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QUANTITATIVE EVALUATION OF BUFFALO SEMEN BY CASA DURING CRYOPRESERVATION PROCESS

DHARMENDRA KUMAR[1*], PRADEEP KUMAR[2] AND P.S. YADAV[3]

Animal Physiology and Reproduction Division
ICAR-Central Institute for Research on Buffaloes, Hisar - 125 001

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ABSTRACT

The present study evaluated the quantitative impact of cryopreservation on buffalo bull sperm with the use of computer assisted semen analyzer (CASA). The motility and kinetics parameters were not affected during equilibration ($p>0.05$), except curvilinear velocity (VCL), average lateral head displacement and viability, but total motility (83.07±0.57%), progressive motility (47.56±0.70%) and rapid motility (64.17±1.17%) of fresh semen reduced to 57.41±0.92%, 23.56 ±0.60% and 30.47 ±0.88%, respectively in frozen-thawed semen ($p<0.05$). Freezing and thawing procedures reduced average path velocity (27.5%), straight linear velocity (27%), VCL (21%), and beat cross frequency (11.5%) of sperm ($p<0.05$). In conclusion, motility and kinetics of buffalo sperm remained unaffected at equilibration stage, whereas, freeze-thawing process caused damage to motility apparatus and reduced total, progressive and rapid sperm motility.

Keywords: CASA, Semen, Sperm motility, Sperm kinetics, Viability

INTRODUCTION

Assessment of sperm motility is usually done subjectively under phase contrast microscope. The results largely depend on experience of technicians, thus implying great variation between laboratories making poor estimations of fertility (Rodriguez-Martinez, 2003). The variations in motility were 30-60% in subjective assessment of buffalo semen in the same ejaculates (Pant *et al.,* 2003 and Koonjaenak *et al.,* 2007). In order to decrease this variation, computer-assisted semen analysis (CASA) instruments were developed with software to analyze and record every sperm characteristic that improved the semen evaluation. Their advantage is that they are considered to be more 'objective' and not only determine the proportion of motile spermatozoa, but also assess the kinetics of individual sperm (Rasul *et al.,* 2000 and Mandal *et al.,* 2003). Furthermore, few scattered reports are available to know the quantitative evaluation of motility parameters of spermatozoa during different stages of cryopreservation. Therefore, the present study was conducted for CASA-based quantitative evaluation of motility parameters of buffalo bull sperm during different stages of cryopreservation.

MATERIALS AND METHODS

Fifty ejaculates (10 ejaculates/bull) from five breeding Murrah buffalo bulls (age, 3-5 yr), used under progeny testing program of the institute and maintained under identical milieu, were collected using artificial vagina and conventionally assessed for volume, color and sperm concentration with Accucell bovine photometer as well as assessed for mass activity and motile spermatozoa percentage. Sperm motility was subjectively assessed under phase contrast microscope equipped with a warm stage (37°C) at 400X magnification and only ejaculates with e"70% sperm motility were used for cryopreservation.

The fresh semen was extended in tris-egg yolk extender containing tris (3.02% w/v), citric acid (1.67 %w/v), fructose (1% w/v), egg yolk (20% v/v), glycerol (6.4% v/v), penicillin (500 IU/ml) and streptomycin (500 µg/ml) plus 6.4% glycerol, to make a final concentration of $80x10^6$ spermatozoa/ml. Thereafter, the extended

[1,2]Scientist, [3]Principal Scientist; *vetdmkl@yahoo.co.in

semen was slowly cooled to 4°C and kept for a period of 3-4 h for equilibration. The equilibrated semen was loaded into 0.25 ml plastic straws and frozen into a programmable biological freezer for cooling down from 4°C to -140°C. Each semen sample was initially cooled at the rate of -5°C/ min from 4° to -10°C. Between -10° to -100°C, freezing rate was -40°C/min and then from -100°C to -140°C, its rate was -20°C/min. After reaching -140°C, semen straws were immediately plunged into liquid nitrogen at -196°C for storage.

The semen was evaluated at fresh, equilibrated and frozen-thawed stages of cryopreservation for sperm motility, kinetics and viability. For semen analysis, semen samples were washed twice in tris buffer to remove seminal plasma and extender. Single experienced operator carried out the motility assessments. The whole experiment was repeated thrice and atleast 200 sperm were counted for each analysis from each bull.

Sperm motility was assessed using CASA system (Kumar *et al.,* 2014). Briefly, the semen sample was diluted with pre-warmed tris buffer to give a sperm concentration of 2 - 6 x 10^6 spermatozoa/ml. The CASA software settings for recording sperm motility were set as; Frame rate 60Hz, Frames acquired 30, Minimum contrast 35, Minimum cell size, 5 pixels, Cell size, 9 pixels, Cell intensity 110 pixels, Path velocity (VAP) 50 µm/s, Straightness (STR) 70%, VAP cut-off, 30 µ/s and VSL cut-off 15 µ/s. In a pre-warmed (38°C) Leja® eight chamber slide (depth 20 µm), 1 µl prepared semen sample was loaded and analyzed for sperm motility characteristics. For each sample, five optical fields around the central reticulum of the chamber were used to count spermatozoa. The motion characteristics recorded were total motility (%), progressive motility (%), rapid motility (%), straight linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), average lateral head displacement (ALH, µm/s), beat cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), elongation (%) and area (µm sq) of spermatozoa.

The Viadent option of CASA was used to determine sperm viability. Hoechst 33258 (5 µg/ml) dye that stains only the sperm cells DNA with damaged membrane. Semen samples were diluted properly at a concentration of 20 - 60 x 10^6/ml then 500 ìl of diluted semen samples were added to 500 ìl of stain solution to make the final stain concentration of 5ìg/ml. The stained samples were incubated at 37°C for 2 min and analyzed with blue light and Viadent filter block under CASA.

Statistical analyses were performed using statistical analysis system (SAS) 9.2 for window. One-way ANOVA followed by Duncan multiple range post-test was used to assess differences among mean of different stages of cryopreservation on sperm motion characteristics and viability.

RESULTS AND DISCUSSION

The semen characteristics of five bulls revealed on an average 3.78±0.24 ml ejaculate volume, 3.8±0.11 mass activity and 1365.1±88.0x10^6/ml spermatozoa concentration. The progressive motility observed under phase contrast microscope was higher before cryopreservation in comparison to after frozen-thawed evaluation (78.8±1.0 and 60.3±1.0%, respectively; $p<0.05$). About 18% reduction in the motility was also obtained in a previous report of subjective assessment (Kumar *et al.,* 2012).

The CASA obtained comparison of different stages of cryopreservation process on buffalo sperm revealed no major impact ($p>0.05$) at equilibrated stage on the motility and kinetics parameters except the values ($p<0.05$) of VCL, ALH and viability in comparison to the fresh semen (Table). However, the total motility, progressive motility and rapid motility of fresh semen sample was reduced by about 25.5, 24 and 33.7%, respectively in frozen-thawed samples ($p<0.05$, Table). The sperm motility is an important feature associated with sperm fertilizing capacity in the female reproductive tract (Verstegen *et al.,* 2002). In a previous study, the total motility was reduced by 49% after freezing and thawing in buffalo

Table: Buffalo sperm motility, kinetics and viability at different stage of cryopreservation evaluated under CASA (mean±SE)

Parameter	Fresh	Equilibrated	Frozen-Thawed
Total Motile, %	83.07±0.57[a]	81.50±0.67[a]	57.41±0.92[c]
Progressive motility, %	47.56 ±0.70[a]	48.66±0.63[a]	23.56±0.60[b]
Rapid, %	64.17±1.17[a]	66.79±0.65[a]	30.47±0.88[b]
Slow, %	11.08 ±0.28[a]	10.57±0.23[a]	23.01±0.48[b]
VAP, µm/s	109.5±0.5[a]	109.9±0.7[a]	82.0±0.8[b]
VSL, µm/s	91.70±1.17[a]	91.24±0.79[a]	66.79±0.77[b]
VCL, µm/s	185.8±1.0[a]	180.6±0.8[b]	146.5±1.4[c]
ALH, µm	6.89±0.06[a]	6.54±0.05[b]	6.67±0.05[b]
BCF, Hz	37.29±0.56[a]	36.32±0.27[a]	33.00±0.27[b]
Straightness, %	82.94±0.63[a]	80.24±0.34[b]	80.22±0.30[b]
Linearity, %	51.95±0.53[a]	49.94±0.34[b]	46.21±0.43[c]
Elongation, %	55.47 ±0.56	54.94±0.46	54.44±0.29
Area, µm sq	8.13±0.11[a]	6.59±0.05[a]	6.40±0.10[a]
Viability, %	85.85±0.54[a]	80.72±0.57[b]	74.39±0.58[c]

$p<0.05$, values with different superscripts within a row are different; VAP - Average path velocity, VSL - Straight linear velocity, VCL - Curvilinear velocity, ALH - Average lateral head displacement, BCF - Beat cross frequency

sperm (Rasul *et al.,* 2001). In cattle, the reduction in total motility was about 9% after cryopreservation (Thomas *et al.,* 1998).

The percentage of slow motile sperms was increased and rapid motile sperms decreased in frozen-thawed semen ($p<0.05$, Table), due to adverse effect of cryopreservation on motility apparatus of spermatozoa. The measurement of sperm velocity has been considered as an indirect indicator of mitochondrial function of spermatozoa and is associated with fertility (Graham *et al.,* 1984 and Budworth *et al.,* 1987).

In kinetics parameters, the relative values of VAP, VSL and BCF were not changed at equilibration stage ($p>0.05$, Table). However, in frozen-thawed buffalo semen compared to fresh semen, VAP, VSL, VCL and BCF were reduced by about 27.5, 27, 21 and 11.5%, respectively ($p<0.05$, Table). In case of ALH, the values of fresh semen declined at equilibration stage followed by an improvement in frozen-thawed semen ($p<0.05$, Table). Similar results due to freezing-thawing were reported in buffalo (Rasul *et al.,* 2001). The reduction in VCL in frozen-thawed semen could be due to cryoinjuries to mitochondrial apparatus and axoneme of spermatozoa (Jones and Stewart, 1979 and Courtens *et al.,* 1989). This suggested that buffalo sperm mitochondria and axoneme are more sensitive to cryopreservation.

In brief, motility and kinetics of buffalo sperm remained unaffected at equilibration stage reflecting their ability to withstand dilution and cooling stresses efficiently. A loss in total motility (25.5%), progressive motility (24%) and rapid motility (33.7%) was observed after freezing and thawing. This study has provided information on impact of cryopreservation on buffalo sperm characteristics that help in making better strategy for improving freezing and fertilizability.

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