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Research article

Motility degradation rate, plasma membrane integrity, and kinematics during cryopreservation of boar *(Sus scrofa domesticus)* spermatozoa in different freezing extenders and thawing temperatures

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Abstract

Despite the very limited use of frozen-thawed semen (FTS) in pig artificial insemination, FTS in some instances can be truly beneficial as it is not constrained by time (shelf-life) and space (regional quarantine) restrictions unlike fresh-extended semen (FES). It also allows long-term banking of highly valuable genetics particularly during epidemics. This study compares existing and currently available freezing extenders used in boar semen cryopreservation aimed to optimize protocols useful for in-country local swine industry with special focus on the motility degradation rate (MDR), and plasma membrane structural (percent live) and functional (HOST reactive) integrity. Treatment samples from ten freezing runs using five different sperm-rich fractions were frozen using three different cooling/freezing extenders (CE/FE): A) LEYGE, B) BF5, and C) Cryoguard (~500 x 10^6 spz/mL) in liquid nitrogen (LN2) vapor, thawed either at ~38°C or ~50°C for 20 sec, and examined using the Sperm Class Analyzer® CASA system. LEYGE had significantly the highest MDR from about 50% reduction post-thawing to 70% one hour thereafter. Cryoguard consistently had the lowest MDR although closely similar to BF5. There was a minimal effect on the plasma membrane functional integrity and was primarily limited to LEYGE and BF5. A fertility trial is recommended to attest the performance of FTS vs FES in terms of conception rates and the litter size following post-cervical AI before full-scale production and potential adoption by the breeder swine industry.

Keywords: Boar semen, Cryopreservation, Freezing extenders, Frozen-thawed boar semen, Motility degradation rate.

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INTRODUCTION

Pig *(Sus scrofa domesticus)* artificial insemination using frozen-thawed semen (FTS) significantly lags behind fresh-extended semen (FES; Knox, 2011a; Knox, 2015; Knox, 2016). This is largely due to the uniquely sensitive nature of boar spermatozoa, long and complex processes involved with boar sperm freezing, and the additional equipment and facilities required during cryopreservation. There are also logistical challenges associated with the voluminous nature of boar semen along with specific temperatures by which processed boar semen must be maintained to avoid cold shock. These often result in FTS having reduced viability and poor conception rates, among others, thus its minimal use.

Nevertheless, development of FTS particularly in swine is becoming increasingly important due to its inherent practical benefits (Knox, 2011b). The implications of such biotechnology cannot be overstated amid an epidemic brought about by the African Swine Fever (ASF), and similar outbreaks, pandemics, and natural disasters. In particular, frozen semen is not constrained by time (shelf-life) and space (regional restrictions) unlike FES or liquid-stored semen with very limited shelf-life (3-5 days). Moreover, cryopreservation biotechnology allows long-term banking of highly valuable genetic materials or breeder lines needed to preserve genetically selected stocks for repopulation purposes (Bailey et al., 2008; Yánez-Ortiz et al., 2021).

Success in boar sperm cryopreservation appears to be related to several factors before, during, and after the sperm freezing process. These include the use of only the sperm-rich fraction, freezability and ejaculate quality, exposure and holding time in the seminal plasma, cryoprotectants, thawing extenders and temperatures, and straw size among others (Yeste, 2015). Delaying the time of cryopreservation of semen samples while stored at 15–17°C also appears to help stabilize the plasma membrane of spermatozoa by equilibration or preserving the lipid architecture of the sperm (Casas and Althouse, 2013). Furthermore, supplementing antioxidants and other additives in the freezing media may also contribute to sperm quality post-thaw including reproductive performance following AI (Yeste et al., 2010).

One important consideration in the future is that our laboratory will consider freezing semen coming from other regions in the country that will require extra time for transport either by land, sea, or air. Seeing the quality outcomes of frozen-thawed boar semen at different equilibration times could provide baseline data for our potential collaborators to safely send their semen samples for freezing purposes.

This study particularly aims to revisit the basic impact of existing and current freezing extenders used in boar semen cryopreservation on boar sperm cryosurvival with special focus on combined quality parameters including the motility degradation rate (MDR), sperm kinematics, and the plasma membrane structural and functional integrity. In essence, results of this study are projected to propel developmental initiatives on cryopreservation using boar semen with a goal to optimize suitable protocols useful for in-country local breeder swine industry including those of the national animal genetic conservation programs aimed to preserve native pig species particularly in light with onslaught of ASF.

MATERIALS AND METHODS

Semen Samples and Experimental Design

Boar semen were outsourced from sexually mature boars owned by a local AI operator located about 12 km from the PCAARRD-VSU boar semen laboratory of the College of Veterinary Medicine, Visayas State University, Baybay City, Leyte, Philippines (10°44'44.5"N 124°47'48.5"E). These boars aged 1.5 years old are

proven breeders and were collected regularly as sources of AI doses for insemination of sows by local pig growers within Baybay City. The boars are housed in individual stalls and fed with about 3 kg/head (14% CP) given twice per day. Briefly, sperm-rich fractions (~90 mL each collection) filtered using a plastic semen collection bag (US Bag® with filter and sprout, Minitube, Germany) were brought to the laboratory in a 100-mL AI bottle placed inside the BotuFLEX® portable semen box (without ice block). Upon arrival, the semen sample was frozen using three different freezing extenders along with comprehensive monitoring of semen quality prior to freezing and post-thawing at different thawing temperatures as described below. No experimental manipulation was done to constitute ethical consideration as these boars are owned by a smallhold boar operator and used for local pig AI following their normal AI routine and management practices. Moreover, semen samples were only purchased from the AI operator and proper coordination was conducted so that semen collection and animal management were guided following the principles of the Animal Welfare Act of the Philippines (RA 8485).

In this study, five boars (five different sperm-rich fractions) were used and collection per boar was scheduled at 7:00AM every Friday from March to April 2023. To make the most of the available semen and the limited liquid nitrogen supply, sperm freezing was conducted twice each time; one, immediately following the arrival of the sample (day run), and replicated 12 hours after storage (~15° C using Klimabox, Minitube, Germany; evening run) for a total of 10 separate freezing runs (n=10). As the sperm-rich fraction reached the laboratory, an initial evaluation was conducted to determine the motility and concentration. This is done while the sample was still cooling down inside a polystyrene foam box protected from light and placed on tabletop for 1 hour. All raw samples that were used in the study had at least 90% motility upon arrival and examination at the laboratory. After cooling down, the whole sample (90 mL) was diluted 1:1 (v/v) with Androstar Plus (at ~ 29-30 °C; same temperature for both semen and extender) in a 250-mL Erlenmeyer flask and divided equally into six 50-mL conical tubes (30 mL/tube) identified as $A_{1.5hr}$, $B_{1.5hr}$, $C_{1.5hr}$, and A_{12hr}, B_{12hr}, C_{12hr} designated for three different extenders (Lactose egg yolk glycerol Equex STM, LEYGE, A; Beltsville F5, BF5, B; and CryoGuard®, Minitube, Germany, C) and stored at ~15° C (Klimabox, Minitube Germany) for either 1.5 hours or 12 hours prior to the freezing process as described below.

Table 1 Schedule of evaluation and tests conducted used to monitor the quality of boar spermatozoa before and after cryopreservation in liquid nitrogen.

Parameter	Extender	Fresh - extended	Pre-freezing	Immediately Post-thawing		1 Hour Post-thawing	
				38° C	50° C	38° C	50° C
1.5 hr post-storage (~15 °C)	LEYEGE BF5 Cryoguard	MOT, VIT, HOST	MOT	MOT, VIT, HOST		MOT	
12 hr post-storage (~15 °C)	LEYEGE BF5 Cryoguard	MOT, VIT, HOST	MOT	MOT, VIT, HOST		MOT	

MOT, motility; VIT, vitality; HOST, Hypo-osmotic swelling test; n = 10 freezing runs/treatment (5 sperm-rich fractions frozen twice)

Boar Semen Cryopreservation

During storage of the extended semen at ~15°C (Klimabox, Minitube Germany), the three different freezing extenders were prepared following the

respective compositions as described in Table 2. For both LEYGE and BF5, about 50 mL of CE was prepared each time by mixing respective ingredients in a volumetric flask using a magnetic stirrer. Thereafter, the CE was centrifuged at 12,000 g for 12 min to remove the egg yolk colloids and stored at ~15 °C until use. The Cryoguard extender was prepared following the manufacturer's instructions to the amount needed for that day in order to have fresh extender every freezing run. The composition and the steps involved with the preparation of LEYGE and BF5 were based on several past publications (Pursel and Johnson, 1975; Purdy, 2008; Peña et al., 2022).

Following storage at ~15°C, the semen samples in individual 50-mL conical tubes were centrifuged at 800 g for 10 min (Z 446 K Hermle, Labortechnik GmbH, Germany) and the supernatant was decanted. The sperm pellet was then reconstituted using the CE from each of three different extenders to two-thirds of the final volume for LEYGE and BF5 or 50% of the final volume for Cryoguard. The final volume was calculated based on the total spermatozoa/ejaculate (TSE) that was determined earlier so that the final concentration at the time of freezing is ~500 x 10^6 spz/mL. The reconstituted sperm pellet was then stored at ~5°C for another 2.5 hours before the FE was added (the other one-third for LEYGE and BF5, or 50% for Cryoguard), followed by loading in individual 0.5 mL straws (Minitube, Germany). The straws were then exposed to liquid nitrogen (LN2) vapor about three cm above the LN2 level in a modified medical polystyrene foam box for 20 min. The box has an external dimension of about 21 cm x 18.5 cm x 17.5 cm, and an internal dimension of 18 cm x 15 cm x 11 cm. Thereafter, the straws were plunged into the LN2 and allowed to stay submerged for 1 hour. Thereafter, individual straws were thawed at either 38°C or 50°C for 20 seconds and resuspended using Beltsville Thawing Solution (BTS, Minitube, Germany) before downstream semen quality analysis was done as described earlier (Table 1).

Table 2 Composition of three different extenders used for cryopreservation of boar semen.

Extenders	LEYEGE	BF5	Cryoguard®
Cooling extender	- 310mM Lactose monohydrate - 20% Egg yolk (v/v) in deionized water	- 52 mM TES - 16.5 mM Tris - 178 mM Glucose - 20% Egg yolk (v/v) in deionized water	- Cryoguard A
Freezing extender	- 91.5% CE (v/v) - 6.0% Glycerol - 2.5% Equex paste	- 91.5% BF5 - 6.0% Glycerol - 2.5% Equex paste	- Cryoguard B

Semen Quality Assessment

Sperm kinematics and motility degradation rate

A comprehensive assessment of the semen quality was conducted at four major points including 1) Fresh-extended - following extension 1:1 using Androstar Plus and storage (~15 °C); 2) Pre-freezing - following 2.5 hours of storage with CE and FE (~5 °C); 3) Immediately post-thawing - following 1 hour in liquid nitrogen (-196 °C); and 4) 1 hour post-thawing - following 1 hour in hot-water bath (~38 °C and ~50 °C). Boar sperm motion parameters were characterized following the comprehensive analysis using the Sperm Class Analyzer® (SCA® version 6.6.15.0, Microptic S.L., Barcelona, Spain) computer-aided sperm analysis (CASA) system following the manufacturer's recommendations and related protocols (Mortimer, 2000; Peña et al., 2015; van der Horst et al., 2018) and as previously conducted in our laboratory (Peña et al., 2023). The SCA® software used in the CASA system allows automated analysis of sperm images captured by a Basler acA1300-200uc camera that is connected to the Nikon Eclipse E200 microscope, thereby giving an objective estimation of general sperm motility and kinematic characteristics.

For sperm motility, an aliquot was prepared (~50 x 10^6 spz/mL) in a 4-mL plastic tube and warmed to 38 °C (water bath) for about 20 min prior to analysis. This procedure was followed throughout the schedule of evaluation as indicated in Table 1. Thereafter, about 10 µL of the diluted sample was dropped on a pre-warmed microscope slide (38 °C, Goldcyto slide warmer) and covered with a 22mm x 22mm cover slip. The motility analysis was then commenced using the SCA® MOT module (normal slide setting) with at least 500 spermatozoa per treatment slide analyzed including the MDR as previously described (Baldaniya et al., 2020).

Plasma membrane structural and functional integrity

To determine the sperm plasma membrane integrity, two separate tests were conducted using the supravital staining technique and the hypo-osmotic swelling test (HOST) (Peña et al., 2024) for the percent live and functional integrity, respectively. For the percent live, smears were prepared after staining an aliquot of semen sample with Eosin G stain (2% solution) and Nigrosin stain (4% solution) stains following the manufacturer's recommendations (Minitube, Tiefenbach, Germany) in two slides using Hemaprep (J.P. Gilbert Co., USA), and were air dried. Thereafter, 200 spermatozoa were examined thoroughly (400x magnification) and classified either live (white/opaque sperm heads) or dead (pink/purplish/colored sperm head), and expressed as percentage live.

The HOST assay was based on procedures from earlier studies (Jeyendran et al., 1992; Vazquez et al., 1997; Lechniak et al., 2002; Malo et al., 2012). Initially, BTS was prepared following the recommended dilution and stored at 5°C as stock solution (labelled BTS Stock Solution). On the day of the experiment, an aliquot of the BTS Stock Solution was diluted 1:3 using distilled water (labelled BTS-HOST Test Solution) for use in the HOST assay. Thereafter, 30 µL semen sample and 100 µL BTS-HOST Test Solution was mixed thoroughly in a 1.5-mL tube and incubated at 37°C for 30 minutes in warm blocks. A smear was prepared on a clean slide and covered with a coverslip with the edges sealed with a clear nail polish. The slides were examined immediately by analyzing 200 spermatozoa per slide, classified between HOST-positive and HOST-negative. HOST-positive spermatozoa were identified by curled/swollen tails and expressed as percentage of the total number of spermatozoa examined.

Statistical analyses

All data were consolidated in a spreadsheet for use in JASP (Version 0.16.2), a free and open-source statistical computer software (Team JASP, 2022) for the required statistical analyses. Significant differences (p value of ≤ 0.05) between MDR were determined using a repeated measures ANOVA with Greenhouse-Geisser correction and Bonferroni post hoc analysis applied. Otherwise, a classical ANOVA or Students' T-test was used to determine significant differences for all other treatment groups, as deemed appropriate. Where data is not suitable for a parametric test, the Dunn's test was used instead (Dinno, 2015).

RESULTS

Both total and progressively motile spermatozoa were found to be relatively similar between fresh-extended (at ~15°C) and the pre-freezing stage when spermatozoa have undergone a series of processing procedures including centrifugation, reconstitution in CE/FE, and equilibration time at ~5 °C (2.5 hours). However, significantly lower total (38°C, 61.89 ± 4.72%; 50 °C, 57.74 ± 5.88%) and progressively motile (38 °C, 28.49 ± 4.19%; 50 °C, 22.53 ± 5.54%) spermatozoa were found with LEYGE, post-thawing. A significant improvement was observed with Cryoguard (total motility = 38°C, 82.37 ± 3.24%; 50°C, 92.31 ± 1.67%; progressive motility = 38°C, 49.10 ± 3.91%; 50°C, 58.38 ± 3.43%, respectively) but

was not significantly different from the BF5 extender. A similar trend can be observed in one-hour post-thawing, with BF5 still in the middle between the LEYGE and Cryoguard, with total (38°C, 66.47 ± 7.03%; 50°C, 67.42 ± 5.57%) and progressively motile (38°C, 31.05 ± 6.10%; 50°C, 35.26 ± 5.04%) spermatozoa, respectively. These results are reflected in Figure 1 which considers the rate by which boar sperm loses its motility along the cryopreservation process from the time boar semen was extended to pre-freezing, and immediately post-thawing including one-hour thereafter. Overall, the reduction in the motility of spermatozoa occurs at the rate of about 40%-70% for LEYGE, 20%-30% for BF5, and 15%-25% for Cryoguard, immediately post-thawing and one-hour thereafter, respectively.

Table 3 Mean (±SEM) percentage of sperm motility and kinematic characteristics of boar spermatozoa before and after cryopreservation in different freezing extenders and thawing temperatures.

Parameter	Extender	Fresh - extended	Pre-freezing	Immediately Post-thawing		1 Hour Post-thawing	
				38° C	50° C	38° C	50° C
Total motility	LEYEGE	95.83 ± 0.98	94.88 ± 0.78	61.89 ± 4.72	57.74 ± 5.88	47.34 ± 7.10	47.16 ± 6.32
	BF5	96.50 ± 0.73	96.42 ± 0.91	77.73 ± 3.78	77.89 ± 4.44	66.47 ± 7.03	67.42 ± 5.57
	Cryoguard	96.34 ± 0.71	96.74 ± 0.86	82.37 ± 3.24	92.31 ± 1.67	70.65 ± 6.54	82.90 ± 3.89
Progressive motility	LEYEGE	63.53 ± 4.28	67.53 ± 1.53	28.49 ± 4.19	22.53 ± 5.54	18.14 ± 4.81	17.98 ± 5.70
	BF5	67.54 ± 2.87	62.27 ± 3.09	38.27 ± 0.03	42.10 ± 3.97	31.05 ± 6.10	35.26 ± 5.04
	Cryoguard	64.21 ± 3.58	72.44 ± 2.28	49.10 ± 3.91	58.38 ± 3.43	39.11 ± 6.68	49.55 ± 4.97
VAP	LEYEGE	43.27 ± 7.02	66.68 ± 4.04	3.36 ± 2.57	26.24 ± 3.48	21.28 ± 2.43	23.09 ± 3.30
	BF5	43.90 ± 5.10	75.23 ± 3.56	39.15 ± 3.60	45.06 ± 2.87	31.08 ± 3.60	34.96 ± 2.87
	Cryoguard	43.92 ± 5.42	83.69 ± 4.83	39.51 ± 2.41	45.67 ± 3.68	32.38 ± 3.95	39.83 ± 3.09
VCL	LEYEGE	69.74 ± 7.57	93.95 ± 4.38	52.96 ± 3.46	42.38 ± 5.44	38.33 ± 3.33	37.54 ± 4.29
	BF5	72.02 ± 7.79	110.67± 5.27	59.76 ± 4.19	68.95 ± 5.51	50.28 ± 4.82	56.03 ± 4.23
	Cryoguard	68.75 ± 6.62	94.79 ± 4.67	56.39 ± 3.13	65.36 ± 3.57	45.47 ± 3.31	58.36 ± 3.43
VSL	LEYEGE	28.14 ± 4.84	44.55 ± 2.95	22.61 ± 2.13	17.48 ± 2.10	13.90 ± 1.82	15.69 ± 2.59
	BF5	27.47 ± 2.89	44.31 ± 1.82	24.95 ± 1.71	29.40 ± 1.66	20.37 ± 2.57	24.06 ± 1.96
	Cryoguard	29.80 ± 4.16	50.87 ± 3.07	28.24 ± 1.94	32.12 ± 3.02	24.37 ± 3.48	29.14 ± 1.94
STR	LEYEGE	59.58 ± 3.40	62.14 ± 1.59	58.30 ± 2.66	60.36 ± 3.14	53.87 ± 3.40	58.54 ± 2.15
	BF5	59.28 ± 1.83	57.17 ± 2.25	58.56 ± 2.22	57.45 ± 2.34	57.97 ± 3.40	58.93 ±1.84
	Cryoguard	61.16 ± 2.23	65.25 ± 0.86	62.84 ± 1.14	62.66 ± 1.54	64.66 ± 3.27	63.58 ± 1.77
LIN	LEYEGE	37.55 ± 3.69	45.73 ± 2.60	40.06 ± 2.83	42.58 ± 3.44	32. 47 ± 3.74	39.85 ± 3.09
	BF5	11.04 ± 0.63	11.04 ± 0.63	11.04 ± 0.63	11.04 ± 0.63	11.04 ± 0.63	11.04 ± 0.63
	Cryoguard	41.23 ± 3.44	51.05 ± 1.42	46.12 ± 1.22	45.37 ± 1.95	47.44 ± 4.55	45.12 ± 1.22
ALH	LEYEGE	1.70 ± 0.12	1.87 ± 0.08	1.35 ± 0.04	1.18 ± 0.07	1.12 ± 0.05	1.11 ± 0.06
	BF5	1.71 ± 0.12	2.24 ± 0.11	1.47 ± 0.07	1.61 ± 0.10	1.33 ± 0.08	1.41 ± 0.08
	Cryoguard	1.66 ± 0.08	1.81 ± 0.07	1.41 ± 0.04	1.57 ± 0.03	1.21 ± 0.03	1.43 ± 0.06
BCF	LEYEGE	11.24 ± 1.40	15.83 ± 0.59	9.28 ± 0.84	7.28 ± 1.23	6.29 ± 0.93	6.21 ± 1.03
	BF5	11.98 ± 1.43	17.60 ± 0.76	10.08 ± 0.83	11.25 ± 0.67	8.93 ± 1.07	10.49 ± 0.73
	Cryoguard	11.03 ± 1.21	15.24 ± 0.81	10.05 ± 0.83	11.04 ± 0.63	8.54 ± 1.05	10.67 ± 0.69
WOB	LEYEGE	58.50 ± 4.36	69.50 ± 2.45	61.54 ± 0.68	62.47 ± 3.25	61.54 ± 2.68	59.87 ± 3.21
	BF5	60.31 ± 3.51	67.31 ± 1.66	64.88 ± 2.15	63.08 ± 1.55	60.11 ± 3.99	59.60 ± 1.54
	Cryoguard	62.15 ± 3.78	75.31 ± 2.22	68.57 ± 1.35	68.49 ± 1.73	67.15 ± 4.63	65.78 ± 1.92

Figure 2 provides the viability profile between fresh-extended and FTS in 38°C and 50°C. While expectedly more dead sperm were found with FTS regardless of the thawing temperature used, the percentage live spermatozoa was still considerably high regardless of the extenders or thawing temperatures (Figure 2A). Moreover, there was no significant difference in the percentage live spermatozoa between extenders used post-thawing. As regards to the plasma membrane functional integrity using the HOST, a minimal difference can be observed between fresh-extended and the FTS for LEYGE and BF5 but only at 50°C. No difference was observed when using Cryoguard (Figure 2B).

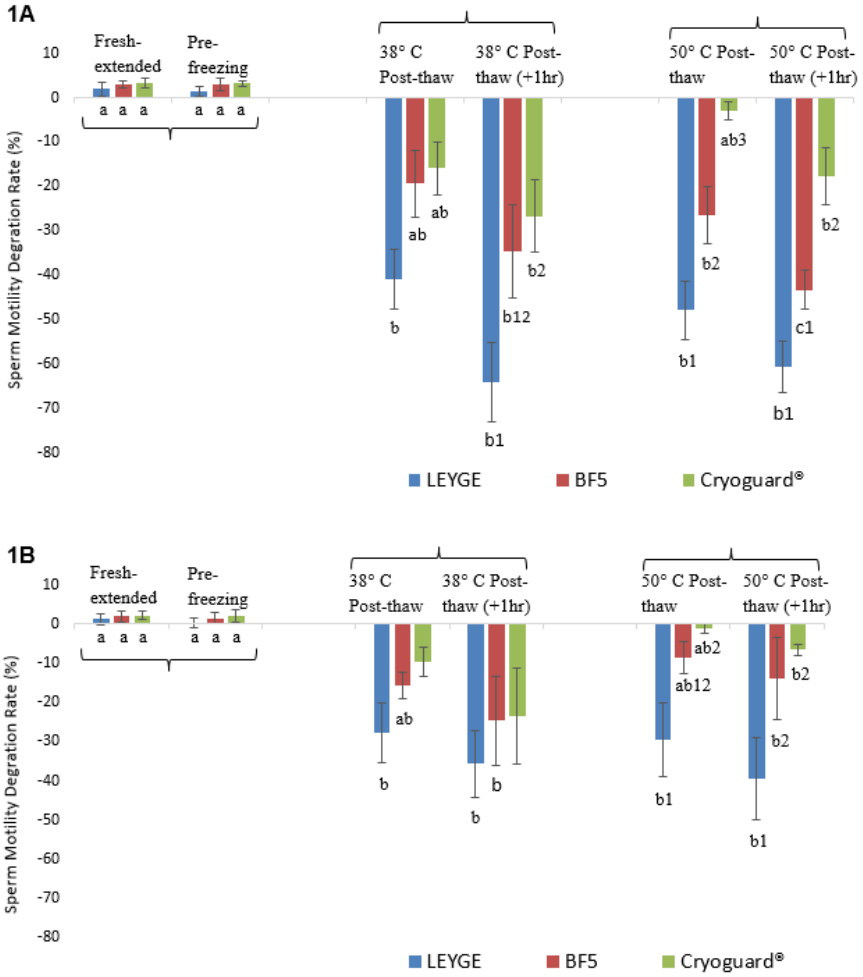

Figure 1 Motility degradation rate (MDR) post-thawing between semen samples stored for 1.5 hr (1A) and 12 hr (1B) at ~15°C prior to freezing. n= 5 different sperm-rich fractions/ treatment); different letters signify differences between processing stages for individual extenders while different numbers signify differences between extenders used.

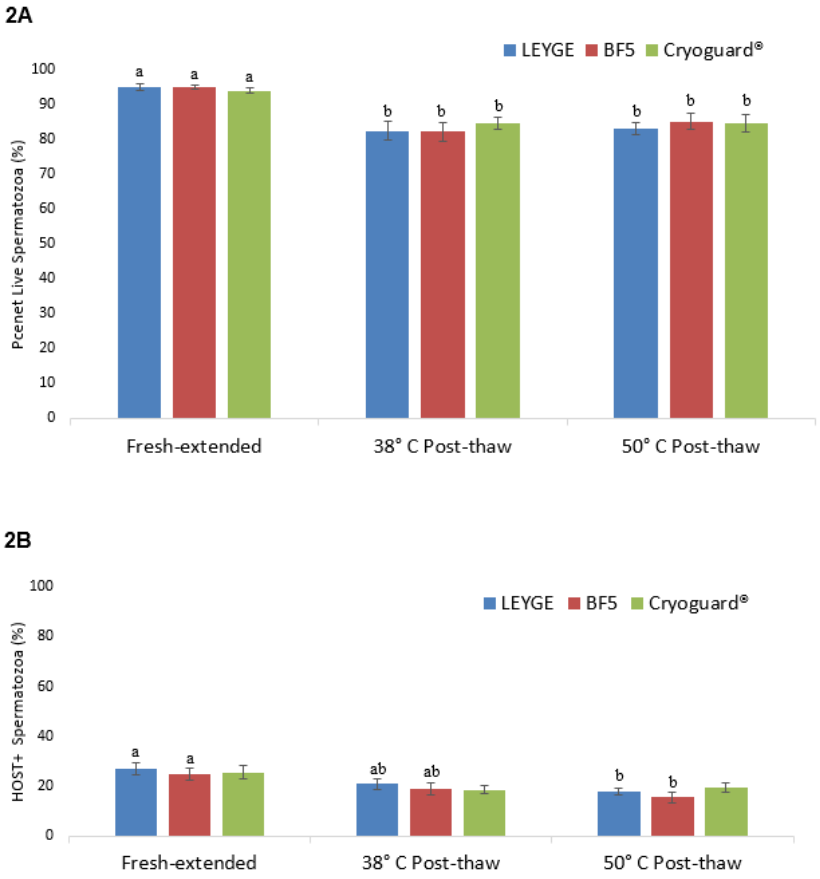

Figure 2 Mean (±SEM) percentage of live (2A) and HOST reactive (2B) spermatozoa either between freezing extenders or thawing temperatures. Different letters indicate significant difference between fresh extended and thawing temperature for each extender type ($p > 0.05$; n= 10 per extender per thawing temperature).

DISCUSSION

The literature provides extensive evidences showing the negative impact of freezing and thawing against boar sperm. Potential damage to the structural integrity and functions resulted in reduced motility, damage to sperm membrane functions and the mitochondrial architecture, and sperm DNA fragmentation, among others (Watson, 1995; Thurston et al., 2001; Roca et al., 2006). This is on top of the fact that some 70% of the variability in the survival rates of frozen-thawed boar sperm can be attributed to individual boar effects (Holt, 2000; Roca et al., 2006). Nevertheless, using proven breeder boars and the sperm-rich fractions made it possible to generate reasonable motility and viability from ten freezing runs conducted in this experiment. However, the impact of the composition of freezing extenders appears to clearly differentiate sperm cryosurvival immediately post-thawing and one hour thereafter, regardless of the thawing temperature and storage time at ~15°C.

Essential elements for successful sperm freezing lie in the composition between non-permeating and permeating cryoprotectants. Non-permeating cryoprotectants normally include the basic sugars, amino acids, and other macromolecules that do not necessarily pass through the sperm plasma membrane. These substances may be found in the glucose or lactose, and egg yolk which form major ingredients in the freezing media. On the other hand,

permeating cryoprotectants reach intracellularly and thus are normally considered critical to the overall survival and quality of spermatozoa post-thawing. Of note, while there are a number of permeating cryoprotectants tested in boar semen including DMSO and dexamethasone, glycerol still remains to be the cryoprotectant of choice in boar semen cryopreservation (Malo et al., 2012).

There are obvious logistical challenges using boar sperm for cryopreservation. Perhaps the most important among these is the availability of appropriate temperature-controlled devices to ensure that boar spermatozoa do not succumb to reduced viability post-thawing associated with temperature changes. Every freezing run of this experiment entailed about six hours from the time semen samples arrive in the laboratory to the final quality assessment post-thawing. During this long process, boar spermatozoa underwent a series of temperature changes from the time the semen was collected at the farm (rectal temperature, ~39°C); followed by 1:1 dilution with an extender upon arrival in the laboratory (~30°C); during storage after dilution for either 1.5 hours or 12 hours (~15°C); then prior to freezing following reconstitution of the sperm pellet with CE and FE for 2.5 hours (~5°C); during manual exposure at liquid nitrogen vapor for 20 minutes (about -40°C), while frozen in liquid nitrogen for 1 hour (-196°C); at thawing either in 38°C or 50°C for 20 seconds; and finally during the time when FTS are at 38°C water bath for quality assessment using the CASA system. Despite the limitations in the equipment used to maintain such temperature requirements and given the constant level of glycerol included between LEYGE and BF5, it can be argued that components other than the basic elements present in LEYGE including their respective concentrations may have provided better conditions for sperm survival in BF5 and Cryoguard.

Furthermore, this study tested both thawing temperatures as a matter of technical and logistical aspects as some small laboratories may have limitations on the availability of multiple water baths. Since the standard holding temperature for evaluating boar semen is 38 °C, results of this study further support the use of 38°C for thawing without compromising the post-thawing quality nor the need of another thawing equipment set at different temperature thus, lessening logistical costs. In addition, the literature suggests the need to equilibrate boar semen by allowing extra time for spermatozoa to be exposed to the seminal plasma after collection and prior to the freezing process. This equilibration time is believed to provide extra protection by stabilizing the sperm plasma membrane for better post-thaw quality outcomes, thus the two freezing runs. Another very important aspect to consider in future is that our laboratory will embark on freezing semen coming from other regions that require extra time for transport. Given the relatively similar results between 1.5 hours and 12 hours storage, it simply means that it is highly feasible for potential collaborators to send their semen samples for freezing purposes.

The greatest challenge to sperm survival is the negative impact of liquid nitrogen on spermatozoa upon freezing. To ensure that this impact is mainly limited upon and during storage in liquid nitrogen, it was necessary to monitor the performance of spermatozoa from the very start after semen collection and processing until the time just prior to the exposure of the reconstituted semen to liquid nitrogen vapor. Prior to this exposure stage, boar spermatozoa have undergone multiple processing procedures including centrifugation, reconstitution in CE/FE, and the long equilibration time at ~5°C (2.5 hours). Nevertheless, both total and progressively motile spermatozoa remained relatively similar with reduction in sperm quality only became apparent post-thawing. These results suggest the apparent stability of boar spermatozoa prior to liquid nitrogen exposure but also imply the crucial role freezing extenders have on the maintenance of sperm viability considering the significant reduction in sperm motility particularly with LEYGE. Overall, this reduction in sperm motility is reflected in the MDR which occurs at the rate of about 40%-70% for LEYGE, 20%-30% for BF5, and 15%-25% for Cryoguard, immediately post-thawing and one-hour thereafter,

respectively. Despite this however, the considerably higher percent live spermatozoa is worth noting, regardless of the extenders used and the minimal effect on the plasma membrane functional integrity post-thawing. Thus, the composition of freezing extenders should be directed towards improving the MDR post-thawing to allow some time and other logistical considerations to settle in place until insemination.

Motility evaluation of spermatozoa is an important aspect to assess the baseline quality potential of a particular sperm sample (Rodríguez-Martínez, 2006; Vyt et al., 2008). Using CASA system could provide thorough and objective evaluation of sperm motion characteristics. Furthermore, using an automated estimation of the progression and velocity of spermatozoa along with other kinematic parameters offers a more credible indicator in predicting the fertilizing capacity of the sperm sample. Results of the study indicate parallel outcomes in kinematic results and progressivity and velocity of spermatozoa prior to and after freezing using different types of extenders used.

In conclusion, LEYGE significantly had the highest MDR from about 50% reduction post-thawing to 70% one hour thereafter. Cryoguard consistently had the lowest MDR although closely similar to BF5. There was a minimal effect on the plasma membrane functional integrity and was primarily limited to LEYGE and BF5. A fertility trial is recommended to attest the performance of FTS vs FES in terms of conception rates and the litter size following trans-cervical or post-cervical AI before full-scale production and potential adoption by the breeder swine industry. This study further highlights the need for deeper understanding of appropriate protocols and logistics required for optimal boar semen cryopreservation particularly of native pig breeds that are particularly exposed to the threats of ASF and swine epidemics and natural calamities.

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AUTHOR CONTRIBUTIONS

STPJ contributed to project administration, funding acquisition, study conceptualization, original manuscript writing, review, and editing, and data consolidation and analysis. BTPY, and MEBJ equitably contributed to methodology, sampling and laboratory works, data management, and review of the manuscript.

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

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