ORIGINAL ARTICLE

BOAR SPERMATOZOA MAINTAIN DNA INTEGRITY AFTER CRYOPRESERVATION USING DIFFERENT CONCENTRATIONS OF GLYCEROL

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ABSTRACT

Despite its potential benefits, the application of frozen-thawed boar spermatozoa in commercial pig production is still very limited. Cryopreservation could induce sperm DNA damage causing reduced fertility and early embryo loss. Glycerol is a popular cryoprotectant used to improve motility and plasma membrane integrity during sperm freezing but the optimal concentration needed to prevent DNA damage without causing cell toxicity is unknown. Thus, the aim of this study was to determine the cryoprotective effect of different concentrations of glycerol on DNA integrity and motility of frozen-thawed boar spermatozoa. Using the TUNEL assay and flow cytometry, there was no significant difference in the percentage of sperm DNA damage between fresh or frozen-thawed sperm at 3%, 6%, and 8% glycerol (1.9 ± 0.4 *vs.* 3.5 ± 0.8 *vs.* 2.8 ± 0.5 *vs.* 3.0 ± 0.8% respectively; $P > 0.05$). While total and progressively motile spermatozoa were higher in fresh samples, other CASA motion parameters such as straight-line velocity, average path velocity, straightness, and linearity were generally higher in 3% and 6% glycerol-frozen than fresh samples ($P \leq 0.05$). At the concentrations tested, glycerol appears to protect DNA integrity of boar spermatozoa during cryopreservation while slightly enhancing some sperm motility parameters.

Keywords: *boar sperm, cryopreservation, DNA damage, glycerol, TUNEL*

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INTRODUCTION

The quality of frozen boar spermatozoa is still insufficient for extensive use in commercial pig production despite its potential to support long -term banking, faster distribution of superior genetic materials, and control disease transmission (Bailey *et al.*, 2000; Johnson *et al.*, 2000; Knox, 2011). This is mainly due to the complex processing and expensive equipment required for freezing along with significantly reduced survival and fertility of frozen-thawed spermatozoa. Damage to the structural integrity and function of the sperm (including sperm membranes, mitochondrial architecture, motility, and possibly DNA integrity) during freezing and thawing procedures can lead to greatly reduced fertilisation success (Watson, 1995; Thurston *et al.*, 2001; Roca *et al.*, 2006). Moreover, up to 70% of the variability in pig sperm cryosurvival is due to individual boar effects (Holt, 2000; Roca *et al.*, 2006). To overcome this, AI doses containing greater semen volumes, higher sperm number or pooled semen are commonly required to

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compensate for low fertility in frozen-thawed boar semen (Foxcroft *et al.*, 2008).

While standard protocols for boar sperm cryopreservation have been used extensively (Pursel and Johnson, 1975; ARS-USDA, 2007), cellular stress occurs when the temperature drops below 0 ^{o}C. Damage is mainly induced by osmotic dehydration of the cell and crystallisation of the extracellular components (Watson, 1995). In order to overcome this, freezing media typically include cryoprotective agents (Fuller, 2004) like glycerol (1,2,3-propanetriol or glycerine), the main by-product upon transesterification of vegetable oils or animal fats that is abundant in nature as a structural component of many lipids (Pagliaro *et al.*, 2007; da Silva *et al.*, 2009). Glycerol has been commonly used as a cryoprotectant at different concentrations ranging from 2-4% in many different freezing protocols across species including bovine (Awad, 2011), porcine (Almlid and Johnson,1988; Das *et al.*, 2016), equine (Graham, 1996), and caprine (Fiser and Fairfull, 1984) to improve the motility and plasma membrane integrity of spermatozoa after thawing. When spermatozoa are mixed with hypertonic medium containing glycerol, water leaves the cells and is replaced by glycerol. This mechanism protects the cells from ice crystal formation during freezing (Fowler and Toner, 2005).

Moreover, glycerol has proven to be superior to other cryoprotectants in preserving boar spermatozoa (Watson, 1995; Kim *et al.*, 2011; Silva *et al.*, 2015) but boar spermatozoa appear to be more sensitive to the standard concentrations of glycerol used in sperm cryopreservation protocols of other domestic species (Almlid and Johnson, 1988). High concentrations can be toxic to cells (Buhr *et al.*, 2001; Macias Garcia *et al.*, 2012), affecting sperm motility and acrosomal integrity. This high sensitivity has been attributed to hypersensitivity of boar sperm to cold shock including the high levels of unsaturated phospholipids and low levels of cholesterol on the boar sperm plasma membrane thus, increasing the likelihood of oxidative damage (Rath *et al.*, 2009). The freezing process itself may also cause damage to sperm DNA which could potentially cause reduced fertilisation and/or blastocyst formation rates *in vitro* (Royere *et al.*, 1988; Hamamah *et al.*, 1990; Johnson *et al.*, 2000), early embryonic loss, interrupted embryo development, genetic abnormalities in offspring, lower pregnancy rates (Ahmadi and Ng, 1999; Henkel *et al.*, 2004; Paul *et al.*, 2008), and ultimately negatively impact *in vitro* fertilization outcome (Simon *et al.*, 2013). Sperm DNA fragmentation has been observed in the boar (Hamamah *et al.*, 1990), human (de Paula *et al.*, 2006), ram (Peris *et al.*, 2004), and mouse (Yildiz *et al.*, 2007) as a result of cryopreservation. Boar and human spermatozoa had significantly reduced Feulgen-DNA content and sperm nuclear surface area as a result of freeze-thawing leading to a state of 'overcondensation' (Royere *et al.*, 1988; Hamamah *et al.*, 1990) which may explain the reduced fertilising potential of frozen spermatozoa (Royere *et al.*, 1991). Current evidence indicates that sperm DNA damage is mediated by free radicals generated due to oxidative stress associated with the freezing and thawing processes (Gavriliouk and Aitken, 2015; Bisht and Dada, 2017). This is compounded by the limited endogenous antioxidant systems inherent in spermatozoa and the loss of cytosolic machinery during spermatogenesis making sperm transcriptionally and translationally inactive and thus, deficient in repair mechanisms (Henkel *et al.*, 2004; Aitken and De Iuliis, 2010; Aitken *et al.*, 2012).

There is still more to be known about the optimal concentration of glycerol to protect DNA integrity during cryopreservation of boar spermatozoa. In boars, 2-4% glycerol results in higher sperm motility and acrosome integrity, while 8% reduces motility and normal acrosome morphology (Almlid and Johnson, 1988; Buhr *et al.*, 2001; Kim *et al.*, 2011). Moreover, 3% glycerol (in either lactose-hen egg yolk or extender with lactose and lyophilized lipoprotein fractions isolated from ostrich egg yolk) significantly reduced DNA damage (determined by comet assay) in frozen-thawed boar spermatozoa than those cryopreserved without glycerol (Fraser and Strzezek, 2007). This study aimed to compare the ability of 3, 6, and 8% glycerol to protect DNA integrity without inducing toxic effects on function as measured by motility in frozen-thawed boar spermatozoa.

MATERIALS AND METHODS

Boars and Location

Six Large White boars were purchased at 11-12 months of age from a commercial piggery and reared in an open, gable roof-type facility within individual 3 x 3 meter pens at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Queensland, Australia (19°19'46.4"S, 146°45'40.3"E). Boars were exposed to prevailing winds and ambient temperatures throughout the day. Each boar was fed 1.8 - 2.3 kg/day of a commercial pelleted diet (Barastoc, Ridley AgriProducts, Victoria, Australia) to maintain a body score between

3 - 3.5. Water was provided *ad libitum* via an automatic pig nipple waterer. Experiments were approved by the James Cook University Animal Ethics Committee (Approval Number A1998).

Semen Collection and Processing

The procedures used for semen collection and processing were according to Peña *et al.* (2019a). Briefly, sexually mature boars, using the gloved hand technique (Hancock and Hovell, 1959), were collected by the same person at regular intervals prior to experimental sampling. This was necessary to maintain regular turnover of mature epididymal sperm populations while maintaining the boars training to the dummy mount (Minitube, USA). One experimental semen sample was collected from each of the six boars (late dry season), diluted 1:3 in Beltsville Thawing Solution (BTS; pH 7.2; Pursel and Johnson, 1976; Gadea, 2003) containing 205 mM D-glucose, 20 mM sodium citrate tribasic dihydrate, 3 mM ethylenediamine tetraacetic acid (EDTA) disodium salt dihydrate, 10 mM potassium chloride, 15 mM sodium bicarbonate and 0.1% (v/v) gentamicin reagent solution (Life Technologies) in nanopure deionised water, and split into three cryopreservation treatments: 3%, 6% and 8% glycerol-containing medium (see below). The late dry period in Townsville is characterised by having relatively low ambient temperatures and humidity (Peña *et al.*, 2019a), as well as minimal sperm DNA damage compared to the peak wet season (January), where tropical summer conditions induce significant sperm DNA damage (Pena *et al.*, 2019a). For inclusion in the study, boars selected produced semen with these minimum standards: having spermatozoa of at least 70% motility, 65% normal morphology, and an ejaculate volume of at least 100 ml. Semen samples were maintained at 38 ºC using a water bath from the time of collection to dilution in 1:3 BTS. Concentration of sperm was determined using a Neubauer haemocytometer following standard protocols.

Freezing and Thawing of Semen Samples

Semen was frozen following standard procedures adapted from Pursel and Johnson (1975), ARS-USDA (2007) and Purdy (2008). Initially, each 1:3 BTS-diluted boar semen was split into 3 cryopreservation treatments and equilibrated at room temperature for 1 h before storage at 15 °C for not more than 5 h until needed. Thereafter, samples were centrifuged and resuspended in 8 mL BF5 cooling extender (CE; 52 mM TES, 16.5 mM Tris(hydroxymethyl) aminomethane, 178 mM glucose, 20% egg yolk; ~300 × 10^6 sperm/mL), and cooled to 5 °C over 2.5 h. Samples were then further diluted drop-wise with 4 mL BF5 freezing extender (FE; containing 2.5% Equex Paste - Minitube, Tiefenbach, Germany, and either 3, 6 or 8% glycerol in BF5 CE). Sperm samples of ~200 × 10^6 sperm/mL were then manually loaded into 0.5 mL CBS straws (IMV Corporation, Minneapolis, MN, USA) using a modified sterile syringe (Braun) with a pipette tip attached into it. Straws were then frozen in liquid nitrogen vapour using an IceCube programmable freezer (Minitube, Tiefenbach, Germany) at the following freeze rate: –20 °C/min from 5 to –8 °C; –69 °C/min from –8 to –120 °C; –20 °C/min from –120 to –140 °C. Straws were grouped in goblets according to treatment then stored in liquid nitrogen for approximately three months. Prior to downstream analysis of sperm motility and DNA integrity, samples were thawed by submerging two 0.5 mL semen straws in a 38 °C water bath while gently agitating for 30 seconds (Buranaamnuay *et al.*, 2011) and gently mixed into 9 mL of BTS. Thereafter, the straws were maintained at 38 °C and processed immediately for analysis.

Determination of motility characteristics by CASA

About 3 µl of 20 x 10^6 sperm/ml of fresh or frozen-thawed semen in BTS was loaded into each chamber of 38 ºC pre-warmed Leja Standard Count 4 Chamber Slides (Leja Products, Nieuw-Vennep, Netherlands) and loaded into a computer-assisted sperm analyser (CASA; IVOS version 10, Hamilton Thorne Research. Beverly, MA, USA). Motility characteristics of spermatozoa were analysed as previously described (Peña *et al.*, 2015).

Sperm DNA Integrity Assay and Flow Cytometry

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) has been reported to provide more robust measures of actual DNA damage than neutral comet or other similar assays (Ribas-Maynou *et al.*, 2013; Cui *et al.*, 2015). Thus, sperm DNA integrity was evaluated using TUNEL (*In Situ* Cell Death Detection Kit, Fluorescein, Version 17, Nov 2012, Roche Diagnostics, Mannheim, Germany) according to Peña *et al.* (2019a; 2019b). The TUNEL reaction labels DNA damaged cells positive for Fluorescein isothiocyanate (FITC). Multiple control and reaction treatments were prepared including: Unlabelled controls (U1 and U2) incubated in 50 µl PBS; Negative controls (N1 and N2) incubated in 50 µl TUNEL labelling solution without the enzyme; and DNase-treated Positive controls (P1 and P2) and treatment samples incubated in 50 µl TUNEL reaction mixture containing enzyme. In addition, U2, N2, P2 and all treatment

samples were counter-stained with 5 µg/mL of the nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) to ensure that only nucleated TUNEL-positive spermatozoa were accounted for as DNA damaged cells during analysis by flow cytometry. Fluorescent microscopy of stained boar sperm was conducted to confirm specificity of the staining technique showing FITC/DAPI positive DNA damaged sperm in green alongside DAPI positive intact nucleated boar sperm in blue (Peña *et al.*, 2019a; 2019b)

Twenty-thousand cells per sample at about 150 events/sec were evaluated using a CyanADP flow cytometer (Dako Cytomation, Glostrup, Denmark) after filtering samples through a 60 µm nylon woven net filter into 5 mL round-bottom polystyrene tubes. Before treatment samples were analysed, control samples were used to accurately define the different cell staining populations delineated into four distinct quadrants by adjusting both vertical and horizontal thresholds: (i) R3, FITC-positive cells only; (ii) R4, both FITC and DAPI-positive cells; (iii) R5, unstained cells; and (iv) R6, DAPI-positive cells only. Sample N2 (Negative control in Label Solution with DAPI) was used to set a 0.5% threshold cut-off before running all treatment samples, while cells in R4 were considered as nucleated DNA damaged spermatozoa, expressed as a percentage of the total number of cells analysed within the gated area (Peña *et al.*, 2019a; 2019b).

Data Presentation and Statistical Analyses

Data were analysed using IBM SPSS Statistics version 22 (IBM Corporation, NY, USA). Graphs were plotted using Microsoft Excel 2016. The Shapiro-Wilk test was used to evaluate normality of the data while Levene's test was used to determine if variation between groups was homogeneous. Data were Log_{10} transformed for parameters in which the distribution was not normal and variance was heterogeneous. Fresh, 3%, 6%, and 8% frozen-thawed semen samples for each boar were compared by using one-way ANOVA followed by a post-hoc Tukey's HSD test to determine significant differences in sperm DNA damage. A Kruskal Wallis test was used to determine differences in sperm motility (total motility, progressive motility, and motion parameters determined by CASA). If the Kruskal Wallis test was significant, then a post hoc Mann-Whitney U test was used to determine which means differed significantly. The level of significant difference was set to $P \leq 0.05$.

RESULTS

While sperm DNA damage appeared to be slightly lower in fresh spermatozoa, there was no significant difference between fresh or frozen-thawed spermatozoa at each concentration of glycerol (Figure 1a).

Both total and progressively motile spermatozoa were higher in fresh than frozen-thawed spermatozoa ($P \leq 0.05$). There was no difference observed in either motility parameters between frozen-thawed samples cryopreserved in either 3, 6, or 8% glycerol ($P > 0.05$; Figure 1b and 1c). Sperm motility and head shape characteristics determined by CASA are shown in Table 1. There was no difference observed in curvilinear velocity, lateral head displacement, beat cross frequency, and elongation between fresh and cryopreserved samples ($P > 0.05$). By contrast, straight-line velocity, average path velocity, straightness and linearity were significantly higher after cryopreservation using 3 and 6% glycerol (as well as 8% glycerol for straight-line and average path velocities) compared to fresh samples ($P \leq 0.05$), but these parameters did not differ significantly between glycerol treatments ($P > 0.05$; Table 1).

DISCUSSION

Cryopreservation using boar semen is not new however, our study demonstrates that despite the expected reduction in total and progressive motility, DNA damage in boar spermatozoa does not increase when frozen in 3% to 8% glycerol-containing medium. Moreover, it is important to select a glycerol concentration low enough to prevent deleterious effects on sperm motility. Our study suggests that several post-thaw CASA motility parameters are consistently higher using 3% to 6% glycerol. It is yet to be determined whether the motility of such frozen-thawed sperm is of sufficient quality for artificial insemination, but it is clear that the glycerol-based cryopreservation process itself does not induce additional DNA damage that may compromise embryo viability.

The freeze-thaw process can cause reduction of glutathione (GSH) content (Gadea *et al.*, 2004), tyrosine phosphorylation associated with capacitation (Kumaresan *et al.*, 2012), calcium imbalance, and acrosome damage among others (reviewed by Yeste, 2015). Freezing can also compromise sperm DNA integrity (Yildiz *et al.*, 2007) and boar spermatozoa appear to be highly susceptible to cryoinjury and oxidative

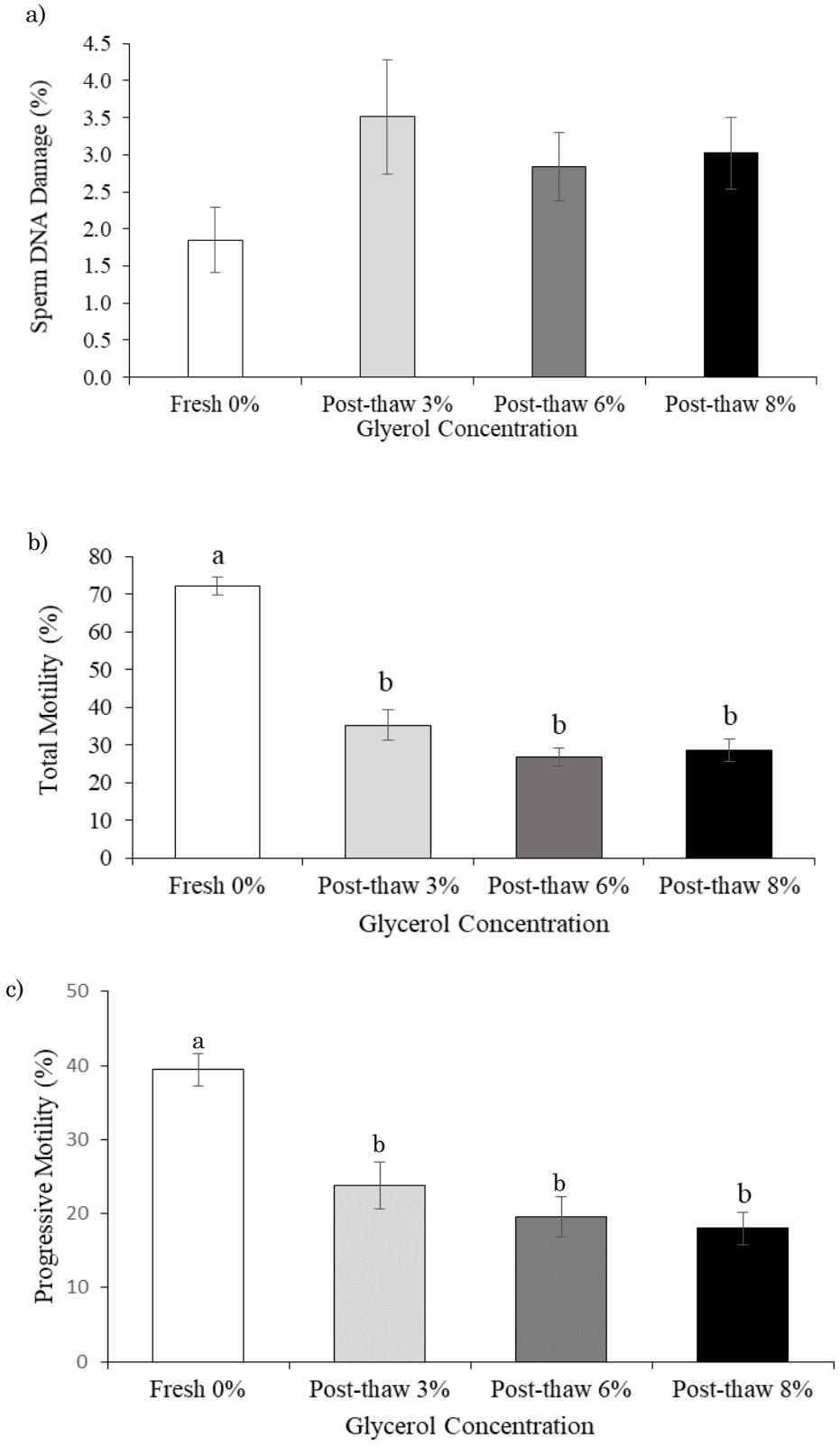

Figure 1. Mean (± SEM) percentage of (a) DNA damage, (b) total motility and (c) progressive motility in fresh and frozen-thawed boar spermatozoa cryopreserved using different concentrations of glycerol. Values not sharing the same letter are significantly different ($P \leq 0.05$); n=6 boars.

Table 1. Mean (± SEM) sperm motility and head shape characteristics between fresh and frozen-thawed boar spermatozoa cryopreserved using different concentrations of glycerol.

CASA Parameter	Fresh (n=6)	Post-thaw		
		3% Glycerol (n=6)	6% Glycerol (n=6)	8% Glycerol (n=6)
VCL	51.7 ± 5.2	55.6 ± 3.0	63.7 ± 5.0	65.9 ± 6.5
VSL	25.3 ± 2.1[b]	33.0 ± 2.3[a]	35.8 ± 2.7[a]	35.6 ± 3.2[a]
VAP	30.2 ± 2.9[b]	37.7 ± 2.3[a]	41.8 ± 3.0[a]	40.9 ± 3.5[a]
ALH	2.6 ± 0.2	2.5 ± 0.2	2.7 ± 0.2	2.7 ± 0.2
BCF	21.4 ± 0.9	18.1 ± 1.8	16.0 ± 2.0	16.6 ± 1.9
STR	79.9 ± 1.9[b]	83.4 ± 2.3[a]	83.9 ± 2.6[a]	83.9 ± 2.2[ab]
LIN	49.6 ± 2.5[b]	58.6 ± 3.1[a]	57.8 ± 2.7[a]	54.7 ± 2.5[ab]
ELONG	79.0 ± 1.8	79.2 ± 3.3	81.0 ± 3.9	82.9 ± 3.7

Values with different letters differ significantly between treatments for each parameter ($P \leq 0.05$); Numbers in parentheses indicate sample size. VCL, curvilinear velocity (μm/sec); VSL, straight-line velocity (μm/sec); VAP, average-path velocity (μm/sec); ALH, amplitude of lateral head displacement (μm); BCF, beat cross frequency (Hertz); STR, straightness (ratio of VSL/VAP); LIN, linearity (ratio of VSL/VCL); ELONG, elongation (ratio in % of head width to head length)

damage due to high levels of unsaturated phospholipids and low levels of cholesterol on the plasma membrane (Rath *et al.*, 2009). In fact, boar sperm with greater than 6% DNA fragmentation was found to cause both decreased farrowing rates and average number of piglets born (Didion *et al.*, 2009) with up to 0.5 to 0.9 fewer piglets born per litter when sperm DNA fragmentation was above 2.1% (Boe-Hansen *et al.*, 2008). Overcondensation of sperm chromatin can also lead to poor conception rates in cryopreserved boar and human sperm (Hamamah *et al.*, 1990). Hence, it is important to protect sperm DNA integrity during sperm cryopreservation via the use of cryoprotectants. In addition, evaluating sperm DNA integrity before and after freezing could provide valuable information about individual boar susceptibility to the freeze-thaw process (Holt *et al.*, 2005). Using TUNEL, it was found that there is no difference in the proportion of DNA damage between fresh and frozen-thawed spermatozoa treated with 3, 6, and 8% glycerol, respectively. The study appears to support earlier results using neutral comet assay that 3% glycerol in either a lactose-hen egg yolk or a lactose-ostrich egg yolk extender protects sperm DNA integrity better than the extender alone (Fraser and Strzezek, 2007). Although not significant, 6% glycerol provided the lowest rate of post-thaw sperm DNA damage (2.8%) across the n=6 boars that were tested. This study thus confirms the protective effect of glycerol in maintaining sperm DNA integrity during freezing using these standard protocols.

Evaluation of sperm motility is one important parameter to detect semen with poor potential fertility (Holt *et al.*, 1997; Tardif *et al.*, 1999; Vyt *et al.*, 2008). Moreover, motility can be a sensitive indicator of any deleterious effect that high concentrations of glycerol may have during the freeze-thaw process. Motility and acrosomal integrity of boar spermatozoa frozen with 0% and 8% glycerol were significantly lower than those frozen with 2% and 4% glycerol (Buhr *et al.*, 2001) while 5% glycerol exhibits maximum toxicity in stallion sperm possibly due to osmotic and non-osmotic effects (Macias Garcia *et al.*, 2012). Glycerol at 3% yielded better motility and plasma membrane integrity than other cryoprotectants such as dimethylacetamide (DMA) and dimethyl sulfoxide (DMSO; Kim *et al.*, 2011) but produced poorer post-thaw sperm quality than the non-permeable cryoprotectant trehalose (Athurupana *et al.*, 2015).

In this study, motility of spermatozoa before and after freezing in different concentrations of glycerol was evaluated using CASA. As expected, there was a significant drop to less than 35% in total and 25% in progressively

motile spermatozoa post-thaw at each glycerol concentration. Although not significant, the highest post-thaw total (35%) and progressive motility (24%) was achieved at 3% glycerol. Interestingly, straight-line velocity, average path velocity, linearity, and straightness parameters were higher in frozen-thawed than fresh boar spermatozoa, particularly at 3% and 6% glycerol. While we did not examine the capacitation status in our post-thawed samples, the freeze-thaw process may have initiated physiological changes to the sperm plasma membrane leading to capacitation and early upregulation of motility (Gillan *et al.*, 1997; Thundathil *et al.*, 1999). Cryopreservation-induced capacitation has been reported in bovine spermatozoa (Pons-Rejraji *et al.*, 2009) as well as capacitation-like changes in equine spermatozoa (Thomas *et al.*, 2006).

While utilisation of frozen-thawed semen in pig AI is very limited, it is not clear whether this slight improvement in some post-thaw motility parameters would be an advantage. Under field conditions, multiple intra-cervical inseminations of boar spermatozoa with post-thaw motility greater than 50% resulted in farrowing rates of nearly 80% and a litter size of 12.5 (Didion *et al.*, 2013), showing the potential of frozen boar semen in the pig industry. However, lower rates of motility should still be suitable for use in pig IVF, where spermatozoa with average post-thaw total motility of 38% has been used (Daigneault *et al.*, 2014). Interestingly, post-thaw motility of cryopreserved boar spermatozoa does not appear to predict penetration rates nor IVF success (Martínez *et al.*, 1993; Suzuki *et al.*, 1996). More recently however, increased monospermic penetration rates and blastocyst formation in porcine IVF were further improved by freezing spermatozoa at lower concentrations than normal (20×10^6 *vs.* 1000×10^6 sperm/ml) at about 60% motility (Martinez *et al.,* 2019). Collectively, this suggests that the success of porcine IVF using frozen-thawed spermatozoa is not limited by low sperm motility, with factors like sperm concentration appearing to play an important role. That said, more research is needed to further improve post-thaw sperm motility in the boar to facilitate commercial application in the pig industry.

In conclusion, the results demonstrate that boar semen can be successfully cryopreserved using 3% to 8% glycerol as cryoprotectant in boar freezing medium without inducing further sperm DNA damage. A glycerol concentration of 6% tends to provide slightly better DNA protection and 3% to 6% glycerol generally provides post-thaw improvement across several motility parameters. As such, glycerol appears to mitigate freezing-induced sperm DNA damage that may compromise early embryo survival.

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STATEMENT ON COMPETING INTEREST

The authors have no competing interest to declare.

AUTHORS' CONTRIBUTION

All authors equally contributed to this work.

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