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Supplementation with Different Concentrations of Dithiothreitol and Glutathione During Cryopreservation of Semen from Large White Boars

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Abstract: The study aims to compare different concentrations (0, 2.5, 5, 7.5 and 10 mM) of antioxidants, Dithiothreitol (DTT) and Glutathione (GSH), on sperm parameters following the freeze-thawing of semen from large white boars. Three large white boars were selected for semen collection and the collected semen was sent to the lab for analysis. Following dilution with Beltsville thawing solution and 120 min of equilibration at 17°C, the semen was centrifuged at 800 g for 10 min at 15°C. Following supplementation of semen with DTT or GSH at different doses (0, 2.5, 5, 7.5 and 10 mM), the semen was transferred into 0.25 mL freezing straws. After 20 min in Liquid Nitrogen (LN_2) vapor, the straws were submerged in the LN_2 tank, which was at a temperature of -196°C. Following thawing at 37°C, the semen was assessed for several sperm characteristics. Data were analyzed using the GenStat® statistical program with the generalized linear model procedure. Treatment means were separated using Fisher's protected t-test least significant difference at a 0.05 level of significance (Mean ± SD). When compared to all treatments, the proportion of sperm with total motility and progressive motility was higher in control (51.1±12.7; 27.1±10.8), 5 mM GSH (45.2±16.1; 24.7±11.3) and 5 mM DTT (43.8±10.3; 23.9±10.7) ($p<0.05$). When compared to all treatments, sperm rapid motility was highest in the control (21.3%) and 5 mM DTT (21.5%) treatments ($p<0.05$). The control (46.8±6.5), 5 mM GSH (44.8±8.2) and 5 mM DTT (40.3±2.20) had a high percentage of normal live sperm following thawing as compared to all the treatments ($p<0.05$). In the hypo-osmotic swelling test, the percentage of the swollen tail was greater in the control treatment (38.8±6.1) than in the treatments with 5 mM DTT (33.6±4.9) and 5 mM GSH (32.8±4.5) ($p<0.05$). However, the differences were not statistically significant. In conclusion, the quantities of 5 mM GSH and 5 mM DTT added to freezing extenders were appropriate for cryopreserving large white boar semen.

Keywords: Dithiothreitol, Glutathione, Cryopreservation, Semen, Boars

Introduction

The most important management technique for increasing the productivity of the herd is artificial insemination (Bailey *et al*., 2008). For artificial insemination, liquid-preserved semen is widely utilized in the pig business; however, the length of time liquid semen is stored *in vitro* and the type of extender used both significantly affect the percentage of viable sperm while simultaneously raising the percentage of non-viable sperm. (Thema *et al*., 2022). Cryobanking is essential in reproductive facilities for the preservation of endangered

species' gametes for later use (Mphaphathi *et al*., 2023). Using cryopreserved semen is essential to the pig business since it would prevent the spread of certain infections and safeguard the herd's health (Grötter *et al*., 2019; Mphaphathi *et al*., 2023). Moreover, it has previously been detected that bacteria can be found in 43-100% of raw boar ejaculates (Tvrdá *et al*., 2021). Furthermore, according to Jäkel *et al*. (2021), about 75% of prolonged semen specimens may show signs of bacterial contamination. This could potentially harm the effectiveness of artificial insemination in the production of pigs. Therefore, the use of cryopreserved semen by the pig business will aid in preventing bacterial barriers and other potential problems in the near future.

Many factors have an impact on the achievement of boar cryopreserved semen, as well as the semen's preliminary state of quality, the make-up of a cryomedia, a cryoprotectant and diluting factor, the rate of cryopreservation and thawing and physical characteristics of sperm (Aramli *et al*., 2015; Linhartova *et al*., 2013; Shishanova *et al*., 2012). The studies on boar sperm cryopreservation technology showed that sperm are prone to molecular damage during the cryopreserving and thawing procedures (Pérez-Cerezales *et al*., 2010), including significant physical and osmotic damage to the sperm, which later resulted in a reduction in sperm functions (Ledwaba *et al*., 2022). Oxidative damage that sperm encounter through seminal freezing reduces their viability, motility and acrosomal integrity (Reyes *et al*., 2022). Therefore, one of the key factors leading to sperm cryodamage is an imbalance between the sperm's capacity for antioxidants and the amount of ROS (Ball, 2008; Li *et al*., 2010).

To prevent these damages, antioxidants are supplemented in the process of freezing. Some antioxidants, whether synthetic or natural extracts were used and they have had a major and beneficial influence on the parameters of viable sperm both *in vitro* and *in vivo* (Pezo *et al*., 2021). The antioxidant defense mechanism in sperm and the enzymes that provide antioxidants in seminal plasma provide some defense by searching the ROS (Kankofer *et al*., 2005; Dutta *et al*., 2019) dilution, freezing and thawing processes used in freezing can disrupt the equilibrium amongst ROS formation and removal, which can cause a variety of difficulties with sperm structure and productiveness (Nesci *et al*., 2020). Several research has revealed that incorporating exogenous antioxidants into the freezing medium is a successful method for reducing oxidative stress and enhancing sperm quality (Amidi *et al*., 2016). The inclusion of antioxidants in the sperm extender may assist the sperm to survive freezing and thawing (De Albuquerque Lagares *et al*., 2020). However, the issue of poor fertility rate has not yet been fully resolved, even though on frozen-thawed boar sperm, these broad-spectrum antioxidants provide protective advantages.

Antioxidants like Glutathione (GSH) have been added to extenders to prevent oxidative impairment to sperm motility and integrity. Antioxidants can also be used to neutralize reactive oxygen species created during cryopreservation (Jacob *et al*., 2003). According to Atmaca (2004), GSH is the main thiol molecule not a protein in human cells and directly contributes to the neutralization of ROS in addition to the preservation of exogenous antioxidants like active versions of vitamins C and E (Gadea *et al*., 2004). Under these circumstances, lower GSH was found to increase boar sperm tolerance during freezing, protect nucleoprotein structure, maintain improved sperm motility and viability (Yeste *et al*., 2013) and enhance the capacity of cryopreserved sperm to fertilize (Estrada *et al*., 2014). It is important to note that this antioxidant is among the most prevalent thiols in living cells and is crucial for preserving intracellular redox balance (Jacob *et al*., 2003). Dithiothreitol (DTT) reduces protamine disulfide bonds and functions as an antioxidant. (Watanabe and Fukui, 2006). It inhibits sulfhydryl group oxidation as well as the breakdown of mucolytic disulfide bonds in mucoprotein, which might harm the cryopreserved membranes (Ollero *et al*., 1998). The DTT has a preventive impact on damage from oxidation and apoptosis in lymphocytes brought on by toxicity (Deshpande and Kehrer, 2006). Supplementation of DTT seemed to improve the motility of bull and human sperm while stored in liquid or when frozen (Bucak *et al*., 2012). As far as we know, no study has been conducted on the function of DTT as a cryoprotectant extender or against cryodamage to boar sperm. As an outcome, this research is aimed at comparing the impact of various DTT and GSH concentrations (0, 2.5, 5, 7.5 and 10 mM) on sperm characteristics following the freezing and thawing of semen from large white boars. Antioxidant intervention is a useful tactic for fending off oxidative stress and protecting boar semen against peroxidation damage (Amidi *et al*., 2016). Furthermore, using frozen and thawed boar semen could enhance biosafety and lessen the negative consequences of an unexpected natural disaster or abrupt outbreak of an infectious disease. Moreover, effective gene banking requires the proper cryopreservation of boar semen (Giaretta *et al*., 2015).

Materials and Methods

Ethical Clearance

All experimental large white boars utilized in this research were permitted and cared for according to the criteria of the Agricultural Research Council, Animal Production Ethics Committee (APAEC) [2020/06]. and Tshwane University of Technology Ethics Committee

(AREC AREC2021/10/009). All large white boars that will be used for the study will be monitored daily. Any boar that shows any abnormal behavior will be housed in separate pens and treated according to the vet's instructions. The boars will be trained by the qualified animal technician, also semen from the boars will be collected by the qualified animal technician.

Chemicals and Reagents

Unless otherwise specified, all chemicals and reagents were bought from Sigma Chemical Co. (St. Louis, Missouri, the United States of America). Consumables and semen-freezing straws were bought from Embryo Plus® (Brits, South Africa).

Study Site

The study was done at the agricultural research council's germplasm conservation and reproductive biotechnologies unit in Irene, South Africa. The Agricultural Research Council-Irene campus is located at 25°55'South; and 28°12'East. The campus is situated at a height of 1,525 meters above sea level in the South African Highveld region. For this study, three large white boars (around 3 years old) were used. On the farm, these pigs were raised and trained to produce semen for artificial insemination. They were kept under consistent housing and feeding arrangements. Large white boars were housed in the same house but in separate single pen sizes 4×0,8 meters for each boar (to prevent them from fighting). The pen size allows each boar to turn around and display their usual postural adjustment. The house where the boars are kept has windows that may be opened and closed to allow for ventilation and humidity (21°C). Ventilation is managed to provide the boars with uniform or constant fresh air. The house was dry cleaned three times each week and wet cleaning was done twice per week for hygiene and to prevent toxicity or contamination. The house is intended to facilitate manure disposal and cleaning to prevent infection. To justify biosecurity and avoid contamination, water baths were placed at each dwelling entry. The boars were not given bedding, as the dry surface of their pen floor comforted them. The temperature in the residence was constantly checked to avoid extremes. The boars received one meal each day and water at ad libitum. The dummy sow was used to train the boars for 30 min per boar per day until they began mounting the dummy sow.

Preparation of Freezing Extenders

For semen freezing extenders, two fractions (A and B; Table 1) of chicken egg yolk base extenders were prepared using the 50 mL centrifuge tubes (Whitehead Scientific, Cape Town, South Africa) to make the final volume of 25 mL of the freezing extenders. Until they were used, the semen-freezing extenders were maintained at 5°C.

Table 1: Composition of freezing extenders used for cryopreservation of semen from large white boars

Semen extender compositions	Fraction A (mL)	Fraction B (mL)				
		Control	2.5 mM	5 mM	7.5 mM	10 mM
BTS	20	19.25	19.1870	19.1250	19.0620	19.00
CEY	5	5.00	5.0000	5.0000	5.0000	5.00
Glycerol	-	0.75	0.7500	0.7500	0.7500	0.75
DTT/ GSH	-	-	0.0625	0.1250	0.1875	0.25

BTS = Beltsville Thawing Solution; CEY = Chicken egg yolk; DTT = Dithiothreitol; GSH = Glutathione

Semen Collection from Large White Boars

The sheath's hair was first shaved and the prepuce was then cleaned with 70% ethanol and a sterile paper towel before the boars' semen was extracted. This method was carried out to avoid contamination of semen during collection. Three large white boars with proven fertility were used for the collection of semen twice a week (on Monday and Thursday) for five days in a row (10 ejaculates per boar), utilizing a gloved-hand approach, as outlined by Roca *et al*. (2004). The gel fraction was separated from the sperm-rich fraction using a glass beaker coated in a gauze filter (non-sterile Sterilux® ES, 10×10 cm) and a thermos flask (500 mL semen collection, MS Schippers) filled with warm water at 37°C (Roca *et al*., 2004). Semen was taken to the lab for analysis within 10 min of being collected. The semen was kept inside the 100 mL graduated glass reagent bottle (Whitehead Scientific, Cape Town, South Africa) and kept inside the Styrofoam box to prevent semen from being exposed to sunlight. The temperature of the Styrofoam box was kept at 37°C throughout the transportation of semen to the lab.

Cryopreservation of Large White Boar Semen

For cryopreservation, the protocol that was previously described by Ledwaba *et al*. (2022) was used in this study. Semen was pooled (to eliminate individual differences) and then transferred into 50 mL centrifuge tubes (Nest®, biotechnology, China). Then diluted with Beltsville Thawing Solution (IMV technologies, Maple Grove, Minnesota, United States of America) at a ratio of 1:1 v/v. Semen that was diluted was centrifuged at 800 rpm for 10 min at a temperature of 15°C after being equilibrated at 17°C for 2 h. Centrifugation of semen is required for boar sperm cryopreservation to isolate sperm from seminal plasma and concentrate sperm, allowing them to be diluted with freezing extenders. After centrifugation, the supernatant was removed and the sperm pellet was then re-suspended in a 1:1 v/v solution of a semen freezing extender (fraction A) before being subjected to the cooling process at 5°C for an additional hour and 30 min. A 1:1 v/v dilution was used as it is required to appropriately dilute out the seminal plasma in semen with an acceptable volume for the extender to allow for optimum retention of sperm quality. Semen was then supplemented with different semen freezing extenders (fraction B), including

control (without antioxidants), DTT (2.5, 5, 7.5 and 10 mM/mL) and GSH (2.5, 5, 7.5 and 10 mM/mL), to reach a final sperm concentration of roughly 38×10^6 sperm/mL. Semen was diluted and then placed into polyvinyl chloride straws measuring 0.25 mL (Embryo plus, Brits, South Africa). The straws were placed in contact with LN_2 vapor for 20 min at a height of 3 cm above the LN_2 level in an expandable polystyrene box (Sellés *et al*., 2003). The straws were then kept in an LN_2 (-196°C) tank pending thawing.

Thawing of Semen from Large White Boars

Six semen straws from each treatment (to make six replicates) were thawed for 10 sec in the air and 1 min in the thermos flask at 37°C, then semen was transferred into the 15 mL Eppendorf tube (whitehead scientific, Cape Town, South Africa). One straw was thawed at a time to ensure the consistency of the thawing procedure and to avoid compromising post-thaw motility. Following thawing, a drop of 5 µL of the semen was used to check for sperm motility using the computer-aided sperm analyzer.

Evaluation of Sperm Motility and Velocity Traits

Sperm motility and velocity traits from frozen-thawed semen were assessed using a computer-aided sperm analyzer (Microptic S.L, Barcelona, Spain) system with settings (Table 2) for the evaluation of boar semen. A total of 20 µL of thawed semen was diluted with 200 µL of pre-warmed (37°C) Beltsville thawing solution (IMV technologies, Maple Grove, Minnesota, United States of America). A total of 5 µL of the dilution was transferred on a pre-warmed microscope slide (Labchem Pty Ltd, Johannesburg, South Africa) and covered with a coverslip (Labchem Pty Ltd, South Africa), then examined. The sperm motility and velocity traits evaluated are sperm total motility (TM%), Progressive Motility (PM%), non-Progressive Motility (NPM%), Static (STC%), rapid (RAP%), Medium (MED%), Slow (SLW%), Curvilinear (VCL µm/s), straight-line (VSL µm/s), average path Velocity (VAP µm/s), Linearity (LIN%), straightness (STR%), Wobble (WOB%), the Amplitude of Lateral Head displacement (ALH µm/s), Beat Cross Frequency (BCF Hz) and Hyperactivity (HPA%).

Evaluation of Sperm Viability and Morphological Traits

Sperm morphology for frozen-thawed semen samples was evaluated under a microscope after the semen samples were stained on a microscope slide with Eosin/Nigrosin stain. A total of 7 µL of frozen-thawed semen was added into 20 µL of Eosin/Nigrosin (University of Pretoria, Onderstepoort, South Africa) stain solution in a micro-centrifuge graduated tube (Whitehead Scientific, Cape Town, South Africa) and mixed gently. A total of 5 µL mixed sample was smeared on the microscope slide and before evaluation, the slide was allowed to dry at room temperature for 10 min. A phase contrast microscope (Olympus Corporation BX 51FT, Tokyo, Japan) was used at 100× magnification to count 200 sperm per stained slide/treatment. The slides were evaluated for the following morphological defects: Sperm viability (live and dead) and sperm abnormalities (live sperms with head defects, live sperms with tail defects, live sperms with midpiece defects and live sperms with droplet defects).

Preparation of HOST Solution for Sperm Membrane Integrity

0.735 g of Sodium citrate and 1.351 g of Fructose were prepared into 100 mL volume of sterile water (SABAX, Adcock Ingram, Midrand, South Africa) and then stored in the refrigerator at 5°C until use.

Evaluation of Sperm Membrane Integrity

Sperm membrane integrity for frozen-thawed semen was assessed with the aid of a microscope (Nikon®, Japan). Two slides were prepared for each thawed sperm straw. A drop of 10 µL frozen-thawed semen was added and mixed with 100 µL of Hypo-Osmotic Swelling Test Solution (HOST) in an Eppendorf micro-centrifuge tube (Simport, Canada). Later, incubated at 37°C for 30 min. After incubation, a drop of 5 µL of the mixture was placed in the center of the microscope glass slide, spread and assessed. A total of 200 sperm were counted on each slide (n = 6) under the phase-contrast microscope at ×100 magnification and recorded. Sperms were characterized as membrane intact (swollen tail sperm/live) and membrane damaged (Unswollen tail sperm/dead).

Table 2: The Sperm Class Analyzer® (V.6.3.0.41) settings used to analyze sperm motility and velocity traits from large white boars

Traits	Settings
Calibration name	10×
Calibration value (µm/pixels)	0.832649
Capture method	ph-
Grind distance (µm)	100
Frame rate (fps)	50
Area (µm^2) (min)	5
Area (µm^2) (max)	70
Drifting (µm^2)	10
Static (µm/s) <	10
Slow-medium (µm/s) >	25
Rapid (µm/s) >	100
Progressivity (STR >)	70
Connectivity (pixels)	12
VAP points (pixels)	7
VCL/VAP	VCL
Number of images	50

Statistical Analysis

Data were analyzed using the GenStat® statistical program with the general linear model procedure to test the effect of antioxidant concentrations on sperm motility, viability, morphology and membrane integrity traits. The experiment was done using the factorial design of 2×4. The experiment was replicated 6 times per experiment. Fisher's protected t-test's least significant difference was used to differentiate treatment means at the 0.05 level of significance (Mean ± SD). Percentage data are presented as mean ± standard deviation (Mean ± SD) values. Statistical Product and Services Solutions were used for all statistical analyses (SPSS 11.5 for Windows, SPSS).

Results

Table 3 covers the analysis of post-thaw sperm motility and velocity traits in Large White sperm treated with various antioxidants. Sperm Total Motility (TM) ranged from 12.4-51.1% ($p<0.05$). When compared to all treatments, a larger proportion of sperm TM and progressive motility was observed on Control (51.1±12.7; 27.1±10.8), 5 mM GSH (45.2±16.1; 24.7±11.3) and 5 mM DTT (43.8±10.3; 23.9±10.7) ($p<0.05$). Moreover, the treatments with control (21.3%) and 5 mM DTT (21.5%) had a higher proportion of sperm moving rapidly than the other treatments combined ($p<0.05$). The lowest percentage of sperm with static motility was recorded on control (48.9±12.7), 5 mM DTT (56.2±10.3) and 5 mM GSH (59.1±13.6) in contrast to every treatment ($p<0.05$).

Table 4 represents the characterization of post-thaw sperm velocity traits in large white semen supplemented with different antioxidants. Sperm curvilinear velocity (125.6±34.3), straight-line velocity (29.5±4.7) and average path velocity (70.2±13.9) were high on post-thaw semen supplemented with 10 mM DTT ($p<0.05$). In comparison with 7.5 mM DTT (4.0±5.5) and 5 mM GSH (10.0±7.6; $p<0.05$), a greater proportion of hyperactively moving sperm was observed at 2.5 mM (27.2±14.4) of DTT. In comparison to all other treatments, there was a notable variation in the percentage of linearity of sperm at 5 mM DTT (16.1±4.2). Moreover, the 5 mM DTT (34.8±9.2) have significantly low percentage of sperm moving in straightness as compared to 2.5 mM DTT (45.6±10.3), 7.5 mM DTT (52.3±7.7), 10 mM DTT (44.3±11.6), 2.5 mM GSH (51.9±5.7), 5 mM GSH (50.4±10.0), 7.5 mM GSH (46.0±7.5) and 10 mM GSH (46.7±8.3).

Table 3: Characterization of post-thaw sperm motility traits in large white semen supplemented with different antioxidants

TRT	CONC (mM)	TM (%)	PM (%)	NPM (%)	RAP (%)	STC (%)	MED (%)	SLW (%)
Control		51.1±12.7^{a}	27.1±10.8^{a}	24.0±6.1^{a}	21.3±9.4^{a}	48.9±12.7^{c}	16.8±3.6^{ab}	2.9±4.9^{a}
DTT	2.5	14.4±04.5^{bc}	8.8±02.6^{c}	5.6±2.1^{b}	6.7±3.4^{b}	85.6±04.5^{ab}	6.3±3.4^{cd}	1.4±1.6^{b}
	5.0	43.8±10.3^{a}	23.9±10.7^{ab}	19.9±9.2^{a}	21.5±9.5^{a}	56.2±10.3^{c}	11.3±6.2^{bc}	11.0±6.6^{a}
	7.5	12.4±04.1^{c}	8.2±03.0^{c}	4.3±2.2^{b}	6.4±1.3^{b}	87.6±04.1^{a}	3.3±2.6^{d}	2.7±1.5^{b}
	10.0	16.8±07.4^{bc}	12.2±03.5^{c}	5.1±4.3^{b}	10.1±1.2^{b}	82.1±07.7^{ab}	3.7±2.2^{d}	3.1±4.8^{b}
GSH	2.5	25.2±13.1^{b}	15.9±10.2^{bc}	9.3±8.5^{b}	10.6±6.1^{b}	74.8±13.1^{b}	11.0±6.9^{bc}	3.6±3.6^{b}
	5.0	45.2±16.1^{a}	24.7±11.3^{ab}	20.5±8.1^{a}	12.9±7.7^{b}	59.1±13.6^{c}	17.9±7.9^{a}	10.4±8.0^{a}
	7.5	19.8±10.7^{bc}	10.9±06.7^{c}	8.8±4.7^{b}	8.3±5.1^{b}	80.2±10.7^{ab}	7.8±4.5^{cd}	3.6±2.6^{b}
	10.0	17.2±08.7^{bc}	8.9±04.0^{c}	8.2±6.8^{b}	6.4±2.9^{b}	82.9±08.7^{ab}	7.5±5.9^{cd}	3.3±1.9^{b}

$^{a-c}$Values with different superscripts within the column are different statistically ($p<0.05$). TM = Total Motility; PM = Progressive Motility; NPM = Non-Progressive Motility; RAP = Rapid; MED = Medium; SLW = Slow; STC = Static

Table 4: Characterization of post-thaw sperm velocity traits in large white semen supplemented with different antioxidants

TRT	CONC (mM)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)	HPA (%)
Control		98.3±18.9^{ab}	19.9±3.3^{cd}	44.4±07.1^{cd}	22.9±4.5^{a}	46.8±06.6^{a}	46.1±3.9^{bc}	2.9±0.7^{b}	16.2±5.1^{c}	12.8±10.1^{abc}
DTT	2.5	112.9±30.7^{ab}	25.6±7.5^{abc}	59.5±15.7^{ab}	25.3±7.3^{a}	45.6±10.3^{a}	50.1±6.6^{ab}	2.6±0.7^{b}	25.6±6.2^{ab}	27.2±14.4^{a}
	5.0	101.9±17.2^{ab}	16.5±6.1^{d}	47.4±10.6^{bcd}	16.1±4.2^{b}	34.8±09.2^{b}	42.0±3.3^{c}	2.9±0.4^{b}	17.5±4.3^{c}	20.1±08.5^{ab}
	7.5	121.9±27.1^{a}	26.8±4.9^{ab}	49.5±08.9^{bcd}	23.8±4.6^{a}	52.3±07.7^{a}	43.3±2.9^{c}	3.6±0.7^{a}	17.3±3.1^{c}	4.0±05.5^{c}
	10.0	125.6±34.3^{a}	29.5±4.7^{a}	70.2±13.9^{a}	25.1±6.2^{a}	44.3±11.6^{ab}	53.5±5.0^{a}	2.8±0.7^{b}	27.6±8.4^{a}	23.3±19.0^{ab}
GSH	2.5	106.6±18.9^{ab}	25.9±4.4^{abc}	53.8±11.1^{bc}	26.7±3.1^{a}	51.9±05.7^{a}	49.1±2.9^{ab}	2.9±0.4^{ab}	19.6±4.8^{bc}	22.1±13.9^{ab}
	5.0	92.4±23.2^{b}	21.2±5.7^{bcd}	40.7±09.5^{d}	23.9±6.1^{a}	50.4±10.0^{a}	43.7±2.9^{c}	2.8±0.6^{b}	14.8±3.3^{c}	10.0±07.6^{bc}
	7.5	107.6±22.9^{ab}	22.5±3.6^{bcd}	49.1±10.4^{bcd}	22.5±4.8^{a}	46.0±07.5^{a}	45.3±3.8^{bc}	3.0±0.7^{ab}	18.3±4.8^{c}	17.9±13.9^{abc}
	10.0	98.8±16.5^{ab}	22.5±6.2^{bcd}	45.8±11.4^{cd}	24.3±5.2^{a}	46.7±08.3^{a}	46.3±5.5^{bc}	3.0±0.2^{ab}	16.4±6.2^{c}	13.8±12.1^{abc}

$^{a-d}$Values with different superscripts within the column are different statistically ($p<0.05$). VCL = Curvilinear Velocity; VSL = Straight-line velocity; VAP = Average path velocity; LIN = Linearity; STR = Straightness; WOB = Wobbled

Table 5: Characterization of post-thaw sperm viability and morphology traits in large white semen supplemented with different antioxidants

		Viability (%)		Live sperm with abnormalities (%)			
Treatments	Concentrations (mM)	Live	Dead	Head defects	Tail defects	Proximal droplets	Distal droplet
Control		46.8±6.5^{a}	50.1±5.7^{d}	0.5±0.5^{ab}	1.8±0.7^{ab}	0.3±0.8^{ab}	0.3±0.5^{a}
DTT	2.5	23.1±7.2^{c}	74.8±8.4^{a}	0.3±0.5^{ab}	1.1±1.9^{b}	0.1±0.4^{b}	0.1±0.4^{a}
	5.0	40.3±2.2^{a}	57.0±2.8^{cd}	0.1±0.4^{b}	1.8±0.9^{ab}	0.3±0.5^{ab}	0.3±0.5^{a}
	7.5	27.0±7.6^{bc}	69.0±6.6^{ab}	1.1±1.6^{a}	1.5±1.6^{b}	0.8±0.9^{ab}	0.5±.05^{a}
	10.0	25.5±6.8^{bc}	69.6±7.6^{ab}	0.1±0.4^{b}	3.1±1.9^{a}	0.8±1.3^{ab}	0.6±0.8^{a}
GSH	2.5	32.0±7.0^{b}	64.5±7.7^{bc}	0.6±0.8^{ab}	1.6±1.0^{ab}	0.3±0.8^{ab}	0.8±0.7^{a}
	5.0	44.8±8.2^{a}	51.6±6.2^{d}	0.3±0.5^{ab}	1.5±1.3^{b}	1.0±0.8^{ab}	0.6±0.5^{a}
	7.5	28.0±5.4^{bc}	68.6±8.5^{ab}	0.1±0.4^{b}	1.0±1.0^{b}	1.1±1.8^{ab}	1.0±1.5^{a}
	10.0	28.6±6.7^{bc}	65.6±6.8^{b}	0.8±1.1^{ab}	2.3±1.5^{ab}	1.5±1.6^{a}	1.0±0.8^{a}

$^{a-d}$ Values with different superscripts within the column are different statistically ($p<0.05$). GSH = Glutathione; DTT = Dithiothreitol

Table 6: Characterization of post-thaw sperm membrane integrity in large white semen supplemented with different antioxidants

		HOST (%)	
Treatments	Concentrations (mM)	Intact membrane	Membrane damaged
	Control	38.8±6.1^{a}	61.1±6.1^{c}
DTT	2.5	28.8±6.4^{bc}	71.1±6.4^{ab}
	5.0	33.6±4.9^{ab}	66.3±4.9^{bc}
	7.5	22.8±3.1^{c}	77.1±3.1^{a}
	10.0	22.8±8.1^{c}	77.1±8.1^{a}
GSH	2.5	27.3±8.3^{bc}	72.6±8.3^{ab}
	5.0	32.8±4.5^{ab}	67.1±4.5^{bc}
	7.5	30.6±4.2^{b}	69.3±4.2^{b}
	10.0	29.6±5.8^{bc}	70.3±5.8^{ab}

$^{a-c}$ Values with different superscripts within the column are different statistically ($p<0.05$). GSH = Glutathione; DTT = Dithiothreitol

Table 5 represents the characterization of post-thaw sperm viability and morphological traits in large white semen supplemented with different antioxidants. After thawing, a higher proportion of normal living sperm was observed in the control group (46.8±6.5), 5 mM GSH (44.8±8.2) and 5 mM DTT (40.3±2.20) compared to all other treatments ($p<0.05$). The lowest percentage of normal live sperm was observed on 2.5 mM of DTT (23.1±7.2; $p<0.05$). There was no significant difference in the sperm with distal droplets for all the treatments. The 5 mM DTT (0.1±0.4), 10 mM DTT (0.1±0.4) and 7.5 mM GSH (0.1±0.4) had a significantly lower percentage of live sperm with head defects as compared to 7.5 mM DTT (1.1±1.6).

Table 6 represents the characterization of post-thaw sperm membrane integrity in large white semen supplemented with different antioxidants. The proportion of the swollen tail in the HOST was higher in the control treatment (38.8±6.1) than in the treatments with 5 mM DTT (33.6±4.9) and 5 mM GSH (32.8±4.5) ($p<0.05$). However, the differences were not statistically significant. The lowest percentage of swollen tails was recorded on treatments supplemented with 7.5 mM DTT (22.8±3.1) and 10 mM DTT (22.8±8.1; $p<0.05$).

Discussion

The current investigation was carried out to determine the optimal antioxidant concentration for usage during the cryopreservation of boar semen. Boar semen is well recognized for its high concentration of membrane polyunsaturated fatty acids, as well as its sensitivity to cold shock and peroxidative damage (Pilane *et al*., 2016). Boar sperm are particularly sensitive to oxidative damage during freezing. The influence of oxidative stress on sperm function, membrane integrity and motility was documented. By incorporating exogenous antioxidants into the freezing extender, this process could be stopped, at least in part. Therefore, to test the above hypothesis, different concentrations of DTT and GSH were studied to inhibit oxidation.

Glutathione is a tripeptide ubiquitously distributed in living cells and is crucial for the intracellular defense against oxidative stress. The findings of the current study showed that supplementation of 5 mM GSH improved sperm TM and RAP. These results are low as compared to the results recorded by Trzcińska and Bryła (2015) whereby supplementation of 5 mM GSH improved sperm TM by 71.5%. However, in the current study, GSH concentration was the best in improving sperm following freezing. A study by Gadea *et al*. (2004) reported that the addition of 5 mM GSH to the freezing extender did not result in any improvement in standard semen traits. Based on our results, we can conclude that 2.5, 7.5 and 10 mM GSH were insufficient to preserve sperm from cold shock during freezing. In experiments carried out by Gadea *et al*. (2005); Yeste *et al*. (2014), this favorable impact was shown when 1 and 2 mM GSH were added to the freezing extender. In the present study, it was also observed that supplementation of 5 mM GSH improved sperm PM and RAP. These findings comply with the results of the present study showing that the addition of GSH to the freezing extender improved the survival of sperm following cryopreservation. Sperm function, membrane integrity and motility were documented.

This investigation aimed to determine whether DTT would offer the best defense against sperm cryopreservation damage. To date, no research has been

done to evaluate whether DTT may be utilized as an antioxidant to freeze boar sperm. The results from the present study showed that supplementation of 5 mM DTT improved sperm TM and RAP. This increase in sperm motility may be brought on by the addition of GSH to the freezing extender, which causes better preservation of the sulfhydryl group content in the membrane protein (Chatterjee *et al*., 2001). These results were in agreement with the study done by Büyükleblebici *et al*. (2014) who demonstrated that supplementing the freezing extender with 5 mM of DTT improved bull sperm TM by 46.8%. However, when 2.5, 7.5 and 10 mM of DTT were supplemented with freezing extenders; the concentrations were detrimental to the survival rate of post-thaw sperm TM and RAP. This data reveals that boar semen samples with lower antioxidant levels exhibit a higher exposure to cryoinjury. Furthermore, a high concentration of antioxidants may have been ineffective in lowering LPO and boosting sperm motility. Boar sperm may be susceptible to free radical damage due to their unique cell structure, including their plasma membrane, numerous mitochondria, low cytoplasm and low antioxidant content in the cytoplasm (Amidi *et al*., 2016).

Sperm analysis includes the measurement of sperm velocity characteristics on a significant level. The results from the present study showed that 10 mM of DTT had high sperm VCL, VSL, VAP, WOB and BCF. Moreover, treatments with 7.5 mM of DTT and 5 mM of GSH had a lower percentage of sperm with hyperactivity. Therefore, since there is low sperm with hyperactive traits at the time of analysis, we can assume that better fertility is associated with a less significant amount of sperm with such traits. It is crucial to keep in mind that sperm with early hyperactivation/capacitation traits during IVF (De Andrade *et al*., 2011) have a lower survival time in the female reproductive tract because they are unable to fuse to the sperm reservoir in the isthmus (Andrade *et al*., 2022; Alkmin *et al*., 2014). Therefore, it is important to identify the right concentration that will improve sperm motility, velocity and viability as well as reduce post-thaw ROS formation, which increases the sperm's ability to fertilize matured oocytes. Sperm fertility is an important issue to consider during both *in vitro* and *in vivo* fertilization. A study was done by Ledwaba *et al*., (2022) to compare the efficacy of DTT and GSH antioxidants on oocyte fertilization by cryopreserved semen from large white boars. A total fertilization rate of 31.94% for 5 mM GSH, 44.74% for 5 mM DTT and 48.72% for 2.5 mM DTT +2.5 mM GSH was recorded. These results gave evidence that post-thaw semen supplemented with either GSH or DTT can fertilize mature pig oocytes.

Although sperm cryopreservation is a useful treatment for treating infertility, it may have an impact on the viability and morphology of sperm following thawing. In the current study, post-thawed sperm viability, morphology and membrane integrity were evaluated. The results showed that supplementation of 5 mM of GSH and DTT to freezing extenders improved sperm viability. These results were low compared to the results found by Trzcińska and Bryła (2015), where they discovered that supplementation of 5 mM GSH improved sperm viability to 68.5% following cryopreservation of bull semen. The present study also revealed sperm viability was low when 2.5 mM of DTT was added to freezing extenders. These findings suggest that sperm viability and functioning were not maintained by low concentrations of freezing extenders with antioxidant capability.

A functioning membrane is required for the sperm to fertilize since it is involved in sperm capacitation, acrosome response and sperm binding to the oocyte surface (Ramu and Jeyendran, 2013). It can be hypothesized that antioxidants may help maintain post-thawed sperm membrane integrity, as determined by the HOST. The present study's findings demonstrated that 38.8% of the sperm in the control treatment had intact membranes or enlarged tails. Guo *et al*. (2021) earlier investigation, which found that 38.76% of the sperm in the control treatment had an undamaged membrane, corroborated these findings. The HOST measures the sperm's plasma membrane's structural and functional integrity and it is also being utilized as a helpful predictor of sperm fertility as the effective fusion of the male and female gametes depends on the correct change of membrane dynamics (Ramu and Jeyendran, 2013). The present study showed that 5 mM of GSH and DTT had a high percentage of sperm with intact membranes. According to these findings, the suggested treatments did not affect membrane integrity. This way, it will be feasible to verify that some of the damage to the sperm that happens during the cryopreservation process has nothing to do with the sample's oxidative state. However, the proportion of sperm with intact membranes decreased when 7.5 and 10 mM of DTT were added. These findings suggest that the cryopreservation method and high levels of ROS can source axonemal damage that worsens sperm motility, morphological function and membrane integrity due to ATP depletion. The mitochondria that wrap the axonemal and related fibers of the central parts of sperm provide energy from ATP stored intracellularly, which is necessary for sperm movement (Başpınar *et al*., 2011).

Conclusion

In conclusion, the addition of 5 mM DTT and 5 mM GSH to the freezing extender maintained Large White sperm motility, viability, morphology and membrane integrity after cryopreservation. Thus, it was found that adding 5 mM GSH and 5 mM DTT to freezing extenders would be appropriate for cryopreserving large white boar semen.

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Author's Contributions

Mahlatsana Ramaesela Ledwaba: Conception and design, acquisition interpreted and analysis of data, drafting the article and giving final approval of the version to be submitted and any revised version.

Masindi Lottus Mphaphathi, Cyril Mpho Pilane and Tshimangadzo Lucky Nedambale: Conception and design, supervision, reviewed the article, gave final approval of the version to be submitted and any revised version.

Mamonene Angelinah Thema: Assisted in data collection and analysis of data, reviewed the article and gave final approval of the version to be submitted and any revised version.

Tlou Caswell Chokoe: Reviewed the article and gave final approval of the version to be submitted and any revised version.

Ethics

The experiments were evaluated and approved by the Animal Production Ethics (APAEC 2020/06) and Tshwane University of technology ethics committee (AREC2021/10/009).

Conflict of Interest

All authors have reviewed and approved this final version of the manuscript. There is no competing interest in our submission.

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