

Original Research Paper

# Frozen Semen Quality of Kolbroek Boars Concerning Glutathione Concentrations

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**Abstract:** Glutathione (GSH) is an antioxidant that is being studied for its ability to improve cellular responses to semen preservation challenges. The research goal aimed to evaluate the impact of introducing different glutathione concentrations to boar semen freezing extender (0, 1, 5, and 10 mM) on the Kolbroek boar sperm's quality after being frozen and thawed. For two hours, the collected sperm-rich fraction was chilled at 17°C. Following the 1 h of equilibration, fraction B: control (egg yolk 20% + BTS 72% + glycerol 8%), 1 mM (egg yolk 20% + BTS 70% + glycerol 8% + GSH 2%), 5 mM (egg yolk 20% + BTS 62 + 8% glycerol + GSH 10%) and 10 mM (egg yolk 20% + BTS 52% + glycerol 8% + GSH 20%) was added into the semen samples. Following that, 0.5 mL straws with the diluted, cooled semen samples were submerged in liquid nitrogen (LN<sub>2</sub>) vapor and then stored inside the LN<sub>2</sub> tank (-196°C). After thawing (37°C), parameters of boar sperm motility, velocity, morphology and viability, membrane permeability, and Malondialdehyde content were assessed and recorded. Using the analysis of variance, the data were examined. The results revealed a difference in total motility (%) on fresh semen as compared to post-thawed control and GSH treatments. A significant difference was revealed in sperm progressive motility (%) in fresh semen (27.07±4.5). Significant differences were recorded in live sperm morphology and viability on fresh semen (81.8±2.8%) compared to control, 1, 5, and 10 mM. In the 10 mM diluted semen (78.5±6.8), the proportion of sperm with an intact plasma membrane integrity was highest ( $P<0.05$ ), while the percentage in the 1 mM diluted semen (73.0±2.7) was lower. Malondialdehyde levels were lowest ( $P<0.05$ ) in the group receiving 5 mM treatment. In conclusion, 5 mM of GSH is the required amount to be added to the freezing extender while cryopreserving semen from Kolbroek boars.

**Keywords:** Antioxidant, Kolbroek Semen, Cryopreservation, Glutathione

## Introduction

An indigenous pig breed from South Africa known as Kolbroek is distinguished by its extraordinary adaptability features. This pig breed is popular amongst local South African pig farmers and their stock is protected as national treasures in order to preserve the genetic purity of the genotypes (Halimani *et al.*, 2010; Netshirovha *et al.*, 2020). In comparison to other pig genotypes, there has not been much research done on the ability of native Kolbroek boars to reproduce (Masenya *et al.*, 2011). Semen conservation has been

extensively studied and semen banking offers an effective method for maintaining higher male gametes for future use (Bucci *et al.*, 2018). Regardless of these possible advantages, due to cell cryodamage that happens during the freezing and thawing processes, cryopreserved sperm is not frequently employed for artificial insemination in the swine sector (Giaretta *et al.*, 2015). Such cryodamage eventually results in a decrease in sperm fertilizing ability (Didion *et al.*, 2013). Furthermore, a sudden outbreak of an infectious disease or a natural disaster could have less of a negative impact if frozen-thawed boar semen could be

used (Giaretta *et al.*, 2015). Many studies have attempted to understand the mechanisms driving cryodamage to enhance cryopreservation technologies for boar sperm (Parrilla *et al.*, 2019; Yeste *et al.*, 2014). According to Crisóstomo *et al.* (2019), the most obvious effects of freeze-thawing techniques affect plasma and acrosome membranes, the mitochondrial midpiece, and the axoneme. Such sperm membrane injury, which is detrimental to the process of fertilization causes cryopreservation degradation in the quality of the boar semen (Estrada *et al.*, 2014). This membrane damage is most likely caused by the fact that boar sperm contains more unsaturated phospholipids and less cholesterol distribution is uneven and higher in some species than in others (Casas and Flores, 2013). According to Subczynski *et al.* (2017), Cellular resilience to heat shock and cholesterol go hand in one which has a substantial effect on the fluidity, membrane lipid, positioning of protein chains, and permeability which fall under the physical characteristics of membranes. The effectiveness of *in vitro* liquid perseveration for prolonged periods might be affected by the latter sperm cryo efficacy (Mphaphathi *et al.*, 2016), the kind of semen extender employed, thawing temperature, storage temperature, and semen re-dilution (Sathe, 2012). Quantifying sperm characteristics using a computer-assisted sperm analyzer system (Neumann *et al.*, 2017) has become critical to obtain objective data both before and after freezing. To achieve uniform results, artificial insemination centers (AI) and cryo biologist can make decisions (Alqu  zar-Baeta *et al.*, 2019).

The cryopreservation technique is defined as a successful strategy for long-term gamete preservation (Yeste *et al.*, 2016). Following AI, sperm viability, motility, and conception rates are routinely evaluated to determine the success of cryopreservation (Mphaphathi and Nedambale, 2021). After the cryopreservation process, frozen sperm can be thawed and, hopefully, biological activity can be resumed (Mphaphathi and Nedambale, 2021). One of the improvements of cryopreservation technology is the introduction of antioxidants in the boar's sperm cells (Giaretta *et al.*, 2015). Different anti-oxidants, including  $\alpha$ -tocopherol (Chatiza *et al.*, 2018), decreased Glutathione (GSH) (Estrada *et al.*, 2014), superoxide dismutase and catalase (Bucci *et al.*, 2018) have been included to freezing semen extenders to reduce sperm cryodamage. Impact of antioxidants are believed to have positive influences on boar semen in sperm motility, survivability, and capacity to fertilize both *in vitro* and *in vivo* after thawing (Estrada *et al.*, 2014). In this case, sperm is protected by GSH against oxidative damage. Silvestre *et al.* (2021), maintaining redox balance (Pac  la *et al.*, 2017) and a defense mechanism against oxidative lipid peroxidation

brought on by reactive oxygen species (ROS; Silvestre *et al.*, 2021). The supplementation of various concentrations of glutathione is, however, not well understood in frozen-thawed Kolbroek boar's semen. The current study's goal was to find out how introducing glutathione to boar sperm freezing extenders affected the quality of frozen-thawed Kolbroek sperm.

## Materials and Methods

### *Study Site and Experimental Kolbroek Boars*

The study was undertaken at the Agricultural Research Council (ARC) Irene's Germplasm Conservation and Reproductive Biotechnologies laboratory. Located 1525 meters above sea level, in Pretoria, South Africa's highveld at 25  53' 59.6 south latitudes and 28  12' 51.6 east longitudes. A total of 3 Kolbroek boars were adhere to the ARC's animal production ethical committee's standards of care (REF: APAEC2020/11) and the Tshwane University of Technology (REF: AREC-2021/11/006). The boars were  $\pm$ 1-2 years and kept at the ARC pig testing. The pen of each boar was cleaned before semen collection. Hygienic practices such as washed and drying hands were carefully applied, the collection glass was sterilized and the dummy sow was cleaned thoroughly to avoid semen contamination. The boars were housed in pens that included mechanical water nipples and self-feeders (Netshirovha *et al.*, 2020). Feed was provided throughout the study and unlimited water was supplied during the experiment.

### *Chemicals and Reagents*

Unless indicated otherwise, sigma-chemical Co, USA, and TOCRIS bioscience, UK were used to purchase all of the chemicals and reagents. Solutions were prepared with highly purified water (Adcock Ingram critical care, SA).

### *Preparation of Beltsville Thawing Solution and Chicken Egg Yolk*

Beltsville Thawing Solution (BTS) (IMV technologies, France) was the primary extender utilized in this study, which was composed of 1-liter deionized water and a 50 g BTS sachet. By moving the egg yolk from one half of the egg shell to the other and removing the albumin, the chicken egg yolk was separated from the albumen. An 18-gauge needle (Neoject, UK) was used to pierce the egg yolk, separating it from its membrane and transferring some albumin to a 50 mL (Plastro, SA) graduated tube. The egg yolk was then placed in a gauze swab (Johnson's, SA).

### *Kolbroek Boar's Semen Collection*

Semen was collected twice weekly. The gloved-hand method was used to collect a total of ten ejaculates from

each boar in a 500 mL glass bottle (10 ejaculates = 10 replicates/treatments). The filtered semen portion was contained inside an insulated thermo-flask that had been pre-warmed to 37°C. For a microscopic semen analysis, the sperm was taken to the laboratory.

#### *Evaluation of Kolbroek Boar's Sperm Motility and Velocity Traits*

A Sperm Class Analyzer<sup>®</sup> system (Microptic, Spain) was used to determine the parameters of sperm motility and velocity under a microscope (Nikon, Eclipse Ci-L, Japan) at the magnification of 10 ×. A swim-up (BTS) method was used to dilute semen before analysis. A drop of 10 µL was taken from the semen sample and diluted with 200 µL of BTS. Using a microscope with × 10 magnification, a drop of 5 µL was deposited on a 37°C preheated microscopic glass slide (Labocare, UK) and mounted with a cover slip (Thermo Scientific, USA) (Nikon, Japan). Estimates of sperm motility and velocity traits were carried out on each semen sample in two separate microscopic fields. The movement of sperm traits was measured in percentages and velocity traits in micrometers per second and percentages.

#### *Evaluation of Kolbroek Boar's Sperm Morphology and Viability*

Boar sperm morphology and viability (live or dead) were analyzed using eosin nigrosine (university of Pretoria, SA) staining according to Brito *et al.* (2011). Smear microscope glass slides were assessed on a phase microscope (Olympus, Japan) at 100 × magnification, live sperm abnormalities were distinguished as unstained and dead sperm as stained. A smear was created at 37°C using a pipette tip after gently combining a drop of 7 µL of semen with a 20 µL stain using the tip of a hand pipette. On the clear end of a microscope slide, a drop of a mixed sample of 5 µL was placed. On the smeared microscope glass slide, an oil drop (UNILAB, SA) for the immersion lens was placed, and about 200 sperm cells per slide were counted.

#### *Evaluation of Kolbroek Boar's Sperm Plasma Membrane Permeability*

The hyperosmotic swelling test (HOST) was performed to assess sperm membrane permeability. A drop of 100 µL HOST (distilled water, 1000 mL, 7.35 g sodium citrate, and 13.51 g fructose) (Zhang *et al.*, 2016) and 10 µL of semen were placed in a centrifuge tube (Symport, Canada) at 37°C for 60 min. A drop of 5 µL of the semen mixture was placed on a microscope glass slide. Sperm swelling was observed as coiled sperm tails, indicating good membrane function. A total number of 200 sperm were analyzed from each microscope glass slide. The sperm cells were classified as positive or negative based on the presence or absence of a coiled tail.

#### *Evaluation of Kolbroek Boar's Sperm Malondialdehyde Content*

Malondialdehyde was based on the Thiobarbituric Acid (TBA) test used by Esterbauer and Cheeseman, (1990). Diluted sperm (1 mL of BTS extender) from each treatment and replicate was to precipitate protein, along with 1 mL of cold trichloroacetic acid. A boiling water bath at 100°C was used to incubate 1 mL of the supernatant with 1 mL of 0.67% (wt/vol) TBA after the precipitate had been centrifuged (1500 × g for 10 min). Once the semen samples were cooled to room temperature, a spectrophotometer (JENWAY<sup>®</sup> 6310, UK) was used to calculate the absorbance at 534 nm. The results were expressed as nmol/mL.

#### *Kolbroek Boar's Semen Freezing Process*

The sperm-rich fraction was cooled at 17°C for 2 h. The extended sperm suspension was then centrifuged in a chilled centrifuge at 800 × g for 10 min, while still at this temperature. The pellets were expanded again after the supernatants were removed. At 1:1 ratio using Fraction A, control (egg yolk 20% + BTS 80%), 1 mM (egg yolk 20% + BTS 78% + GSH 2%), 5 mM (egg yolk 20% + BTS 70% + GSH 10%) and 10 mM (egg yolk 20% + BTS 60% + GSH 20%). After further 60 min of cooling semen at 5°C, Fraction B extender at a ratio of 1:2, control (egg yolk 20% + BTS 72% + glycerol 8%), 1 mM (egg yolk 20% + BTS 70% + glycerol 8% + GSH 2%), 5 mM (egg yolk 20% + BTS 62% + 8% glycerol + GSH 10%) and 10 mM (egg yolk 20% + BTS 52% + glycerol 8% + GSH 20%) were added into semen samples at 5°C for 60 min. Subsequently, samples were placed into 0.5 mL labeled plastic straws (Monotube, Germany) and sealed with polyvinyl chloride powder after 1 h of equilibration with Fraction B. (Breeders choice, USA). A Styrofoam box (29.5 × 18.7 × 24 cm<sup>3</sup>) was used and filled with liquid nitrogen vapor (LN<sub>2</sub>), a rack with two bars that were positioned 7 cm below the LN<sub>2</sub> surface and was submerged to a depth of 5 cm. The straws were then placed on the rack in the LN<sub>2</sub> vapor and horizontally aligned for 20 min before being submerged into the LN<sub>2</sub> tank for storage.

#### *Kolbroek Boar's Semen Thawing Process*

Two straws per treatment were taken after at least 4 days of frozen semen straw storage at -196°C from the LN<sub>2</sub> tank. For 10 sec on air, the straws were defrosted at 37°C and for 1 min in warm water. The semen samples were diluted with a BTS extender at a ratio of 1:2 after thawing. Kolbroek sperm motility traits, velocity traits, morphology traits, plasma membrane, and MDA content were evaluated.

#### *Statistical Analysis*

The data underwent analysis of variance. The generalized linear model procedure was used. At a 5% level of significance, Student's Least Significant

Differences (t-LSDs) were computed to compare the means of significant source effects between treatments (Snedecor and Cochran, 1980). Statistical software SAS 9.4 (SAS, 1999) was used to carry out the stated analysis.

## Results

Table 1 displays how varying glutathione concentrations affected sperm motility in frozen-thawed semen from Kolbroek boars. The results showed that a difference was recorded in sperm total motility (%) on fresh semen compared to control, 1, 5, and 10 mM. There was a significant difference ( $P<0.05$ ) in sperm progressive motility (%) in fresh semen compared to other treatments.

Table 2 displays the impact of various glutathione concentrations on the sperm velocity rates of frozen-thawed semen from Kolbroek boars. Sperm wobble (%) in 5 mM was significantly different from 1 mM ( $P<0.05$ ). Sperm straight-line velocity ( $37.9\pm 27.2 \mu\text{m}/\text{sec}$ ) was lower at 10 mM compared to the control group, fresh semen, and 1 mM. In the control group, 1 mM and fresh semen on sperm straightness (%) indicated a higher percentage ( $P<0.05$ ) in contrast to 10 mM.

Sperm morphology and viability parameters in Kolbroek boars supplemented with glutathione following frozen-thawed are shown in Table 3. The findings demonstrated a distinction in the morphology and viability of live sperm on fresh semen compared to control, 1, 5, and 10 mM. Live sperm abnormalities are shown in Fig. 2 and dead sperm are shown in Fig. 1. However, in comparison to all other treatments, 10 mM had a decreased ( $P<0.05$ ) level of live sperm viability and morphology.

Table 4 displays the findings of the study on the impact of glutathione concentration on the integrity of the sperm plasma membrane in frozen-thawed boar semen from Kolbroek. On HOST+, 10 and 5 mM had the largest sperm membrane concentrations (%) without affecting the integrity of the plasma membrane, while 1 mM of glutathione had a lower ( $P<0.05$ ) percentage of intact plasma membrane integrity. The control group was numerically lower on HOST+ than all other groups.

The impact of different glutathione concentrations on Kolbroek boar sperm MDA content during freezing is demonstrated in Fig. 3. The least improvement was shown in the 5 mM treated group MDA levels in all groups

( $P<0.05$ ). The difference in 1 mM concentration was significant in contrast to the control group and 10 mM.

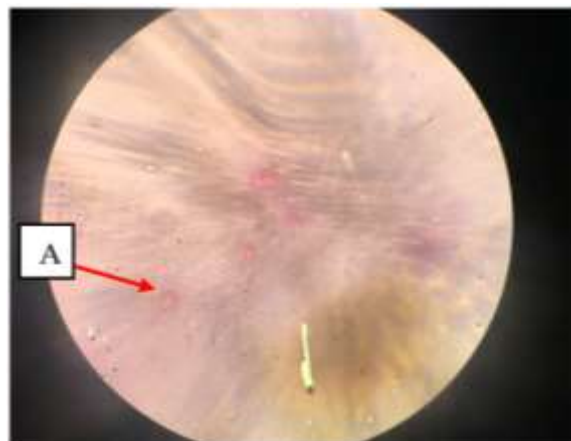


Fig. 1: A-stained sperm cell (dead sperm)

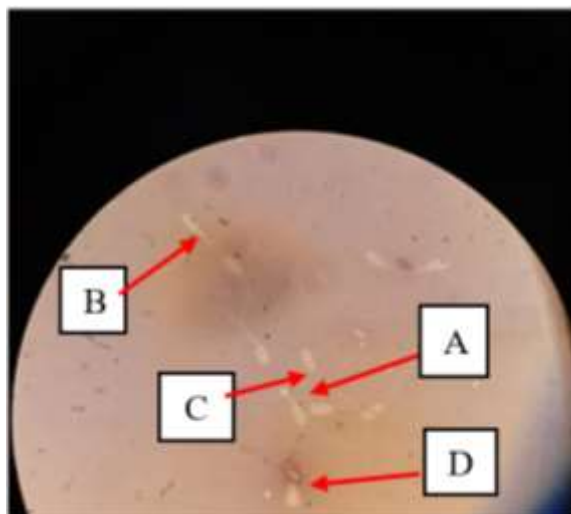


Fig. 2: (A) Unstained sperm cell with hairpin tail and distal droplet abnormality (live sperm); (B) Unstained sperm cell with hairpin tail and proximal droplet abnormality (live sperm); (C) Unstained sperm cell (live sperm); (D) Unstained sperm cell with exploded head and coiled tail abnormality (live sperm)

**Table 1:** Influence of different concentrations (0, 1, 5, and 10 mM) of glutathione in sperm motility parameters on frozen-thawed boar sperm following nitrogen vapor (mean  $\pm$  SD)

Treatment	TM (%)	PM (%)	NPM (%)	SLW (%)	MED (%)
Fresh semen	90.7 $\pm$ 2.5 <sup>a</sup>	27.7 $\pm$ 4.5 <sup>a</sup>	56.10 $\pm$ 12.7 <sup>a</sup>	14.1 $\pm$ 9.8 <sup>ab</sup>	57.9 $\pm$ 9.1 <sup>a</sup>
Control (0 mM)	25.7 $\pm$ 9.6 <sup>c</sup>	9.8 $\pm$ 3.2 <sup>b</sup>	15.9 $\pm$ 9.9 <sup>c</sup>	9.1 $\pm$ 7.1 <sup>b</sup>	8.8 $\pm$ 4.7 <sup>b</sup>
1 mM	23.9 $\pm$ 9.9 <sup>c</sup>	7.1 $\pm$ 2.6 <sup>b</sup>	16.8 $\pm$ 9.9 <sup>cb</sup>	13.0 $\pm$ 8.6 <sup>ab</sup>	6.0 $\pm$ 3.1 <sup>b</sup>
5 mM	33.6 $\pm$ 10.9 <sup>b</sup>	8.1 $\pm$ 3.3 <sup>b</sup>	25.6 $\pm$ 10.2 <sup>b</sup>	19.5 $\pm$ 9.4 <sup>a</sup>	7.8 $\pm$ 3.1 <sup>b</sup>
10 mM	23.1 $\pm$ 5.8 <sup>c</sup>	7.5 $\pm$ 3.5 <sup>b</sup>	15.5 $\pm$ 6.4 <sup>c</sup>	11.6 $\pm$ 6.2 <sup>b</sup>	5.7 $\pm$ 2.1 <sup>b</sup>

<sup>abc</sup>Different superscripts within the same column demonstrate significant differences ( $P<0.05$ ). Values are mean  $\pm$  SD of boar sperm in different concentrations of glutathione. TM - Total Motility, PM - Progressive Motility, NPM - Non-Progressive Motility, SLW - Slow, MED - Medium

**Table 2:** Influence of different concentrations (0, 1, 5, and 10 mM) of glutathione in sperm velocity parameters on frozen-thawed boar sperm following nitrogen vapor (mean ± SD)

Treatment	RAP (%)	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	WOB (%)	Hyperactive (%)
Fresh semen	19.9±12.2 <sup>a</sup>	154.8±43.0	63.6±12.1 <sup>a</sup>	78.6±13.8	43.2±11.3	80.3±04.6 <sup>a</sup>	53.4±12.4 <sup>ab</sup>	5.1±3.8 <sup>a</sup>
Control (0 mM)	7.8±2.5 <sup>b</sup>	157.4±32.9	64.5±23.9 <sup>a</sup>	78.0±19.4	41.0±10.5	79.9±12.9 <sup>a</sup>	50.2±07.6 <sup>ab</sup>	2.1±2.2 <sup>b</sup>
1 mM	4.8±2.7 <sup>b</sup>	145.9±32.4	60.8±21.8 <sup>a</sup>	76.9±18.2	43.1±09.1	78.6±13.8 <sup>a</sup>	53.9±07.9 <sup>a</sup>	1.7±1.6 <sup>b</sup>
5 mM	6.2±3.6 <sup>b</sup>	177.6±56.4	52.3.9±27.1 <sup>ab</sup>	70.9±22.5	31.9±13.5	69.2±19.4 <sup>ab</sup>	43.9±10.6 <sup>b</sup>	1.8±1.5 <sup>b</sup>
10 mM	5.7±4.1 <sup>b</sup>	150.1±49.8	37.9±27.2 <sup>b</sup>	63.3±20.1	31.9±18.4	63.6±21.7 <sup>b</sup>	47.8±13.8 <sup>ab</sup>	2.1±2.5 <sup>b</sup>

<sup>ab</sup> Different superscripts within the same column demonstrate significant differences ( $P<0.05$ ). RAP - Rapid, VCL - Velocity on the curve line, VSL - Velocity on the straight line, VAP - Velocity on the average path, LIN - Linearity, STR - Straightness, WOB - Wobble

**Table 3:** Influence of different concentrations (0, 1, 5, and 10 mM) of glutathione on frozen-thawed boar sperm morphology and viability parameters following nitrogen vapor (mean ± SD)

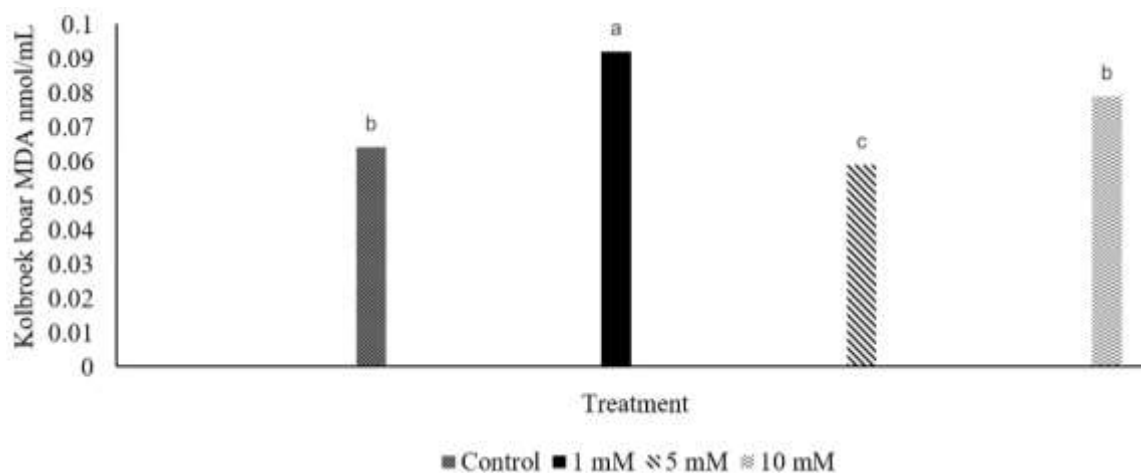
Live sperm abnormalities							
Treatment	Live (%)	Dead (%)	Head (%)	Tail (%)	Midpiece (%)	Proximal droplets (%)	Distal droplets (%)
Fresh semen	81.8±2.8 <sup>a</sup>	12.5±2.8 <sup>c</sup>	0.4±0.5	0.6±0.6 <sup>c</sup>	0.0±0.0 <sup>c</sup>	3.9±1.4 <sup>a</sup>	1.0±0.9 <sup>b</sup>
Control (0 mM)	18.3±3.5 <sup>b</sup>	72.2±4.8 <sup>b</sup>	0.5±0.6	3.6±1.7 <sup>a</sup>	0.3±0.3 <sup>a</sup>	1.9±2.3 <sup>b</sup>	2.2±1.9 <sup>ab</sup>
1 mM	18.1±3.3 <sup>b</sup>	76.6±3.6 <sup>a</sup>	0.5±0.7	1.8±1.8 <sup>bc</sup>	0.1±0.2 <sup>abc</sup>	1.8±1.2 <sup>b</sup>	1.2±1.5 <sup>b</sup>
5 mM	18.8±3.4 <sup>b</sup>	74.8±4.7 <sup>ab</sup>	0.4±0.4	2.5±1.9 <sup>ab</sup>	0.1±0.2 <sup>bc</sup>	1.2±1.1 <sup>b</sup>	1.6±1.5 <sup>ab</sup>
10 mM	13.9±2.7 <sup>c</sup>	78.4±5.1 <sup>a</sup>	0.6±0.5	2.5±2.0 <sup>ab</sup>	0.2±0.3 <sup>ab</sup>	0.8±0.9 <sup>b</sup>	2.9±2.4 <sup>a</sup>

<sup>abc</sup> Different superscripts within the same column demonstrate significant differences ( $P<0.05$ )

**Table 4:** Sperm plasma membrane permeability in frozen-thawed Kolbroek boar's semen supplemented with GSH (mean ± SD)

Treatment	HOST+	HOST-
Fresh	77.4±6.0 <sup>ab</sup>	22.6±6.0 <sup>b</sup>
Control (0 mM)	69.5±5.2 <sup>c</sup>	30.5±5.2 <sup>a</sup>
1 mM	73.0±2.7 <sup>bc</sup>	26.3±3.3 <sup>ab</sup>
5 mM	74.4±4.5 <sup>a-c</sup>	25.6±4.5 <sup>ab</sup>
10 mM	78.5±6.8 <sup>a</sup>	21.5±6.8 <sup>b</sup>

<sup>abc</sup> Different superscripts within the same column demonstrate significant differences ( $P<0.05$ ). HOST - Hyperosmotic swelling test



**Fig. 3:** Malondialdehyde content in frozen-thawed Kolbroek boar's semen supplemented with GSH. <sup>abc</sup>Different superscripts demonstrate significant differences on the bar graph ( $P<0.05$ ). MDA-Malondialdehyde

## Discussion

The primary conclusion reached by this investigation was that there was a decrease in boar sperm motility after cryopreservation. The results obtained in sperm motility showed differences in TM (%) of GSH between the treatments. However, no difference was observed in the

control group, 1, 5, and 10 mM. These differences could be related to differences in the fertility potential of boars used. According to Ansari *et al.* (2010); Câmara *et al.* (2011), GSH at high doses of 3, 5, and 10 mM showed no positive impact on semen quality indicators, which is similar to the results of the present investigation. Nonetheless, comparisons between studies are typically

challenging to understand because of variations in research techniques and sperm processing regimens. This study describes a reduction in GSH content in swine sperm brought on by freezing methods, however, it is not the first to do so.

Human GSH content has decreased by a certain proportion of 64% (Gadea *et al.*, 2011), while in boar it was 32% (Silvestre *et al.*, 2021) and in bull, it was 58% or 78% (Estrada *et al.*, 2017). Glutathione levels in mouse sperm have been found to be substantially greater than other mammalian species (90 mM GSH/ $10^8$  cells) (Chanapiwat *et al.*, 2010). Furthermore, excessive glutathione and glutathione peroxidase, concentrations may compromise the functional integrity of the sperm axosome, which is linked to the motility of sperm cells (Salmani *et al.*, 2013).

Glutathione deficiency may lead to mitochondrial DNA degradation (Gadella, 2008). An essential component of sperm, mitochondria provide the energy needed for sperm motility (Hu *et al.*, 2010). Hence, damage to the mitochondria and axosomes may contribute to the deleterious effects of high GSH levels on progressive motility (%) and other motion characteristics. (Allai *et al.*, 2018). Our findings contrast with previous research by Vozaf *et al.* (2021) who, in contrast to the control group, demonstrated enhanced sperm motility. This variance could be due to various extenders used or supplemented with various animal breeds.

The results of the current investigation demonstrated that greatly improving sperm motility by adding 5 mM GSH to freezing media diminishes viable sperm.

According to the data, sperm movement patterns are altered by increases in values that drop along the straight-line and average route for curved trajectory wobbling, this was controversial to what (Gadea *et al.*, 2011) obtained. Sperm speed on the average path and speed on the curve line, both expressed in percentages remained constant and steady between the treatment groups. The swine genetic industry relies on the cryobanking of boar sperm hence the cryopreservation process needs to be enhanced. In addition to its function, superior male sperm cryobanking is an important backup for proper insemination dosages in the event of sickness, infertility, or death (Vozaf *et al.*, 2021). Cryopreservation has been shown to damage some structural and ultrastructural sperm components.

In comparison to dead sperm (%) and in fresh sperm, there was a high percentage of live normal sperm morphology (%). However, a high percentage of dead sperm was recorded in all treatments. Although the dead sperm (%) was significantly higher, the results were still satisfactory. There are several different types of defective sperm and some of them are hereditary (Morrell *et al.*, 2018). These include primary sperm head abnormalities, such as an acrosomal cap deficiency (Hirohashi and Yanagimachi, 2018), and secondary sperm tail abnormalities

(Bó and Mapletoft, 2014). In this study, these primary sperm abnormalities variables were taken into account.

In this study, in the presence of various glutathione concentrations upon cryopreservation, MDA levels were measured and examined. The MDA concentration was higher in the treatments than that of the control group, except for 5 mM. According to Chatiza *et al.* (2018), cells stimulate their maintenance and survival under low lipid peroxidation rates. This was due to activated antioxidant defense systems (Chatiza *et al.*, 2018). The rate of lipid peroxidation when high, oxidative damage exceeds the potential for repair, and cells trigger apoptosis (Chatiza *et al.*, 2018). Whichever process ultimately causes cell damage, which can lead to the development of various accelerated aging and pathological states (Halliwell and Gutteridge, 2015).

According to (Silvestre *et al.*, 2021), plasma membrane functional integrity is related to sperm motility. These findings suggested that supplementation with glutathione in a freezing extender could enhance sperm motility by shielding the plasma membrane from ROS. The sperm membrane's capacity for function can be damaged when introduced to high concentrations of antioxidants (El-Seadawy *et al.*, 2022).

Little is known about the mechanisms underlying cryodamage to boar sperm and the major contributors, which include ROS (Vaughan *et al.*, 2020). Human sperm cells are assumed to be multifactorial (Vaughan *et al.*, 2020). Significant contributions have previously been identified as increased ROS production caused by freezing and thawing, as well as oxidative stress (Vaughan *et al.*, 2020).

In mammalian cells, GSH is the most prevalent non-protein thiol molecule (Gadea *et al.*, 2011). Multiple cellular activities rely on glutathione, including the transfer of amino acids, DNA, protein synthesis, and disulfide bond depletion (Gadea *et al.*, 2011). Glutathione has been demonstrated to keep certain domestic animals' sperm moving (Masoudia *et al.*, 2020) and to guard sperm against oxidative damage (Silvestre *et al.*, 2021) it is anticipated that adding this antioxidant to extenders can improve the sperm's quality and ability to fertilize eggs through the freezing and thawing processes. The GSH's sulphhydryl groups have been demonstrated to protect cells from oxidants, electrophiles, and free radicals (Perumal *et al.*, 2011).

## Conclusion

In comparison to fresh semen, the freezing extender combined with various GSH concentrations lowered sperm motility parameters. The amount of 5 mM of GSH, was however found to be the most effective concentration to be added to the sperm-freezing extender.

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## Author's Contributions

All authors equally contributed to this study

## Ethics

The agricultural research council ethics committee (REF: APAEC2020/11) and Tshwane University of Technology (REF: AREC-2021/11/006) both granted their approval for the study.

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