al.,* 2010; Tabarez *et al.,* 2017). Nevertheless, the use of non-animal product extenders is thus becoming a popular choice for semen cryopreservation (Masoudi *et al.,* 2016; Toker *et al.,* 2016). Therefore, studies involving appropriate alternative extenders of non-animal origin is still of utmost importance and on-going. A suggestion to a valuable replacement option for EY component is soybean lecithin (SL) or an antioxidant such as butylated hydroxytoluene (BHT).

The advantages of SL extenders over EY as regards to biosecurity issues are unquestionable but the efficacy of SL is still a matter of debate (El-Sisy *et al.,* 2016; Sun *et al.,* 2020). Furthermore, the addition of antioxidants to semen diluents has been reported to be beneficial in maintaining sperm motility and viability during hypothermic storage (Bansal and Bilaspuri, 2010; Amidi *et al.,* 2016). One of such antioxidants is BHT, a synthetic analogue of vitamin E which has positive effects on semen quality towards minimizing cold shock during cryopreservation (Naijian *et al.,* 2013; Patel *et al.,* 2015; Palomo *et al.,* 2017) as well as increasing antioxidant (Suttiyotin *et al.,* 2011). Therefore, replacing EY component with SL or BHT in extenders may suggest to maintain frozen-thawed semen quality, especially with the growing interest towards the use of non-animal components for semen cryopreservation (Khalifa *et al.,* 2008, Papa *et al.,* 2011; Toker *et al.,* 2016). However, controversies still abound as regards the efficacy of SL or BHT, specifically on ram frozen-thawed semen quality.

Thus, the present study was aimed at comparing and evaluating the motion kinematics and qualitative characteristics of washed sperm frozen from our Catalonian endangered ram breeds (*Aranesa* and *Xisqueta*) in three different types of extenders (PEY, SL or BHT). Furthermore, their resilience following a 4-hour post-thawing incubation period was tested in-view towards understanding any sperm physiological or behavioral changes that may not have been apparent immediately after thawing. In this context, we aim that exposing these frozen-thawed sperm for a period thermal incubation time may provide some useful information on their probability for a long-term survival within the female genital tract and capacitation status towards fertilization.

MATERIALS AND METHODS

CHEMICALS AND REAGENT

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA), unless otherwise stated. The powdered egg yolk was obtained from NIVE (Nunspeet Holland Eiproducten).

PREPARATION OF EXTENDERS

In this study, the basic media used for the extender was Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) solution defined by Salamon and Maxwell, 2000. It consisted of Tris (0.3M), citric acid anhydrous (94.7 mM), and D (+)-glucose (27.75 mM) and adjusted to 7.25 ± 0.05 and osmolarity to 333.0 ± 2.8 mOsm. Thereafter, glycerol (5 % v/v, final concentration) and antibiotics (1000 UI/mL sodium penicillin and 1.0 mg/mL streptomycin sulfate) were added to a final pH 7.0–7.17 and 1327 ± 234 mOsm. Afterwards, PEY, SL or BHT was added to the basic extender at final concentration of 15 %, 1 % and 0.6 mM, respectively.

Preparation of the extender based in powdered egg yolk was performed as described by Marco-Jiménez et al. (2004). This was done by diluting powdered egg yolk (1:1.25) with Milli-Q water and stirred for 20 min, while the soy lecithin extender was prepared by adding 1% (w/v) of lecithin (Sigma 11145) to the basic media, as described by Forouzanfar (2010) in ram sperm. The protocol for BHT addition was done as described by Khalifa *et al.* (2008) by dissolving BHT in dimethyl sulphoxide (DMSO) with a final concentration of DMSO in the extender at 0.25 % (v/v).

SEMEN COLLECTION AND FREEZING PROTOCOL

Collection of semen was done twice weekly with two ejaculates per collection from each male, using an artificial vagina from eight rams (4 *Aranesa* and 4 *Xisqueta* breeds) during the breeding season. The rams were housed in a farm at *Institut de Recerca de Tecnologia Agroalimentaria*, (IRTA, Caldes de Montbui, Barcelona, Spain) with uniform nutritional conditions. In each ejaculate, sperm volume, mass motility, progressive motility and concentration were assessed immediately after collection through conventional methods. Only ejaculates with good semen quality (mass motility: ≥ 4; progressive motility: ≥ 80 %; sperm concentration: ≥ 3000 x 10^6 sperm/mL) were selected and pooled (to avoid individual variability).

Pooled semen sample was washed by dilution (1:5) in TCG by centrifuging twice at 600 x *g* for 10 min in order to remove the seminal plasma. Thereafter, the supernatant was carefully removed and the pellet was divided into three aliquots and re-suspended in an extender (1:4) containing 15% (v/v) of PEY, 1% (w/v) of soy lecithin or 0.6 mM of BHT. Sperm concentration of the samples was evaluated by Neubauer haemocytometer and adjusted at a final concentration of 400 × 10^6 sperm/mL. All samples were equilibrated for 4 h at 5 °C before freezing. Sperm was then packaged in 0.25-mL straws (IMV Technologies, L'Aigle, Cedex, France) and sealed with polyvinyl alcohol. Straws were frozen in liquid nitrogen vapor for 10 min. before plunged into the liquid nitrogen for storage.

THERMAL RESILIENCE TEST

Thermal resilience test was performed to determine any behavioral changes and longevity of the post-thawed sperm

samples within the various treatments/extenders following a procedure as described by Osuagwuh and Palomo (2017). Briefly, two straws per treatment and replicate were thawed (30 sec at 37 ºC) and diluted in a modified phosphate buffer solution (PBS supplemented with 36 µg/mL pyruvate and 0.5 mg/mL BSA, 280-300 mOsm and pH 7.3-7.4.) to 40 x 10^6 sperm/mL. Thereafter, samples were incubated at 37 °C in a dry bath in the dark. All samples were evaluated immediately after thawing (0h) and at 4 h of thermal incubation.

SPERM EVALUATION

MOTION PARAMETERS ANALYSIS

Sperm motility parameters were assessed using a computer-assisted sperm analysis (CASA) system ISAS® (PROISER S.L., Valencia, Spain). Briefly, aliquots of frozen-thawed sperm samples were diluted (1:100) in PBS, and a 10 µL drop of sperm dilution was placed on a slide and covered with a coverslip (24×24mm) on a warm stage (37°C). Sperm motility was assessed 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 recorded. 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).

FLOW CYTOMETRIC ANALYSIS

Plasma and acrosome membrane integrity as well as mitochondrial function of thawed sperm were evaluated by flow cytometry using a quadruple-staining technique from our laboratory and 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 done 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 just before analysis. 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).

After evaluation, sperm populations taken into consideration for this study were sperm viability (total alive sperm cells), viable cells with intact acrosome and active mitochondria (SYBR14+/ IP-/PE-PNA-/Mitotracker +), viable cells with damaged acrosome and active mitochondria (SYBR14+/IP-/PE-PNA+/Mitotracker +) and viable cells with intact acrosome and inactive mitochondria (SYBR14+/IP-/ PE-PNA-/Mitotracker-). Also, the total acrosome damage was recorded.

MALONDIALDEHYDE (MDA) CONCENTRATIONS

Lipid peroxidation (LPO) of thawed sperm was assessed by the formation of malondialdehyde (MDA). The concentration of malonaldehyde (MDA) was determined spectrophotometrically according to the method described by Buege & Aust (1978). Briefly, 500 µL of thawed semen was mixed with 1 mL of the TBA reagent solution (trichloracetic acid 15 %, (w/v), hydrochloric acid 0.25 N, thiobarbituric acid 0.375 %, (w/v) and BHT 1 % (v/v) and boiled at 100 °C for 15 min before been cooled in an ice bath. After cooling, the suspension was centrifuged at 1000 × *g* for 10 min and the supernatant was separated following determination through spectrophotometric analysis at a wavelength of 532 nm at 25 °C. The MDA concentration was determined by the specific absorbance coefficient (1.56 x 10^5/mol/cm3).

STATISTICAL ANALYSIS

Statistical analyses were carried out using the statistical program JMP10.0 (SAS Institute Inc., Cory, NC, USA). Results are presented as means and standard error of the mean (± S.E.M.). Data obtained from the analysis of all sperm parameters were subjected to tests of normality and homoscedasticity, Shapiro-Wilk and Levene, respectively. Dependent variables with normal distribution were evaluated using analysis of variance. Differences between means were analyzed by Tukey's test and Student's t-test. In all statistical analyses, the minimal level of significance was set at $P < 0.05$.

RESULTS

The analysis of sperm kinematic parameters within each post-thawed period/time (0h or 4h) evaluated by CASA showed that sperm preserved in PEY and SL extenders had

no statistical differences in the total and progressive sperm motility and both differed significantly ($P < 0.05$) from those in BHT extender (Table 1). As regards other motion parameters, no differences were observed between extenders/treatment groups at any assessment time, except for ALH where a significant difference ($P < 0.05$) was observed between PEY and SL sperm samples after 4 h of incubation (Table1). Furthermore, all sperm motion parameters in all extenders were affected by the thermal incubation ($P < 0.05$) except for LIN and STR as well as in ALH for only SL and BHT extenders where no differences were observed.

As regards sperm functionality analysis by flow cytometer (Table 2), the effect of the different composition of extenders showed that sperm viability differed statistically between SL and BHT at any given time of analysis. However, viable sperm cells with intact acrosome and active mitochondria at 0h for PEY was significantly higher ($P < 0.05$) when compared to sperm in SL and BHT extenders. However, at 4 h, sperm in BHT differed significantly from SL or PEY samples which had no differences between them. As regards viable cells with damaged acrosome and active mitochondria activities which was considered in this study as "cryo-acrosome reacted" sperm, results showed that no effect amongst the extender at 0h. Nevertheless, at 4h, SL differed significantly ($P < 0.05$) from PEY and BHT. This trend was also observed in sperm cells with intact acrosome and inactive mitochondria where SL samples differed significantly from PEY and BHT irrespective of time of evaluation (Table 2).Furthermore, the total sperm acrosome damage was significantly lower ($P < 0.05$) in SL sperm samples when compared to PEY and BHT samples at 0 h or after 4 h of thermal incubation. As regards the effect of incubation, results showed adverse effect ($P < 0.05$) on all sperm functionality parameters except only in the progressive motility of sperm extender with SL.

Finally, the MDA levels of sperm preserved in the studied extenders after thawing (0 h) and 4 h of incubation are presented in Fig. 1. Immediately after thawing, the lowest MDA level was observed in SL sperm samples and statistically differed ($P < 0.05$) with levels showed in PEY samples. However, at 4 h post-thawing incubation, sperm preserved in PEY extender further showed significant higher MDA values when compared with SL and BHT samples (Fig 1). However, the effect of a thermal incubation period was only observed on sperm extended in SL and BHT media which showed lower values of MDA concentration after 4h post-thawing.

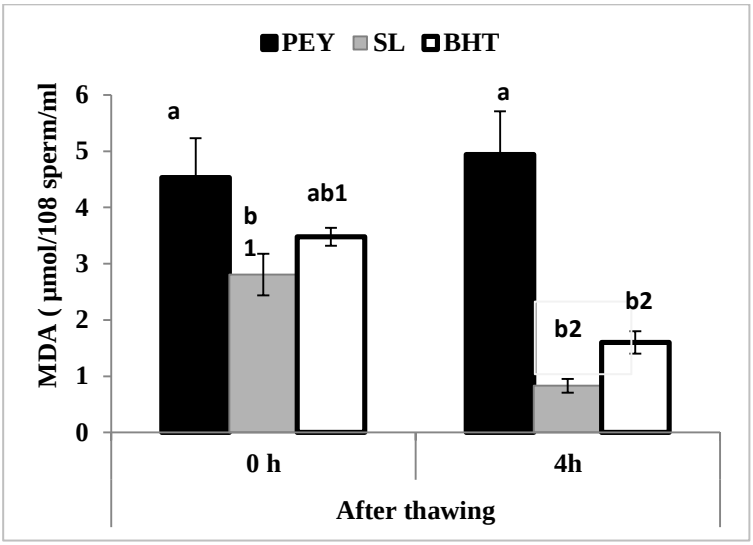

Fig. 1. **MDA concentrations of sperm frozen in various extenders assessed by TBARS test immediately after thawing (0h) and 4 h post-thawing incubation period. PEY: Powdered egg yolk; SL: Soya bean lecithin; BHT: Butylated hydroxytoluene**. Different superscripts (a, b) indicate significant differences ($P < 0.05$) amongst the extenders within each incubation time (0h or 4h). Different numbers (1, 2) indicate significant effect of thermal incubation within each extender ($P < 0.05$).

DISCUSSION

With the ongoing work in our laboratory towards improving ram sperm cryopreservation for some endangered ram breeds, we compared the effectiveness and possible outcome of washed frozen-thawed sperm quality PEY, as a cryoprotectant, was replaced with SL or BHT in the extenders, and their various sperm resilience after a 4 h post-thawing thermal period.

Results from our study showed that BHT does not seem to be an adequate cryoprotectant or a good substitute of PEY for ram sperm freezing as it was proposed. The sperm preserved in BHT showed a significant lower total motility, progressive motility and viability compared to SL or PEY extenders. Although BHT as an antioxidant has been widely used as a supplementation on egg yolk extenders, its different effects on sperm quality has been alluded to species, concentration, and the type of extender composition used for cryopreservation (Ijaz *et al.,* 2009; Memon *et al.,* 2012; Naijian *et al.,* 2013; Patel *et al.,* 2015; Palomo *et al.,* 2017). However, according to Khalifa *et al.* (2008) demonstrated that 0.6 mM concentration of BHT as a substitute for a non-cryoprotectant and replacing egg yolk showed an improvement in sperm motility and other parameters studied.

Nonetheless, the present study showed that total and progressive motility were similar in our powdered egg yolk and soy lecithin-based extenders at post-thaw, and similarly

Table 1.Motility parameters measured by CASA following a 4 h thermal resilience test of frozen-thawed ram spermatozoa cryopreserved in PEY, SL or BHT extenders.

Parameters	Time(h)	PEY	SL	BHT
TM (%)	0	26.34 ± 2.85^{a*}	23.34 ± 2.29^{a*}	14.96± 2.47^{b*}
	4	13.70 ± 4.68^{a*}	17.90 ± 2.32^{a*}	9.30 ± 2.09^{b*}
PM (%)	0	18.50 ± 2.32^{a*}	17.80 ± 3.13^{a}	9.82 ± 1.74^{b*}
	4	13.06 ± 1.94^{a*}	15.62 ± 2.69^{a}	5.14 ± 0.81^{b*}
VCL (µm/s)	0	79.42 ± 6.91*	84.96 ± 6.87*	93.24 ± 9.09*
	4	62.43 ± 4.52*	72.32 ± 9.89*	75.76 ±10.00*
VSL (µm/s)	0	50.94 ± 5.98*	57.79 ± 8.73*	42.04 ± 4.88*
	4	37.98 ± 4.27*	40.06 ± 13.61*	32.86 ± 8.86*
VAP (µm/s)	0	68.04 ± 6.84*	72.41 ± 8.52*	55.26 ± 5.99*
	4	47.98 ± 4.78*	56.44 ± 8.04*	44.28 ± 8.31*
LIN (%)	0	50.08 ± 2.92	55.92 ± 6.52	43.34 ± 3.53
	4	50.82 ± 3.53	40.23 ± 5.28	41.84 ± 7.57
STR (%)	0	62.84 ± 1.99	68.66 ± 3.85	58.44 ± 4.25
	4	63.94 ± 2.91	60.48 ± 5.53	53.88 ± 9.22
ALH (µm)	0	3.70 ± 0.17*	3.72 ± 0.32	3.84 ± 0.32
	4	2.56 ± 0.23^{a*}	3.84 ± 0.48^{b}	3.32 ± 0.14^{ab}
BCF(Hz)	0	8.98 ± 0.65*	9.07 ± 0.68*	7.14 ± 0.94*
	4	7.48 ± 1.07*	6.52 ± 1.84*	5.18 ± 1.16*

PEY: Powdered egg yolk; SL: Soya bean lecithin; BHT: Butylated hydroxytoluene. TM: Total motility; PM: Progressive motility; VCL: Curvilinear velocity; VSL: Linear velocity; VAP: Mean velocity; LIN: Linear coefficient; STR: Straightness coefficient; ALH: Lateral head displacement; BCF: Beat cross frequency. Data are shown as mean ± S.E.M. Different superscripts (a, b) in the row indicates significant differences ($P < 0.05$) amongst the extenders within each incubation time (0h or 4h). *In the column indicate significant effect of thermal incubation within extender ($P < 0.05$).

even after 4 h of a thermal resilience test. Our post-thawed results are in accordance with some studies (Forouzanfar et al., 2010; Sun et al., 2020) but in contrast with other works which showed higher sperm motility in lecithin compared to egg yolk-based extenders (Aires et al., 2003; Amirat et al., 2005; Emamverdi et al., 2013). This discrepancy may be due to the type of egg yolk used unlike in our study where powdered egg yolk was used. We suggest that the viscosity of the extender after reconstituting the powdered egg yolk in the media seems higher due to the high temperatures used during the pasteurization process, and may have denaturalize egg yolk proteins giving higher gelatinous consistency (Miranda et al., 2000).

A surprising observation in the present work was the significant superiority on viability defined as plasma membrane integrity detected in the sperm samples preserved in SL extender after thawing, although only very few of these "viable" sperm showed functional mitochondria. These specific types of mitochondrial inactiveness induced by SL have been described during the freeze-thawing process of ram sperm (Del Valle et al., 2012, Mata-Campuzano et al., 2015). Studies have demonstrated that freeze/thawing and prolonged thermal incubation procedure can induce mitochondrial damage, resulting in the decline of motility and functional integrity of sperm (O'Connell et al., 2002). Based on the current sperm population results, we observed that SL induced certain mitochondrial membrane variations that do not reflect on sperm motility evaluated immediately after thawing contrary to an observation in goat thawed semen (Tabarez et al., 2020). This support the idea that sperm motility may be relatively independent of mitochondrial activities, suggesting that mitochondrial activity may not be the only source of energy for sperm motility. Indeed, glycolysis could be the ATP provider in this case, with mitochondrial activity at this level possibly related to other aspects (Amaral et al., 2013). This finding may explain the similarity in motility results presented in samples extended with SL when compared with PEY samples despites low active mitochondria in SL samples. Nevertheless, since viability and mitochondria function are considered to be good indicators of sperm fertility indices, more studies should be performed in order to assess the consequences of this low mitochondrial activity in other sperm function for capacitation or fertilization.

In the current study, the resilience test with a 4 h thermal incubation period after thawing showed that some sperm motility parameters were negatively affected except for LIN and STR regardless of treatments, as well as SL for PM. This observation was similar to a report by Osuagwuh and Palomo (2017), though on just PEY samples. However, and to our knowledge, no study on the effect of thermal incubation on sperm extended in SL or BHT except for in PEY. In this context, a suggestion is that the adverse incubation time effect on post-thawed may be as a result of the initial sperm washing or seminal plasma removal before freezing. Whether the presence of seminal plasma in our

Table II. Sperm physiological parameters following a 4 h thermal resilience test of frozen-thawed ram spermatozoa cryopreserved in PEY, SL or BHT extenders.

Sperm physiology parameters	Time(h)	PEY	SL	BHT
Viability (%)	0	$11.84 \pm 2.13^{ab*}$	$16.49 \pm 2.05^{a*}$	$8.81 \pm 2.60^{b*}$
	4	$6.52 \pm 0.60^{ab*}$	$11.62 \pm 1.38^{a*}$	$3.38 \pm 1.81^{b*}$
% Viable cells with intact acrosome and active mitochondria	0	$10.79 \pm 1.93^{a*}$	3.61 ± 0.82^{b}	$5.29 \pm 2.66^{b*}$
	4	$4.82 \pm 0.93^{a*}$	4.32 ± 0.31^{a}	$1.22 \pm 1.58^{b*}$
% Viable cells with damage acrosome and active mitochondria	0	$0.32 \pm 0.02^{*}$	$0.76 \pm 0.44^{*}$	$0.49 \pm 0.33^{*}$
	4	$0.83 \pm 0.03^{a*}$	$0.18 \pm 0.04^{b*}$	$1.11 \pm 0.04^{a*}$
% Viable cells with intact acrosome and inactive mitochondria	0	$0.95 \pm 0.34^{b*}$	$12.12 \pm 3.89^{a*}$	$2.19 \pm 0.11^{b*}$
	4	$0.34 \pm 0.06^{b*}$	$6.30 \pm 2.32^{a*}$	$0.15 \pm 0.28^{b*}$
Total acrosome damage (%)	0	$56.65 \pm 1.42^{a*}$	$32.61 \pm 1.73^{b*}$	$52.52 \pm 2.29^{a*}$
	4	$43.62 \pm 1.37^{a*}$	$21.92 \pm 2.39^{b*}$	$41.55 \pm 2.71^{a*}$

PEY: Powdered egg yolk; SL: Soya bean lecithin; BHT: Butylated hydroxytoluene. Data are shown as mean ± S.E.M. Different superscripts (a, b) in the row indicates significant differences ($P < 0.05$) amongst the extenders within each incubation time (0h or 4h). *In the column indicate significant effect of thermal incubation within extenders ($P < 0.05$).

samples before freezing may have improved post-thawed sperm motility (Morrier et al., 2003) or not, cannot be ascertained in our present study. Nonetheless, we can speculate that this decrease in motility could be as a result of the inability of sperm to produce ATP from mitochondrial respiration due to mitochondria ageing (Viswanath and Shannon, 1997).

Literature abounds on the deleterious effect of cryopreservation on sperm physiology, capacitation status and acrosome integrity (Bailey et al., 2000). Consequently, the degree of sperm damage from freezing and thawing may be further exposed during a thermal incubation period, which ultimately may determine the life expectancy of frozen-thawed sperm within the female genital tract. In this study, results seem to show a slight trend (*P > 0.05*) of certain resilience of mitochondria activities when SL is used, thus increasing the proportion of sperm with acrosome membrane and active mitochondria after 4 h of incubation. However, the general deleterious outcome observed on sperm functionality, irrespective of extenders/treatments, may be as a result of dead cells releasing toxic effect of membrane-bound aromatic amino acid oxidase enzyme thereby leading to increase in apoptotic features (Shannon and Curson, 1972).

Furthermore, our results showed lower MDA levels (as an indirect determination of detection of lipid peroxidation) in sperm preserved in SL and BHT extenders compared to PEY samples either at 0 h or at 4 h post-thawing incubation. Therefore, SL and BHT reduced the production of reactive oxygen specie (ROS) during cryopreservation, thus protecting the spermatozoa from damage. This is in agreement with published works on SL (Mata-Campuzano et al, 2015; Salmani et al., 2014) and BHT in semen extender media (Naijian et al., 2013; Alcay et al., 2016; El-Khawagah et al., 2020), demonstrating their positive influence in reducing lipid peroxidation or oxidative stress. However, from general results on our studied sperm parameters, lipid peroxidation release does not seem to be the most important influential factor on sperm parameters due to the fact that BHT samples had the lowest values on all sperm parameters at any given period.

Nonetheless, we can only speculate about the suitability of the different cryoprotectant tested, since no clear differences on their capacity to preserve the sperm during the cryopreservation especially between PEY and SL. It is also worthy to note that the general low values observed in the frozen-thawed semen parameters, irrespective of extenders, may be as a result of age of males (1 year old) used of this study (Lymberopoulos et al., 2010; Garcia et al., 2017). Another possible explanation could be that washing by centrifugation to remove seminal plasma prior to freezing may have had an adverse effect on sperm cryopreservation. Nevertheless, further investigation is needed in order to elucidate the strength of the hypothesis of which lecithin or BHT as a non-animal component could improve frozen-thawed ram sperm quality due to the fact that the exact mechanisms still remains unclear.

CONCLUSION

In conclusion, Butylated hydroxytoluene may not be a good substance for substitution while soy lecithin could be a suitable replacement for powdered egg yolk, although its effects on sperm mitochondria activities have to be strongly considered. The current results show the complexity in

designing a good cryopreservation extender and the need for future investigation to improving frozen-thawed ram semen.

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