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Study the Efficacy of Different Concentrations of Coconut Water on Boar Semen Following Equilibration at 18°C for Different Hours

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Abstract: A rich natural resource such as Coconut Water (CCW) features high concentrations of antioxidants, nutrients, amino acids, sugars, and electrolytes. These herbal extract infusions are used in semen extenders to preserve animal sperm. In addition, CCW is important for *in vitro* production of embryos. The purpose of this study was to investigate the use of coconut water as an alternative bulking agent for boar semen after equilibration at 18° for 8, 16, 24, and 48 h. Three 2- to 3-year-old Large White x Landrace boars were used for semen collection, boar semen was collected by a glove and hand technique, 24 ejaculates were collected and this study was replicated eight times. A complete randomized design was used in this study. Data were analyzed by ANOVA using Stata V12 statistical software (Stata Corp., College Station, Tex.) and treatment measures were separated using Fisher's protected t-test at significance values of $P < 0.05$. Sperm diluted in 60% CCW and equilibrated for 8 h showed improved sperm motility (86.1 ± 1.7), percentage of viable sperm (84.5 ± 2.0), plasma membrane (83.3 ± 2.3), and acrosome integrity (84.00 ± 2.13) compared to boar semen diluted with 80 and 100% CCW. Notably, semen diluted in 80 and 100% CCW and equilibrated for 8 h showed improved sperm motility (76.2 ± 2.5 ; 63.5 ± 2.7), viable sperm (74.5 ± 2.0 ; 63.3 ± 2.1), intact plasma membrane (73.3 ± 2.3 ; 63.5 ± 2.0) and acrosomal membrane integrity after equilibration (74.0 ± 2.1 ; 63.5 ± 2.7). However, sperm diluted in 80 and 100% CCW had reduced sperm motility, viability, and plasma and acrosomal membrane integrity after 8, 16, 24 and 48 h of equilibration at 18°C. In conclusion, equilibrated boar semen diluted with 60% CCW was able to maintain boar semen parameters such as sperm motility, viability, plasma membrane, and acrosomal membrane integrity following 8, 16, 24, and 48 of equilibration at 18°C. However, CCW can be used as an alternative extender for the equilibration of boar semen. Therefore, further studies are necessary to determine the *in vitro* and *in vivo* fertilizing capacity of equilibrium boar semen diluted in CCW.

Keywords: Acrosome Integrity, Boar Semen, Coconut Water, Equilibration, Sperm Viability Plasma Membrane Integrity

Introduction

Boar semen fertility can be maintained at 10–18°C for several days before being used for artificial insemination (Johnson *et al.*, 2000). However, long-term equilibration of boar semen gradually reduces fertility due to the stress

experienced *in vitro*. As a result. Many changes occur during equilibration, along with reduced motility and changes in membrane permeability. Sperm cells also need to be stored at a selected temperature for several days for the sperm cells to survive. This is because the phospholipids in the plasma membrane contain excess

polyunsaturated fatty acids, which pose a risk of oxidative damage to boar semen (Cerolini *et al.*, 2001). Additionally, pig semen is very sensitive to cold temperatures and is usually stored in a liquid state. Interestingly, the metabolic activity of boar spermatids continued during storage at 18°C and this is accompanied by the accumulation of metabolites including Reactive Oxygen Species (ROS) in semen. In addition, elevated ROS levels induce sperm oxidative stress and reduce sperm fertility (Bansal and Bilaspuri, 2007; Alvarez and Storey, 1989). During equilibration, there is a decline in semen fertility and boar semen fertility can be maintained by the use of a semen extender.

Therefore, regardless of the extender used, this becomes a major drawback of liquid storage of boar semen. Boar semen has been diluted with various extenders, including short-term extenders such as Beltsville Thawing Solution (BTS), Illinois Variable Temperature (IVT), and long-term extenders such as Modena, Kiev, Androhep, Acromax, and Zorlesco (Gadea, 2003; Vyt *et al.*, 2004; Dube *et al.*, 2004; Mapeka *et al.*, 2012). Despite the massive use of short-term extenders, there may still be a demand for long-term extenders that can be used to preserve boar semen (Frydrychová *et al.*, 2010). By using an extender at an appropriate volume, the volume of boar semen can be increased to service more sows from a single ejaculate. This is because these semen extenders can be able to maintain boar semen fertility. Therefore, Artificial Insemination (AI) professionals are facing rising prices for high-quality lab equipment and chemicals, as well as rising prices of commercial extenders used for the equilibration of boar semen. As a result, commercial extenders can be replaced by cheaper natural agents such as coconut water. However, CCW is well known and used in laboratories, especially in tissue culture. CCW has been shown to contain high levels of free sugars, antioxidants, and minerals (Vasconcelos *et al.*, 2009). In addition, CCW has successfully been used in the culturing and freezing of goats (Vasconcelos *et al.*, 2009), dogs (Martins *et al.*, 2005; Cordeiro *et al.*, 2006), and buck embryos (Cardosoc *et al.*, 2005). However, they are limited information about the optimum concentration of CCW that can be used to equilibrate boar semen. Therefore, in the interest of developing an effective and more natural extender and in consideration of its cost and benefit, a study was undertaken to assess the effectiveness of different concentrations of CCW on boar semen quality following equilibration.

Materials and Methods

Study Site

This study was conducted at the Agricultural Research Council, Animal Production, Germplasm Conservation, and Reproductive Biotechnologies laboratory. The area is

located at 25°53'59.6"S, 28°12'51.6"E, in Pretoria, South Africa, at an altitude of 1525 m above sea level. The Tshwane University of Technology (AREC2021/06/001) and the Agricultural Research Council Ethics Committee (APIEC15/041) approved the experimental procedures.

Boar Management

A dummy sow was used to train 3 Large White x Landrace boars (n = 3), which were between 2-3 years of age, for semen collection. All the boars were housed in an automated housing system, fed a commercial diet, and given unlimited access to water.

Boar Semen Collection and Handling

Using a gloved hand technique, semen samples from three Large White x Landrace boars were collected twice a week for 4 weeks. Following semen collection, the sperm-rich fraction was collected into a glass beaker covered with a gauze filter to separate the gel fraction from the sperm-rich fraction and stored in an insulated Thermos flask containing warm water at (37°C) before being transported to the laboratory. Upon arrival at the laboratory, collected semen samples were evaluated for macro and microscopic semen parameters. This experiment was replicated 8 times.

Preparation of Extenders

BTS a commercial extender (T1) used was in powder form, which was packed in a 50 g plastic sachet and prepared by dissolving it in 1000 ML of distilled water. However, fresh green CCW was purchased from a local fruit and vegetable shop. Coconut water was washed and dried before extracting water. Extraction was performed by opening a small hole in the shell and water was decanted into a beaker lined with filter paper. The filtered CCW was evaluated for pH, which ranged between 6.8-7.4, and was prepared in different concentrations of 60, 80, and 100%, and used for semen dilution.

Boar Semen Assessment

Collected boar semen samples were evaluated for volume, concentration, and pH. Briefly, 5 µL of diluted semen was pipetted into a microscope slide, covered with a warm coverslip, placed on a microscope warming plate (Omron) set at 37°C, and placed on the Sperm Class Analyzer® system. (Microptic, Spain). Freshly and equilibrated boar semen samples were analyzed at 100x magnification (Nikon, China). The sperm morphology of boar semen was recorded after staining the semen samples with eosin-nigrosine. A Hypo-Osmotic Swelling Test (HOST) was used to assess the membrane integrity of boar sperm, and Isothiocyanate-Fluorescein-Conjugated Pea Agglutinin (FITC-PSA) was used to assess the acrosome integrity of boar sperm. However, stained glass

slides were air-dried and placed on the microscope stage for evaluation. Before evaluation, stained glass slides were coated with a drop of immersion oil and placed under a fluorescence microscope (Olympus, Inc. BX 51 FT, Tokyo, Japan) at 100 x magnification and a total of 200 sperm cells were counted per slide.

Boar Semen Dilution, Processing, and Equilibration

Freshly collected boar semen samples were divided into four equal segments. The initial segment was diluted (1:1) with BTS (control) and stored at 18°C for 8, 16, 24, and 48 h. Subsequently, the three remaining segments were diluted (1:1) with 60, 80, and 100% of CCW and equilibrated at 18°C for 8, 16, 24, and 48 h. However, the equilibrated semen samples were evaluated for sperm motility traits using the Sperm Class Analyzer® system, and morphological traits were evaluated using a fluorescence microscope.

Evaluation of Live and Dead Sperm

Live and dead sperm from fresh and equilibrated boar semen were determined using eosin-nigrosine stain (Onderstepoort, Veterinary Medicine Pharmacy, South Africa). However, 5 µL of freshly collected boar sperm was mixed with 20 µL of eosin-nigrosine staining solution. As a result, 7 µL of the mixture was smeared onto the slide. Therefore, the stained slides were dried at room temperature and placed on a microscope stage, and examined under a fluorescence microscope (Olympus, BX 51FT, Tokyo, Japan) at 100 x magnification. Live and dead sperm were determined by assessing the percentage of sperm that did not absorb the dye and those that absorbed the stain. Those that did not absorb the stain were considered alive, and those that absorbed the stain were considered dead. Therefore, 200 sperm cells were counted per slide.

Evaluation of Sperm Membrane Integrity

Boar sperm membrane integrity was determined using HOST before and after equilibration. A semen volume of 0.1 ml was mixed with 1 mL of HOST. (8.71 g/L) fructose and (4.47 g/L) sodium chloride, pH (8.05) (Sigma-Aldrich, South Africa) (Pty) (Ltd) and incubated at 37°C for 1 h (Naing *et al.*, 2010). After incubation, diluted boar semen was placed on glass slides, covered with coverslips, and analyzed by phase-contrast microscopy using 100x magnification. Sperm with swollen and curled tails were scored as intact, sperm with normal tails were scored as having damaged membranes (Naing *et al.*, 2010) and 200 sperm cells were counted per slide.

Evaluation of Sperm Acrosome Integrity

Boar sperm cell acrosome integrity was determined using FITC-PSA (Partyka *et al.*, 2010). Briefly, 20 µL of diluted

semen was suspended in 500 µL Phosphate-Buffered Saline (PBS), centrifuged at 100 g for 10 min, and the supernatant was discarded. The sperm pellet was resuspended in 250 µL PBS. A drop of resuspended semen sample was placed onto a glass slide and allowed to air dry. Air-dried slides were then fixed in acetone for 10 min at 4°C. the diluted semen samples were fixed with FITC-PSA solution (50 µg/mL in PBS). Therefore, the fixed semen samples were placed under a fluorescence microscope (Olympus, Inc. BX 51FT, Tokyo, Japan) at 100 x magnification. Sperm cells that fluorescence green was recorded as having an intact acrosome while those that fluorescence red was recorded as having a damaged acrosome. A total of 200 sperm cells were counted per slide.

Statistical Analysis

The study was undertaken by using a Complete Randomized design and it was replicated eight times for each treatment group. The findings were expressed as mean values ± standard error. The comparison of the percentage of motile sperm was performed by utilizing the t-test. The average values of the percentage of sperm with different stains in each experiment, the live and dead spermatozoa, plasma membrane integrity, and acrosome integrity were compared by using Duncan's multiple range test by ANOVA procedure, once the *P*-value was significant (*P*<0.05). All statistical evaluations were performed using Statistical Product and Services Solutions (SPSS 11.5 for Windows; SPSS, Chicago, IL, U.S).

Results and Discussion

The results of the effect of different concentrations of CCW on sperm motility parameters of equilibrated boar semen are presented in Table 1. There was a significant difference (*P*<0.05) observed in semen diluted with BTS and 60, 80, and 100% of CCW. For all the sperm motility parameters measured, both 80 and 100% CCW were the worst substitutes for BTS (control) following 8, 16, 24, and 48 h of equilibration. However, the highest sperm motility was recorded in semen diluted with BTS following 8 (87.0±3.7), 16 (68.9±5.29), 24 (45.3±7.5), and 48 (20.9±2.9) h of equilibration, followed by semen diluted with 60% CCW following 8 (86.1±1.7), 16 (74.9±3.0), 24 (65.1±2.1), and 48 (43.42±4.59) of equilibration. However, lower sperm motility parameters were recorded with semen diluted with 100% CCW following 8 (63.5±2.7), 16 (44.86±2.9), 24 (26.74±4.2), and 48 (7.7±3.2) h of equilibration. Moreover, these demonstrate that a high concentration of CCW results in a decreased motility rate following 8, 16, 24, and 48 h of equilibration. furthermore, semen diluted with 60% of CCW was able to maintain sperm motility rate following 8, 16, 24 and, 48 h of equilibration compared to semen diluted with 80 and 100% and equilibrated for 8, 16, 24 and, 48 h.

Table 1: The effect of different CCW concentrations on the motility parameters of equilibrated boar semen (Means ± SEM)

Treatments	Equilibration hours (h)	Total motility (%)	Progressive (%)	Non-progressive (%)	Rapid (%)	Medium (%)	Static (%)
Raw	0 h	95.9±2.7 ^{7a}	57.5±8.1 ^a	38.3±8.7 ^{abc}	29.9±12.4 ^a	65.9±11.8 ^a	4.0±2.7 ^b
BTS (Control)	8 h	87.0±3.7 ^a	61.8±11.4 ^a	24.5±10.4 ^a	48.0±17.0 ^a	36.0±16.4 ^{ab}	13.4±3.5 ^a
	16 h	68.9±5.2 ^b	41.7±16.6 ^b	27.2±16.2 ^a	26.1±15.5 ^b	47.2±14.2 ^a	31.0±5.2 ^b
	24 h	45.3±7.5 ^c	23.5±11.9 ^c	21.7±14.9 ^a	10.8±7.9 ^c	33.3±9.3 ^b	54.6±7.5 ^c
	48 h	20.9±2.9 ^d	4.4±4.1 ^a	16.4±3.8 ^a	1.2±2.2 ^c	19.6±3.9 ^c	79.0±2.9 ^d
60% CCW	8 h	86.1±1.7 ^b	42.1±13.7 ^b	41.3±13.9 ^a	30.3±11.3 ^a	52.5±15.5 ^b	13.8±1.7 ^e
	16 h	74.9±3.0 ^c	33.2±10.0 ^{bc}	41.6±10.3 ^a	22.5±11.5 ^{abc}	52.4±12.5 ^b	25.0±3.0 ^f
	24 h	65.1±2.1 ^d	25.8±10.2 ^d	39.3±8.5 ^{7abc}	17.2±10.6 ^{bcd}	47.8±8.7 ^b	34.8±2.1 ^e
	48 h	43.4±4.5 ^c	14.8±4.7 ^{def}	28.6±6.7 ^{cd}	10.0±5.2 ^{def}	32.9±6.8 ^{de}	56.7±4.3 ^d
80% CCW	8 h	76.2±2.5 ^c	34.8±22.3 ^{bcab}	40.1±20.5 ^{ab}	28.5±22.3 ^{cde}	47.7±22.2 ^{bc}	23.7±2.5 ^f
	16 h	64.6±3.7 ^d	33.5±15.6 ^{bc}	31.1±14.3 ^{abc}	22.9±18.1 ^{ef}	41.7±15.2 ^{bcd}	35.3±3.7 ^e
	24 h	45.2±2.8 ^{8e}	29.0±8.7 ^c	16.2±7.3 ^c	24.5±9.3 ^f	20.7±8.0 ^f	54.7±2.8 ^d
	48 h	22.4±2.5 ^{8e}	4.4±2.7 ^{8e}	18.0±2.5 ^{ed}	1.4±1.48 ^f	21.0±2.2 ^f	77.5±2.5 ^b
100% CCW	8 h	63.5±2.7 ^d	23.9±16.1 ^{cde}	39.5±14.8 ^{ab}	15.8±12.7 ^{cde}	47.7±11.5 ^{bc}	36.4±2.70 ^e
	16 h	44.8±2.9 ^e	14.5±7.1 ^e	30.3±8.7 ^{bc}	5.8±6.3 ^f	39.0±8.2 ^{bcd}	55.1±2.9 ^d
	24 h	26.7±4.2 ^f	8.1±3.0 ^{8f}	18.5±3.7 ^{ed}	1.3±2.3 ^f	25.3±4.5 ^{ff}	73.2±4.2 ^d
	48 h	7.7±3.2 ^h	2.8±1.0 ^{8f}	4.9±2.5 ^f	0.0±0.0 ^f	7.7±3.2 ^f	92.2±3.2 ^a

^{a-g} values with different superscripts within the column differ significantly at $P<0.05$

BTS = Beltsville Thawing Solution (control); CCW= Coconut water

Table 2: The effect of different CCW concentrations on the percentage of live and dead sperm of equilibrated boar semen (Means ± SEM)

Equilibration h	Live		Live			Dead		
	BTS (control)	Dead	60% CCW	80% CCW	100% CCW	60% CCW	80% CCW	100% CCW
8 hrs	85.2±1.8 ^a	14.7±1.8 ^d	84.5±2.0 ^a	74.5±2.0 ^e	63.3±2.1 ^d	15.5±2.0 ^{8e}	25.5±2.0 ^f	36.6±2.1 ^c
16 hrs	74.3±1.9 ^b	25.6±1.9 ^b	74.6±2.2 ^c	64.6±2.1 ^d	53.5±1.9 ^e	24.8±2.2 ^f	35.3±2.1 ^e	46.7±1.6 ^d
24 hrs	64.2±1.7 ^c	35.7±1.7 ^c	64.0±2.0 ^d	53.6±1.6 ^e	43.1±2.2 ^f	15.5±2.0 ^e	46.3±1.6 ^d	56.8±2.2 ^c
48 hrs	35.1±2.5 ^d	64.8±2.5 ^d	43.1±2.9 ^f	35.3±2.6 ^{8e}	25.1±3.1 ^h	56.8±2.9 ^c	64.6±2.6 ^b	74.8±3.1 ^a

^{a-h} values with different superscripts within the column differ significantly at $P<0.05$

BTS = Beltsville Thawing Solution (Control); CCW = Coconut water

Table 3: The effect of different CCW concentrations on the plasma membrane integrity of equilibrated boar semen (Means ± SEM)

Equilibration h	Damaged plasma membrane		Intact plasma membrane			Damaged plasma membrane		
	BTS (control)	Intact plasma membrane	60% CCW	80% CCW	100% CWW	60% CCW	80% CCW	100% CCW
8 hrs	84.8±1.7 ^a	15.1±1.7 ^d	83.3±2.3 ^b	73.3±2.3 ^c	63.5±2.0 ^e	16.6±2.3 ^h	26.6±2.3 ^{8e}	36.5±2.0 ^e
16 hrs	73.7±2.1 ^b	26.2±2.1 ^c	73.7±1.9 ^c	65.8±1.8 ^d	53.6±2.1 ^f	26.2±1.9 ^{8e}	34.1±1.8 ^f	46.6±1.9 ^d
24 hrs	62.2±2.1 ^c	37.2±1.7 ^b	63.5±3.6 ^e	54.3±1.6 ^f	43.5±1.6 ^{8e}	36.5±3.6 ^e	45.6±1.6 ^e	56.5±1.6 ^e
48 hrs	34.0±2.6 ^d	65.8±2.8 ^a	57.1±2.5 ^{8e}	45.6±1.6 ^h	25.3±1.9 ^h	57.1±2.5 ^c	64.1±2.1 ^c	74.6±1.9 ^a

^{a-i} values with different superscripts within the column differ significantly at $P<0.05$

BTS = Beltsville Thawing Solution (control); CCW= Coconut water

Table 4: The effect of different concentrations of CCW on the sperm acrosome integrity percentage of equilibrated boar semen (Means ± SEM)

Equilibration hrs	Intact acrosome		Intact acrosome integrity			Damaged acrosome integrity		
	BTS (control)	Damaged acrosome	60% CCW	80% CCW	100% CWW	60% CCW	80% CCW	100% CCW
8 hrs	84.8±1.6 ^a	15.1±1.6 ^d	84.0±2.1 ^h	74.0±2.1 ^{8e}	63.0±1.8 ^f	16.0±2.1 ^b	26.0±2.1 ^c	37.0±1.8 ^d
16 hrs	74.3±1.9 ^b	25.6±1.9 ^c	74.1±2.3 ^{8e}	64.8±1.8 ^f	52.8±1.5 ^c	25.8±2.3 ^c	35.1±1.8 ^d	47.1±1.6 ^e
24 hrs	63.7±2.1 ^c	36.2±2.1 ^b	63.6±2.0 ^f	53.8±2.2 ^c	45.2±2.7 ^d	36.3±2.0 ^d	46.1±2.2 ^e	54.7±2.7 ^f
48 hrs	35.6±2.7 ^d	64.3±2.7 ^a	42.8±3.2 ^c	53.8±2.2 ^b	26.6±1.0 ^{8e}	57.1±3.2 ^{8e}	63.7±3.1 ^h	73.3±1.0 ⁱ

^{a-i} values with different superscripts within the column differ significantly at $P<0.05$

BTS = Beltsville Thawing Solution (control); CCW= Coconut water

The results of the effect of different concentrations of CCW on the percentage (Means ± SEM) of live and dead equilibrated boar sperm are presented in Table 2. However, there was a significant difference ($P<0.05$) observed in the percentage of live sperm diluted with BTS, 60, 80, and 100% CCW following 8, 16, 24, and 24 of equilibration. There was no significant difference ($P>0.05$) observed in the percentage of live and dead sperm on semen diluted with BTS and 60% CCW. The highest percentage of live sperm was recorded on semen diluted with 60 CCW following equilibration at 8 (84.50±2.07, 16 (74.6±2.2), 24 (64.0±2.0) and 48 (43.1±2.9) h compared to semen diluted with 80% CCW following 8 (74.5±2.0), 16 (64.6±2.1), 24 (53.6±1.6) and

48 (35.3±2.6) h of equilibration. However, the highest percentage of dead sperm was recorded on semen diluted with 80% CCW following 8 (25.50±2.07), 16 (35.37±2.13), 24 (46.37±1.68) and 48 (64.62±2.66) h of equilibration followed by semen diluted with 100% CCW following 8 (36.6±2.1), 16 (46.7±1.6), 24 (56.8±2.2) and 48 (74.8±3.1) h of equilibration as compared to semen diluted with BTS (control) equilibrated for 8 (14.75±1.83), 16 (25.6±1.9), 24 (35.7±1.7) and 48 (64.8±2.5) h and semen diluted with 60% 8 (15.5±2.0), 16 (24.8±2.2), 24 (15.5±2.0) and 48 (56.8±2.9) h. Therefore, it is demonstrated that higher concentrations of live sperm decrease with an increase in the concentration CCW following 8, 16, 24, and 48 h of equilibration.

The results of the effect of different concentrations of CCW on plasma membrane integrity percentage (Means \pm SEM) of equilibrated boar sperm are presented in Table 3. There was a statistical difference ($P<0.05$) observed in semen diluted with BTS (control), and semen diluted with 60, 80, and 100% of CCW following 6, 8, 24, and 48 h of equilibration. However, semen diluted with BTS resulted in a higher percentage of sperm with intact plasma membrane following 8 (84.8 \pm 1.7), 16 (73.7 \pm 2.1), 24 (63.7 \pm 2.1), and 48 (34.0 \pm 2.6) h of equilibration. A higher percentage of sperm with an intact plasma membrane integrity was also recorded on semen samples diluted with 60% CCW following 8 (83.3 \pm 2.3) 16 (73.7 \pm 1.9), 24 (63.5 \pm 3.6), and 48 (57.1 \pm 2.5) h of equilibration as compared to semen diluted with 80% CCW equilibrated for 8 (73.37 \pm 2.32), 16 (65.87 \pm 1.88), 24 (54.37 \pm 1.68) and 48 (45.62 \pm 1.68) and semen diluted with 100% CCW following 8 (63.50 \pm 2.07), 16 (53.62 \pm 2.13), 24 (43.50 \pm 1.69) and 48 (25.37 \pm 1.99) h of equilibration. Furthermore, semen diluted with 100% CCW resulted in a high percentage of sperm with a damaged plasma membrane following 8 (36.50 \pm 2.07), 16 (46.62 \pm 1.92), 24 (56.50 \pm 1.69), and 48 (74.62 \pm 1.99) h of equilibration as compared to semen diluted with BTS, 60, and 80% of CCW. Nonetheless, it was observed that a higher concentration of 100% CCW, leads to a decrease in the percentage of sperm with an intact plasma membrane and an increase in the percentage of sperm with a damaged plasma membrane. The results of the effect of different concentrations of CCW on the acrosome integrity percentage (Means \pm SEM) are presented in Table 4. However, there was a statistical difference ($P<0.05$) observed in the percentage of sperm with intact acrosome on semen diluted with BTS (control), 60, 80, and 100% following 8, 16, 24, and 48 h of equilibration. Consequently, semen diluted with BTS resulted in a higher percentage of sperm with an intact acrosome following 8 (84.8 \pm 1.6), 16 (74.3 \pm 1.9), 24 (63.7 \pm 2.1), and 48 (35.6 \pm 2.7) h of equilibration. This was followed by semen diluted with 60% CCW following 8 (84.00 \pm 2.13), 16 (74.1 \pm 2.3), 24 (63.6 \pm 2.0), and 48 (42.8 \pm 3.2) h of equilibration compared to semen diluted with 80% CCW following for 8 (74.00 \pm 2.13), 16 (64.8 \pm 1.8), 24 (53.8 \pm 2.2), 48 (53.8 \pm 2.2) h of equilibration. Moreover, semen diluted with 100% CCW had a high percentage of damaged acrosomes following 8 (37.0 \pm 1.8), 16 (47.1 \pm 1.6), 24 (54.7 \pm 2.7) and 48 (73.3 \pm 1.0) of equilibration compared to semen diluted with BTS (control), 60 and 80% of CCW following 8, 16, 24 and 48 h of equilibration. However, it is recorded that a high concentration of 100% CCW resulted in an increase in the percentage of sperm with a damaged acrosome following an increase in equilibration time.

It was debated that CCW could be an actual replacement for commercial extenders. However, pig breeders would be offered a cost-effective extender for boar semen. This study was undertaken to determine whether CCW could be used as an alternative extender for boar semen and to determine the optimum concentration of CCW that can be used to equilibrate boar semen on sperm motility, percentage of live sperm, plasma membrane, and acrosome integrity. There was a significant difference ($P<0.05$) observed in the total sperm motility of the equilibrated boar semen diluted with BTS (control) when compared to the semen diluted with different concentrations (60, 80, and 1100% of CCW. However, semen diluted with BTS had higher sperm motility followed by semen diluted with 60% CCW. Lower sperm motility was recorded on semen diluted with 80 and 100% CCW. Semen diluted with 100% CCW had the highest percentage of dead sperm following 8 (36.6 \pm 2.1), 16 (46.7 \pm 1.6), 24 (56.8 \pm 2.2), and 48 (74.8 \pm 3.1) h of equilibration compared to semen diluted with BTS, 60 and 80% CCW. However, they were no significant difference observed in semen diluted with BTS and 60% CCW on the percentage of live sperm and with a high percentage of dead sperm recorded when semen was diluted with 100% CCW following 48 h or equilibration. These findings are in agreement with the results of Roca *et al.* (2000) who found a high percentage of dead sperm in rabbit semen following 48 h of equilibration, which is in agreement with our results. Moreover, they were a significant difference ($P<0.05$) in the percentage of sperm with an intact acrosome, and semen diluted with BTS had a higher percentage of sperm with an intact plasma membrane following 48 h of equilibration. Consequently, semen diluted with 60% of CCW had the highest percentage of sperm with an intact plasma membrane following 8 (83.3 \pm 2.3), 16 (73.7 \pm 1.9), 24 (63.5 \pm 3.6), and 48 (57.1 \pm 2.5) h of equilibration compared to semen diluted 80 and 100% of CCW. This may be due to the major essential amino acids found on CCW, which play an important role in cell membrane integrity (Yancey, 2005; Purdy, 2006; Naing *et al.*, 2010). Therefore, semen diluted with 100% of CCW water had the highest percentage of sperm with a damaged plasma membrane following 8 (36.5 \pm 2.0) 16 (46.6 \pm 1.9), 24 (56.5 \pm 1.6), and 48 (74.6 \pm 1.9) h of equilibration compared to semen diluted with BTS (control), 60 and 80% CCW. However, they were a significant difference ($P<0.05$) in the percentage of sperm with an intact acrosome on semen diluted with BTS, 60, 80, and 100% CCW. Semen diluted with BTS resulted in a higher percentage of sperm with an intact acrosome as compared to semen diluted at 60, 80, and 100% of CCW following 8, 16, 24, and 48 h of equilibration. Nonetheless, semen diluted with 60% of CCW resulted in a higher percentage of sperm with an

intact acrosome following 8 h (84.0±2.1), 16 (74.1±2.3), 24 (63.6±2.0) and 48 (42.87±3.27) h of equilibration as compared to semen diluted with 80 and 100% of CCW. Semen diluted with 100% CCW resulted in a higher percentage of sperm when with a damaged acrosome following 8 (37.00±1.85), 16 (47.1±1.6), 24 (54.7±2.71), and 48 (73.3±1.0) h of equilibration as compared to semen diluted with BTS, 60, 80, and 100% CCW. Therefore, an improvement in sperm parameters witnessed in the current study could be due to the low toxicity, good water solubility of an antioxidant such as pyridoxine and vitamin C derived from coconut water (Kannan and Jain, 2004; Arabi and Seidaie, 2008). Moreover, Shen *et al.* (2010) reported an antioxidant potential and amelioration of oxidative stress with pyridoxine and vitamin C. Therefore, an improvement in sperm parameters witnessed in the current study could be due to the low toxicity, good water solubility of an antioxidant such as pyridoxine and vitamin C derived from coconut water (Kannan and Jain, 2004; Arabi and Seidaie, 2008). Moreover, Shen *et al.* (2010) reported an antioxidant potential and amelioration of oxidative stress with pyridoxine and vitamin C. However, these biological properties are required for the protection of sperm against oxidative damage due to increased production of reactive oxygen species (or free radicals) linked within *in vitro* storage at low temperature, particularly the polyunsaturated fatty acids in the cell membrane, or due to the nucleic acids in the cell nucleus.

Conclusion

In conclusion, equilibrate boar semen diluted with 60% CCW resulted in higher semen motility, viability, sperm plasma membrane, and acrosome integrity following 8, 16, 24, and 48 h of equilibration compared to the semen diluted with 80 and 100% CCW. Therefore, 60% CCW can be used as an alternative substitute to BTS a commercial extender for the equilibration of boar semen and it could be a cheap alternative for use in artificial insemination programs for pig breeders. This study gives encouraging evidence to continue the diluent exploration for semen storage and it may accelerate genetic improvement. Therefore, more studies are required to determine the *in vitro* and *in vivo* fertilizing ability of boar semen diluted with CCW.

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Author's Contributions

Mduduzi M. Tshabalala: Conception, designed, data collection, analysis, interpretation, drafted, and review of the article.

Masindi L. Mphaphathi and Khathutshedzo A. Nephawe: Conception, designed, review of the article.

Thivhilaheli R. Netshirovha: Review of the article.

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Conflict of Interest

All authors have received and approved this final version of the manuscript. Our submissions have no conflict of interest.

Ethics

This experiment was reviewed and approved by the Agricultural Research Council Ethics Committee under the Cryo Gene Bank program and by the Tshwane University of Technology Ethics Committee under project number AREC2021/06/001.

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