Original Research

Stallion Semen Cooling Using Native Phosphocaseinate-based Extender and Sodium Caseinate Cholesterol-loaded Cyclodextrin-based Extender

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ABSTRACT

The objective of this study was to compare semen parameters and embryo recovery rates of cooled stallion semen extended with INRA 96 or BotuSemen Gold. In experiment 1, 45 ejaculates from nine mature stallions were collected, assessed, and equally split between both extenders and then extended to 50 million sperm/mL. Then, the extended semen was stored in three passive cooling containers (Equi-Tainer, Equine Express II, and BotuFlex) for 48 hours. In experiment 2, the same ejaculates extended in experiment 1 were cushion-centrifuged, the supernatant was discarded, and the pellets were resuspended at 100 million sperm/mL with their respective extender. Semen was then cooled and stored as in experiment 1. In both experiments, sperm motility parameters, plasma membrane integrity, and high mitochondrial membrane potential were assessed at 0, 24, and 48 hours post cooling. For experiment 3, 12 mares (n = 24 cycles) were bred with 48 hour-cooled semen from one stallion. Semen was processed as described in experiment 1. Mares had embryo flushing performed by 8-day post-ovulation. In experiment 1, BotuSemen Gold displayed superior total and progressive motility relative to INRA 96 (P < .05). There were no significant differences between the types of containers in any experiment. In experiment 2, INRA 96 and BotuSemen Gold extenders had similar total and progressive motility, but BotuSemen Gold had superior sperm velocity parameters at all timepoints. Embryo recovery was identical for both extenders (50%). Finally, the results obtained herein suggest that BotuSemen Gold is a suitable alternative to be included in semen cooling tests against INRA 96 in clinical practice.

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1. Introduction

Cooled-transported stallion semen is a widely used approach to breed mares in North America, Western Europe, Australia, New Zealand, and Brazil. It first became popular in the 1990s, when most American breed registries began accepting cooled-transported equine semen. In the 1970s, a skim milk–based extender was introduced by Dr. Robert Kenney to be infused in the uterus of mares immediately before mating [1]. After this, the extender was used for on-farm artificial insemination with fresh semen, for the cooling and transporting semen, and as a centrifugation extender before semen freezing. Kenney’s extender has been commercialized with a variety of antibiotics (e.g., amikacin, penicillin, and gentamicin) added by the manufacturer or to be added to the extender immediately before use. Stallions would have semen collected and extended in Kenney’s extender containing different combinations of antibiotics to determine the best antibiotic(s) to sustain sperm motility longevity during cooling for a given stallion [2]. Kenney’s extender spread worldwide and was the main extender used to transport stallion cooled semen until the early 2000s.

In the 1990s, French investigators working at the Institut National de La Recherche Agronomique (INRA) identified that native

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phosphocaseinate was cryoprotective for stallion sperm during cooling [3], and this molecule was used to replace the skim milk component in INRA 82, a traditional French extender, to create the novel extender INRA 96 [3]. About a decade later, the extender became commercially available and then widely used around the world. Given the superior results extending stallion semen with INRA 96 over any Kenney combination, many practices and breeding centers discontinued cooling tests [4,5].

As other extenders (e.g., Ghent and Equi-Pro) were introduced, investigators performed cooling tests [5]; however, INRA 96 remained the superior and most widely used extender overall. One concern with INRA 96 is that the concentration of antibiotics present may be insufficient to prevent bacterial overgrowth during cooling [6]. *Pseudomonas aeruginosa* in particular, a highly contagious cause of chronic endometritis in mares, may not be inhibited; thus, it became an industry standard to include ticarcillin with clavulanic acid, an anti-*Pseudomonas* beta-lactam [7]. In addition, although INRA 96 is a suitable extender for most stallions, there is still a portion of stallions that do not cool well with this extender. Therefore, an alternative extender to serve the needs of this stallion population is warranted.

Recently, a new semen cooling extender was introduced in the market containing sodium caseinate in combination with cholesterol-loaded cyclodextrin (BotuSemen Gold). Caseins are milk proteins that can presumably prevent sperm cryodamage by competitively binding to seminal plasma proteins involved in this process [8,9]. Caseins also interact with ionized calcium, which plays a paramount role in tyrosine phosphorylation during sperm capacitation [10,11]. Cyclodextrin works as a carrier to incorporate cholesterol-rich microenvironment with cholesterolic acid, an anti-*Pseudomonas* beta-lactam [7]. In addition, although INRA 96 is a suitable extender for most stallions, it maintains the temperature for up to 48 hours [19,20]. Because the Equitainer is expensive, cheaper alternative passive cooling semen devices (e.g., Equine Express II) were developed. Although less expensive than the Equitainer, disposable boxes have questionable cooling in this type of stallion [12,16–18]. However, to date, a study comparing this novel extender with INRA 96 is lacking.

Equitainer, a passive cooling device, was developed in the mid-80s to transport equine semen [19]. It provides excellent temperature insulation and a suitable cooling curve for equine sperm, and it maintains the temperature for up to 48 hours [19,20]. Because the Equitainer is expensive, cheaper alternative passive cooling semen devices (e.g., Equine Express II) were developed. Although less expensive than the Equitainer, disposable boxes have questionable insulation from extreme temperature variations [20]. Recently, another passive cooling device was developed using high-density styrofoam material (BotuFlex), which can presumably allow for a cooling curve similar to the Equitainer [21]. However, these three passive cooling devices have not been simultaneously compared.

The objective of this study was to compare the most widely used semen extender based on native phosphocaseinate (INRA 96) and a new extender based on sodium caseinate associated with cholesterol-loaded cyclodextrin (BotuSemen Gold), and three semen cooling containers by assessing semen parameters and embryo recovery rates of cooled stallion semen. We hypothesized that the combination of cholesterol and sodium caseinate results in superior parameters for cooled stallion semen than native phosphocaseinate.

2. Materials and Methods

The experimental protocols were approved by the Animal Care and Use Committee, the Institutional Animal Care Unit Committee of the University of Illinois, under protocols # 19134 and #17140. Three experiments were carried out at the College of Veterinary Medicine of the University of Illinois Urbana-Champaign, IL, USA. Experiments 1 and 2 were conducted between August and September 2019, and Experiment 3 was conducted from November to December 2019. Seven of nine stallions were client-owned, and all the owners signed a consent form permitting us to use their animals in this study. The remaining two stallions belonged to the College of Veterinary Medicine, University of Illinois.

2.1. Stallions, Semen Collection, and Initial Evaluation

Nine mature stallions (4 Quarter Horses, 2 Standardbreds, 1 Paint Horse, 1 Morgan, and 1 Arabian), ranging from 8 to 17 years, were enrolled in the study. Stallions were kept in stalls at the Illinois Veterinary Teaching Hospital in Urbana, IL for the duration of the study. All animals were fed mixed alfalfa-grass hay and had free access to water. Before the beginning of the study, three washout semen collections were performed daily before the beginning of the study to standardize the extra-current ducts sperm reservoirs. Twenty-seven collections (3/stallion) were discarded as part of the washout; the remaining 45 collections (5/stallion) were processed and used in the experiments 1 and 2 described below. Thereafter, semen collections were performed at 48 to 72 hour intervals on a dummy mount in the presence of an estrus mare. A Missouri model (Nasco, Fort Atkinson, WI, USA) artificial vagina coupled with a collection bag (Whirl-Pak, Nasco) and inline filter (Har-Vet, Spring Valley, WI, USA) was used. The artificial vagina was lubricated with a sterile, nonpermiscidal gel (Clarity A.I. Lubricating Jelly Aurora Pharmaceutical, LLC, Northfield, MN, USA).

Immediately after semen collection, the total gel-free volume of the ejaculate was weighed and loaded in 50 mL conical tubes (Corning, CentriStar, Corning, NY, USA). Then, raw semen was immediately extended at 1:1 (v:v) ratio with temperature-matched BotuSemen Gold (Botupharma USA, Phoenix AZ, USA) or INRA 96 (IMV, Maple Grove, MN, USA). The sperm concentration was determined using an automated cell counter (NucleoCounter SP-100, ChemoMetec, Denmark) following the manufacturer instructions. Briefly, 50 µL of semen was diluted in 5 mL of lysis buffer (Reagent S100, ChemoMetec, Denmark) and loaded into the cassettes before the assessment. After the evaluation of concentration, semen was further extended to 50 million sperm/mL with INRA 96 or BotuSemen Gold.

2.2. Experiment 1: Stallion Semen Cooling Extended with INRA 96 or BotuSemen Gold

The extended semen was hermetically packed in a disposable, plastic Whirl-Pak bag (2–5 mL/sample) and stored in three containers: Equitainer (Equitainer II; Hamilton Research, Inc., Ipswich, MA, USA), Equine Express II (Exodus Breeder Supply, York, PA, USA), or BotuFlex (Botupharma USA). All semen samples were stabilized for about 30 minutes at room temperature before cooling. Two units of each container were prepared for evaluations at 24 and 48 hours post cooling, with one unit being open at each timepoint. Each passive cooling container was prepared in accordance with manufacturer recommendations. For the study, 16 to 20 semen samples were stored on each container. This number of samples filled each container’s well (Equine Express II or BotuFlex) or iso-thermometer (Equitainer) to their full capacity. All containers were stored at room temperature (−21°C) in the investigator’s laboratory.

Equitainer’s ice cans and ice packs for BotuFlex and Equine Express II were deep-frozen for at least 24 hours at −20°C. The Equitainer (46 × 25 cm, height × width × height, 5.4 kg) consists of two metal frozen cans; whereas, the BotuFlex (30 × 24.5 × 24.5 cm, length × width × height, 1.4 kg) consists of two ice packs to be placed on each side of the device and a well in the center of the device where semen can be placed (Fig. 1). Equine Express II (35.5 ×
25 × 20 cm, length × width × height, 1.8 kg) consists of only one large ice pack (Fig. 1). According to the manufacturer, the Equitainer is designed to have a cooling curve of −0.07°C/min and maintain semen at 4 to 8°C for 48 hours [22]. BotuFlex is designed to have a curve of 0.05°C/min and maintain semen at 5°C for ~48 hours when two ice packs are included. Whereas Equine Express II is designed to have a cooling curve of −0.03°C/min and maintain semen below 10°C for 48 hours [22]. It is worth noting that Equitainer is supposed to have 120 to 180 mL of semen inside the isothermolizer to have the proper cooling curve, whereas BotuFlex holds 150 to 200 mL volume of semen in the well. The ideal volume for a cooling curve is not specified by the manufacturer of Equine Express II, but it holds the capacity to have two syringes of 60 mL. For this experiment, sperm motility parameters, the percentile of sperm with the intact plasma membrane, and those with intact plasma membrane also having high mitochondrial potential were assessed at times 0, 24, and 48 hours after cooling-storage, as described below.

2.2.1. Sperm Motility Evaluations

Assessment of the sperm motility parameters was carried out using computer-assisted sperm analysis (CASA) with default settings recommended by the manufacturer (Spermvision, Minitube of America, Verona, WI, USA) for equine sperm. The preset values for the CASA were as follows: static cell area 14 to 80 µm², straightness threshold for progressive motility 90%, average path velocity threshold for static cell =9.5 µm/second, cell intensity 106, and light-emitting diode illumination intensity 1800 to 2550. Each sample was incubated for 10 minutes at 37°C before each evaluation. A small aliquot (10 µL) of extended semen was placed on a preheated slide with a coverslip for the assessments. Total percent of sperm motility (%), progressive sperm motility (%), sperm velocity parameters assessed included curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), and straight-line velocity (VSL, µm/s).

2.2.2. Membrane Integrity and Mitochondrial Potential

The evaluation of the percentage of sperm with intact membranes and percentiles of sperm with the intact plasma membrane with high mitochondrial potential was conducted using a spectral flow cytometer as previously described [23]. Briefly, the staining solution of Zombie Green dye (#423112 BioLegend, San Diego, CA, USA) was freshly prepared with 100 µL of dimethyl sulfoxide (DMSO) added to each vial of dye; similarly, MitoTracker Deep Red FM (M22426, Molecular Probes, Eugene, OR, USA) stock solution was prepared by adding DMSO to create a 10 µM solution. The stock solution was aliquoted and frozen at −20°C until the use.

One milliliter containing 50 million sperm/mL was centrifuged (600g × 10 minutes) and then resuspended in phosphate-buffered saline (PBS) to a concentration of 3 to 5 million sperm/mL. A 100 µL aliquot of this solution was stained with both dyes (1 µL of Zombie Green and 1 µL MitoTracker Deep Red) at the same time. After mixing, the sample was incubated for 30 minutes at room temperature in the dark. The incubation was followed by a centrifugation (400g × 5 minutes). The supernatant was discarded, and each pellet was fixed with 500 µL of buffered formalin 2% until flow cytometry evaluation. The fixed samples were stored in the dark at room temperature until the assessment. The flow cytometric analyses were carried out within 72 hours from the fixation. Before the flow cytometric analyses, samples were washed with 1 mL of PBS, centrifuged at 400 g × 5 minutes, and resuspended in PBS (250 µL). The analyses of the stained samples were conducted using a full-spectrum detector—based (filter-less) Cytek Aurora Flow Cytometer (Cytek Biosciences Inc., Fremont, CA, USA). The analysis was concluded when at least 10,000 fluorescent gated events were recorded. Zombie Green was excited and detected with a 488 nm fluorescence detector, whereas MitoTracker Deep Red was excited with a 644/665 nm detector. Unstained and single-stained controls were used to unmix the signals. As previously described [23], four subpopulations of sperm were identified. The populations of sperm with intact (low Zombie Green signal) or damaged (high Zombie

Fig. 1. Passive cooling devices used to transport cooled stallion semen. (A) Equitainer (Hamilton Research, Inc., Ipswich, MA, USA), * denotes the two frozen cans and ± isothermolizer. (B) Equine Express II (Exodus Breeder Supply, York, PA, USA), * denotes the ice packs, and the two arrows denote the pit where semen can be placed for cooled-shipped. (C) BotuFlex (Botupharma, Phoenix, AZ, USA), * indicates the ice packs, and the arrow is pointing to the semen well.
Cushion centrifugation was performed as previously described extended to 50 million sperm/mL in INRA 96 or BotuSemen Gold. Processed with cushion centrifugation after being initially

2.3. Experiment 2: Cooling Test of Stallion Semen, Cushion-Centrifuged and Re-extended with INRA 96 and BotuSemen Gold

The same 45 ejaculates obtained in experiment 1 were further processed with cushion centrifugation after being initially extended to 50 million sperm/mL in INRA 96 or BotuSemen Gold. Cushion centrifugation was performed as previously described [24–27]. Briefly, extended semen in both extenders was loaded in 50 mL conical tubes (Corning, CentriStar) and then added 1 mL of cushion fluid (RedCushion, Botupharma) placed at the bottom of the tube with a blunted spinal needle (18G × 13.5 cm). Centrifugation was carried out at 1,000 g × 20 minutes at room temperature. After centrifugation, the supernatant and cushion solution were discarded. The concentration of the remaining pellet was assessed with NucleoCounter and then resuspended in fresh extenders, INRA 96 or BotuSemen Gold, to a final concentration of 100 million sperm/mL. Semen aliquots extended in INRA 96 or BotuSemen Gold pre-centrifugation were re-extended with the same extender. There was no cross over between extenders post-centrifugation. Therefore, the extended semen was loaded in the three types of containers as described in experiment 1. Samples was then assessed for sperm motility parameters, plasma membrane integrity, and sperm with intact plasma membrane and high mitochondrial potential at 0, 24, and 48 hours after cooling-storage, as described above for experiment 1.

According to the BotuSemen Gold’s manufacturer, once stallion semen is centrifuged, it should be resuspended to at least 100 million sperm/mL. If a lower dilution is used, the high cholesterol/seminal plasma ratio may prevent sperm from undergoing capacitation, despite satisfactory motility. There are no manufacturer recommendations regarding sperm concentration after centrifugation with INRA 96.

2.4. Experiment 3: Embryo Recovery Rates of 48 hour—Cooled Stallion Semen Extended in INRA 96 or BotuSemen Gold

A 13-year-old Quarter Horse stallion belonging to the College of Veterinary Medicine, University of Illinois enrolled in experiments 1 and 2 had semen collected (n = 15) for a fertility test. All collections yielded ejaculates with at least 75% total motility and 65% of progressive motility. The stallion was selected based on full availability to the investigators to conduct the study. In previous years, this stallion had pregnancy rates of 50 to 60% per cycle in cooled-stored semen extended in INRA 96. This stallion was kept at the Illinois Veterinary Teaching Hospital, fed mixed alfalfa-grass hay, and given free access to water. Semen collections and initial evaluations were performed as previously described. After collection, two semen samples were prepared simultaneously, one was extended with BotuSemen Gold and another was extended with INRA 96, and both samples were extended at 50 million sperm/mL and then stored in an Equitainer for 48 hours before insemination. Twenty-four estrous cycles (BotuSemen Gold n = 10 cycles and INRA 96 n = 14 cycles) were used to inseminate 14 light breed mares (ranging from 5 to 18 year old). Mares enrolled in the study have been using as embryo donor mares in the principal investigator’s laboratory for at least two breeding seasons before the present study. The mares are known to be marginally fertile (40%–50% per cycle conception rates) when bred with fresh semen with standard breeding management. The mares were kept on grass pasture and monitored by transectal ultrasonography every other day until at least one preovulatory follicle (35 mm of diameter in the presence of endometrial edema) was detected. Then mares were checked daily, and ovulation was then induced with histrelin acetate (500 µg/IM, Strelin, Botupharma USA). At 24 hours post induction of ovulation, all mares were artificially inseminated with 1 billion total sperm previously stored for 48 hours in Equitainer. Mares were examined 24 hours after insemination to check for ovulation and the presence of free intrauterine fluid accumulation. If a mare did not ovulate 24 hours after breeding, the cycle was discarded. Two mares did not ovulate at 24 hours post ovulation; three mares stopped displaying normal ovarian cyclicity after being flushed for one cycle. Mares were treated, if necessary, with oxytocin or uterine lavage. Uterine flushing for embryo recovery was performed with 4 L of sterile lactated Ringer’s solution 8 days post ovulation. Immediately after uterine flushing, mares received an injection of 250 µg of cloprostenol sodium.

2.5. Statistical Analyses

Data analyses were carried out with RStudio v.0.99.489 (RStudio Team, Boston, MA). Data were analyzed by mixed models with extender, passive cooling devices, and time considered as fixed effects and stallion and ejaculate number as random effects. Tukey’s test was used for posthoc comparisons. Data are expressed as mean ± SEM. Statistical significance was set at P < .05. The embryo recovery rates were identical between extenders, and results are descriptively presented below.

3. Results

3.1. Experiment 1

Total motility and progressive motility decreased over time for semen extended in BotuSemen Gold and INRA 96 (P < .05) (Figs. 2A–2F). Overall there were no differences between the three passive cooling devices used in this experiment (P > .05). Semen extending with BotuSemen Gold, stored in Equitainer or Equine Express II, had superior sperm motility when compared with INRA 96 at 48 hours (P < .05) (Figs. 2A, 2B, 2D, and 2E). There were no differences between INRA 96 and BotuSemen Gold when semen was stored in BotuFlex (P > .05) (Figs. 2C and 2F). There were no effects due to time, passive cooling device, or extender in the percentage of sperm with intact plasma membrane or sperm with intact plasma membrane with high mitochondrial membrane potential (P > .05) (Figs. 2G–2I). There were no differences in curvilinear velocity, average path velocity, and straight-line velocity across passive cooling devices, extenders, or time (P > .05) (Table 1). Representative images of spectral flow cytometry for plasma membrane integrity and mitochondrial membrane potential are portrayed in Fig. 3.

3.2. Experiment 2

Total motility and progressive motility decreased over time for semen extended in BotuSemen Gold and INRA 96 (P < .05) (Figs. 4A–4F). There were no differences between the three passive cooling devices used for the transport of cooled stallion semen (P > .05). There were no effects due to time, passive cooling devices, or extenders in the percentile of sperm with intact plasma membrane...
Fig. 2. Parameters for stallion semen extended in INRA 96 or BotuSemen Gold and cooled for 48 hours in three different passive cooling devices (Equitainer, Equine Express II, and BotuFlex, rows 1–3). (A–C) Total sperm motility; (D–F) Percentage of progressive motility; (G–I) Intact plasma membrane; and (J–L) Percentage of sperm with intact plasma membrane with high mitochondrial membrane potential (intact HMMP). Different superscripts denote effects of time (abc) and differences between extenders for within each time point (ABC) (P < .05).
or percentile of sperm with intact plasma membrane and high mitochondrial membrane potential \((P > .05)\) \((\text{Figs. 4G–4L})\). The only exception being that semen extended in BotuSemen Gold stored in Equitainer had a reduction in plasma membrane integrity between 0 and 48 hours of cooled storage \((\text{Fig. 4G})\). Curvilinear velocity, average path velocity, and straight-line velocity were similar across passive cooling devices \((P > .05)\); however, BotuSemen Gold had superior sperm velocity parameters relative to INRA 96 for all timepoints assessed in all three passive cooling devices \((P < .05)\) \((\text{Table 2})\).

### 3.3. Experiment 3

Embryo recovery rates were identical between extenders 50% \((\text{Fig. 5})\). All embryos recovered were expanded grade 1 blastocysts. Although this was not an endpoint assessed in the present study, mares did appear to develop a similar postbreeding inflammatory response to both extenders.

### 4. Discussion

This is the first study aimed to compare the most widely used equine extender, INRA 96, and BotuSemen Gold, a new commercially available equine semen extender. In addition, the present study examined the most traditional passive semen cooling device (i.e., Equitainer), with the most widely used device (i.e., Equine Express II), and a new device (i.e., BotuFlex). In addition, embryo recovery rates were assessed in mares bred with 48 hour–cooled equine semen. The results obtained with both extenders were largely similar for most endpoints assessed, except that BotuSemen Gold was superior to INRA 96 for total and progressive motility during cooled storage when semen was not centrifuged, and BotuSemen Gold had superior sperm velocity parameters to INRA 96 after cushion centrifugation for all time points in all three passive cooling devices. These findings suggest that there was an interaction between components of BotuSemen Gold and seminal plasma, as once seminal plasma was removed via cushion centrifugation, the differences for total and progressive motility between extenders disappeared. However, removal of seminal plasma enhanced all sperm velocity parameters for cooled semen extended in BotuSemen Gold when compared with INRA 96 in the various time points. Although we cannot be certain of the mechanisms of action, centrifugation is a known method to remove plasma membrane-bound proteins in other species [28,29]. Sperm velocity has been suggested to be essential for the assessment of frozen stallion semen [30], but its relevance for cooled-transported semen has not been clear.

The mechanism by which total and progressive motility were enhanced in the cooled semen extended with BotuSemen Gold not undergoing centrifugation was not studied herein. Presumably, the cholesterol present in this extender can be responsible for the differences in extenders. It has previously been demonstrated that cholesterol-loaded cyclodextrin helps to promote cryotolerance in horses, cattle, sheep, goats, and dogs [12,31–33]. Therefore, shifting the lipid composition of the sperm plasma membrane can be useful to enhance the cryotolerance of sperm at low temperatures [17]. Cholesterol/ phospholipid ratio in the plasma membrane is essential to maintain the fluidity and stability of the sperm membrane at low temperatures [16–34]. Higher concentrations of cholesterol in the plasma membrane are known to result in a reduction in cryodamage through the transition phase during cooling [14]. Sperm from certain species (e.g., rabbits and dogs) have higher cholesterol/phospholipid ratio in the plasma membrane and display higher resistance to freezing than stallions [15]. In addition, sperm quality and longevity are highly variable across animals [12,36] which may, in part, explain the variability of sperm resistance to the cooling in this species. Thus, because native phosphocasein present in INRA 96 and sodium caseinate present in BotuSemen Gold are similar molecules, these two molecules may provide equivalent protection during cooling, and the addition of cholesterol via cyclodextrin in BotuSemen Gold could partially explain the difference in results for total and progressive motility. Regardless of the differences in motilities, both extenders supported excellent semen parameters up to 48 hours of cooled storage.

Surprisingly, plasma membrane integrity and percentage of sperm with both intact plasma membranes and high mitochondrial membrane potentials were not different between extenders with or without centrifugation. Other studies have shown a strong correlation between these variables and sperm motility parameters [37]; however, the previous studies used different staining methods for flow cytometric analyses. Herein, we have used a novel protocol using MitoTracker Red and Zombie Green, which was fixed and read within 72 hours as stated above and recommended by the original study [23]. It is possible that the lack of difference for sperm with both intact plasma membrane and high mitochondrial membrane potential was due to the fact that the mechanism enhancing motility parameters for semen extended in BotuSemen Gold was not directly mediated by plasma membrane or mitochondrial functions. Alternatively, the flow cytometric technique used herein may not have been sensitive enough to detect subtle differences between extenders.

The cholesterol-loaded cyclodextrin extender used in the present extender was reported to be a useful extender for stallions displaying poor semen cooling ability when compared with a standard skim milk–based extender [12]. However, because none of the stallions enrolled in this experiment had poor semen cooling ability, we were unable to compare INRA 96 and BotuSemen Gold for such a population of stallions.

### Table 1

<table>
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<th>Passive Cooling Devices</th>
<th>Time (h)</th>
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<th>VAP (μm/s)</th>
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<td>BG</td>
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<td>143.0 ± 4.3</td>
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Abbreviations: VAP, average path velocity (μm/s); VCL, curvilinear velocity (μm/s); VSL, straight-line velocity (μm/s).
Fig. 3. Representative flow cytometry images of one stallion ejaculate extended in INRA 96 or BotuSemen Gold and cooled in three passive cooling devices (Equitainer, Equine Express II, and BotuFlex) for 48 hours. (A) Semen was not cushion centrifuged. (B) Semen was cushion centrifuged. On each subset of images, the samples were stained with MitoTracker Deep Red and Zombie Green. The left quadrants display sperm with intact plasma membrane and high mitochondrial membrane potential (HMMP) (upper) or low mitochondrial membrane potential (LMMP) (lower). The right quadrants display sperm with damaged plasma membrane and HMMP (upper) or LMMP (lower). Abbreviations: Intact, sperm with intact plasma membrane; PMI, plasma membrane integrity; Damaged, sperm with damaged plasma membrane.
Fig. 4. Parameters of stallion semen cushion centrifuged extended in INRA 96 or BotuSemen Gold and cooled for 48 hours in three different passive cooling devices (Equitainer, Equine Express II, and BotuFlex, rows 1–3). (A–C) Total sperm motility; (D–F) Progressive motility; (G–I) Percentage of sperm with intact plasma membrane; and (J–L) Percentage of sperm with intact plasma membrane and high mitochondrial membrane potential (intact HMMP). Different superscripts denote effects of time (abc) (P<.0).
Cryodamage results in capacitation-like changes that alter the sperm structure and metabolism, which results in loss of sperm motility [38]. Caseins can prevent cryodamage by competitively binding with seminal plasma proteins, such as sequestering binder membrane lipids during capacitation [9,39]. Caseins can prevent cryodamage by competitively binding with seminal plasma proteins, such as sequestering binder membrane lipids during capacitation [9,39]. In pigs, the addition of casein to semen extenders prevented the cholesterol efflux from sperm membranes caused by seminal plasma proteins [42]. As both extenders assessed in the present study contain caseins, we believe that a similar mechanism of protection happens for both extenders, but the exact mechanism remains to be determined [8].

Centrifugation of semen precooling is routinely applied for semen from stallions presenting less than 100 million sperm/mL in raw semen and for stallions known to produce semen with poor cooling ability [43]. In the present study, cushion centrifugation was applied to test these two extenders under typical practical settings. However, because none of the stallions needed centrifugation before cooling, it is unknown if we would have obtained different results with the present experimental design if stallions with low semen concentration or semen with poor tolerance to cooling were used. In the present study, only one horse had slightly worse semen quality than the remaining, even so his semen was not poor enough to be classified as having poor semen cooling.

Previously, semen cooling tests were widely applied in North America to determine the best extender and antibiotics for stallion semen. This practice has been largely discontinued by most breeding centers since INRA 96 outperforms other extenders and became the most popular extender used commercially. The findings of the present study placed the BotuSemen Gold extender at the similar level to INRA 96, which suggest that a semen cooling test could be beneficial to determine the most suitable extender for a given stallion. In addition, the results obtained with BotuSemen Gold provide evidence to have this extender included in any semen cooling test against other extenders available in the market.

The embryo recovery obtained in the present study was satisfactory for 48 hour–cooled semen. The present results were similar to those obtained by French investigators [3], breeding mares with 48 hour–cooled semen extended with INRA 96. As the mares used in the present experiment had been known to have marginal fertility, the results could have been superior if mares of high fertility were used.

5. Conclusion

In conclusion, our results indicated that INRA 96 and BotuSemen Gold result in satisfactory semen parameters upon cooled storage in all three passive cooling devices tested herein. However, for the group of stallions used in our study, BotuSemen Gold extended semen had superior total and progressive motility than INRA 96 without cushion centrifugation. Cushion centrifugation resulted in similar total and progressive motility between extenders. However, BotuSemen Gold had superior sperm velocity parameters for all time points assessed in all three passive cooling devices after cushion centrifugation. Cushion centrifugation and its absence in the processing of semen resulted in similar plasma membrane integrity and sperm with intact plasma membrane and high mitochondrial membrane potential. In addition, embryo recovery rates of 48 hour–cooled semen resulted in similar percentiles for

![Fig. 5. Representative blastocysts harvested from a mare bred with 48 hour–cooled stallion semen extended with INRA 96 (A) or BotuSemen Gold (B).](image-url)
both extenders. It remains to be determined if semen from stallions with poor cooling ability behaves similarly to that from stallions with satisfactory semen cooling ability. Nonetheless, the present study suggests that BotuSemen Gold could be included in semen cooling tests against INRA 96 and other extenders to optimize the identification of the best extender for a given stallion.

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