ORIGINAL ARTICLE

ANALYSIS OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY IN ITIK PINAS–ITIM (*Anas platyrhynchos* L.) SEMEN CRYOPRESERVED USING EITHER GLYCEROL OR DIMETHYL SULFOXIDE (DMSO) AS CRYOPROTECTANT

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ABSTRACT

Itik Pinas (IP), an improved Philippine native breeder duck, is gaining popularity due to a better egg laying performance. In aid of its local production and conservation, this study assessed the suitability of reported semen cryopreservation protocols through evaluation of sperm quality (i.e., plasma membrane integrity, motility, and morphology) and superoxide dismutase (SOD) activity of IP-Itim semen before and after cryopreservation using either 8% glycerol or 10% dimethyl sulfoxide (DMSO) as cryoprotectant in AU extender. Results showed that semen cryopreserved using 10% DMSO has higher post-thawing motility (M) with a lesser motility decrease (DM) compared to 8% glycerol (p = 0.0112). Meanwhile, the average SOD activity of semen cryopreserved with 10% DMSO and 8% glycerol increased from the pre-processing baseline level of 8.50 U/10^9 sperm to 10.45 U/10^9 sperm and 11.76 U/10^9 sperm, respectively, suggesting an increase in the oxidative stress in sperm cells during the freezing process. This study has demonstrated that 10% DMSO is a much suitable cryoprotectant than 8% glycerol for IP-Itim semen. However, testing the SOD activity from seminal plasma is recommended to acquire the total SOD activity of the cryopreserved semen and determine if SOD enzymes within the spermatozoa leak to the seminal plasma.

Keywords: *cryopreservation, Itik-Pinas, oxidative stress, semen, superoxide dismutase*

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INTRODUCTION

Over the years, several attempts to preserve avian semen had been conducted for the purpose of artificial reproduction, animal breeding, genetic diversity conservation, and genetic improvement (Wishart, 1985; Han *et al*., 2005; Barbas and Mascarenhas, 2009; Gerzilov, 2010; Blanch *et al*., 2014; Çiftci and Aygün, 2018; Thélie *et al*., 2019). Such goals drove the development of cryopreservation procedures that would allow storage of semen for a longer period before recovering them again for their intended purpose. Cryopreservation is a process used to store cells or tissues at a very low temperature while maintaining their biological function. This involves a step-by-step cooling process and use of cryoprotectants to protect any biological form from the damaging effects of exposing them to extremely low temperatures (Jang *et al*., 2017). The success of this procedure relies on optimum cooling conditions, appropriate freezing medium, and the type and inclusion rate of cryoprotectant (Pegg, 2015).

Cryoprotectants, such as glycerol and DMSO, are solutes which regulate intracellular ice crystal formation to reduce severe cell structural damage during freezing (Elliott *et al*., 2017). Apart from intracellular ice crystal formation, overaccumulation of reactive oxidative species (ROS) such as superoxide $O_2^{\bullet-}$ anion during and after semen processing can also compromise cell structures (Len *et al*., 2019). ROS are normally produced by sperm cells to regulate functions such as maturation, hyperactivation, acrosome reaction, capacitation, movement, and fertilization (Dutta *et al*., 2020). To balance its generation and

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utilization, the cell has its own antioxidant enzyme system to catalyze the dismutation of these ions (Bansal and Bilaspuri, 2011) however, antioxidant enzymes can only react to physiological levels of ROS.

Superoxide dismutase (SOD), one of the antioxidant enzymes in a cell, catalyzes the dismutation of $O_2^{\bullet-}$ to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) to prevent cell damage (Siddique *et al.*, 2013). It has three isoforms namely, cytosolic (Cu, Zn-) SOD, mitochondrial (Mn-) SOD, and extracellular SOD (EC-SOD) (Surai, 2015; Surai *et al.*, 2019). The function of these enzymes is crucial in preventing highly reactive $O_2^{\bullet-}$ from oxidizing, and thereby damaging fats, proteins, and DNA (Aitken, 2000) in a cell. When SOD and the whole antioxidant system of a cell is overwhelmed by excessive ROS generation, oxidative stress which results in structural damage occurs. Along with membrane integrity, sperm motility, and sperm morphology evaluation, the activity of this enzyme is a good indicator of sperm quality. The involvement of this enzyme in scavenging superoxide anions makes it a good indicator of oxidative stress in cryopreserved semen.

Meanwhile, the differences in response of sperm to several cryopreservation procedures make it difficult to develop one standard protocol for avian species. One species of interest in developing semen cryopreservation protocol for genetic resource conservation is the Philippine native duck called Itik Pinas (IP)-Itim. The IPs are breeder ducks that were cultivated to achieve greater egg production with low-cost expenditure (Parungao, 2016). These ducks can acclimate to local environmental conditions and produce well even with plain housing and inexpensive feeds. Their strains resulted from systematized breeding and selection that concentrated on improved physical attributes, enhanced and predictable egg production performance, and consistent egg quality (Parungao, 2016). IP has three strains: the two purelines IP-Itim and IP-Khaki, and one commercial hybrid line IP-Kayumanggi (Parungao, 2017). The overarching goal of this study is to contribute information that can be used in the wider production and conservation of IP ducks through semen processing technologies and AI. Cryopreservation of tIP drakes' semen can increase the drake-to-duck ratio, and this increase the probability of egg being fertilized. In the long term, the increase in hatchability may increase the duck and egg production that will positively affect the food sufficiency and sustainability. Specifically, the aim of this study was to evaluate the sperm quality (i.e., plasma membrane integrity, motility, and morphology) and to determine the SOD activity of Itik Pinas (IP)-Itim semen processed via adaption of reported cryopreservation protocols by Han *et al.* (2005) using either 8% glycerol or 10% DMSO as cryoprotectant in AU extender. Han *et al.* (2005) studied post-thawing semen motility using different cryoprotectants and concentrations where 8% glycerol and 10% DMSO showed the highest post-thawing motility.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños (UPLB) with assigned protocol number CAFS-2018-006. The study was conducted in University Animal Farm (UAF) in Putho-Tuntungin, Los Baños, Laguna, Region IV-A, Philippines (14°09'24.4"N, 121°15'06.6"E) and Animal Physiology Laboratory at the Institute of Animal Science, College of Agriculture and Food Science (IAS-CAFS), University of the Philippines Los Baños from September 2020 to January 2021.

Animals and sample collection

Ten 29-month old IP-Itim drakes were housed in individual cages and labeled from Itim 1 to Itim 10. A caretaker was assigned to maintain proper husbandry conditions and a stress-free environment, wherein the drakes followed an optimized daily routine and were fed with standard commercial diets. Semen samples were collected individually using a sterilized glass funnel through abdominal massage method (Burrows and Quinn, 1935). The collected semen samples were siphoned with a sterile 1cc syringe and placed in a sanitized insulated box before transporting at room temperature from the UAF to the Animal Physiology Laboratory for evaluation and processing. A total of 12 collections were done in the entire experiment.

Fresh Semen Evaluation

The collected semen samples per animal were characterized based on volume, color, consistency, and pH. The volume of individual samples was measured using 1cc syringe while the color (i.e., white, cream or yellow) and consistency (i.e., thick, thin or watery) were recorded through visual observation. The pH was determined using commercially available pH strips. Only individual semen samples with pH $\geq$ 7.0 were pooled.

An aliquot (20 µL) from each pooled semen sample was diluted in AU extender at 1:23 ratio (v/v) and was examined using the computer-assisted

sperm analyzer (CASA) (Ceros II, IMV Technologies, Shanghai, China) to determine the total sperm motility (%). The total sperm motility (%) is computed by CASA based on static, progressive, motile, and slow movements of the sperm cells. Briefly, about 1.0 µL of the diluted semen sample was placed on a clean glass slide. The sample was then viewed under the microscope which was equipped with a high-performance camera connected to the Animal Breeder Software of the CASA system. A total of five captured frames per sample were used for sperm motility analysis. Meanwhile, the composition of the AU extender used in this study was based on the optimized protocol of Gerzilov *et al.* (2011) in Muscovy ducks, which is composed of 0.2 g D-glucose, 0.4 g D-fructose, 0.4 g sugar, 0.45 g sodium citrate, 0.42 g monosodium glutamate, 0.2 g glycine, 0.02 g ethylenediaminetetraacetic acid (EDTA), and 50 ml sterile distilled water.

Sperm concentration was determined by manually counting the cells using a Neubauer haemocytometer (Capitan and Palad, 1999). Sample was drawn from the pooled semen up to the 0.5 mark of the RBC dilution pipette. Then, the staining solution (0.1% eosin in 3.0% NaCl solution) was drawn until it reached the 101 mark of the same pipette. The pipette was gently swirled to mix the sample thoroughly. Stained sample was loaded onto the Neubauer chamber (Max Levy, Philadelphia, USA). The cells within the four corner boxes and the center box with 5 × 5 counting chambers were counted under a high-power objective (400×).

Sperm Plasma Membrane Integrity Evaluation

Sperm plasma membrane integrity or percent (%) cells with intact membrane was evaluated using the hypo-osmotic swelling test (Jeyendran *et al.*, 1984; Balogun *et al.*, 2020). An aliquot (20 µL) of fresh or cryopreserved semen was added to 200 µL of hypo-osmotic solution (0.46 g sodium citrate and 0.8775 g fructose in 100 mL) that was prewarmed at 37°C. The sperm suspension was incubated in a water bath at 37°C for an hour and 30 minutes. After incubation, 5 µL of sperm suspension was mixed with 45 µL eosin stain. A drop of sperm suspension + stain was placed on a glass slide and gently covered with a coverslip. The prepared slide was observed under oil immersion objective (1,000×), then a minimum of 200 spermatozoa was counted to observe different swelling types. Results were expressed in percentages.

Semen cryopreservation

Pooled semen samples with CASA-assessed total sperm motility of ≥ 70% were used in cryopreservation. The general freezing procedure of Han *et al.* (2005) was optimized for the semen of IP-Itim drakes. Here, the pooled semen sample was diluted in AU extender at 1:3 ratio (v/v) and incubated at 5ºC for 2 hours as initial equilibration. While equilibrating the sample, the freezing extender was prepared by diluting glycerol (Life Technologies Corp., California, USA) or DMSO (Vivantis Inc., California, USA) in AU extender to a final inclusion level of 8% and 10%, respectively. The 8% glycerol and 10% DMSO were selected as they demonstrated the highest post-thawing motility based on the study made by Han *et al.* (2005). The volumes of the AU extender added in 8% glycerol and 10% DMSO were adjusted to make the final sperm count per 500 µL of samples to 200 million. After 2 hours of initial equilibration, the freezing extender was added to the samples. A 500 µL aliquot of samples were pipetted in 1.5-mL microcentrifuge tubes and sealed with laboratory film. The samples were then refrigerated (5ºC) for another 2 hours. After this, the tubes were placed 5 cm above the liquid nitrogen surface for 10 minuts before plunging and storing in liquid nitrogen for at least 24 hours.

Post-thawing sample evaluation

All cryopreserved samples (i.e., with either 8% glycerol or 10% DMSO) were thawed at 37°C for 1 minute. Post-thawing total sperm motility was determined using the CASA. The plasma membrane integrity of post-thawed semen samples was also evaluated using the hypo-osmotic swelling test as previously described.

Superoxide Dismutase (SOD) Assay

Fresh semen samples were stored according to the procedure by Partyka *et al.* (2012). A 100 µL semen was centrifuged at 6,000 x g for 1 minute. The sperm pellet was obtained by discarding the seminal plasma. The sperm pellet was washed twice with phosphate-buffered saline (PBS) and centrifuged. After centrifugation, 500 µL sterile distilled water was added to the sperm pellet and the tubes were immediately stored at -80°C until analysis. Similarly, thawed semen samples were also centrifuged at 6,000 x g for 1 minute to remove the freezing medium, washed twice with PBS, and centrifuged. The same amount, 500 µL of sterile distilled water, was also added to the sperm pellet. The spermatozoa (fresh and cryopreserved) were frozen-thawed thrice to lyse the cells and centrifuged at 8000 rpm for 1 minute to pellet some cell debris. The supernatant

or the cell lysate was used for the assay.

Superoxide dismutase activity (U/10^9) in fresh and cryopreserved sperm was determined using commercially available SOD Colorimetric Assay Kit (Life Technologies Corp., California, USA). The SOD standards were prepared by diluting the SOD concentrate (2 U/mL) with the assay buffer to a final concentration (U/mL) of 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0. Prior to the assay, cell lysates from both fresh and cryopreserved samples were diluted 1:10 with the assay buffer. Ten (10) µL of each sample and standard were added to each well, followed by 50 µL of 1X substrate and 25 µL of 1X xanthine oxidase. Then, the reaction was incubated for 20 minutes at room temperature. Using the Thermo Scientific™ MultiSkan Sky Microplate Spectrophotometer (Life Technologies Holdings Pte Ltd, Singapore), the absorbance at 450 nm was measured. The SOD quantification protocol in the SkanIt™ Software for Microplate Readers was selected to calculate the SOD activity (U/mL) in each sample. SOD activity in U/10^9 cells was calculated based on the final sperm count of the samples.

Statistical analysis

All data for semen parameters like Motility (M), Membrane Integrity (MI), SOD activity (SOD), Decrease in % Motility (DM), Decrease in Membrane Integrity (DMI), and Change in SOD Activity (CSOD) were first tested for normality and homoscedasticity using Shapiro-Wilk's test and Levene's test, respectively. All data parameters satisfied both assumptions except for Change in SOD Activity which was heteroscedastic. Data parameters satisfying both assumptions were analyzed using the parametric two-sample t-test while data for Change in SOD Activity were analyzed using the Welch's t-test. Moreover, after testing for normality and homoscedasticity of residuals, the Pearson Correlation Coefficient was used to measure the relationship between semen parameters through R statistical software (Lucent Technologies, Inc., New Jersey, USA).

RESULTS AND DISCUSSION

Samples were collected from ten (10) IP-Itim drakes maintained in UAF. Qualitative and quantitative semen characteristics such as color, consistency, volume, pH, concentration, motility, and membrane integrity were evaluated from the collected semen samples. All individually collected semen samples appeared cream in color with 79.75% thick and 20.25% thin consistency. Individual semen samples with pH $\geq$ 7.00 were pooled. The observed pH of individual semen samples used for pooling ranges from 7.00 to 7.60 with a mean of 7.30 ± 0.08. These pH values were slightly higher from the reported values of Pekin drakes (Taşkin *et al.*, 2020) and Mallard drakes (Zawadzka, 2015). Meanwhile, the volume of pooled semen samples ranges from 0.24 mL to 0.84 mL with a mean volume of 0.48 ± 0.20 mL. Other semen parameters like concentration, motility, and membrane integrity were also observed from the pooled semen samples. The obtained sperm concentration ranges from 3.58 × 10^9 cells/mL to 6.96 × 10^9 cells/mL with a mean of 5.52 ± 1.1 × 10^9 cells/mL. The total sperm motility observed using CASA ranges from 73.2% to 87.2% with a mean of 77.85 ± 4.83%. In this study, the observed sperm concentration was higher than the reports in Muscovy and Mallard ducks with an average of 5.20 × 10^9 cells/mL and 4.27 × 10^9 cells/mL, respectively (Oguntunji *et al.*, 2019). While for motility, the observed values were higher compared to the reported average in Muscovy drakes (Chen *et al.*, 2016) and Mallard drakes (Zawadzka *et al.*, 2015). On the other hand, the observed membrane integrity percent ranges from 51.30% to 91.40% with a mean of 75.5%, ± 14.62%. There are no reported membrane integrity values using HOS Test for duck semen. However, compared to roosters (Najafi *et al*., 2019), the observed average membrane integrity in IP-Itim drakes is higher. All mentioned characteristics are summarized in Table 1.

DMSO as CPA. As shown in Table 2, the mean % Motility of semen cryopreserved using DMSO is significantly higher than glycerol (p-value=0.0112) with concomitant lesser % Decrease in Motility (p-value=0.0075) (Figure 1). These findings agree with the study of Han *et al.* (2005), which showed higher post-thawed sperm motility using 10% DMSO than 8% glycerol. DMSO has decreased toxicity when combined with sugars (Pires *et al.*, 2018). The AU extender contains glucose, fructose, and sucrose. The sugar-loaded extender probably lessened the cytotoxic effects of DMSO. Bhattacharya (2018) also stated that DMSO along with electrolytes and sugar have minor cytotoxicity effects when cold and freezes at 18.5°C. These freezing properties of DMSO make it a suitable cryoprotectant. Thus, during the equilibration period or slow cooling process, DMSO transforms into solid and easily freezes the sperms. Meanwhile, the observed values for % Membrane Integrity and % Decrease in Membrane Integrity showed no significant differences between CPAs suggesting that both cryoprotectants comparatively stabilize the sperm

Table 1. Range and mean ± SD values of semen characteristics of IP – Itim.

Traits	Parameter Range/Value	Mean ± SD
Volume (mL)	0.24 – 0.84	0.48 ± 0.20
pH[a]	7.00 – 7.60	7.30 ± 0.08
Color[a] (%)	Cream - 100	
Consistency[a] (%)	Thick - 79.75 Thin - 20.25	
Concentration (× 10^9 cells/mL)	3.58 – 6.96	5.52 ± 1.10
Motility (%)	73.20 – 87.20	77.85 ± 4.83
Membrane Integrity (%)	51.30 – 91.40	75.50 ± 14.62
SOD Activity (U/10^9 sperm)	4.98 – 15.15	8.50 ± 2.75

[a]samples before pooling; SD, standard deviation; SOD, superoxide dismutase

membrane against damage during freezing (Cabrita *et al.*, 2001).

Diluted pooled semen samples were equally divided and cryopreserved using either 8% glycerol or 10% DMSO as CPA. As shown in Table 2, the mean % Motility of semen cryopreserved using DMSO is significantly higher than glycerol (p-value=0.0112) with concomitant lesser % Decrease in Motility (p-value=0.0075) (Figure 1). These findings agree with the study of Han *et al.* (2005), which showed higher post-thawed sperm motility using 10% DMSO than 8% glycerol. DMSO has decreased toxicity when combined with sugars (Pires *et al.*, 2018). The AU extender contains glucose, fructose, and sucrose. The sugar-loaded extender probably lessened the cytotoxic effects of DMSO. Bhattacharya (2018) also stated that DMSO along with electrolytes and sugar have minor cytotoxicity effects when cold and freezes at 18.5°C. These freezing properties of DMSO make it a suitable cryoprotectant. Thus, during the equilibration period or slow cooling process, DMSO transforms into solid and easily freezes the sperms. Meanwhile, the observed values for % Membrane Integrity and % Decrease in Membrane Integrity showed no significant differences between CPAs suggesting that both cryoprotectants comparatively stabilize the sperm membrane against damage during freezing (Cabrita *et al.*, 2001).

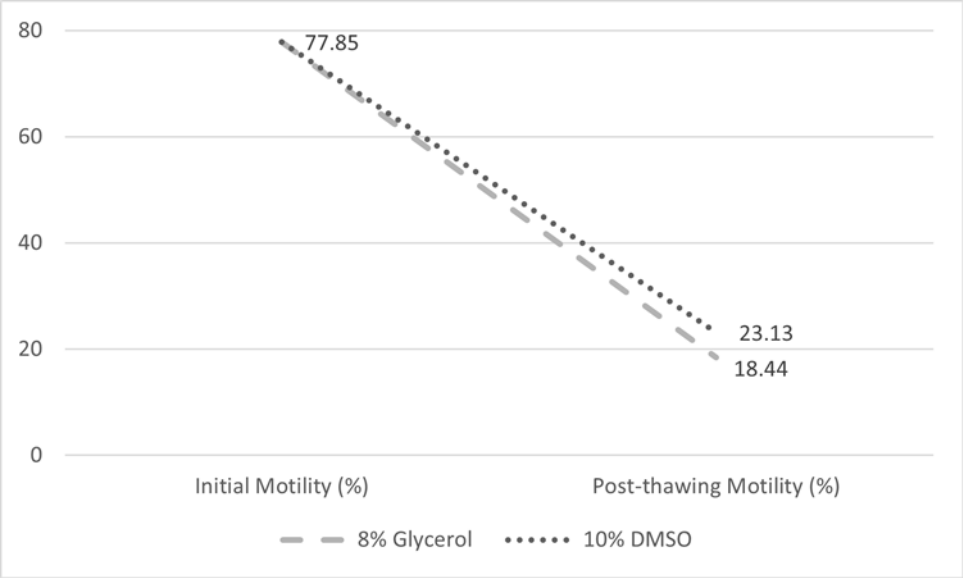

Figure 1. Percentage decrease in total sperm motility of IP – Itim semen after cryopreservation using either 10% dimethyl sulfoxide (DMSO) or 8% glycerol as CPA. The percentage decrease in motility was found to be significant ($p<0.05$) between CPAs.

Table 2. Mean ± SD values of semen characteristics of IP-Itim cryopreserved using different CPA.

PARAMETERS	CRYOPROTECTANT		*P*-VALUE
	10% DMSO (Mean ± SD)	8% Glycerol (Mean ± SD)	
Motility (%)	23.13 ± 2.84	18.44 ± 5.12	0.0112*
Membrane Integrity (%)	20.28 ± 9.12	18.88 ± 9.29	0 .7146
Superoxide dismutase Activity ($U/10^9$)	10.45 ± 1.70	11.76 ± 2.03	0.1012
Decrease in Motility (%)	69.92 ± 4.92	76.34 ± 5.73	0.0075*
Decrease in Membrane Integrity (%)	73.40 ± 11.93	75.36 ± 11.94	0.6924
Change in SOD Activity (%)	28.19 ± 22.38	49.54 ± 53.25	0.2189

*$p \leq 0.05$, significant; SD, standard deviation; DMSO, dismutase sulfoxide; SOD, superoxide dismutase

In this study, SOD assay was used to assess the oxidative stress level of fresh and processed spermatozoa. The observed mean SOD Activity in the fresh semen samples was 8.50 ± 2.75 $U/10^9$ (Table 1). After cryopreservation, the observed mean SOD Activity of post-thawed semen using DMSO was 10.45 ± 1.70 $U/10^9$ while 11.76 ± 4.67 $U/10^9$ for glycerol (Figure 2). However, these results showed no significant difference between groups for both the SOD Activity and Change in SOD Activity (Table 2).

Results of this study are different from previously observed Change in SOD Activity of other processed semen. Studies in chicken (Partyka *et al.*, 2012), human (Lasso *et al.*, 1994), and fish (Huang *et al.*, 2021) showed a decrease in SOD activity after cryopreservation suggesting degradation or damage of the SOD enzymes due to the freeze-thaw process where the sperm plasma membrane ruptures, leading to the leakage of the SOD enzymes to the seminal plasma. In this current study, results showed an increase in SOD activity for both treatments. The report of Ismail *et al.* (2020) demonstrated that SOD activity increases during the early stages of stress to prevent lipid peroxidation thus, an increasing SOD activity suggests an increasing cellular stress level. However, SOD concentration is limited inside the cell and its activity is depleted during prolonged stress as characterized by overwhelming concentration of ROS (Yan *et al.*, 2014). The observed increase in the SOD

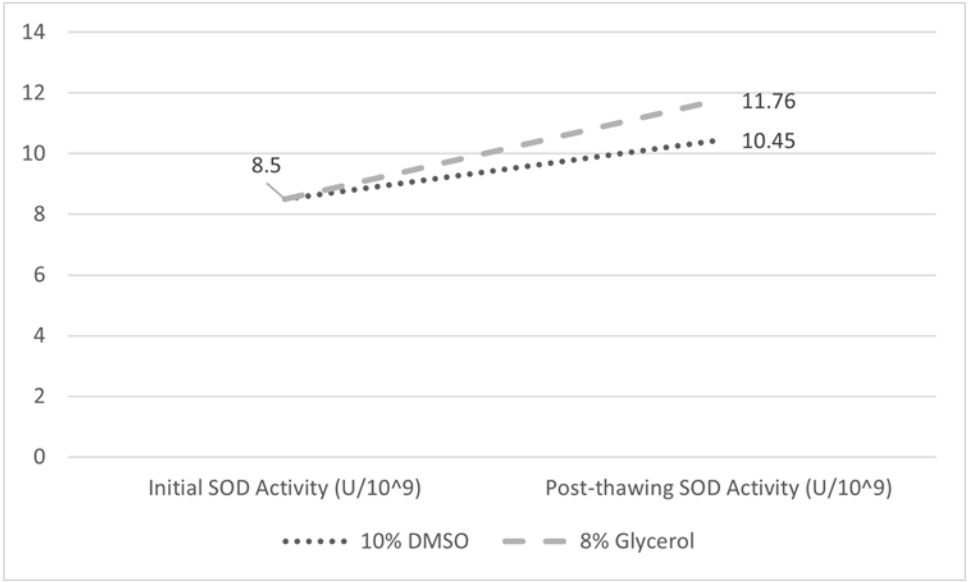

Figure 2. Percentage increase in superoxide dismutase (SOD) activity of IP–Itim semen after cryopreservation using either 10% dimethyl sulfoxide (DMSO) or 8% glycerol as CPA. The percentage increase in SOD activity was comparable ($p>0.05$) between CPAs.

activity in the current study may be attributed to the possible substantial concentration of SOD enzymes and/or a more resilient sperm plasma membrane in IP-Itim resulting in more intact sperm after thawing. This led to a higher activity of SOD enzymes that was captured and detected during assay. Ideally, sampling from each step including final equilibrium and before subjecting the processed semen in liquid nitrogen, should have been done for stepwise stress level assessment however, the low semen volume and kit availability are limitations of the study. Thus, the overall stress effect of the processes involved was considered. Pearson correlation coefficient was used to determine the relationship between semen parameters (i.e., Motility (M), Membrane Integrity (MI), SOD Activity (SOD), Decrease in Motility (DM), Decrease in Membrane Integrity (DMI), and Change in SOD Activity (CSOD)) of samples cryopreserved within each experimental group (i.e., 10% DMSO or 8% glycerol). Results are summarized in Tables 3 and 4.

Table 3. Pearson correlation coefficient of semen characteristics in IP-Itim cryopreserved using 10% DMSO.

TRAIT	M	MI	SOD	DM	DMI	CSOD
M	-					
MI	0.3448	-				
SOD	0.5013	0.1057	-			
DM	-0.9375*	-0.0754	-0.4326	-		
DMI	-0.1441	-0.8792*	-0.0268	-0.0332	-	
CSOD	0.0012	0.2324	-0.0089	0.0794	-0.2926	-

*$p \leq 0.05$, significant; M, motility; MI, membrane integrity; SOD, superoxide dismutase activity; DM, decrease in motility; DMI, decrease in membrane integrity; CSOD, change in superoxide dismutase activity

Table 4. Pearson correlation coefficient of semen characteristics in IP-Itim cryopreserved using 8% glycerol.

TRAIT	M	MI	SOD	DM	DMI	CSOD
M	-					
MI	0.1337	-				
SOD	0.0947	-0.2235	-			
DM	-0.983*	-0.1223	-0.1355	-		
DMI	-0.0706	-0.9040*	0.2226	0.0685	-	
CSOD	0.2779	-0.2003	0.7395*	-0.3082	0.1167	-

*$p \leq 0.05$, significant; M, motility; MI, membrane integrity; SOD, superoxide dismutase activity; DM, decrease in motility; DMI, decrease in membrane integrity; CSOD, change in superoxide dismutase activity

Table 3 shows a significant (p-value ≤ 0.05) very strong inverse relationship between M and DM. A similar relationship was also depicted between MI and DMI among frozen-thawed semen samples with DMSO as CPA. On the other hand, the correlation between M and SOD showed a moderate positive relationship and demonstrated a trend (p-value ≤ 0.10). Other observed correlations of varying magnitude (i.e., very weak to weak) were found to be insignificant.

As shown in Table 4, there is also a significant very strong inverse relationship between M and DM among semen samples cryopreserved using glycerol. Similar very strong inverse relationship was also observed between MI and DMI. In contrast, the correlation between SOD and CSOD had a significant strong positive relationship while the rest of the observed correlations were insignificant.

M and MI both showed a significant decrease after cryopreservation for both experimental groups. Cryopreservation may result in decreased sperm motility due to change in cellular structure and function (Ozkavukcu *et al.*, 2008). Studies on human sperm by Oberoi *et al.* (2014) proved that sperm motility decreased after the freeze-thaw cycle. Other studies on avian species like chicken (Shanmugam and Mahapatra, 2019), peregrine falcon (Cardoso, 2020), and capercaillie (Kowalczyk and Lukaszewicz, 2015) also reported a decrease on sperm motility after cryopreservation. Several damaging processes, such as formation of intracellular and extracellular ice crystals leading to thermal shock, cellular dehydration, osmotic shock, and damaged mitochondrial membrane can occur during freezing and thawing of sperm (Oberoi *et al.*, 2014). Plasma membrane integrity of spermatozoa is important for motility, but this is often damaged during cryopreservation (Zhang *et al*., 2021). Semen with low membrane integrity due to lipid peroxidation, rupturing, or apoptosis, will result in low motility (Ramirez *et al.*, 1992). The stress that spermatozoa underwent because of cryopreservation can decrease their plasma membrane integrity thus, decreasing their motility (Lin *et al*., 1998). Overall, sperm morphology, motility, and plasma membrane integrity are susceptible to cryopreservation-induced damage (O'Connell *et al.*, 2002). Within glycerol, SOD and CSOD exhibit a correlation. A trend (p-value ≤ 0.10) with positive correlation was also observed between M and SOD within DMSO. Other studies showed that motility has a strong correlation with SOD activity (Yan *et al*., 2014). Semen cryopreservation is considered as a stressful process that leads to the generation of ROS. These ROS are neutralized by cellular antioxidants like SOD (Surai, 2016; Surai *et al*., 2019) which stabilizes the sperm by preventing oxidation of its components and DNA (Aitken, 2000). Although, despite the observed increase in the SOD activity, the demonstrated post-thawing sperm motility was still relatively low for both experimental groups. This suggests the need to further optimize the protocol for semen cryopreservation of IP-Itim and to consider other important factors affecting their sperm motility and viability during processing.

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STATEMENT ON COMPETING INTEREST

The authors have no competing interests to declare.

AUTHOR'S CONTRIBUTION

JLMS took care of methodology, investigation, formal analysis, visualization, writing, and editing**.** MML was involved with methodology, investigation, writing, and editing. GAD handled methodology and PPS participated in the conceptualization, supervision, writing, and editing.

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