Comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of boar semen

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Abstract

This study investigated the effects of using egg yolk and/or lecithin combinations in semen extender on frozen-thawed boar sperm quality. A total of 19 ejaculates from 8 Duroc boars were included. Each ejaculate was aliquoted and cryopreserved in three different extenders containing only egg yolk (20.0%; group I), only lecithin (6.0%; group II) and a combination of egg yolk (10.0%) and lecithin (3.0%) (group III). Frozen-thawed sperm motility, motion characteristics, viability, acrosome integrity, membrane permeability and mitochondrial activity were evaluated using a computer assisted sperm analysis system, SYBR-14/ Ethidiumhomodimer-1, FITC-PNA, sHOST test and JC-1 staining. The frozen-thawed sperm motility in groups I and III did not differ significantly ($P>0.05$), though the combination of extenders was better than group II ($P<0.05$). Motion characteristics, including straight-line velocity (VSL), linearity (LIN) and wobble coefficient (WOB), were higher in groups I and III than in group II ($P<0.05$). Likewise, sperm viability, membrane permeability and mitochondrial activity were higher in groups I and III than in group II ($P<0.05$). In conclusion, the use of lecithin without egg yolk in cryopreserved boar semen extender impaired frozen-thawed sperm quality. Thus, using either 20.0% egg yolk or a combination of 10.0% egg yolk and 3.0% lecithin is recommended for cryopreserved boar semen extender.

Key words: *boar; lecithin; sperm cryopreservation; sperm quality*

Introduction

Cryopreservation techniques have been successfully developed to preserve mammalian semen for almost 70 years (Polge et al., 1949). Nevertheless, boar semen cryopreservation is still not completely successful due to poor frozen-

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thawed semen quality (Knox et al., 2015). This is mainly due to the fact that the plasma membrane of boar sperm contains a high level of polyunsaturated fatty acids (PUFA), resulting in a high risk of sperm damage after cryopreservation (Maldjian et al., 2005). Therefore, substrates, e.g. egg yolk and/or lecithin, are included in the boar semen cryopreservation extender to protect the sperm plasma membrane from cryo-damage. Egg yolk (15% to 20%) has been used for over 40 years as a standard membrane-modifying agent that is added to traditional semen cryopreservation extender (Phillips and Lardy, 1940; Visser and Salamon, 1974). However, the use of egg yolk as a semen extender ingredient has been considered a risk in terms of bacterial contamination. Moreover, the composition of egg yolk also varies depending on its initial sources (Bousseau et al., 1998). Thus, alternative ingredients, e.g. lecithin and other unsaturated lipids, have been considered as replacements for egg yolk (Aires et al., 2003; Pillet et al., 2012; Moraes et al., 2015).

Lecithin or phosphatidylcholine is a phospholipid extracted from soybean (Layek et al., 2016). The use of lecithin as an egg yolk substitute has been successfully developed for many species, *i.e.* rams and goats (Forouzanfar et al., 2010, Salmani et al., 2014), bulls (Aires et al., 2003), cats (Vick et al., 2012), dogs (Dalmazzo et al., 2018) and pigs (Zhang et al., 2009). However, the use of lecithin to replace egg yolk in the cryopreserved boar semen extender is still rare (Zhang et al., 2009). One reason for its underuse could be the limited number of references for pigs and the lack of clearly defined optimal concentrations of lecithin. Theoretically, egg yolk is a source of cholesterol (Faitarone et al., 2013), which can assist the cell membrane in resisting phase transition temperatures (Muller et al., 2008, van Meer et al., 2008). In boar sperm, up to 34% of the total membrane lipids consist of cholesterol, which is necessary for sperm viability, motility and morphology (Am-in et al., 2011; Žura Žaja et al., 2016a). Therefore, the total replacement of egg yolk with other ingredients might diminish the protective effects of cholesterol. To our knowledge, there are no studies on the effect of the combination of lecithin and egg yolk on frozen-thawed boar semen quality. Therefore, the present study aimed to investigate the effects of lecithin in combination with egg yolk in semen extender on frozen-thawed boar sperm quality.

Materials and methods

The experimental procedure was performed according to the Ethical Principles and Guidelines for the Use of Animals, National Research Council of Thailand and was approved by the Chulalongkorn University Animal Care and Use Committee (IACUC) (protocol number 1731012).

Animals and experimental design

A total of 19 ejaculates from 8 proven Duroc boars (aged 2 to 4 years) were included in the experiment. Each ejaculate was aliquoted and cryopreserved in three different extenders containing only egg yolk (20.0%; group I; Buranaamnuay et al., 2009) or only lecithin (6.0%; group II; Zhang et al., 2009) or containing a combination of egg yolk (10.0%) and lecithin (3.0%) (group III). All ejaculates were frozen and thawed according to our previous protocol (Buranaamnuay et al., 2009). The frozen-thawed sperm qualities were evaluated and compared between groups. Semen samples were obtained from proven sires routinely used at the Livestock Research and Breeding Centre, Nakornratchasima Province, Thailand. The experiment was carried out from November to December 2017. On average, the interval between semen collections was 8.2 ± 2.2 days (range: 5 to

9 days). The sperm rich fraction of boar semen was collected using the gloved hand method. The ejaculate was kept in a thermos flask and the gelatinous fraction was eliminated using filter paper. After semen collection, sperm were evaluated for subjective motility under a light microscope at 200 x magnification. Semen with a subjective motility below 65.0% and/or with a total abnormal sperm morphology ≥ 20.0% were excluded.

Extender

The cryopreserved boar semen extenders used in the present study consisted of extenders I, II and III. Extender I was a commercial boar semen extender (Duragen®, Magapor, Zaragoza, Spain) in the ratio of 4.23 g in 100 mL distilled water. Extender II was divided into three groups (*i.e.* groups I, II and III), which contained different amounts of egg yolk, *i.e*. only 20.0% egg yolk (group I), only 6.0% lecithin (group II) or a combination of 10.0% egg yolk plus 3.0% lecithin (group III). Group I (only 20.0% egg yolk) was prepared by adding 20 mL egg yolk into 80 mL 11.0% lactose solution. Group II (only 6.0% lecithin) was prepared by adding 6.0 g lecithin (L-α-Phosphatidylcholine, p3644, Sigma®, MO, USA) and 20 mL distilled water into 80 mL 11.0% lactose solution. Group III (a combination of 10.0% egg yolk plus 3.0% lecithin) was prepared by adding 10 mL egg yolk, and 3.0 g lecithin in 10 mL distilled water, into 80 mL 11.0% lactose solution. The preparation of lecithin solution in distilled water was performed at 50 °C and mixed using a homogeniser (HG-15A, Daihan, Gangwon-do, Korea). The lecithin solution was the treated with an ultrasonic machine (Sonorex Super, Bandelin, Berlin, Germany) at 50 °C for 3 h. Extender III was prepared by mixing together extender II (89.5% for each group), glycerol (9.0%) and Equex STM Paste (1.5%, Nova Chemical Sales Inc., MA, USA). The osmolarity of extender III in Groups I, II and III were evaluated. The average osmolalities of extender III were 1971.3 ± 5.1, 1844.6 ± 6.1 and 1906.7 ± 4.1 mOsmol/kg in groups I, II and III, respectively.

Freezing and thawing procedure

After collection, the semen was diluted (1:1 [v/v]) using extender I (Duragen®, Magapor, Zaragoza, Spain). The diluted semen was transferred to 50 mL centrifuge tubes, equilibrated at 15 °C for 120 min and centrifuged at 800 × g for 10 min to separate seminal plasma from sperm cells. The supernatant was discarded, and the sperm pellet was re-suspended (about 1-2:1) using extender II to a concentration of 1.5×10^9 spermatozoa per mL. The diluted semen was cooled to 4.0 °C for 120 min. Then, two parts semen were mixed with one part extender III. The final concentration of semen was approximately 1.0×10^9 spermatozoa per mL and contained 3.0% glycerol (Buranaamnuay et al., 2009). The processed semen was loaded into 0.5-mL straws (Bio-Vet, Z.I. Le Berdoulet, France). The straws were sealed with PVC powder before being placed in liquid nitrogen vapor at 3 cm above the level of liquid nitrogen for 20 min and then plunged into liquid nitrogen. Thawing was achieved by immersing the straws in water at 50 °C for 12 sec. Post-thawed sperm qualities were evaluated after incubation in a 37 °C water bath for 15 min.

Semen evaluation

Sperm motility and motion characteristics were evaluated using the computer assisted sperm analysis (CASA) system (SCA® CASA System, MICROPTIC S.L., Barcelona, Spain). Images were taken under a phase contrast microscope with a green filter at 100 x magnification. The frozen-thawed semen was diluted with phosphate buffer saline solution (PBS) at a ratio of 1:20. The

diluted semen (8 μL) was dropped onto a warmed slide (37 °C) and covered with a coverslip. A total of 1500 sperm cells from five different fields of each sample were randomly selected to determine sperm motility and motion characteristics. The motion characteristics of the spermatozoa, including straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, %), straightness (STR, %), wobble coefficient (WOB, %), mean lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz), were obtained using the CASA software.

Sperm viability was evaluated with SYBR-14/Ethidiumhomodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, the Netherlands). Thawed semen (10 μL) was diluted with PBS (140 μL). Then, 50 μL diluted semen was gently mixed with fluorescence solution, composed of SYBR-14 (2.7 μL) and EthD-1 (10 μL). The mixed semen sample was incubated at 37 °C for 20 min. Two hundred stained sperm were evaluated under a fluorescent microscope at 1000 x magnification with an oil objective lens. Under the fluorescent microscope, sperm heads with green luminescence were defined as having intact plasma membranes (live), while red luminescence was defined as damaged membranes (dead). Sperm viability was presented as the percentage of live sperm.

Acrosome integrity was determined using fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA). Briefly, the frozen-thawed semen sample (10 μL) was mixed with 10 μL working EthD-1 solution (4.65 μM/mL), then incubated at 37 °C for 15 min. After incubation, the semen sample (10 μL) was smeared on a glass slide and air dried at room temperature. The dried slides were placed into 95% ethanol for 30 sec prior to staining with working FITC-PNA solution (100 μg/mL) at 4 °C for 30 min in a moist chamber. The FITC-PNA-stained slides were rinsed with 4 °C PBS and air dried at room temperature. A total of 200 sperm were evaluated under a fluorescent microscope at 1000 x magnification using an oil objective lens. The criteria for assessing the acrosome integrity of the sperm was modified from the previous methodology (Cheng et al., 1996). Acrosome integrity was classified as intact (expressed bright fluorescence of the acrosome, which indicated outer acrosome membrane integrity) or damaged. Acrosome integrity was presented as percentages.

The hypo-osmotic swelling test (sHOST) was used to evaluate the sperm plasma membrane permeability (Perez-Llano et al., 2001; Dobranić et al., 2005; Samardžija et al., 2008). The hypo-osmotic solution was prepared with fructose and Na-citrate in distilled water until the final osmolality was 75.0 mOsm/kg. The osmolality of the solution was measured by freezing point depression. The frozen-thawed semen sample (100 μL) was mixed with 1000 μL hypo-osmotic solution and incubated at 38.0 °C for 30 min. Then, sperm were fixed with 1000 μL hypo-osmotic solution with 5.0% formaldehyde (Merck, Darmstadt, Germany). A well-mixed sample (10 μL) was placed on a glass slide with a coverslip. A total of 200 sperm were evaluated under a light microscope with 400 x magnification. The coiled tail sperm were defined as sperm with functional sperm membranes. The proportion of sperm with functional sperm membranes was presented as a percentage.

Mitochondrial activity was assessed using tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, Molecular Probes Inc., Eugene, OR, USA). Stock solutions were prepared as follows: 0.153 mM JC-1 in DMSO, 2.4 mM PI in PBS and 0.02 mM SYBR-14 in DMSO. Staining solution was made by mixing the stock solution containing JC-1

Table 1. Means ± standard errors of frozen-thawed sperm motility (%), sperm viability (%), acrosome integrity (%), membrane permeability (%) and mitochondrial activity for three groups (*n*=19 ejaculates per group).

Sperm parameter	Group I	Group II	Group III
Sperm motility parameters			
Total motility (%)	37.8 ± 2.2^{a}	26.2 ± 2.8^{b}	37.9 ± 2.7^{a}
Progressive motility (%)	15.7 ± 1.7^{a}	8.4 ± 1.3^{b}	13.7 ± 1.4^{a}
Rapid motile (%)	10.8 ± 1.4^{a}	5.8 ± 0.8^{b}	9.5 ± 0.9^{a}
Medium motile (%)	8.2 ± 0.7^{a}	5.9 ± 0.7^{b}	8.9 ± 0.6^{a}
Slow motile (%)	18.8 ± 0.6^{a}	15.1 ± 0.9^{c}	21.9 ± 1.0^{b}
Sperm viability (%)	44.2 ± 1.8^{a}	32.6 ± 2.0^{b}	42.1 ± 1.9^{a}
Acrosome integrity (%)	59.8 ± 2.7	52.1 ± 2.6	58.3 ± 3.0
Membrane permeability (%)	31.7 ± 1.3^{a}	23.3 ± 1.3^{b}	31.6 ± 1.3^{a}
Mitochondria activity (%)	49.3 ± 3.1^{a}	35.0 ± 3.0^{b}	47.2 ± 2.6^{a}

Values followed by different letters within the same row were significantly different (P<0.05).

(2 μL), PI (1.6 μL), SYBR-14 (1 μL) and HEPES-buffer solution (95 μL). The semen sample (12.5 μL) was gently mixed with the staining solution (25 μL), then incubated at 37 °C for 30 min. One drop (8 μL) of stained sample was placed on a glass slide covered by a coverslip. Sperm mitochondrial activity was examined under a fluorescent microscope at 1000 x magnification. Green fluorescence of mitochondrial expression was considered low mitochondrial activity, whereas orange fluorescence of mitochondrial expression was considered high mitochondrial activity (Cossarizza et al., 1996; Garner et al., 1997).

Statistical analysis

Statistical analysis was performed using Statistical Analysis Systems version 9.0 (SAS Institute Inc., 1996; Cary, NC, USA). Data were presented as means ± SEM. The normality of all variables was evaluated by the UNIVARIATE procedure. Factors affecting frozen-thawed sperm motility, sperm viability, acrosome integrity, mitochondria activity, sperm membrane permeability and motility characteristics were analysed using general linear mixed models under the MIXED procedure of SAS. The semen extender group (group I, II and III) was included in the model as a fixed effect, and boar identity was included in the model as a random effect. Differences in least squares means between the groups were compared using the least significant difference (LSD) test. A significant difference was defined as P<0.05.

Results

Sperm motility and motion characteristics

Frozen-thawed sperm motility and progressive motility in the different extenders are presented in Table 1. Frozen-thawed total motility of boar sperm was higher in groups I and III than in group II (37.8 ± 2.2%, 37.9 ± 2.7% and 26.2 ± 2.8%, respectively; P<0.01). Likewise, progressive motility was higher in groups I and III than in group II (15.7 ± 1.7%, 13.7 ± 1.4% and 8.4 ± 1.3 %, respectively; P<0.01). Moreover, the percentage of rapid motile sperm was

higher in groups I and III than in group II (10.8 ± 1.4%, 9.5 ± 0.9% and 5.8 ± 0.8%, respectively; $P<0.05$).

The frozen-thawed sperm motion characteristics are demonstrated in Fig. 1. Sperm VSL was higher in group I than in groups II and III (11.8 ± 0.9%, 6.7 ± 0.7%, 9.2 ± 0.9%, respectively; $P<0.05$). However, sperm VSL was higher in group III than in group II ($P<0.05$). Sperm VCL, VAP and STR were higher in group I than in group II ($P<0.05$, Fig. 1). Sperm

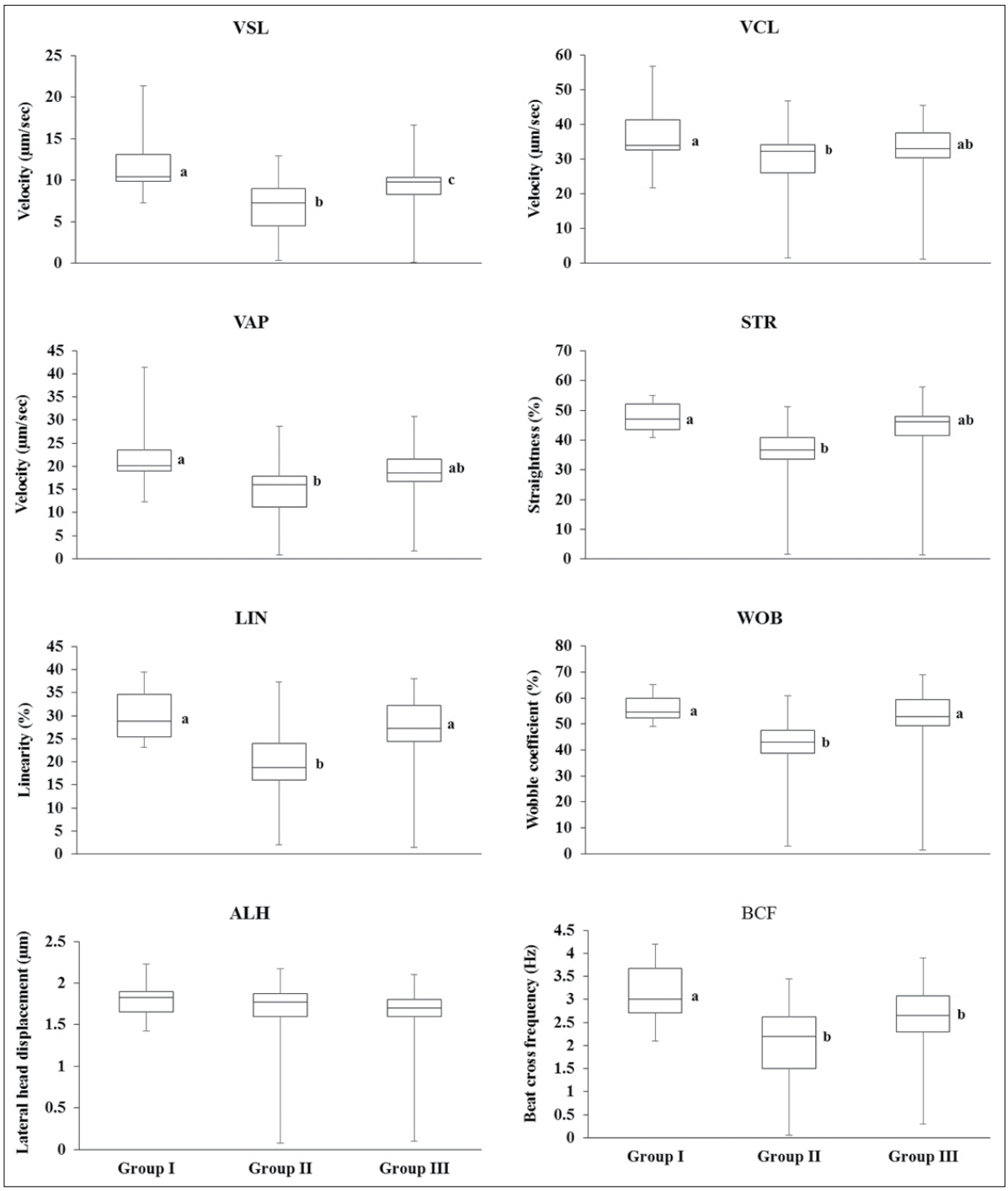

Figure 1. Motion characteristics of frozen-thawed sperm in groups I (20.0% egg yolk), II (6.0% lecithin) and III (10.0% egg yolk and 3.0% lecithin). Different letters within each motion characteristic were significantly different ($P<0.05$).

LIN and WOB in groups I and III were higher than in group II (P<0.05, Fig. 1), and sperm BCF was higher in group I than in groups II and III (P<0.05).

Sperm viability, acrosome integrity, mitochondrial activity

The frozen-thawed sperm quality, assessed by fluorescence staining in different extenders, is presented in Table 1. Frozen-thawed sperm viability was higher in groups I and III than in group II (44.2±7.1%, 42.1±7.2% and 32.6±8.1%, respectively, P<0.05). Likewise, sperm membrane permeability was higher in groups I and III than in group II (P<0.05). Sperm mitochondrial activity was higher in groups I and III than in group II (P<0.05). However, acrosome integrity did not differ among the groups (P>0.05).

Discussion

The present study demonstrated that the use of 6.0% lecithin to replace egg yolk in cryopreservation extender significantly compromised the quality of frozen-thawed boar sperm. This is in contrast with Zhang et al. (2009), who found that using 6.0% soybean lecithin for boar semen cryopreservation extender resulted in higher total sperm motility, higher plasma membrane integrity and higher acrosome integrity than using 20.0% egg yolk. The precise mechanism by which soybean lecithin protects sperm during the cryopreservation process remains unclear (Zhang et al., 2009). However, it was hypothesized that lecithin might reduce the cholesterol/phospholipid ratio of the sperm cell membrane by permeating into the sperm membrane (Žura Žaja et al., 2016b, 2019). As a consequence, capacitation-like changes during the freezing process are controlled and thus, the freezing ability of the boar sperm increases (Gamzu et al., 1997). Another hypothesis is that phospholipids from egg yolk or soybean lecithin might integrate with the sperm membrane to form a protective film against the formation of lethal intracellular ice crystals (Quinn et al., 1980). Therefore, the sperm membrane is protected from mechanical damage during the freezing and thawing processes. However, Zhang et al. (2009) reported that frozen-thawed boar sperm quality was significantly reduced when the concentration of lecithin was increased from 6.0% to 9.0% or 12.0%. In the present study, the use of 3.0% lecithin in combination with 10.0% egg yolk resulted in better frozen-thawed boar sperm motility, sperm viability, acrosome integrity and mitochondria activity, compared with the use of 6.0% lecithin alone. This suggests that lecithin can be used as an egg yolk substitute, but that the concentration of lecithin should be considered. Furthermore, the source of lecithin used in the present study was different from the source of lecithin used in the previous study (Zhang et al., 2009). In the previous study, soybean lecithin (obtained from Unicorn Co., Ltd., Beijing, China) was purified through mixing with ethanol, centrifuged and a drying process, while the source of lecithin used in the present study was a purified lecithin purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Therefore, either the purity or the concentration might be different. These data indicated that the cryo-protective effect of lecithin may also vary depending on the source and the purifying process of the lecithin. Moreover, both the previous study (Zhang et al., 2009) and the present findings indicate that too high of a concentration of lecithin in the semen extender compromises frozen-thawed boar sperm quality.

In the present study, no synergistic effect of egg yolk and lecithin on frozen-thawed sperm quality was found in boars (*i.e.* groups I and II did not differ significantly). However, the synergistic effect of lecithin and egg yolk (1.25:2.00)

and lecithin and cholesterol (4:1) on frozen-thawed sperm motility has been demonstrated in stallions (Nouri et al., 2013) and humans (Mutalik et al., 2014). These data indicate that the cryo-protective effect of the egg yolk and lecithin combination may vary depending on the species. In boars, we could not demonstrate a synergistic effect of egg yolk and lecithin. Blanch et al. (2014) demonstrated that the frozen-thawed boar sperm motility did not differ significantly between extenders containing 20.0% or 10.0% egg yolk. Likewise, the present study also found that the frozen-thawed boar sperm quality did not differ significantly between the extenders containing 20.0% egg yolk and 10.0% egg yolk plus 3.0% lecithin. These data indicate that 10.0% egg yolk is a minimum concentration for protecting boar sperm during the cryopreservation. Additionally, the effect of the lecithin and egg yolk ratios on the frozen-thawed boar sperm quality should be investigated further to determine the optimal concentration of lecithin in combination with egg yolk.

In the present study, lecithin and egg yolk combined with lecithin extenders, did not improve the motion characteristics of the boar spermatozoa, *i.e.* VSL, VCL, VAP, STR, LIN, WOB, ALH and BCF, compared to the traditional egg yolk base extender. This is the first report demonstrating the association between lecithin and motion characteristics of frozen-thawed boar sperm. Interestingly, the use of a high concentration of lecithin (*i.e.* 6.0%) significantly reduced the motion activities of boar sperm, as indicated by VCL, ALH, STR, LIN and BCF. This could be due to the texture of the lecithin extender, having a higher viscosity than the traditional egg yolk extender. Furthermore, in the previous study, lecithin also had a lethal effect on the inner mitochondrial membrane, which interfered with mitochondrial function (Del Valle et al., 2012). Likewise, the egg yolk-based extender also resulted in higher sperm viability and membrane permeability compare to the lecithin-based extender in bulls (Muino et al., 2007; Singh et al., 2018). In the present study, the frozen-thawed sperm viability and sperm membrane integrity was lowest with the lecithin base extender (group II).

In conclusion, the use of lecithin without egg yolk in cryopreserved boar semen extender impaired frozen-thawed sperm quality. Thus, using either 20.0% egg yolk or a combination of 10.0% egg yolk and 3.0% lecithin is recommended as a cryopreserved boar semen extender.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

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Usporedba razrijeđivača za duboko smrzavanje sjemena nerasta temeljenih na dodatku žutanjka jajeta i sojinog lecitina

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U ovom su radu istraživani učinci žutanjka jajeta i/ili lecitina u razjeđivačima sjemena na kakvoću smrznute-odmrznute sperme nerasta. Uključeno je ukupno 19 ejakulata od 8 nerasta pasmine Duroc. Svaki je ejakulat bio raspodijeljen i zamrznut u 3 različita razrijeđivača, koji su sadržavali dodatak žutanjka jajeta (20,0 %) (skupina I), lecitina (6,0 %) (skupina II) i kombinaciju žutanjka jajeta (10,0 %) i lecitin (3,0 %) (skupina III). Nakon odmrzavanja vrednovane su: pokretljivost spermija, karakteristike gibljivosti, vijabilnost, integritet akrosome, permeabilnost membrane spermija i aktivnost mitohondrija pomoću računalno podržanog sustava za analizu sperme (CASA), SYBR-14/etidiumhomodimer-1, FITC-PNA, JC-1 bojenja i HOS testa. Po odmrzavanju, pokretljivost spermija u skupinama I i III nisu se značajno razlikovale (P>0,05), ali su oba razrijeđivača bili bolji od skupine II (P<0,05). Karakteristike gibljivosti, uključujući pravocrtnu brzinu (VSL), linearnost (LIN) i koeficijent kolebljivosti (WOB), bili su viši u skupinama I i III u odnosu na skupinu II (P<0,05). Isto tako, vijabilnost spermija, permeabilnost membrane i aktivnost mitohondrija bili su viši u skupinama I i III u odnosu na skupinu II (P<0,05). Zaključno, uporaba lecitina bez žutanjka jajeta u razrjeđivaču za krioprezervaciju sjemena nerasta pogoršala je kvalitete zamrznuto-odmrznutog sjemena. Stoga se preporučuje uporaba 20,0 % žutanjka jajeta ili kombinacije 10,0 % žutanjka jajeta i 3,0 % lecitina kao dodataka razrijeđivaču za duboko smrzavanje sjemena nerasta.

Ključne riječi: *nerast, lecitin, duboko smrzavanje sperme, kakvoća sperme*