

Research Note

Boar Sperm Performance in Polystyrene Transport Cool Box Under Various Storage Conditions and Holding Times

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When transporting highly valuable boar semen samples over long distances, it is crucial to ensure that boar semen is stored ideally at approximately 15 – 17°C to minimize the impact of cold shock and oxidative damage on sperm quality. This study evaluated the performance of a commercially available semen and embryo polystyrene transport cool box, BotuFLEX®, in preserving the quality of extended boar semen under different storage conditions and holding times. Boar ejaculates were extended at 1:1 or 1:3 using a commercial mid-term extender, stored in a BotuFLEX® semen cool box, and examined 24 vs. 48 h thereafter (Experiment 1; n = 12), or 24 h after storage in air-conditioned room vs. ambient room (Experiment 2; n = 9) conditions. Comprehensive sperm motility using the Sperm Class Analyzer® CASA system revealed no significant difference in either total and progressively motile sperm, and the combined progressivity and velocity characteristics across different treatments. Almost similar results were observed on sperm vitality using the standard supravital staining technique except that percent live spermatozoa were higher at 24 h of storage using the 1:1 dilution.

Keywords: boar sperm, semen cool box, sperm progressivity, velocity, vitality

INTRODUCTION

In the swine industry, processing liquid-stored boar semen for artificial insemination (AI) has been a standard practice with minimal to no significant impact on sperm quality and fertility. Fresh ejaculates may be diluted using an appropriate nutrient-rich extender at certain concentrations (Foxcroft et al. 2008; Knox 2016) and stored to about 17°C for up to a week while preserving semen quality (Johnson et al. 1988; Althouse et al. 1998; Pezo et al. 2019). This practice has brought several advantages, particularly when applied directly on-farm and in commercial pig production settings, increasing production efficiency (Knox 2011; Knox 2016) and allowing AI to attract more pig farmers (Singh and Mollier 2020). With AI, as many as 1000 – 2000 sows per year (Colenbrander et al. 1993; Knox et al. 2008) may be inseminated from a single boar with good farrowing rates (Porth 2016). Moreover, good quality semen may be collected from disease-free boars of good genetic merit

and used to inseminate multiple sows across regions including remote locations. The ability to extend and preserve boar semen provides greater flexibility in selecting the best boars, collecting and transporting AI doses, and synchronizing recipient sows for increased efficiency.

Boar sperm, however, could still be subject to irreversible damage due to cold shock and oxidative stress which could significantly impact fertility outcomes (Pursel 1979; Althouse et al. 1998; Johnson et al. 2000; Peña et al. 2017). In this case, cold shock may be caused by rapid cooling and/or exposure of boar sperm to critical temperatures below 12°C (Althouse et al. 1998). This may result in irreversible loss of motility and metabolic activity, increased membrane permeability, and loss of intracellular proteins and enzymes, among others (Pursel et al. 1972), thus making the use of household refrigerators unsuitable when storing raw or extended boar semen.

Cooling devices used for storing and transporting livestock semen use either passive or active cooling systems to provide appropriate temperatures required during storage. While active cooling systems use electricity and may provide better control to regulate the temperature, passive containers that use ice blocks/packs or other forms of coolants may provide better flexibility when transporting small amounts of semen samples by land, sea, or air without the need for electricity.

This study tested the capacity of a commercially available semen and embryo polystyrene transport cool box (BotuFLEX®, Botupharma, USA) in preserving the quality of boar semen under different storage conditions and holding times. Such a transport box has been used in previous studies involving semen from stallions (Maciel et al. 2017), donkeys (Lago-Alvarez et al. 2020), dogs (Araujo et al. 2022), bulls (Sebastião et al. 2018), rabbits (Romero et al. 2020), and pig-like white-lipped peccaries (Barros et al. 2019). So far, only one study has used BotuFLEX® in boar semen (Bernardino et al. 2022); hence, it was deemed necessary to establish the motility and viability characteristics of boar sperm during storage in the BotuFLEX® cool box. Results of this study will support the researchers' use of this semen cool box when transporting boar semen samples from collaborating swine breeder farms located several kilometers away from the team's laboratory for further downstream quality analyses and/or cryopreservation purposes.

MATERIALS AND METHODS

Semen Samples and Experimental Design

Two separate experiments were conducted using 12 (Experiment 1) and 9 (Experiment 2) semen samples collected from sexually mature boars owned by smallholder AI operators. As the number of available boars was limited and the animals varied in age (1.5 – 2 yr old), breed (mostly crossbreeds), and management practices, only boars that were regularly collected from and had at least 70% total motile sperm during the preliminary assessment were used in the experiment. The boars were owned by smallholder AI operators from selected municipalities in Leyte located 10 – 50 km from the research laboratory. Prior consultation was conducted with the City Agriculture Office of the City of Baybay. The researchers did not have direct contact with the boars as animal care and management were provided by their owners. Semen was collected by the boar owners according to their individual farm practices as part of their routine activity with selling semen doses. No experimental manipulation nor intervention was conducted and only a portion of the ejaculate was purchased each time a sample was needed for the experiment.

Initially, following collection at the boar operator's farm, a portion of the ejaculate was filtered using a plastic semen collection bag (US Bag® with filter and sprout, Minitube, Germany) and extended at either 1:1 or 1:3 using MIII commercial extender (Minitube, Tiefenbach, Germany) at about 38°C, then transferred into individual sterile 50-mL conical tubes labelled appropriately. The conical tubes were placed inside the BotuFLEX® portable semen and embryo polystyrene cool box fitted with a temperature logger (RC-5, Elitech Technology, Inc., USA) located in between the tubes, allowed to settle for about 30 min, and transported via motorcycle to the Visayas State University - Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development 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).

Upon arrival in the laboratory, the individual BotuFLEX® cool boxes were placed on a tabletop in an air-conditioned room. Downstream analyses for motility and viability were done after 24 and 48 h of storage for Experiment 1. For Experiment 2, separate cool boxes labeled Ar-R (air-conditioned room) and Am-R (ambient room) were used to store the extended boar semen prior to semen quality evaluation 24 h after storage. The Ar-R treatment was similar to the storage conditions used in Experiment 1; for the Am-R treatment, the corresponding BotuFLEX® cool box was placed inside an adjacent room without air-conditioning but with open windows for normal lighting and ambient storage conditions. This was done to simulate the possibility of transporting semen samples inside transport conditions without air-conditioning. Separate temperature loggers were placed on top of each box to monitor the temperatures between different storage conditions.

Sperm Motility and Vitality Evaluations

Comprehensive analysis of boar sperm motility was done 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 standard protocols on CASA (Mortimer 2000; van der Horst et al. 2018; Peña 2023). The CASA system is composed of a computer hardware connected to a microscope (Nikon Eclipse E200) fitted with a camera (Basler acA1300-200uc). The installed SCA® software system allows an automated analysis of sperm images captured by the camera for an objective estimation of sperm motility characteristics including the total and progressively motile spermatozoa. A combined measure of the velocity and progressivity of spermatozoa was also evaluated and expressed as rapid, medium, non-progressive, and immotile spermatozoa.

For sperm motility, 2 mL of semen sample (about 37.5×10^6 spz/mL) was first prepared for each treatment aliquoted into a 4-mL plastic tube and allowed to stay in a warm water bath (38°C) for about 20 – 30 min before the motility analysis commenced. Thereafter, about 10 μ L semen aliquot was placed on a plain microscope slide also pre-warmed to about 38°C (Goldcyto slide warmer, Microptic S.L., Barcelona, Spain) covered with a 22 x 22 mm cover slip and examined accordingly using the normal slide setting of the SCA® MOT module which was set to analyze at least 500 spermatozoa per treatment slide. The concentration for sperm motility was standardized based on the initial sperm concentration of the raw sample diluted at 1:10 in a 0.9% NaCl solution, using a disposable SCA® counting chamber (Microptic S.L., Barcelona, Spain) and examined accordingly using the SCA® MOT module.

To determine sperm vitality, direct smears were prepared using Hemaprep (J.P. Gilbert Co., USA) in two glass slides and allowed to dry in air. Staining was conducted using a combination of Eosin G (2%) and Nigrosin (4%) staining solutions following the manufacturer's recommendations (Minitube, Tiefenbach, Germany) and about 200 spermatozoa were examined thoroughly (400x magnification) thereafter. Spermatozoal heads that were clear of coloration (white/opaque) were considered vitally normal and expressed as percentage of live spermatozoa, while spermatozoa that were dark red/purplish/dicolored were considered dead (having broken plasma membrane allowing the entry of the eosin stain).

Statistical Analyses

All data were entered into a spreadsheet using Google Sheets and converted into a Comma Separated Values (CSV) file required by JASP (Version 0.16.2), a free and open-source statistical computer software for appropriate statistical analyses. Significant differences (p -value of ≤ 0.05) between treatment groups were determined using a 2 x 2 repeated measures ANOVA for Experiment 1 and a 2-way ANOVA for Experiment 2. On either case, at least two levels of factors and/or conditions were considered including the holding times (Expt. 1) and storage conditions (Expt. 2) at different dilution rates (1:1 vs 1:3) in both experiments (Goss-Sampson 2020). Data were presented as mean values \pm standard error of the mean (\pm SEM).

RESULTS

Boar Sperm Motility Characteristics

Motility characteristics including the total and progressively motile spermatozoa after 24 and 48 h of storage time and between Ar-R and Am-R storage conditions are presented in Tables 1 and 2, respectively. While some reduction was

observed in the motility of spermatozoa 48 h after storage in 1:3 dilution, this change was not significant. Similarly, no significant change was observed in the motility of spermatozoa 24 h after storage between air-conditioned and ambient storage conditions. Moreover, no statistical interaction can be observed between the dilution rates and storage times, or between the dilution rates and storage conditions on the motility of spermatozoa.

Tables 1 and 2 also present the combined velocity and progressivity characteristics of boar spermatozoa following

Table 1. Motility characteristics (mean \pm SEM) of boar spermatozoa at 24 and 48 h of storage in the BotuFLEX® semen cool box at 1:1 or 1:3 dilution.

Dilution	Sperm Motility	24 h (%)	Coefficient of Variation	48 h (%)	Coefficient of Variation
1:1	Total	93.64 \pm 1.58	0.06	93.88 \pm 1.51	0.050
	Progressive	56.17 \pm 3.83	0.24	53.89 \pm 6.07	0.037
	Rapid progressive	43.30 \pm 4.87	0.39	43.40 \pm 5.47	0.420
	Medium progressive	12.87 \pm 1.86	0.50	10.49 \pm 1.89	0.600
	Non-progressive	37.47 \pm 2.48	0.23	32.89 \pm 2.89	0.290
	Immotile	6.37 \pm 1.58	0.86	13.23 \pm 7.24	1.820
	Total	92.25 \pm 2.00	0.07	88.41 \pm 7.72	0.140
1:3	Progressive	52.07 \pm 4.30	0.28	51.95 \pm 5.44	0.350
	Rapid progressive	39.57 \pm 4.91	0.43	38.44 \pm 4.80	0.410
	Medium progressive	12.49 \pm 1.80	0.50	13.52 \pm 1.66	0.410
	Non-progressive	40.18 \pm 2.60	0.23	36.46 \pm 2.75	0.250
	Immotile	7.75 \pm 2.00	0.90	11.60 \pm 3.72	1.060

Table 2. Motility characteristics (mean \pm SEM) of boar spermatozoa 24 h after storage in the BotuFLEX® semen cool box between Ar-R (air-conditioned) and Am-R (ambient) storage conditions at 1:1 or 1:3 dilution.

Dilution	Sperm Motility	Aircon (%)	Coefficient of Variation	Ambient (%)	Coefficient of Variation
1:1	Total	96.87 \pm 0.91	0.03	95.98 \pm 2.16	0.07
	Progressive	60.53 \pm 2.75	0.14	59.61 \pm 2.33	0.11
	Rapid progressive	49.01 \pm 3.70	0.23	48.27 \pm 2.02	0.13
	Medium progressive	11.53 \pm 2.53	0.66	11.35 \pm 2.14	0.57
	Non-progressive	35.84 \pm 2.67	0.23	36.39 \pm 3.02	0.25
	Immotile	3.15 \pm 0.91	0.87	4.00 \pm 2.16	1.62
	Total	89.52 \pm 6.61	0.22	90.47 \pm 4.38	0.15
1:3	Progressive	49.54 \pm 6.90	0.42	52.27 \pm 4.50	0.26
	Rapid progressive	37.39 \pm 5.14	0.41	39.69 \pm 4.38	0.33
	Medium progressive	12.15 \pm 2.57	0.63	12.58 \pm 0.89	0.21
	Non-progressive	39.96 \pm 3.33	0.2	38.21 \pm 2.55	0.20
	Immotile	10.51 \pm 6.61	1.89	9.53 \pm 4.38	1.38

storage in the BotuFLEX® semen cool box at different holding times and storage conditions. These motility data present the potential impact on the velocity and progressivity of spermatozoa upon exposure to different experimental treatments. Results show no significant effect on the velocity and progressivity of spermatozoa while kept inside the BotuFLEX® semen cool box for up to 48 h or while exposed to ambient storage conditions for at least 24 h.

Boar Sperm Vitality

In terms of sperm vitality (Fig. 1), the percentage of live spermatozoa remained high across different treatments in either experiment (Table 3). However, a significant reduction can be observed after 48 h of storage in 1:1 dilution ($p \leq 0.05$).

Temperature Monitoring

In Experiment 1, temperatures inside the BotuFLEX® semen cool box were 15.50 – 26.88°C (mean of 17.64°C) in the first 24 h and 15.76–27.44°C (mean 20.79°C) within 48 h of storage. In Experiment 2, temperatures in Ar-C and Am-R were 27.29 – 29.27°C (mean of 28.12°C) and 29.19 – 31.87°C (mean of 30.82°C), respectively.

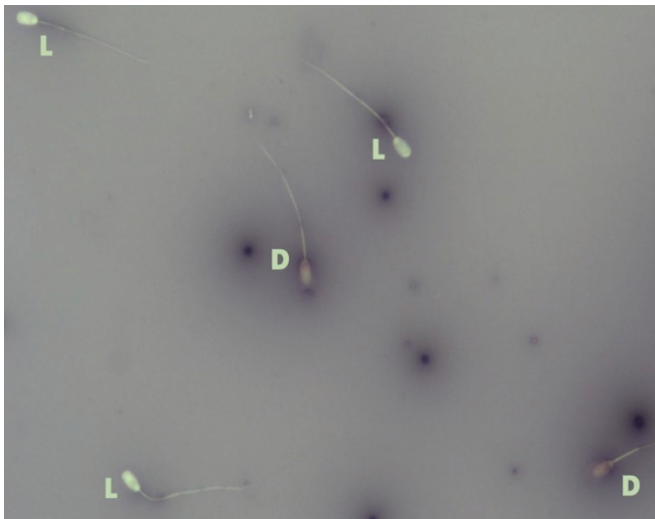


Fig. 1. Representative images for sperm vitality performed using the eosin-nigrosin staining technique for determining the percentage live (L) vs. dead (D) spermatozoa (400x magnification).

Table 3. Percentage vitality (mean ± SEM) of boar spermatozoa at different storage times (Expt. 1) and conditions (Expt. 2) in the BotuFLEX® semen cool box at 1:1 or 1:3 dilution. Different letters indicate significant difference between storage times.

Treatment Conditions	Percent Vitality (mean ± SEM)				
	1:1 Dilution	CV	1:3 Dilution	CV	
Expt. 1 (n = 12)	24 h	91.00 ± 0.73 ^a	0.03	92.17 ± 0.67	0.02
	48 h	89.46 ± 0.90 ^b	0.03	91.27 ± 0.88	0.03
Expt. 2 (n = 9)	Aircon	89.56 ± 1.07	0.04	90.11 ± 1.92	0.06
	Ambient	92.11 ± 0.84	0.03	90.11 ± 1.34	0.04

Different letters indicate significant difference between storage times. CV: Coefficient of variation.

DISCUSSION

As boar spermatozoa are particularly susceptible to cold shock and oxidative damage (Althouse et al. 1998; Peña et al. 2017), several factors need to be considered to maintain semen quality for further downstream analyses and/or cryopreservation purposes. In the case of cryopreservation, essential steps include identification of boars with good freezability, selection of high-quality ejaculates, and optimization of the freezing protocol (Rath et al. 2009; Pezo et al. 2019; Yáñez-Ortiz et al. 2021). In protocol optimization, it is crucial to ensure that candidate boar semen is transported safely and securely in a semen transport box at appropriate temperatures (15 – 17°C) without compromising quality. This is a critical step to cryopreserving highly valuable boar semen provided by collaborating accredited swine breeder farms located several kilometers from the region. This study demonstrated the potential capacity of the BotuFLEX® semen cool box in maintaining desirable motility and viability of boar spermatozoa at different storage conditions and holding times.

A variety of semen shippers are available in the market—from a basic polystyrene transport box that uses ice blocks/packs for cooling to thermo-regulated containers equipped with more sophisticated cooling systems and temperature control such as the Micro Q iQ1 CR (Micro Q Technologies, Arizona, USA), Equitainer (Hamilton Biovet, Ipswich, Massachusetts, USA), and the Klimabox (Minitube, Tiefenbach, Germany), among others. BotuFLEX® is a commercially available transport container designed for the transport of animal semen where 5 or 15°C is required. It is made of about 4.5-cm thick polystyrene foam with external and internal dimensions of about 28.4 × 23.6 × 23.2 cm, and 19 × 14 × 13 cm, respectively and can accommodate three 100-mL semen bottles fitted at the center of the box. Cooling is provided by either one ice block if 15°C is required or two ice blocks placed on both sides for 5°C. In this case, semen samples may be transported by air, sea, or land, or a combination of these with possible exposure to some adverse handling and transport conditions such as delays and weather changes. Thus, this study monitored the motility and viability of liquid-stored boar semen diluted at 1:1 or 1:3 in a commercial extender during storage in a BotuFLEX® semen cool box 24 or 48 h after storage in the 1st experiment, and 24 h after exposure to either air-conditioned or ambient conditions in the 2nd experiment.

An appropriate and stable cool box is necessary to properly transport boar semen. Specifically, for extended boar semen, 15 – 18°C (Johnson et al. 2000) appears to be the most optimal temperature for storage without risking boar sperm to the deleterious effects of cold shock while also slowing down chemical reactions, thus extending its storage life. Using different extenders, Pezo et al. (2019) observed that boar sperm could maintain viability, motility, and chromatin integrity for up to

4 d of storage at 17°C. Similarly, Bielas et al. (2017) demonstrated the maintenance of boar sperm plasma membrane, acrosome status, and mitochondrial function during storage in a long-term semen extender at the same cooling temperature. Essentially, storage in a lower temperature immobilizes spermatozoa and decreases the sperm's metabolic activity (Johnson et al. 2000), thus reducing the accumulation of metabolic by-products such as lactic acid and CO₂ (Gibb and Aitken 2016) which can be damaging to spermatozoal health. Results of this study support the use of the BotuFLEX® semen cool box as indicated in earlier studies where a certain storage temperature is required.

Cryopreservation could significantly impact the motility and viability of spermatozoa. Reduction of sperm motility by 45.2% has been reported in raw human sperm post-thawing (Oberoi et al. 2014), while a ≤ 35% sperm viability and motility after freezing may already signal poor freezability in boars (Rath et al. 2009). Hence, it is important that boar ejaculates outsourced from remote locations have outstanding motility and viability prior to cryopreservation. Sperm motility not only provides a general measure of the viability of a spermatozoa (Brinsko et al. 2011) but may also be associated with fertility or pregnancy rate (Love 2011). Among many studies on the cryopreservation of boar semen, only those ejaculate samples that displayed ≥ 70% motility appear to be the ideal minimum requirement for ejaculates to qualify for further processing (Eriksson and Rodriguez-Martinez 2000; Sancho et al. 2007; Alkmin et al. 2014; Ratchamak et al. 2019). A similar requirement has been used in rabbit sperm (Mocé et al. 2015), goat sperm (Marco-Jiménez et al. 2006), and bull sperm (Awad 2011). Moreover, given the higher-percentage results of live spermatozoa across the different treatments, results of this study imply that boar semen may be stored safely in the BotuFLEX® cool box for at least 48 h without compromising both motility and viability of spermatozoa. Further studies may include the impact of vibration emissions that spermatozoa are exposed to during transport, particularly as influenced by intensity and duration during shipment (Hafemeister et al. 2023). Reduction in sperm motility, mitochondrial activity, and plasma membrane/acrosome integrity have been demonstrated as affected by vibration emissions during long-term storage (Schulze et al. 2018).

This study also highlights the combined progression and velocity results of boar spermatozoa using the SCA® CASA system and provides more objective motility characteristics of boar spermatozoa during storage. Similarly, the results show no significant variations in the velocity and those spermatozoa with forward progression between treatments. Progressivity of spermatozoa is considered a better indicator when using motility in assessing the fertilizing capacity of sperm (Brinsko et al. 2011). In one study, semen doses were prepared from boars with at least 30% progressive motility post-thawing

(Lacalle et al. 2021). Similarly, a progressive motility of ≥ 30% was considered normal for motility and vigor in frozen-thawed bull sperm (Verón et al. 2021).

CONCLUSION

Extended boar semen may be safely stored in the BotuFLEX® cool box for at least 48 h without compromising both motility and viability of spermatozoa. Moreover, while similar results were observed on sperm vitality using the standard supravital staining technique, percent live spermatozoa were higher at 24 h of storage using the 1:1 dilution.

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