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Variation in sperm cryosurvival is not modified by replacing the cryoprotectant glycerol with ethylene glycol in bulls

Short running title: Cryoprotectants fail to modify sperm cryosurvival differences

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Summary

Breed and sire differences in sperm cryosurvival have been noted, with negative implications for sperm cryobanking and assisted reproduction programs. This study hypothesised that these differences could be modified by using lower molecular weight cryoprotectants. Therefore, the effect of replacing glycerol (GLY) with ethylene glycol (EG) on differential cryosurvival of semen from two Sanga cattle breeds (Mashona vs. Tuli) was determined. Three to five ejaculates were collected from each of ten bulls (3-8 years) by electro-ejaculation, diluted in three Tris-egg yolk extenders (Triladyl®, 7% GLY-based and 7% EG-based) and evaluated for sperm motility, viability and morphology at three time periods (fresh – 0 h, pre-freeze – 4 h and post-thaw). Tuli bulls produced larger (11.8±0.31 ml vs. 8.5±0.38 ml) and more concentrated ejaculates of lower fresh semen quality. Breeds differed across time for motility and morphology, but not viability. Mashona bull semen had significantly higher motility and normal morphology values at each sampling time. Bulls classified as poor freezers had lower concentration (0.70±0.09 × 10^9 sperm/ml vs. 1.37±0.10 × 10^9 sperm/ml), sperm motility index (SMI, 35.0±3.4 % vs. 67.8±2.1 %) and viability (69.7±1.1 % vs. 75.7±1.0 %) compared to good freezers. Maintenance of semen quality by GLY and EG did not differ between breeds, poor and good freezers, or age groups. The interaction breed by extender across time did not reach statistical significance for all variables. The study revealed that bull and breed variation in sperm quality and cryosurvival is not modified by replacing GLY with EG, suggesting that cryostress tolerance of sperm may be under control of mechanisms other than differential response to GLY cytotoxicity.

Keywords: bull semen; sperm cryosurvival; poor freezers; glycerol

Introduction

Cryobanking of semen is the most commonly used strategy for conservation and use of valuable animal genetic resources across spatial and temporal scales (van Arendonk, 2011). Sperm cryobank stocks may be used to breed for productivity and adaptation, restock certain areas, manage the diversity of genes in existing populations, minimize inbreeding, reconstruct lost breeds and to address longer term livestock breeding goals (Blackburn, 2018). In order to achieve these objectives, the functional integrity of spermatozoa must be preserved as much as possible.

Cryobanks face the challenge of cryogenic damage to sperm caused by a combination of thermal stress, sperm plasma membrane lipid phase transitions, ice crystallization and osmotic-induced stressors during freezing and thawing (Am-In et al., 2011; Lieberman et al., 2016; Tapia et al., 2012). In routine semen work, sperm is protected from cryogenic damage through inclusion of cryoprotectants in semen extenders; slow and stepwise addition of extenders in order to limit an-

isosmotic cell volume excursions (Yeste, 2016); widening osmotic tolerance of the spermatozoa (Guthrie et al., 2002); and use of optimized cooling and freezing profiles (Rodríguez-Martínez & Peña-Vega, 2013). Glycerol (GLY) and other small, ionic membrane permeating molecules such as dimethyl-sulphoxide (DMSO), ethylene glycol (EG), propylene glycol (propanediol, PND) and dimethyl-acetamide (DMA) are used in bovine semen cryopreservation (Sieme et al., 2016). These molecules achieve cryoprotection because they facilitate cellular dehydration, reduce water crystallization and extend sperm osmotic tolerance limits by reducing cell volume excursions during freezing and thawing (Guthrie et al., 2002; Sieme et al., 2016). However, cryogenic damage is inevitable and up to 40-50% of sperm functionality may be lost (Holt, 2000).

Cryobanking programs also face the challenge of differential cryosurvival of semen of different breeds and individual sires, with serious consequences for cryobanking and assisted reproductive outcomes (Mostek et al., 2018). Cryobanks incur losses due to rejected ejaculates, production of fewer but more concentrated semen doses and discards of frozen straws that fail to meet quality thresholds. Farmers lose out through reduced conception and calving rates. This also means that standard freezing protocols may be optimal for some sires and breeds and suboptimal for others. GLY ($C_3H_8O_3$) remains the cryoprotectant of choice in bovine sperm cryopreservation, owing, probably, to its effectiveness, lower cost and ready availability. However, it has been observed to have lower membrane permeability (Büyükleblebici et al., 2014), cause greater osmotic volume excursions and damage (Guthrie et al., 2002), and to be more cytotoxic to sperm (Sieme et al., 2016) compared to alternatives such as EG ($(CH_2OH)_2$), PND ($C_3H_8O_2$), DMSO ($(CH_3)_2SO$), and DMA ($CH_3CON(CH_3)_2$). As a result, some researchers suggested that these lower molecular weight cryoprotectants may be used for sperm sensitive to GLY cytotoxicity (Büyükleblebici et al., 2014; Seshoka et al., 2016). We hypothesised that bull and breed differences in sperm cryosurvival could be modified by using a lower molecular weight cryoprotectant such as EG (62.07 g/molar) instead of GLY (92.10 g/molar). Therefore, this study sought to determine the effect of using two cryoprotectants, GLY and EG, on breed and bull differences in sperm cryosurvival.

Materials and Methods

Study sites

Zimbabwe is a subtropical country in southern Africa. It experiences a tropical savanna type climate. Ten bulls used in the study were sampled from conservation populations at Grasslands and Makoholi Research Institutes. These two sites differ in their ecological conditions and vegetation type. Grasslands ($18^0$11`S, $31^0$28`E) is located at an altitude of 1,600 m above sea level, 67 km south-east of Harare. It is situated in a cool to warm (average 18^{o}C) high unimodal rainfall (600-900 mm) agro-ecological zone (Vincent et al., 1960). Vegetation is described as *Hyperhenia tall grass-veld*. Makoholi (19^0 50' S, 30^0 46'E) is located in a lower altitude (1,204 m above sea level), lower rainfall (450-650 mm) area, some 32 km north of Masvingo town. Soils are coarse-grained, granitic ferralic arenosols of the Fersiallitic group. Vegetation is described as mixed-veld dominated by annual grass species which remain palatable throughout the year.

Animals

All bulls were maintained on natural grazing with *ad libitum* access to water in the paddocks. A clinical and physical examination of the bulls was performed at the onset of the research to assess feasibility for semen collection. Age (years), body condition score (BCS), scrotal circumference (SC), and testicular consistency (TC) parameters were measured and recorded. SC was measured in cm using a flexible tape at the greatest diameter of the scrotal sac. TC was determined subjectively by palpation of testicular tissue and classified as moderate or soft or hard. During the evaluation, those bulls found to have clinical inflammatory or congenital defects of the reproductive organs or SC of less than 30 cm were excluded from the study. Thus, five Tuli and five Mashona bulls were selected for the study.

Ethical statement

This study was approved by the Academic Board of the Department of Animal Production and Technology, Chinhoyi University of Technology and the Department of Research and Specialist Services (DR&SS), Zimbabwe. Sample collection was carried out under the supervision of a qualified veterinarian and according to the Chinhoyi University of Technology 'Guidelines for Animal Handling and Sample Collection', which conforms to European Union Directive 2010/63 regarding the protection of animals used in scientific experiments.

Semen collection

Semen was collected during the cool dry season (May - August 2017) five times at two week intervals from each bull using an electro-ejaculator (Minitube, Germany) standardised for bovine semen collection (Baracaldo et al., 2007). At ejaculation, a pre-warmed (35 - 37^{o}C) sterile assortment tube connected to a latex rubber cone was used to collect the ejaculate and immediately assessed for colour, consistency, odour and presence of foreign material. Only clean ejaculates with gross sperm motility value of >70% were considered for subsequent processing.

Extenders

All extenders and media were prepared at the Germplasm Conservation and Reproductive Technology Laboratory (GRTL), Chinhoyi University of Technology (Chinhoyi, Zimbabwe) using reagent-grade chemicals purchased from Merck (Darmstadt, Germany), Associated Chemical Enterprises (Johannesburg, South Africa), Irvine's Zimbabwe (Harare, Zimbabwe) and Minitub GmbH (Tiefenbach, Germany). The basic extender used in this study was composed of 2.7 g Tris, 1.0 g fructose, 1.4 g citric acid and 20 ml whole egg yolk, supplemented with 100 IU penicillin and 1 mg streptomycin per ml diluent. Two different extenders were prepared by the addition of cryoprotectants in the form of either 7% GLY or 7% EG (v/v) and made up to 100 ml with double distilled water. Triladyl®, a commercially available egg-yolk Tris-citrate buffered one-step bovine semen extender, was prepared for use as the control. Extenders were filtered through sterile Whatman paper to remove large particles, then adjusted to pH 7.2 and stored at 4^{o}C until use.

Semen processing

Semen processing was carried out in a mobile laboratory in the field at the site of sample collection. Each ejaculate was split into four fractions, diluted 1:1 (v/v) with the three extenders (7% GLY, 7% EG, Triladyl®) maintained at 36°C and cooled to 4^{o}C over 2 h. During this period, the fourth semen fraction was used for gross sperm motility and concentration evaluation. After 2 h, the second extender fraction was added stepwise (0.25 + 0.50 + 0.25) at 3 intervals of 10, 20 and 10 min respectively to bring sperm count down to a final storage concentration of ~100 x 10^{6}sperm cells per ml. Diluted samples were equilibrated for 2 h at 4^{o}C, packaged into 0.25 ml mini-straws by gentle suction and sealed with polyvinyl sealant powder. Straws were then placed on freezing racks and allowed to equilibrate for a further 20 min. semen was frozen using the method of liquid nitrogen (LN_2) vapours. The racks were placed ±4 cm above LN_2 (-140^{o}C) in a

Styrofoam box without ventilation for 10 min, plunged into the LN_2 (-196°C) and held for another 5 min. The straws were then extracted from the LN_2, loaded into canisters and stored in LN_2 flasks for at least one month before analyses.

Semen evaluation

Ejaculate volume, sperm concentration and all fresh and pre-freeze motility variables were determined in the field. Analyses of all post-thaw samples as well as fresh and pre-freeze dry mount smears for viability and morphology were carried out at the Germplasm and Reproductive Technologies Laboratory (GRTL), Chinhoyi University of Technology. Ejaculate volume was directly read off the graduated collection tube. Sperm concentration was determined by a hemacytometer with an improved Neubauer chamber (Boerco, Germany).

For sperm motility evaluation, a bright-field microscope with a pre-heated stage (37°C) was used. A 10 µl aliquot of semen was placed on a pre-warmed (37°C) slide, covered with a cover slip and evaluated at ×400 magnification. Ten fields of view with groups of ~10 sperm per field were counted, estimating the proportion of motile and progressively motile sperm and the rate of forward linear propulsion (1 – very slow, 5 – very fast). A sperm motility index (SMI) was then calculated for each ejaculate using the formula: *SMI = [gross motility % + (20 × rate)]/2* (Chatiza et al., 2011).

For viability and morphology, eosin–nigrosin dry-mount stained smears were assessed using bright field microscopy. The histological dual stain used was made up of 1.67 g eosin Y, 10 g nigrosin, and 2.9 g sodium citrate dissolved in 100 ml distilled water according to Björndahl et al. (2003). A 10 µl semen aliquot was mixed 1:1 (v/v) with the stain, smeared across a microscope slide, quickly air dried and stored for later evaluation. Viability and sperm morphology were determined by counting 200 cells under bright-field microscopy (Amscope, USA) at ×1,000 and ×400 magnifications, respectively. For viability, sperm displaying partial or complete purple staining were considered non-viable and those showing strict exclusion of stain were counted as viable. Spermatozoa having a smooth, oval shaped head, a well-defined cap (acrosome) and a long tail with no visible defects were classified as normal. Cytoplasmic residues exceeding one third of the sperm head were determined and classified as proximal, distal or translocating cytoplasmic droplets. Each normal sperm was counted once, and each defect was counted separately even if multiple defects occurred on the same sperm cell.

Statistical Analyses

The study was analysed as a factorial experiment in which split ejaculates from ten bulls of two breeds were diluted in three different extenders and evaluated at three time periods (fresh, pre-freeze and post-thaw) for motility, viability and morphology. Repeated measures analyses of variance (RM ANOVA) was performed in IBM SPSS Statistics Version 20 for Windows to evaluate within-subjects effects of time period and between-subjects effects of breed and extender on the response variables with age as a covariate. Partial Eta Squared (η_P^2) parameter was used to estimate effect sizes of the model factors and their interactions. Only morphology satisfied the assumption for homogeneity of variances (Bartlett test, $P > 0.01$). Viability and motility also violated the sphericity assumption (Mauchly's test, $P < 0.001$) and for these variables, Greenhouse-Geisser ($\varepsilon < 0.75$) or Huynh-Feldt ($\varepsilon > 0.75$) corrected results were reported (Field, 2013). Where significant interactions were observed, main effects were ignored in favour of simple effects. Bulls were categorized based on their composite post-thaw SMI values (>50% or <50%) into good or poor freezers (Perumal et al., 2014) and compared by one way ANOVA in Minitab 17 (Minitab, 2014). Non-parametric tests were used to compare fresh and post-thaw sperm defects within breed and between breeds at each sampling time. *Post hoc* treatment of data was done using Fisher's LSD at the 5% level of significance.

Results

Semen production parameters

Semen production and quality parameters were summarized according to SC and TC (Table 1). SC averaged 36.6±3.0 cm and did not differ between breeds. Bulls with SC > 38 cm produced significantly greater ejaculate volumes and more concentrated semen but with significantly higher proportion of sperm tail defects compared to those with SC of 30-34 and 35-38 cm. Semen from bulls with hard testicular tissue produced significantly greater ejaculate volumes of higher concentration and viability but with lower normal sperm and higher tail defects relative to those from normal and soft testicular tissue. Significant breed and bull differences were observed for a number of ejaculate characteristics. Semen volume averaged 10.2±2.95 ml, ranging from 6.3 to 13.4 ml per ejaculate. Compared to Mashona, Tuli bulls produced greater (11.8±0.31 ml vs. 8.5±0.38 ml, $P < 0.001$) and more concentrated (1.39±0.09 vs. 0.75±0.10 spermatozoa per ml, $P < 0.01$) semen ejaculates. Mean seminal concentration was 1.09×10^9 spermatozoa per ml, with a range of 0.08×10^9 - 2.87×10^9 spermatozoa per ml. Tuli fresh semen had significantly lower

progressive motility (93.6±0.44 % vs. 94.6±0.46 %) and SMI (92.8±0.64 % vs. 94.2±0.63 %), and higher sperm tail defects (3.98±0.25 % vs. 2.35±0.25 %) and cytoplasmic droplets (0.31±0.05 % vs. 0.01±0.05 %). Age grouping bulls into those 3-5 years and those older than five years had no effect on proportion of morphologically normal and viable spermatozoa. However, younger bulls produced significantly greater (11.1± 0.36 ml vs. 9.4±0.42 ml, $P < 0.01$), more concentrated ejaculates (1.385×10^9 vs. 0.828×10^9 spermatozoa per ml, $P < 0.001$) of better gross motility (98.0±0.21 % vs. 96.9±0.32 %, $P < 0.01$) and SMI (94.4±0.67 % vs. 92.6±0.59 %, $P < 0.05$). However, proportions of sperm tail defects (2.8±0.32 % vs. 3.7±0.20 %) and cytoplasmic droplets (0.1±0.04 % vs. 0.3±0.06 %) were lower ($P < 0.01$) in older compared to younger bulls.

Sperm cryosurvival

Results of the RM ANOVA for the effect of breed and extender on various semen quality variables across time are given in Table 2. Relative to fresh semen, cryopreservation resulted in a progressive decrease of 47%, 56% and 39% in gross sperm motility, progressive motility and SMI, respectively. Time had a significant main effect on the decline in total motility, $F(2,1880) = 3.878$, $P < 0.05$, $\eta_P^2 = 0.039$; progressive motility, $F(2,4320) = 9.670$, $P < 0.001$, $\eta_P^2 = 0.092$; SMI, $F(2,921) = 2.466$, $P < 0.05$, $\eta_P^2 = 0.025$; and normal morphology, $F(2,69) = 4.199$, $P < 0.05$, $\eta_P^2 = 0.044$. Breeds differed across time for all motility variables, with Mashona bull semen having greater values at each sampling time (Figure 1). Over time, the three extenders did not differ in their effects on all variables analysed. However, the control (Triladyl®) had better motility values ($P < 0.05$) in pre-freeze samples. The interaction effects of breed by extender across time did not reach statistical significance for all variables, showing that breed differences were not modified by the type of extender used. Significant individual bull variability ($P < 0.001$) was observed for all motility variables at all sampling times, and this variability was partially explained by bull age ($P < 0.001$).

Among the model terms, only the main effects of time, $F(2,853) = 15.686$, $P < 0.001$, $\eta_P^2 = 0.142$, and extender, $F(2,263) = 3.881$, $P < 0.05$, $\eta_P^2 = 0.078$, reached statistical significance for the viability parameter. Proportion of viable sperm decreased from 94.1±0.24 % in fresh samples to 84.7±0.49 % after extension, cooling and equilibration and then to 73.2±0.81 % in post-thaw samples, with no significant breed differences or breed by extender interactions across time. GLY- and EG-based extenders did not differ in the maintenance of viability, but had significantly lower figures compared to Triladyl® (control). For morphology, only the main effects of time, $F(2,69) =$

4.199, $P < 0.05$, $\eta_P^2 = 0.044$, and breed, $F(1,248) = 4.451$, $P < 0.001$, $\eta_P^2 = 0.194$, reached statistical significance, with small and moderate effect sizes, respectively. A progressive decline of 5% in the proportion of normal spermatozoa was observed across time. Proportions of normal spermatozoa was higher ($P < 0.01$) in Mashona compared to Tuli bull semen at each of the three sampling times (Figure 2). Triladyl® had post-thaw normal sperm morphology values (91.3±0.47 %) similar to GLY (89.9±0.57 %) and significantly higher than EG (89.5±0.51 %). However, GLY and EG did not differ in post-thaw normal sperm morphology.

Table 3 shows the proportion of sperm defects counted in fresh and post-thaw Tuli and Mashona semen samples. Fresh Tuli bull semen had significantly higher proportion of detached heads, coiled tails and proximal cytoplasmic droplets. Significantly higher proportions of coiled ($P < 0.001$) and broken ($P < 0.01$) tails post-thaw were also observed for Tuli bull semen compared to Mashona. Cryopreservation resulted in significant increases ($P < 0.001$) in the proportion of detached heads and coiled tails in both breeds; bent tails in Mashona semen; and broken tails and proximal cytoplasmic droplets ($P < 0.01$) in Tuli semen.

Comparison between poor and good freezers

The hypothesis that EG is a better alternative cryoprotectant to GLY particularly for poor freezers was tested. Four of the bulls (three Tuli and one Mashona) consistently produced semen with poor cryosurvival compared to others and were classified as poor freezers. Poor freezers had significantly lower sperm concentration, sperm motility and sperm viability, and higher proportion of sperm head defects post-thaw (Table 4). Good and poor freezers did not differ in mean ejaculate volume, normal morphology and tail defects. Performance of the two cryoprotectants (7% GLY, 7% EG) was similar ($P > 0.05$) for both poor and good freezers for all quality variables tested.

Discussion

This study revealed that bull age, SC and their interactions are important sources of variation in fresh semen quality in bulls. Age had a positive relationship up to about six years, making the use of younger bulls for semen collection advantageous. D'Andre et al. (2017) and Enciso et al. (2011) reported similar results and concluded that the best semen quality is obtained from bulls not older than five years of age. Earlier, Vilakazi and Webb (2004) observed that semen quality starts to deteriorate in Friesian bulls older than 72 months, with morphological traits being the worst affected. Bulls older than five years produced less concentrated semen with higher proportions of

tail defects and cytoplasmic droplets. Aging is associated with testicular degeneration and fat accumulation, leading to an increase in aberrations of spermatogenesis and shedding off of spermatid cytoplasm during epididymal maturation. This leads to higher proportion of sperm defects and reduced motility in ejaculated semen (Menon et al., 2011). Favourable relationships between SC and both semen production and quality traits in bulls have been demonstrated, with SC above 30 cm being related to the most satisfactory spermiogram (Ahmad et al., 2011; Vijetha et al., 2014). The present study also observed that higher SC and moderate to hard testicular tissue is more desirable for semen production and quality compared to lower SC and softer testicular tissue. Thus, the combination of SC, TC and age parameters could be a reliable tool to use in bull breeding soundness evaluation.

Significant bull and breed variability in semen quality and cryosurvival was observed, with lower cryosurvival for Tuli compared to Mashona bull semen. Breed and bull variation in fresh and cryopreserved semen quality has been reported in a number of studies, including tropical breeds (D'Andre et al., 2017; Enciso et al., 2011; Seshoka et al., 2016). Bulls were classified as good or poor freezers. A pre-freeze semen quality advantage did not necessarily translate into better sperm cryosurvival.

Our findings failed to support the hypothesis that relative to GLY, EG could offer better sperm cryoprotection especially for bulls with compromised sperm cryosurvival. The type of cryoprotectant (7% GLY or 7% EG) did not differ in performance over time or modify differential sperm cryosurvival between breeds or bulls classified as good or poor freezers. Similar to our findings, the use of EG and DMSO depressed sperm motility and other semen quality parameters to the same extend as GLY in Holstein bulls in Turkey (Büyükleblebici et al., 2014). Other researchers (Forero-Gonzalez et al., 2012; Tasdemir et al., 2013) found no advantage in using 5-7% EG or 5-7% DMSO to replace 6-7% GLY in bull sperm cryopreservation. Seshoka et al. (2016) used higher cryoprotectant concentrations (12%) and reported that GLY maintained significantly higher total sperm motility (77.8%) compared to EG (20.4%) and DMSO (15.7%) in Nguni bulls. Contrary to our findings, Guthrie et al. (2002) reported that EG reduced osmotically induced cell volume excursions in extended Angus bull semen compared to GLY, leading to favorable cryoprotection. In the present study GLY was used at typical concentrations (≤7%) and at lower temperature, which may have limited sperm volume excursions within 92-103% (270-360

mOsm) of their isosmotic cell volume for both cryoprotectants. There are, therefore, conflicting findings on the ability of EG to replace GLY in bovine sperm cryopreservation.

Based on the obtained results, it is speculated that differential cryostress tolerance of sperm between sires and breeds of Sanga cattle may be related to mechanisms other than sensitivity to GLY cytotoxicity. The basis for these differences need to be elucidated as a first step towards developing biomarkers for predicting the outcome of semen cryopreservation or optimizing freezing protocols for specific genotypes. Differential cryogenic survival may be understood from a biochemical or genetic standpoint.

Biochemical markers for sperm cryosurvival are mostly related to differences in sperm plasma membrane lipid profiles and compositional stability (Tapia et al., 2012) and resilience to an-isosmotic conditions of cryopreservation (Guthrie et al., 2002). Membrane lipids, which function to protect the sperm cell against extracellular injury and respond to physiological challenges, are dominated by docosahexaenoic (22:6 n-3) and arachidonic (20:4 n-6) acids, accounting for up to 62% of total phospholipid content. Generally, long chain saturated fatty acids increase rigidity, while polyunsaturated fatty acids give flexibility and fluidity to biological membranes, which allows them to perform their physiological functions. Possession of highly unsaturated fatty acids confers great fluidity to the sperm membrane which makes spermatozoa particularly vulnerable to premature cryo-capacitation, attack by reactive oxygen species and loss of sperm functionality (Chakrabarty et al., 2007).

Reviews of work on the genetic explanations for individual and breed variations in sperm cryogenic survival have been done by Fortes et al. (2013) and Hezavehei et al. (2018), among others. A number of quantitative trait loci and genetic variants are already known to be associated with male fertility traits, including semen quality and cryosurvival. Recently, proteomic approaches have started to be used to investigate semen freezability and identify sperm proteins, *m*RNA and *mi*RNA transcripts that could be used as biomarkers of bull semen and fertility traits (Parthipan et al., 2017). For instance, Mostek et al. (2018) observed differences in the protein profile and carbonylation level between high and low-quality bull semen ejaculates. High quality ejaculates were characterised by abundance of extra-nuclear proteins and a low carbonylation level. Thus, a number of tools now exist to investigate possible mechanisms for observed differences between bulls and breeds in sperm cryosurvival.

Conclusions

This study revealed extensive variability in semen production and post-thaw sperm quality between bulls, such that bulls were classified as either good or poor freezers. Relative to Mashona, Tuli bull semen had lower sperm cryosurvival rates. These cryosurvival differences were not modified by replacing the cryoprotectant GLY with EG, suggesting that cryostress tolerance of sperm may be under control of mechanisms other than differential response to GLY cytotoxicity.

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Author contributions

EG and FPC conceived the research, collected data, carried out statistical analyses and wrote the manuscript; SMM and FC reviewed the manuscript; all authors read and approved the final manuscript.

Conflict of interest statement

None of the authors have any conflict of interest to declare.

Data availability statement

The data that support the findings of this study are available from the corresponding author on request

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Table 1: Fresh semen quality variables (Mean±SE) summarized according to testicular parameters in bulls (n=10)

	Semen volume (ml)	Concentration ($\times 10^9$ /ml)	Viability (%)	Normal (%)
Palpation of testicular consistency				

Hard	11.1±0.36[a]	1.385±0.108[a]	94.2±0.37[ab]	94.5±0.40[b]
Moderate	10.4±0.53[a]	0.942±0.130[b]	93.3±0.33[b]	94.5±0.58[b]
Soft	7.6±0.45[b]	0.601±0.094[b]	95.8±0.49[a]	97.7±0.36[a]
Scrotal circumference				
30-34 cm	10.1±0.54	0.673±0.136[b]	95.7±0.30[a]	94.7±0.73[ab]
35-38 cm	10.2±0.46	0.662±0.110[b]	93.5±0.39[b]	95.7±0.52[a]
>38 cm	10.4±0.50	1.494±0.116[a]	94.0±0.31[b]	94.2±0.40[b]

[ab] Means in the same category and column that do not share a common superscript are statistically different ($P < 0.05$)

Table 2: Results of the repeated measures analysis of variance (IBM SPSS 20) for semen quality variables in split ejaculates of ten bulls of two breeds (Mashona and Tuli) diluted in three Tris-egg

yolk extenders (Triladyl®, 7% glycerol-based and 7% ethylene glycol-based) and evaluated at three time periods (fresh – 0 h, pre-freeze – 4 h and post-thaw)

Parameter	Variable				
	Total motility	Progressive motility	SMI	Morphology	Viability
Bartlett test (P-value)	0.000	0.000	0.000	0.020	0.000
Mauchly's test for sphericity					
X^2 value	37.711	24.995	58.323	0.144	29.025
P-value	0.000	0.000	0.000	0.930	0.000
Epsilon (ε)	0.752	0.811	0.684	0.998	0.790
Results interpreted	Greenhouse-Geisser	Huynh-Feldt	Greenhouse-Geisser	Sphericity assumed	Huynh-Feldt
Within subjects effects (*P value*)					
Time	0.034	0.000	0.048	0.016	0.000
Time*Age	0.000	0.000	0.000	0.013	0.052
Time*Breed	0.013	0.016	0.013	0.135	0.738
Time*Extender	0.222	0.095	0.228	0.065	0.241
Time*Breed*Extender	0.926	0.956	0.706	0.580	0.698
Between subjects effects (*P value*)					
Age	0.000	0.000	0.000	0.038	0.000
Breed	0.008	0.011	0.001	0.000	0.100
Extender	0.140	0.157	0.218	0.447	0.024
Extender*Breed	0.730	0.841	0.771	0.745	0.631

Table 3: Frequency (Mean ± SE %) of major morphological sperm defects in Tuli and Mashona bull semen

Sperm defects	Mashona (n = 48)			Tuli (n = 51)		
	Fresh	Post-thaw	P-value	Fresh	Post-thaw	P-value
Detached normal heads	0.92 ± 1.27^{b}	2.63±0.29	***	1.63 ± 1.54^{a}	3.00±0.23	***
Coiled tails	0.88 ± 0.13^{b}	$2.00\pm0.18^{\gamma}$	***	1.63 ± 0.13^{a}	$2.92\pm0.18^{\beta}$	***
Broken tails	0.54±0.11	$0.47\pm0.10^{\gamma}$	ns	0.41±0.11	$1.15\pm0.13^{\beta}$	***
Bent tails	0.38±0.13	1.03±0.13	***	0.71±0.13	1.28±0.20	ns
Proximal droplets	0.00 ± 0.04^{b}	0.04±0.03	ns	0.14 ± 0.03^{a}	0.00±0.00	**

The P value indicates statistical difference between fresh and post-thaw defects within breed – ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Wilcoxon test, $P < 0.05$); Different superscripts indicate significant breed differences for fresh (a, b) and post-thaw (β, γ) semen samples (U Mann Whitney, $P < 0.05$).

Table 4: Comparison of semen production characteristics and post thaw sperm quality (Mean ±SE) between bulls classified as good or poor freezers

Semen parameter	Freezability		Significance
	Good	Poor	
Ejaculate volume (ml)	10.2±0.32	10.3±0.55	ns
Concentration (x10^9 sperm/ml)	1.37±0.10	0.70±0.09	***
Viable sperm (%)	75.7±1.0	69.7±1.1	**
Gross motility (%)	64.7±2.4	29.8±3.5	***
Progressive linear motility (%)	50.3±2.5	18.9±3.0	***
Sperm motility index (SMI, %)	67.8±2.1	35.0±3.4	***
Normal spermatozoa (%)	90.7±0.4	89.5±0.4	ns
Head defects (%)	3.3±0.22	4.1±0.32	*
Tail defects (%)	5.9±0.30	6.0±0.34	ns

Bulls with composite sperm motility index > 50% were classified as good freezers and those < 50% as poor freezers; for each parameter, comparisons were – ns: not significant (P > 0.05), * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure captions

Fig. 1. Sperm motility index (SMI) between fresh, pre-freeze and post-thaw spermatozoa of Tuli and Mashona bulls - Error bars indicate SEM; Significant breed differences at each sampling time are denoted by the Greek letters, β and γ; for comparison between fresh, pre-freeze and post-thaw values within a breed, Latin letters (Mashona – a, b, c; Tuli – A, B, C) are used to indicate dissimilar values ($P < 0.05$).

Fig. 2. Proportion of morphologically normal spermatozoa in fresh, pre-freeze and post-thaw Tuli and Mashona bull semen - Error bars indicate SE; Significant breed differences at each sampling time are denoted by the Greek letters – β and γ; For comparison between fresh, pre-freeze and post-thaw values, Latin small case letters (a, b, c) and upper case letters (A, B, C) are used to indicate dissimilar values in Mashona and Tuli, respectively.

Fig. 1

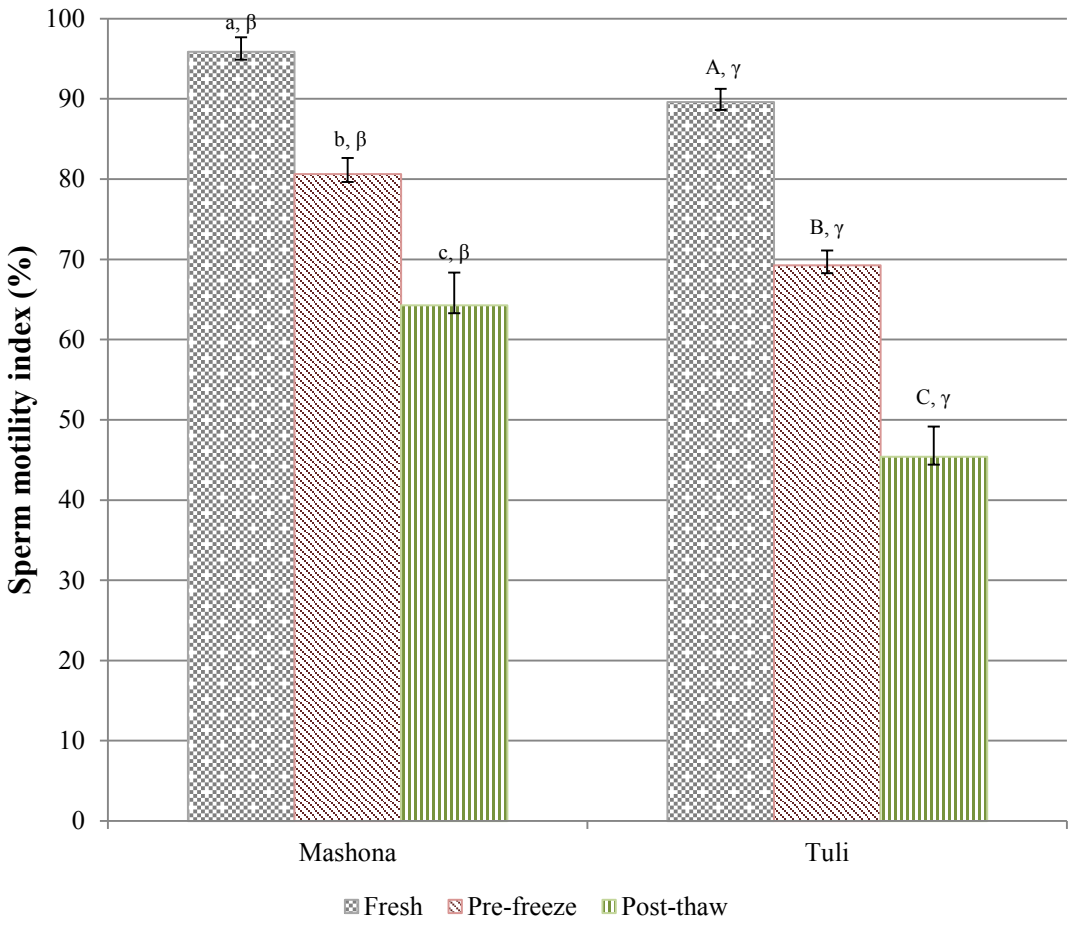

Fig. 2