Original Article

Appraisal of ATP1B1 and GSTM3 proteins as freezability factors in buffalo ejaculated spermatozoa

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Abstract

Sperm cryopreservation intensifies the use of assisted reproductive technologies in food animals. However, variations in freezability confront frozen-thawed semen applications. Hence, this groundwork aimed to identify the fundamental differences between high (HF) and low freezability (LF) ejaculated spermatozoa in swamp buffaloes, which were limitedly described for this agricultural species. Twelve (12) different samples of bubaline semen were collected, cryopreserved and categorized into freezability phenotypes using post-thaw sperm motility as the basis. Sperm kinematics and functional plasma membrane integrity were evaluated in HF and LF samples using computer-assisted sperm analysis (CASA) and hypo-osmotic swelling test, respectively. The relative concentrations of some selected sperm proteins namely ATPase subunit beta 1 (ATP1B1) and glutathione S-transferase Mu 3 (GSTM3) were also comparatively quantified using dot blot and image processing analyses. The results revealed that all CASA parameters except wobble and straightness were significantly higher in HF than LF cluster ($P<0.05$). Moreover, HF spermatozoa also exhibited more intact plasma membranes under a hypo-osmotic state than the LF group ($P<0.05$). However, there was no significant difference in the abundance of ATP1B1 and GSTM3 proteins among freezability phenotypes, suggesting that other proteins related to energy production and oxidative stress protection influence bubaline ejaculated sperm cryoresilience. Nevertheless, these findings reaffirmed the existence of variabilities in sperm cryopreservation outcomes in buffaloes and established the key differences between HF and LF bubaline ejaculated spermatozoa in terms of motility, kinematics and plasma membrane integrity.

Keywords: Buffalo, Cryopreservation, Freezability, Protein, Spermatozoa

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Introduction

Water buffaloes (*Bubalus bubalis*) are integral components of agricultural-based economies as a source of meat, milk and draught power in smallholder farming communities. Compared to cattle, buffaloes present numerous merits – resilience to harsh environmental conditions, resistance to many bovine diseases and the ability to make use of poor quality roughage (Warriach *et al*., 2015). However, these benefits are negated by their inherent poor reproductive characteristics. In males, a slower period of maturity and a longer interval between spermatogenesis and puberty confront current bubaline production systems (Perera, 2008). Hence, attempts have been made to overcome these challenges through the development, improvement, optimization and utilization of assisted reproductive technologies (ARTs) such as cryopreservation and artificial insemination (AI) (Yulnawati *et al*., 2013; Bertol *et al*., 2016).

Yet, these procedures are not without their drawbacks. The successive phases of cryopreservation elicit some deleterious structural and functional changes to the spermatozoa (Ugur *et al*., 2019). Moreover, the use of bubaline cryopreserved semen in artificial breeding does not always yield acceptable outcomes, leading to low conception rates (Gordon, 1996). The sperm's capacity for fertilization and embryonic development also varies between buffaloes of comparable post-thaw semen qualities (Misra *et al*., 1999). Furthermore, the individual variations in sperm cryotolerance, cryoresistance or freezability (Casas *et al*., 2009; Vilagran *et al*., 2014; Rego *et al*., 2016; Hitit *et al*., 2020) limit the contemporary field applications of frozen-thawed semen. Irrespective of species, ejaculates are broadly categorized as either high (or good) or low (or poor) freezability. In the bull, high freezability spermatozoa are typified by higher motility, kinematics, viability and mitochondrial membrane potential than their low freezability counterparts (Rego *et al*., 2016; Ryu *et al*., 2019) although these attributes are yet to be clarified in bubaline ejaculates of contrasting freezability.

With the advent of omics technologies, researchers have steered their investigation towards the identification of molecular entities present in the seminal plasma and/or spermatozoa that may elucidate such andrological variabilities. While it is essential to discover alternative extender additives and/or refine prevailing protocols to enhance spermatozoa's resilience to freezing and thawing, the search for putative freezability biomarkers is also imperative to aid in the prediction of sperm's response to cryopreservation and in the selection of freezing-tolerant semen which could ultimately improve assisted reproductive technology efficiency in buffaloes. To date, diverse protein signatures have been correlated with freezability differences in assorted domestic livestock (Khan *et al*., 2021; Salinas *et al*., 2021) but not in the water buffalo where proteomic studies are rather scarce. These proteins play critical roles in various sperm functions and reproduction-associated events, including motility, maintenance of cell integrity, capacitation, acrosome reaction, sperm-oocyte membrane fusion and embryonic development (Moura and Memili, 2016). In particular, adenosine triphosphatase subunit beta (ATP1B1) involved in energy synthesis and glutathione-S-transferase (GST) implicated in oxidative stress response have been purported to be candidate freezability markers in the bull epididymal spermatozoa (Ryu *et al*., 2019). Taking this into account, it was hypothesized that these same proteins from a related ruminant species may also be employed in buffalo sperm freezability classification.

Therefore, this work aimed to characterize the differences between high and low freezability bubaline ejaculates using conventional parameters and validate the utility of selected protein biomarkers in distinguishing cryoresistance phenotypes in water buffaloes.

Materials and Methods

All experimental procedures carried out in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Chiang Mai University (No. 8393 (18) 5.2/ 2.038) following international standards.

Semen collection and cryopreservation: Ejaculates were collected from 5 to 15-year-old buffalo bulls using an artificial vagina. Twelve samples that reached at least 80% motility were selected and processed. Semen extension was achieved using the usual tris-citric acid-egg yolk mixture. Cryopreservation was executed with the conventional two-step dilution procedure with glycerol as the cryoprotectant (Salinas *et al.* 2022).

Freezability determination: The freezability of each cryopreserved ejaculate was based on its post-thaw motility. Samples of buffalo semen that acquired >40% total motility and >15% progressive motility were classified as high freezability (HF) whereas those that did not attain the set cut-off values were regarded as low freezability (LF) (Vincent *et al*., 2014).

Computer-assisted sperm analysis (CASA): To exclude subjectivity bias, a computer-assisted sperm analyzer (Androvision, Minitube®, Germany) was employed in the post-thaw motility assessment of the buffalo ejaculated spermatozoa. Likewise, the CASA system was used to evaluate the velocity, trajectory and kinematics of bubaline freezability groups. After thawing, a pre-warmed (37 °C) glass slide was loaded with a small drop (5 µL) of the semen and topped with a pre-warmed (37 °C) coverslip. The sample was allowed to settle for a minute before the analysis. The manufacturer's predetermined software setting for buffalo sperm was utilized to establish the following parameters in each ejaculate: total motility (expressed in %), progressive motility (PMOT, %), curvilinear velocity (VCL, µm/sec), straight-line velocity (VSL, µm/sec), average path velocity (VAP, µm/sec), DCL (distance curved line, µm), DAP (distance average path, µm), DSL (distance straight line, µm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm), head activity (HAC, Rad) and beat/cross-frequency

(BCF, Hz). Each measurement was made from at least 500 spermatozoa in five random vision fields.

Hypo-osmotic swelling test (HOST): The plasma membrane integrity of buffalo ejaculate freezability clusters was determined using the hypo-osmotic swelling test. Briefly, 100 µl of the frozen-thawed semen was added to 1000 µl of the HOST solution (150 mOsm/kg) in a 1.5 microcentrifuge tube. After incubation at 37°C for 60 mins, a 10 µl drop was placed on a glass slide topped with a coverslip and examined at 400x magnification under the light microscope. A total of 200 spermatozoa were assessed per sample for their tail swelling or coiling. Such a reaction to the HOST solution indicates intact plasma membranes whereas its absence (straightness of the tails after the assay) otherwise denotes damaged membranes.

Dot blot analysis: Extracted proteins from the buffalo ejaculated spermatozoa were diluted with PBS, vortexed and incubated for 5 mins at 95 °C. A 0.2 µm pore-size Immun-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) was subjected to methanol treatment then tris-buffered saline Tween-20 (TBST) washing. A Whatman filter paper was also prepared by soaking in a TBST solution. The membrane and filter paper were stacked together to avoid drying of the former during the protein spotting procedure. A total of 3 µL of diluted proteins was dropped in the membrane per spot. After drying at room temperature, the PVDF membrane was rinsed again with TBST for 5 mins. Blocking was carried out for 30 mins at RT using 5% bovine serum albumin (BSA, Bio Basic, Markham, ON, Canada). After washing for 3 mins with TBST, the membrane was incubated with the primary antibodies (1:200) namely, mouse monoclonal anti-ATPase subunit beta (ATP1B1) [464.8] (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA), monoclonal rat anti-β-actin (BioLegend, San Diego, CA, USA) and mouse monoclonal anti-Glutathione S-Transferase (Biolegend, San Diego, CA) for 2 hrs at RT with agitation. Subsequently, the membranes were rinsed again with TBST for 3 mins then incubated with the horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG secondary antibody (clone Poly4053, BioLegend, San Diego, CA, USA) for 45 min at RT. Following the final wash with TBST for 3 mins, signal visualization was accomplished with the 3,3′-diaminobenzidine (DAB) protocol. The abundance of ATP and GST proteins in each ejaculate were compared with β-actin as the internal control. Relative expressions of the two proteins of interest were computed from the band optical density ratios generated using Image Studio Lite Ver. 5.2.

Statistical analysis: Data was analyzed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Values obtained for all parameters were expressed as mean with standard error (mean ± SEM). T-test or Mann–Whitney U test was applied to calculate the statistical differences between freezability groups, following the evaluation for normal distribution with Shapiro–Wilk test. The significance level was set at $P<0.05$.

Results

Classification of swamp buffalo ejaculates into freezability groups: Ejaculates from twelve individual buffaloes, which met the minimum requirements for cryopreservation, were categorized into high (HF) and low freezability (LF) clusters based on their post-thaw motility. This classification resulted in 6 HF and 6 LF samples. Table 1 reveals the significant differences in the total (P=0.0001) and progressive (P=0.0037) motilities between the freezability groups as quantified by CASA. Overall, HF buffalo ejaculates demonstrated higher motility values than their LF counterparts.

Variations in motion, velocity, kinematics and plasma membrane integrity of HF and LF buffalo ejaculates: Table 2 differentiates buffalo ejaculate freezability clusters in terms of motion, velocity, kinematics and plasma membrane integrity. Results showed that nearly all CASA parameters including VCL (P=0.0131), VSL (P=0.0041), VAP (P=0.0048), DCL (P=0.0054), DSL (P=0.0091), DAP (P=0.0035), ALH (P=0.0069), BCF (P=0.005), HAC (P=0.0022), and LIN (p=0.0130) were significantly greater in HF compared to LF. On the other hand, WOB (P=0.0541) and STR (P=0.1637) did not considerably vary between freezability groups. Moreover, a substantial number of spermatozoa with intact plasma membranes were observed in HF than LF buffalo ejaculate under hypo-osmotic conditions (P <0.001).

Differences in ATP1B1 and GSTM3 relative expressions of HF and LF buffalo ejaculates: Dot blot results for ATP1B1 and GSTM3 proteins of HF and LF were normalized using β-actin as the internal standard (Fig.1). The normalized amounts of the two proteins of interest are presented in Fig.2 for ATP1B1 and Fig. 3 for GSTM3. The relative expression of ATP1B1 was greater in HF compared to LF, implying a certain trend towards significance (P=0.08) (Fig.1). Likewise, GSTM3 relative concentrations tend to be comparable among freezability groups (P=0.85) (Fig.2), with higher amounts noted in LF than HF.

Table 1 Post-thaw motility of buffalo ejaculates employed in screening for semen freezability

Semen Parameter	Freezability Group	
	HF	LF
Total Motility (%)	53.93±3.77	26.31±2.47***
Progressive Motility (%)	18.39±3.93	3.47±0.56**

Data is presented as mean ± standard error of the mean. Symbol representation: ***$p < 0.001$, **$p<0.01$. Abbreviation: HF= high freezability, LF= low freezability.

Table 2 Differences in motion, velocity, kinematics and plasma membrane integrity of high (HF) and low (LF) freezability buffalo ejaculates

Semen Parameter	Freezability Group	
	HF	LF
VCL)µm/s(	33.92±5.99	15.68±0.93*
VSL)µm/s(	15.51±2.45	6.27±0.48**
VAP)µm/s(	19.84±3.06	8.55±0.70**
DCL)µm/s(	8.58±1.19	4.30±0.24**
DSL)µm/s(	2.96±0.51	1.27±0.10**
DAP)µm/s(	4.27±0.57	2.00±0.18**
ALH (µm)	0.45±0.06	0.24±0.02**
BCF (Hz)	4.36±0.87	1.17±0.16**
HAC (Rad)	0.15±0.02	0.08±0.00**
WOB (%)	58.50±1.18	54.17±1.60
LIN (%)	45.67±1.54	39.80±1.17*
STR (%)	78.00±2.44	73.70±1.54
PMI (%)	67.92±1.02	43.58±2.13****

Data is presented as mean ± standard error of the mean. Abbreviation: VCL=Curvilinear velocity, VSL=Straight line velocity, VAP=Average path velocity, DCL= Distance curved line, DSL= Distance straight line, DAP=Distance average path, ALH=amplitude of the lateral head displacement, BCF=Beat cross frequency, HAC=Head activity, WOB=wobble (VAP/VCL), LIN=Linearity, STR=Straightness (VSL/VAP), PMI= Plasma membrane integrity. Symbol representation: ****$p < 0.0001$, **$p<0.01$, *$p<0.05$.

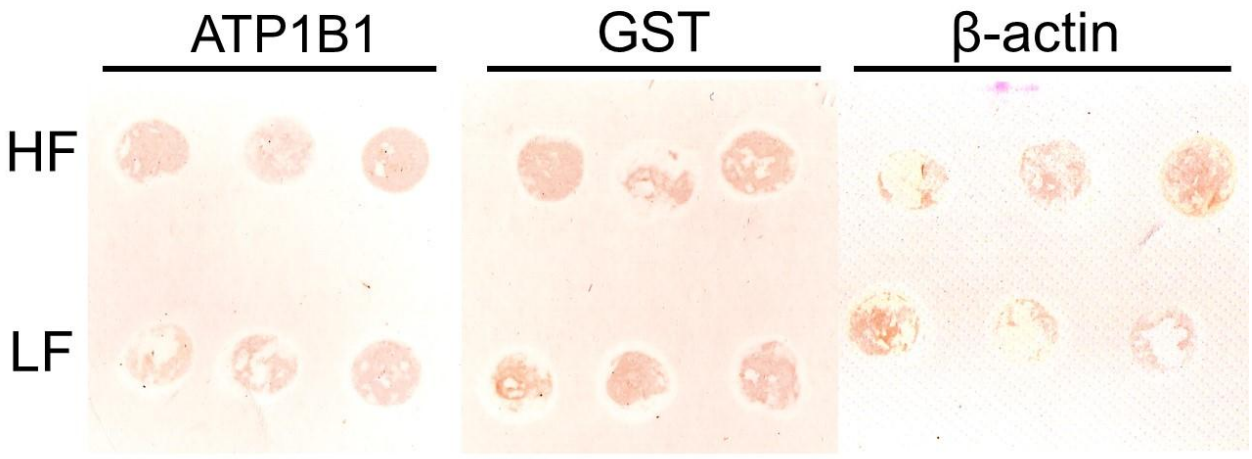

Figure 1 Representative dot blot results of ATP1B1 and GSTM3 from high (HF) and low (LF) freezability buffalo ejaculates (n= 3, each group). Beta-actin was used as the housekeeping protein to normalize protein expression.

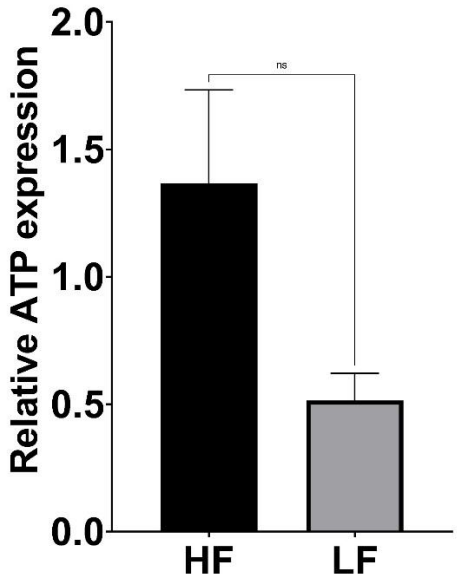

Figure 2 Comparison of ATP1B1 relative expression in high (HF) and low (LF) freezability buffalo ejaculates (n=12). Bars represent the mean ± SEM protein volumes of the freezability groups in two independent experiments normalized with β-actin as the internal control (ns=not significant, analyzed by Mann-Whitney test).

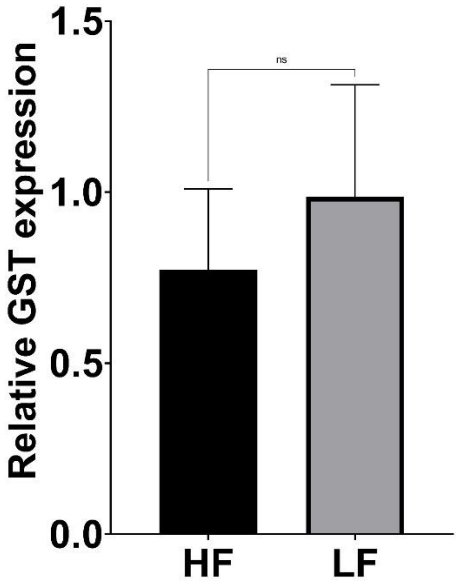

Figure 3 Comparison of GSTM3 relative expression in high (HF) and low (LF) freezability buffalo ejaculates (n=12). Bars represent the mean ± SEM protein volumes of the freezability groups in two independent experiments normalized with β-actin as the internal control (ns=not significant, analyzed by Mann-Whitney test).

Discussion

The ability of the spermatozoa to endure the cryopreservation procedures while sustaining their structural integrity and functionality denotes their inherent freezability (Martínez-Fresneda *et al.*, 2021). This characteristic, however, presents significant individual variations among farm animals such as pigs (Vilagran *et al.*, 2014; Llavanera *et al.*, 2019), cattle (Rego *et al.*, 2016; Hitit *et al.*, 2020), and sheep (Rickard *et al.*, 2016), with the water buffaloes as no exception. Consequently, this was highlighted in this study since such information about sperm freezability differences in the bubaline species remains elusive. Preceding ruminant-centered reports use different gauges to discriminate between high (HF) and low freezability (LF) samples (Salinas *et al.*, 2021). Nevertheless, post-thaw motility and viability proved to be consistent parameters in these works and thus, were employed in this investigation.

Concomitant to the findings from other livestock (Vilagran *et al.*, 2014; Rego *et al.*, 2016; Rickard *et al.*, 2016), HF buffalo ejaculated spermatozoa were also more motile compared to the LF group. This, therefore, substantiates the freezability classification made in this study. Motility remains a mainstay in the post-thaw spermiogram in buffaloes, mainly due to its correlation with a number of semen features such as concentration, morphology and fertilizing capacity. It is also a function of the integrity of the sperm plasma membranes and the optimal production of intracellular adenosine triphosphate (ATP) (Singh *et al.*, 2014). Hence, the maintenance of total and progressive motilities in HF sperm may perhaps reflect their better mitochondrial integrity and activity undeterred by cryopreservation-induced stresses than their LF counterparts. In bovine (Ryu *et al.*, 2019) and equine (Yeste *et al.*, 2015) models, mitochondrial membrane potential (MMP) was also found to be higher in the good rather than poor freezability spermatozoa or quality semen.

The sperm plasma membrane is among the initial structures affected by the freeze-thaw procedures (Peris-Frau *et al.*, 2020), thus, the importance of assessing its integrity in freezability experiments cannot be overstated. In water buffaloes, plasma membranes were found to be more intact in the HF cluster than the LF one. This observation corroborates with earlier works (Rego *et al.*, 2016; Ryu *et al.*, 2019), suggesting that resilience of the sperm membranes against cryopreservation differs among freezability phenotypes. The variability in the fatty acid and sterol constituents of the sperm plasma membranes has long been implied to cause differences in semen processing outcomes among species (Gautier and Aurich, 2021). Such disparities in membrane constitution might likewise impart for the male-to-male plasma membrane integrity and freezability variations in the buffalo ejaculates. In Holstein cattle, greater amounts of arachidic and oleic acids, which are associated with oxidative stress protection and membrane fluidity regulation, were measured in the good freezability spermatozoa (Evans *et al.*, 2020). In addition, differential levels of membrane polyunsaturated fatty acids were implicated in the post-thaw sperm survival variations within and between breeds in Norwegian Landrace and Duroc pigs (Waterhouse *et al.*, 2006).

Aside from low motility, correspondingly low speed (VCL, VSL and VAP), distance (DCL, DSL and DAP), velocity ratio (LIN) and wobble features (ALH, BCF and HAC) were also exhibited by the LF bubaline ejaculated spermatozoa compared to HF. On the other hand, the differences in WOB and STR between freezability categories were negligible. Consistent with these results, investigations in other animals also demonstrated that high freezability sperm tend to be faster and more linear than the low freezability ones (Rego *et al.*, 2016; Bubenickova *et al.*, 2020). The greater fertilizing capacity of the bubaline spermatozoa has

also been associated with higher motion kinematics (Del Rei *et al*., 2007; Ahmed *et al*., 2016). These absolute and derived CASA parameters offer objective and valuable information about the movement characteristics in buffalo semen with contrasting freezabilities, which were limitedly described in the literature.

While there were characteristic variations in the conventional sperm parameters of HF and LF groups as validated by this present study, the definitive factors that impart for such freezability variability need to be elucidated. Differences in the concentrations of specific proteins were initially implicated in the freezing resilience of the buffalo spermatozoa (Asadpour *et al*., 2007). In cattle, these proteins play pivotal functions in energy metabolism, sperm protection from osmotic and oxidative stresses, maintenance of sperm membrane integrity, among others (Asadpour *et al*., 2007; Rego *et al*., 2016; Ryu *et al*., 2019; Gomes *et al*., 2020). Nonetheless, these cannot be detected in standard seminogram and require an in-depth comprehensive and comparative approach such as proteomics.

In this work, selected proteins namely ATPase beta subunit (ATP1B1) and glutathione-S-transferase Mu 3 (GSTM3), which were previously correlated with sperm freezability in cattle (Ryu *et al*., 2019; Gomes *et al*., 2020), were explored for their involvement in the cryoresilience of the bubaline ejaculated spermatozoa. The former was associated with energy production for sperm motility and kinematics in high freezability bovine epididymal sperm (Huang *et al*., 2015; Davila *et al*., 2016; Ryu *et al*., 2019), whereas the latter was an antioxidant enzyme crucial in protecting the spermatozoa from excessive reactive oxygen species production in low freezability bovine epididymal sperm (Kumar *et al*., 2014; Ryu *et al*., 2019; Llavanera *et al*., 2020). Their occurrence in the buffalo ejaculated sperm supports the idea that proteins with such roles are necessary for the functionality of cryopreserved mature spermatozoa in adult ruminants (Westfalewicz *et al*., 2021). However, neither of these proteins differ in abundance between buffalo HF and LF sperm. Interestingly, these findings deviate from the prior reports in pig and cattle (Llavanera *et al*., 2019; Ryu *et al*., 2019), which can be explained by the species-specific peculiarities in sperm and seminal plasma composition. The process of cryopreservation has been shown to alter the proteome of pig (Perez-Patiño *et al*., 2019), bull (Westfalewicz *et al*., 2015) and ram (Lv *et al*., 2020) spermatozoa. These quantitative changes can be exploited to identify novel putative markers for freezability and other sperm or semen attributes. Yet, this is not true in the case of this investigation as ATP1B1 and GSTM3 may not be able to predict or discriminate buffalo ejaculate freezability. This implies that other proteins, molecular entities and their underlying mechanisms and interactions contribute to bubaline sperm survival to freeze-thaw procedures which would require a thorough analysis using omics technologies equipped with bioinformatics.

In conclusion, this work underscored the occurrence of freezability differences in the bubaline ejaculated spermatozoa, as earlier established in other species of agricultural importance. While fundamental variations in motility, viability and kinematics in the buffalo sperm were demonstrated between high and low freezability phenotypes, assessment of alternative parameters such as acrosomal integrity, DNA fragmentation, mitochondrial function and oxidative stress status should be considered in future research. Finally, full characterization of the protein profiles of the seminal plasma and ejaculated and epididymal spermatozoa must also continue in the buffaloes in order to clarify the freezability conundrum for the improvement of cryopreservation and AI outcomes.

Conflicts of Interest: The authors declare no conflicts of interest.

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