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RESEARCH ARTICLE

Changes in the Phospholipid Asymmetry of Buffalo Sperm Plasma Membrane in Relation to Cryopreservation Technology

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ABSTRACT

This study evaluates the integrity of the plasma membrane (PM), and the mitochondrial transmembrane potential and their relationship with the motility and kinematic parameters of buffalo spermatozoa frozen in pellets and straws. Ejaculates (n=20) from eight Bulgarian Murrah buffaloes were frozen in pellets (Nagaze-Niva; medium GH22L®) and in straws (Cassou; medium Triladyl®) at final sperm concentration 80×10^6/ml. The comparative CASA analysis of the motility and kinematic parameters of spermatozoa showed significant differences in the rapid motility and in the VCL values in favor of pellets vs straws ($P<0.05$). The molecular changes in PM phospholipid asymmetry (assessed by double staining Annexin V-Cy3™ Apoptosis Detection Kit) were higher after freezing – thawing, when compared to the fresh semen ($P<0.05$). No significant differences were observed in the number of live and functional spermatozoa with intact PM (6CF+/Ann V Cy3-) and the number of apoptotic spermatozoa (6CF+/Ann V Cy3+) between the two freezing technologies. The percentage of spermatozoa with well preserved and functioning mitochondria visualized by Rhodamine123 (Rh123) in the fresh semen was higher, when compared to spermatozoa after cryopreservation ($P<0.05$). An increase in the percentage of cells with nonfunctional mitochondria (Rh123- or Rh123+) was observed after cryopreservation using both freezing technologies. In conclusion, the freezing of buffalo sperm in pellets, using GH22L® protective medium, preserves high sperm motility, kinematics, PM integrity, and mitochondrial transmembrane potential, which is a guarantee of good fertilization capacity of the spermatozoa.

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INTRODUCTION

The sperm PM is a complex mosaic structure organized in discrete macrodomains that reflect compositional and functional heterogeneity. PM is characterized with unusual proportion of polyunsaturated phospholipids that give a special physical characteristics and compartmentalization of different proteins and lipids into the following domains - acrosome, postracosome, equatorial segment, mid-piece and tail (Holt and North, 1984). The interest in the structural-functional organization and the alterations of sperm surface in cryobiology is caused by the high sensitivity of PM to low temperatures. A delicate reorientation and modification of PM molecules take place during sperm cryopreservation. It has been found that the bilayer lipid structure of the PM is vulnerable to varying degrees but is particularly sensitive in the region of the acrosome domain. Such a seemingly elusive effect may affect the fertilization capacity of the sperm cells at different levels. For example, it could influence the spatially dependent signaling pathways required for the acrosomal exocytosis, the development of membrane fusogenicity and antigen migration (Yanagimachi, 1994).

Low and ultralow temperature storage of spermatozoa is accompanied by structural and functional changes in PM and mitochondria (Celeghini *et al*., 2008; Dziekońska *et al*., 2009). The cryopreservation also affects the PM lipid ratio, such as decrease in the phospholipid content through loss of phosphatidylcholine and phosphatidy-

lethanolamine (Saragusty *et al*., 2005). These molecular changes of PM lead to destabilization of the lipid bilayer and functional disturbance of the membrane proteins. PM lowers its barrier function, which is the cause of abnormal distribution of biologically active ions. Bailey and Buhr (1994) have demonstrated that the access of Ca^{2+} in the cell increases due to the cryopreservation process. Studies on the mechanism of cellular death in blood cells, have found that the elevation of intracellular calcium levels leads to floppase inactivation and scramblase activation. Such alterations in the membrane proteins result in translocation of phosphatidylserine and its expression on the extracellular surface of PM (Jones *et al*., 2013). Under normal physiological conditions, membrane asymmetry is maintained by integral membrane proteins which act as lipid transporters. It is supposed that freezing and thawing result in disturbances in the functioning of these proteins and their enzymatic activity and, accordingly, leads to alterations of the PM surface (Duru *et al*., 2001; Kumar *et al*., 2016). That's why the membrane integrity and mitochondrial activity are important viability parameters of spermatozoa and fluorescent techniques based on membrane permeability to dyes have been developed to determine these parameters.

Although the pellet freezing method is not widely used in practice for a number of reasons, we are interested in the determination of motility and kinematic parameters as well as the integrity of PM and mitochondria of the spermatozoa when compared to these values when using the biotechnology of freezing in straws. Because the structural and functional integrity of PM and mitochondria is critical for fertilization, the aim of the current study was to analyze whether the externalization of phosphatidy-lserine on the outer monolayer of the PM in different regions of the spermatozoa may affect motility, kinematic parameters and functional competence of spermatozoa, and whether these changes are due to the biotechnology for freezing of buffalo spermatozoa.

MATERIALS AND METHODS

Ejaculates (n=20) from eight Bulgarian Murrah buffaloes (2 ejaculates from 4 bulls and 3 ejaculates from the other 4 bulls) were collected all year round in the period 2017-2018 year. The ejaculates were obtained at one week interval between collections. 50µl from each ejaculate was used for fresh semen evaluation. The rest of the ejaculate was divided into two equal parts that were frozen in pellets and in straws at sperm concentration 80×10^6/ml. The pellets (0.5ml) were frozen using cryoprotective medium GH22L® (Kichev and Danov, 1975) on dry ice (Nagaze-Niva method). The straws (0.25ml) were frozen using Triladyl® (Minitube) using programmable freezer (Digitcool®, IMV Technologies; Cassou method). All semen samples (pellets and straws) were stored in liquid nitrogen (-196°C) for a minimum of 72h. The pellets were thawed at 37°C for 5-8 sec using 0.5ml 2.8% sodium citrate. The straws were thawed at 37°C for 30 sec. All thawed samples were incubated at 37°C in air humidified incubator (Binder GmbH, Tuttlingen) for 10 min before CASA.

Computer-assisted sperm analysis (CASA): Motility, progression, velocity and kinematics of fresh and frozen-thawed spermatozoa were analyzed using Leja®20 slides on Sperm Class Analyzer® (Microptic). The studies were performed by "Motility & Concentration" software on: Progressive motility; Non-progressive motility; Rapid motility; Curvilinear Velocity (VCL); Straight-line Velocity (VSL); Average Path Velocity (VAP); Linearity (LIN); Straightness (STR); Wobble (WOB) and Head Area.

Morphological analysis of PM status: The integrity of the PM was evaluated using double staining Annexin V-Cy3™ Apoptosis Detection Kit (Sigma-Aldrich). The studies were performed on fresh and frozen-thawed spermatozoa by protocol adapted for use with spermatozoa. Two-fold washing of sperm cells with binding buffer (1×BB) (Sigma-Aldrich) by centrifugation at 300×g for 3 to 5min was performed to remove seminal plasma and media. The spermatozoa were resuspended in 1×BB up to a final concentration of $0.5\text{-}1\times10^6$/ml. 50µl of the cell suspension were added on Poly-L-Lysine treated slides. The staining procedure followed the Annexin V-Cy3™ Apoptosis Detection Kit (Sigma-Aldrich) protocol. The stained spermatozoa were evaluated using fluorescence microscope Olympus® System Microscope BX51 at 40× and 100× magnification. The protein Annexin V in the Annexin V Cy3 (λ=532 nm) binds specifically to the phosphatidylserine which may be externalized on the outer monolayer of the PM. This binding is visualized as red fluorescence. The 6-carboxyfluorescein diacetate (6CFDA) dye is used to evaluate sperm viability. When this non-fluorescent component enters living cells, the esterases cause its hydrolysis, producing the fluorescent 6-carboxyfluorescein (6CF; λ=492nm), visualized as green fluorescence.

Using cell counter Rathenow® Eltinor, 200 spermatozoa from each sample were evaluated. Each sperm cell was evaluated by successively using filters for 6CF (λ=492nm) and for Cy3 (λ=532 nm). The results were reported in three groups: Live and viable spermatozoa with preserved PM integrity (6CF+/Ann V Cy3-); Apoptotic - live with initial molecular changes in PM (6CF+/Ann V Cy3+); Dead (6CF-/Ann V Cy3+).

Determination of mitochondrial transmembrane potential: Mitochondrial specific fluorescent dye Rh123 (Sigma-Aldrich) was used to determine the mitochondrial bioenergetics in living cells. The staining procedure followed the Rh123 (Sigma-Aldrich) protocol. The stained spermatozoa were evaluated using fluorescent microscopy (λ=534nm). Rh123 is accumulated in the mitochondria in response to the electrochemical gradient created by the mitochondrial transmembrane potential. The classification of the spermatozoa was based on the intensity of Rh123 absorption: spermatozoa with non-functional mitochondria (-; no fluorescence); live spermatozoa with disturbances of the mitochondrial transmembrane potential (+; low fluorescence); normal spermatozoa with functionally preserved mitochondria (++; bright fluorescence). All results are presented as mean±SEM. Statistical significance was determined by Student's T-test.

RESULTS

The ejaculates used in the experiments had the following motility parameters of spermatozoa after collection measured by CASA (in%): static 4.68±1.56; non-progressive motile 7.55±1.89; progressive motile 87.77±2.75. The comparative analysis of the motility after thawing showed no statistical differences in the initial progressive motility of spermatozoa between the two freezing biotechnologies. It is noteworthy that there were significant differences in the rapid motility with high significance level in favor of the spermatozoa frozen in pellets vs straws (P<0.05) (Table 1).

Analysis of the kinematic parameters showed statistically significant differences only in the VCL values between the two biotechnologies (P<0.05), while VSL, VAP, LIN, STR and WOB showed no significant differences. The data of the head area showed a tendency for higher values in the spermatozoa frozen in pellets.

The results from the morphological analysis of the PM status are presented on Table 2. The molecular changes in phospholipid asymmetry of the PM are significantly higher after freezing– thawing, when compared to fresh semen (P<0.05). No significant differences were observed in the number of live and functional spermatozoa with intact PM (6CF+/Ann V Cy3-) and the number of apoptotic spermatozoa (6CF+/ Ann V Cy3+) between the two freezing technologies. However, it was noteworthy that the semen frozen in pellets showed tendency for lower number of apoptotic spermatozoa with commencing changes in the PM: live cells with initial phosphatidylserine externalization - apoptotic (6CF+/Ann V Cy3+) (Table 2). Also, the numbers of dead spermatozoa (6CF-/ Ann V Cy3+) were at close range for the two freezing biotechnologies.

The results from the analysis of mitochondrial transmembrane potential are presented on Table 3. The data showed that the percentage of spermatozoa with well-preserved and functioning mitochondria in the fresh semen was significantly higher, when compared to sperm cells after cryopreservation (P<0.05). An increase in the percentage of cells with nonfunctional mitochondria (Rh123- or Rh123+) was observed after cryopreservation using the two freezing technologies.

Table 1: Comparison of motility, velocity and kinematics of buffalo spermatozoa after freezing-thawing – pellets and straws

Parameters	Pellets	Straws
Progressive motility (%)	36.90±1.77	33.45±1.10
Non-progressive motility (%)	56.33±2.61	50.65±2.67
Rapid motility (%)	66.66±3.99[a]	36.90±3.45[b]
VCL (μm/s)	70.16±4.26[a]	51.33±3.33[b]
VSL(μm/s)	32.63±3.48	26.08±2.82
VAP (μm/s)	49.99±3.15	37.21±2.32
LIN (%)	41.03±1.50	54.20±3.60
STR (%)	66.03±3.60	65.60±2.97
WOB (%)	61.73±2.60	71.63±1.66
Head Area (μm^2)	33.33±2.15	27.20±1.92

Values within row with different superscripts (ab) are statistically different (P<0.05).

Table 2: Comparative analysis of the integrity of PM by double staining Annexin V Cy3™ Apoptosis Detection Kit of fresh and frozen-thawed buffalo spermatozoa

Groups	Ann V $Cy3^{+/-}$/ $6CF^{+/-}$ (n=20)		
	Live 6CF+/Ann V Cy3-	Apoptotic 6CF+/Ann V Cy3+	Dead 6CF-/Ann V Cy3+
Fresh	90.17±5.75[a]	3.30±0.89[a]	6.53±2.13[a]
Pellets	54.84±6.16[b]	20.82±4.08[b]	24.34±3.82[b]
Straws	48.16±9.62[b]	32.85±4.53[b]	18.99±7.76[b]

Values within column with different superscripts (ab) are statistically different (P<0.05)

Table 3: Comparative analysis of mitochondrial transmembrane potential measured by Rh123 of fresh and frozen-thawed buffalo spermatozoa

Groups	Rh123 (N = 8)		
	(-)	(+)	(+ +)
Fresh	9.63±3.73[a]	7.30±2.78[a]	83.07±5.85[a]
Pellets	20.33±2.78[b]	38.32±5.29[b]	41.35±4.43[b]
Straws	23.94±2.77[b]	39.00±4.55[b]	37.06±3.07[b]

Values within column with different superscripts (ab) are statistically different (P<0.05).

DISCUSSION

With the present study we demonstrated that buffalo spermatozoa preserved good motility after cryopreservation, regardless of the cryobiotechnology used. At the same time, however, significantly higher molecular changes in the mid-peace region of the PM were registered after thawing compared to fresh semen. The phospholipid organization of the PM is not statically fixed but rather it is characterized by permanent transport of FL between the two layers (Bennett and Healy, 2008; Fajardo *et al*., 2011; Machnicka *et al*., 2014). The phospholipid asymmetry of the PM is maintained by the activity of three transporter proteins - floppase, translocase and scramblase (Hankins *et al*., 2015; Kodigepalli *et al* 2015). The cryopreservation process may induce changes in the membrane which lead to disturbances in the functions of these transporter proteins. Alterations in PM could generate a collapse of the phospholipid asymmetry, expressed by equalization of the individual classes of phospholipids in the bilayer (Shaikh *et al*., 2001; Vance, 2008; Fadeel and Xue, 2009; Braverman and Moser, 2012). The persistent expression of phosphatidylserine on the membrane surface is considered to be one of the earliest signs of the process of apoptosis in somatic cells (Segawa *et al*., 2011). In this regard, the assessment of molecular changes and function of PM may be a useful indicator for the fertilizing ability of spermatozoa after in vitro storage and cryopreservation.

The alterations in mitochondrial status are associated with decrease in energy potential and functionality of the spermatozoa. The results demonstrated a significant increase in the number of spermatozoa with phosphatidylserine translocation in the mitochondrial region in the form of granular clusters with helical conformation. These changes were also accompanied by disturbances in the mitochondrial transmembrane potential and are expressed by significant lowering of the rapid motility and VCL of the spermatozoa frozen in straws, when compared to those frozen in pellets. The correlation of PM phosphatidylserine translocation and mitochondrial transmembrane potential changes is suggestive of an early apoptosis phenotype as is typically observed in somatic cells. In the present study those changes were identified in sperm population with low rapid motility and VCL. We speculate that such changes may be used as diagnostic markers for dysfunctions of spermatozoa with lower freezability and reduced fertility potential.

It can also be said that the alterations in phosphatidylserine behavior, accompanied by specific structural signs in the PM, correspond to the status of the intracellular structures, such as mitochondria. We suppose that the presence of apoptotic phenotypic traits in the PM (established *in vitro* in our research), is the result of the cryobiotechnology used rather than the physiological processes prior to ejaculation. Some authors suggested that cell apoptosis is the main mechanism by which the spermatozoa die or are eliminated during spermatogenesis (Martin *et al*., 2004; Fathi Najafi *et al.*, 2012; Dogan *et al*., 2013). With the results of this investigation we add new data to the knowledge on programmed cell death in spermatozoa from buffaloes. We suppose that there is a possibility for cell death associated with caspase independent pathway or apoptosis inducing factor (AIF). Probably the mitochondrial transmembrane potential abnormalities resulting from the cryopreservation process are the cause for AIF to exit mitochondria and to cut 50 kb DNA fragments.

Conclusions: Although the pellet freezing method is not widely used in practice for a number of reasons, the present study proves that the freezing of buffalo sperm in pellets, using GH22L® protective medium, preserves rapid sperm velocity and high VCL values of the spermatozoa, and showed tendency for better results on the integrity of PM and mitochondrial transmembrane potential, when compared to straws. These sperm biological parameters are of great importance and guarantee the good fertilization potential of the spermatozoa.

Authors contribution: Conceived and designed the study: MGI and DGG; Executed the experiment: MGI, DGG, TST, MSG and BAG; Analyzed the data: MGI and BAG.

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