Identification of sperm subpopulations in water buffalo ejaculates: Changes in cryopreservation stages and bull variation

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Abstract

The objective of this study is to identify and characterize the sperm subpopulations existing in water buffalo semen using a computer assisted sperm analyzer (CASA), assess the effects of cryopreservation on the sperm subpopulation structure and evaluate bull variability. Eight Bulgarian Murrah bulls were collected with semen, four times in an interval of one week each. The semen was cryopreserved following a standard protocol and sperm kinematics was assessed. Clustering methods were applied to individual sperms forming two significantly different ($P<0.05$) subpopulations. Subpopulation 1 represents those spermatozoa that moved most rapidly and progressively (46.29%) and Subpopulation 2 includes spermatozoa with relatively low velocity or poorly motile but with high progressiveness (53.41%). There is a decline on the population of Subpopulation 1 sperms from fresh (52.52%) to pre freeze (45.73%) to post thaw (35.17%) stages and significant difference on the sperm kinematics between subpopulations. A significant decline in the values of distance, velocity and ALH parameters were observed at post thaw, while an increase is observed on trajectory and BCF kinematics. Values of sperm kinematics are also significantly different ($P<0.05$) among all bulls. The frequency distribution of spermatozoa on both subpopulations was quite similar for all bulls in pre-freeze and post-thaw stages but with significant ($P<0.05$) variability on fresh stage. Bulls with the highest maintained frequency of Subpopulation 1 sperms are denoted as good freezer bulls. In sum, kinematic characterization of water buffalo sperm and clustering into subpopulation enabled identifying bulls that are more resistant to cryopreservation and production of quality semen for genetic propagation.

Keywords: Sperm subpopulations, buffalo semen, sperm kinematics, cryopreservation, CASA

1. Introduction

The intensified use of AI in water buffaloes has put forward the interest in improving the quantitative analysis of semen samples in order to ensure its quality. In the field, it is essential to come up with the predictive capacity of the sperm quality for potential fertility of bulls. The practical use of this study is to devise a method in selecting bulls to become semen donors with a known cryosurvivability and quality for a higher fertilizing capability.

The rise of the Computer Assisted Sperm Analyzer or CASA has brought an advantage in determining the semen quality. Currently, it is the most objective and detailed method, with improved repeatability and enhanced sensitivity in determining the motility not only of the semen sample, but also of individual sperms and can even characterize them to sperm subpopulations within a sample [1]. These sperm subpopulations are based on the kinematics data, which defines the movement of the sperm based on its distance travelled, velocity, head movement and trajectory [2][3].

Sperm subpopulation, commonly devised either by principle component analysis or clustering have been known to have a positive and significant correlation on the ejaculate quality and fertilizing ability in bulls to both in vitro and in vivo [4][5][6]. The cryopreservation process had also been found out to significantly modify the distribution of spermatozoa within subpopulations and that the magnitude of the subpopulation in fresh ejaculates was positively correlated with their resistance to cryopreservation. The cryopreservation process does not only induce a loss of sperm viability but also impair the functionality of the surviving spermatozoa, which accounts for the lower fertilizing capacity of the frozen thawed semen [6]. Realizing the effects, the sperm subpopulations are now being used to identify the quality of the semen and its potential fertilizing ability [4].

The changes during the cryopreservation process and individual variations on the sperms of different mammals are also being documented using the subpopulation structure [7][5][8][9]. However, very few efforts are being given to water buffaloes.

The objective of this research is to identify and characterize the different sperm subpopulations in water buffalo semen and assess the effects of cryopreservation on the kinematic parameters and frequency distribution within the different sperm subpopulations. Finally, to evaluate bull variations on the kinematic parameters and frequency distribution within the different sperm subpopulations structure of fresh, pre-freeze and frozen-thawed semen.

2. Materials and methods

2.1. Semen collection

Semen was collected from eight Bulgarian Murrah bulls with an age ranging from six to ten years old. Four successive collections were performed in a one-week interval using an artificial vagina and teaser bull. The bulls used in the study were regular semen donors of the National Bull Farm, Philippine Carabao Center at Central Luzon State University, Carranglan, Nueva Ecija, Philippines.

2.2. Semen processing cryopreservation

After semen collection and initial evaluation of the ejaculates (volume, color, pH, sperm concentration and subjective motility grading), sample aliquot was taken and placed on a water bath for the fresh semen CASA evaluation. The remaining semen was processed for

cryopreservation following the methods of Mamuad and Venturina (2002) [10] using simple rapid freezer (FHK, FA-1652). A sample aliquot was again taken during the equilibration stage of the semen or before freezing, also for the CASA evaluation for the pre-freeze stage. After 24 hours to one week of processing, frozen semen was thawed in 37°C water for 15secs for the post thaw quality assessment using CASA.

2.3. Sperm motility evaluation using CASA

The Hamilton Thorne IVOS II ver. 14 Computer Assisted Sperm Analyzer (CASA) was used to perform the study. The fresh, pre-freeze and frozen thawed semen were evaluated using the default technical setting for bull semen. The main set of parameters includes 30 consecutive, digitalized photographic images which are taken in a time lapse of 1 sec, which is equivalent to 60 Hz. The evaluation procedure was done using 3 µl of the diluted semen sample with an adjusted sperm concentration ($25x10^6$/ml), and loaded into the chambers of the Leja ® slide for CASA examination. Operation starts by scanning seven randomly allocated fields for each sample, recording at least 100 motile sperms. Semen samples were automatically analyzed by CASA, and evaluated with eleven default sperm kinematic parameters, describing the distance travelled, velocity, head movement and trajectory. For the distance travelled, the average distance of the smoothed cell path is measured as distance average path (DAP), the average distance measured over the actual point to point followed by the cell is measured as distance curved line (DCL), and the average distance measured in a straight line from the beginning to the end of the track is the distance straight line (DSL). For the velocity kinematics, these are the curvilinear velocity (VCL) or the average velocity measured over the actual point to point followed by the cell, straight line velocity (VSL) or the average velocity measured in a straight line from the beginning to the end of the track and average path velocity (VAP) or the average velocity of the smoothed cell path. It also includes the different head movements which are the amplitude of lateral head movement (ALH) or the mean width of the head oscillation as the sperms swim and beat cross frequency (BCF) or the frequency of sperm head crossing the average path on either direction. Finally, for the trajectory, linearity (LIN) or the ratio of straight line velocity over curvilinear velocity, straightness (STR) or the ratio of straight line velocity over path velocity, and wobble (WOB) or the ratio of average path velocity over curvilinear velocity. Progressive sperm motility is defined by the CASA as the percentage of spermatozoa with mean average path velocity above 50 µm/s and straightness of 65%.

2.4. Statistical analysis

In determining the subpopulation composition of semen samples using the sperm kinematics obtained by the CASA analysis, data from all the motile spermatozoa during fresh, pre freeze, post thaw stages collected from the eight bulls were pooled on a single data sheet to represent the whole population. Following the analysis made by Rencher (2002) [11], Ward's Method in Hierarchical Clustering, separate dendograms for all the parameters was made. A multivariate k-means cluster analysis was followed to classify the N number of sperms into a reduced number of subpopulations according to their patterns of movement. The clusters made were based on the dendograms constructed. The k-means clustering model used Euclidean distances computed from the different sperm motion kinematic parameters after normalization of the data so that the cluster centers are the means of the observations assigned to each cluster. The population of sperms belonging to each cluster were also calculated and presented as relative

frequencies. The effects of cryopreservation on the different kinematic parameters were subjected to analysis of variance (ANOVA). Significant differences for all the statistical tests were set at P=0.05. The SSPS Statistics Data Editor was used in the study and with the guidance of a statistician.

3. Results

3.1. Motility characteristics of sperm subpopulations

There are two sperm subpopulations defined with a significantly large distance of 220.24. There are a total of 82,195 sperms that are included in the analysis and their motility characteristics are shown in Table 1, and are described as follows.

Subpopulation 1 represents those spermatozoa that moved most rapidly and progressively as indicated by high VAP, VSL, and VCL together with a high STR, BCF, LIN, WOB and moderate ALH. It also has longer distances travelled as shown by high DAP, DSL and DCL.

Subpopulation 2 includes spermatozoa with relatively low velocity or poorly motile but with high progressiveness as seen with their low VAP, VSL and VCL and high STR, BCF, LIN, WOB and low ALH. It also registered the shortest distances travelled as indicated by low DAP, DSL and DCL.

The sperm subpopulations formed are significantly different ($P<0.05$) from each other on the distance and velocity parameters with Subpopulation 1 being the highest. The values for head movement are also significantly different ($P<0.05$) with each other, however, there is a higher BCF recorded in Subpopulation 2. In terms of trajectory, LIN and WOB are significantly higher in Subpopulation 2 while STR is significantly higher in Subpopulation 1.

Table 1. Overall sperm kinematics and frequency distribution in each subpopulation in water buffalo semen (mean±SD)

Kinematic Parameters	**Sperm subpopulations**	
	1	**2**
No. of sperms (%)	38,162 (46.29)	44,033 (53.41)
DAP (um)	54.29±21.48[a]	24.37±14.41[b]
DSL (um)	45.72±21.76[a]	20.31±14.12[b]
DCL (um)	96.15±39.87[a]	41.39±22.80[b]
VAP (um/s)	153.80±33.10[a]	60.15±32.04[b]
VSL (um/s)	128.22±39.67[a]	50.31±32.10[b]
VCL (um/s)	271.62±64.61[a]	101.20±48.94[b]
ALH (um)	10.32±3.29[a]	4.79±2.85[b]
BCF (Hz)	32.08±9.46[b]	32.37±11.51[a]
STR (%)	83.13±18.25[a]	80.54±21.40[b]
LIN (%)	49.02±16.06[b]	49.94±20.94[a]
WOB (%)	57.92±10.66[b]	59.68±15.16[a]

Means±SD having different superscripts on each row are significantly different from each other at 0.05 level of significance.

3.2. Frequency distribution and sperm kinematics within subpopulations of water buffalo semen during cryopreservation

Table 2 shows the proportion of spermatozoa assigned in the two subpopulations based on their sperm kinematics across the cryopreservation stages. In fresh semen samples, 52.52% belong to Subpopulation 1 (most rapid and progressive) and is seen to be significantly decreasing ($P<0.05$) from 45.73% at pre freeze to 35.17% at post thaw. This in general denotes that proportions of spermatozoa that are moving most rapid and progressive (Subpopulation 1) change during cryopreservation in a declining manner. Conversely, an increasing percentage of low velocity or poorly motile but with high progressiveness (Subpopulation 2) spermatozoa was observed across the cryopreservation stages. A total of 17.35% of the sperms in the Subpopulation 1was lost or re-assigned to Subpopulation 2 after cryopreservation.

Table 2. The effect of cryopreservation on the sperm kinematics and frequency distribution in each subpopulation in water buffalo semen

Kinematic Parameters	**Sperm subpopulations**					
	Fresh		**Pre Freeze**		**Post Thaw**	
	1	**2**	**1**	**2**	**1**	**2**
No. of sperms	15,114	13,664	16,378	19,435	6,191	11,413
(%)	(52.52)	(47.48)	(45.73)	(54.27)	(35.17)	(64.83)
DAP (µm)	60.14 ±22.34^{a}	28.35 ±16.44^{b}	50.57 ±20.80^{a}	23.73 ±14.16^{b}	49.87 ±17.96^{a}	21.90 ±12.51^{b}
DSL (µm)	53.83 ±22.43^{a}	25.38 ±16.16^{b}	39.73 ±19.89^{a}	19.02 ±13.69^{b}	40.81 ±19.14^{a}	18.00 ±12.27^{b}
DCL (µm)	100.84 ±39.00^{a}	46.35 ±26.27^{b}	94.81 ±41.90^{a}	41.08 ±22.27^{b}	89.99 ±35.24^{a}	37.33 ±191.19^{b}
VAP (µm/s)	161.91 ±30.76^{a}	71.94 ±35.33^{b}	154.92 ±33.65^{a}	59.13 ±32.22^{b}	130.75 ±30.74^{a}	51.88 ±27.56^{b}
VSL (µm/s)	144.10 ±36.60^{a}	64.46 ±35.55^{b}	120.78 ±39.24^{a}	47.58 ±31.75^{b}	106.32 ±37.72^{a}	42.83 ±27.67^{b}
VCL (µm/s)	271.29 ±57.89^{a}	116.91 ±55.16^{b}	287.31 ±66.34^{a}	101.15 ±48.46^{b}	234.71 ±62.34^{a}	87.58 ±40.56^{b}
ALH (µm)	9.42 ±2.96^{a}	4.67 ±2.73^{b}	11.65 ±3.09^{a}	5.30 ±3.04^{b}	9.30 ±3.36^{a}	4.15 ±2.45^{b}
BCF (Hz)	35.00 ±9.20^{a}	33.96 ±11.11^{b}	29.29 ±8.68^{a}	30.79 ±10.94^{b}	31.99 ±10.44^{a}	33.35 ±12.28^{b}
STR (%)	88.66 ±14.26^{a}	87.52 ±17.76^{b}	77.98 ±19.41^{a}	77.08 ±22.12^{b}	80.82 ±21.17^{a}	79.48 ±21.51^{b}
LIN (%)	54.81 ±14.83^{a}	56.55 ±19.22^{b}	43.44 ±14.76^{a}	46.69 ±20.75^{b}	47.43 ±17.66^{a}	48.72 ±21.02^{b}
WOB (%)	60.96 ±10.74^{a}	63.02 ±14.48^{b}	54.91 ±9.41^{a}	58.03 ±14.65^{b}	57.35 ±11.86^{a}	59.01 ±15.77^{b}

Means±SD having different superscripts on each row are significantly different from each other at 0.05 level of significance.

Significant differences ($P<0.05$) on the sperm kinematics between subpopulations across the cryopreservation stages are observed. A decrease on the distance travelled (DAP and DCL) by the sperms on both subpopulations from fresh to post thaw stages is also observed. However, values of DSL are decreasing at pre freeze stage but Subpopulation 1 increased at post thaw stage. A decreasing observation in both subpopulations is recorded on the velocity parameters (VAP, VCL and VSL) from fresh to post thaw stage. Meanwhile, an increase in the value of ALH is noticed in both subpopulations at pre freeze stage but eventually decreased at post thaw with values close with fresh stage (9.30 vs 9.42 and 4.15 vs 4.67). On the other hand, BCF decreased at pre freeze stage but eventually increased at post thaw with a close value at fresh stage in both subpopulations (35.00 vs 31.96 and 31.99 and 33.35). In terms of trajectory, STR, LIN and WOB decrease at pre freeze and eventually increase at post thaw for both subpopulations. It was also observed that the values for LIN and WOB are significantly higher in Subpopulation 2 unlike for the other sperm kinematic parameters wherein the values of Subpopulation 1 are always higher.

3.3. Frequency distribution within subpopulations of spermatozoa of individual bulls during cryopreservation

In fresh stage of the semen samples, the proportion of Subpopulation 1 (most rapid and progressive) and Subpopulation 2 (poorly motile or low velocity and high progressiveness) sperms are found to be significantly different ($P<0.05$) among bulls as presented in Figure 3. In Subpopulation 1, bull 2 has significantly the highest frequency while bull 7 is the lowest. Conversely, bull 7 has the significantly highest Subpopulation 2 sperms and bull 2 has the least.

During the pre-freeze stage, a decline in the percentage of Subpopulation 1 sperms is observed among all the bulls with no significant difference ($P>0.05$) from each other. On the other hand, Subpopulation 2 is significantly different from each other among all bulls with bulls 7 and 4 having the highest percentage respectively.

Immediately after thawing, the same decline was observed in the Subpopulation 1 sperms and are significantly different among all bulls. Overall, bull 8 has the highest frequency of Subpopulation 1 while bull 7 has the least. Meanwhile for the Subpopulation 2, all the bulls are also significantly comparable with each other with bulls 7 and 2 with the highest frequency.

The percentage of decline at post thaw of Subpopulation 1 sperms was observed to be highest with bull 1, 46.93%; followed by bull 2, 39.68%; and, bull 3, 37.88%. These bulls could also be denoted as bad freezer bulls due to their more than 25% and almost 50% decline in the distribution of the most rapid and progressive sperms. Meanwhile, bulls 2 and 8 could be described/denoted as good freezer bulls with the lowest decline on the percentage of sperms on Subpopulation 1 of 17.57 and 21.21 respectively.

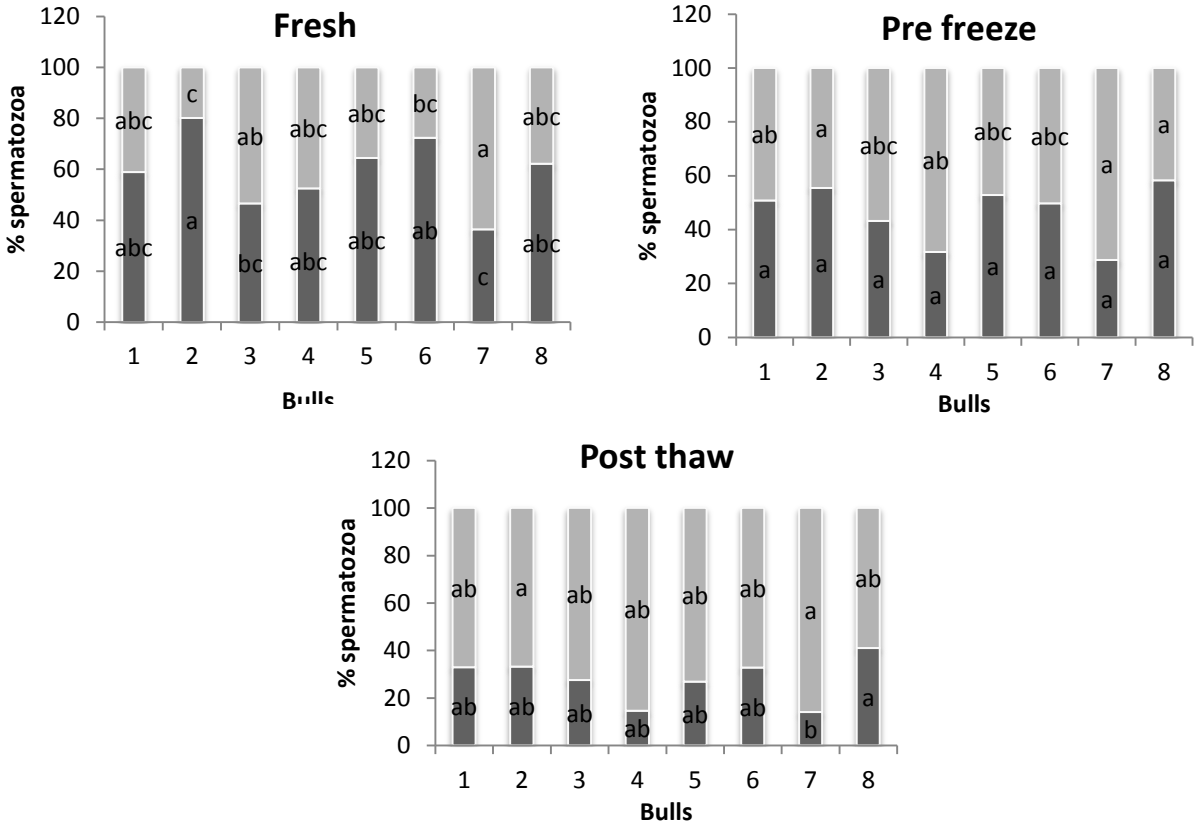

Figure 3. Relative frequency distribution of motile spermatozoa in mean percentages (n=8) within subpopulations (1: gray, 2: light gray) between bulls in fresh, pre-freeze and post-thaw semen. Different letters inside the columns indicate significant differences within subpopulations between bulls at 0.05 level of significance.

3.4. Effect of individual variability on the sperm kinematic parameters within the sperm subpopulations

There is a significant difference observed on the sperm kinematics of the two subpopulations among all bulls. In Subpopulation 1, bull 8 had the significantly ($P<0.05$) highest distance (DAP, DSL and DCL) and velocity (VAP, VSL and VCL) kinematics, while bull 7 had the significantly least ($P<0.05$) were DAP: 58.94±22.22 vs 52.45±19.58 µm/s; DSL: 53.07±20.80 vs 46.82±18.45 µm/s; DCL: 103.09±41.99 vs 90.80±37.07 µm/s; VAP: 162.21±29.62 vs 147.87±29.64 µm/s; VSL: 145.75±29.62 vs 131.36±28.35 µm/s; and VCL: 283.04±64.07 vs 255.14±63.38 µm/s. Meanwhile for the ALH, bull 1 (10.18±3.33 µm) had the highest while bull 4 (8.81±3.37 µm) had the least. For the head movement, in BCF, bulls 8 (34.37±7.90 Hz) and 4 (33.82±9.21 Hz) had the highest and bulls 2 (30.81±8.02 Hz) and 1 (31.29±8.52 Hz) had the significantly least. For the trajectory, bull 4 has the significantly highest STR of 90.74±9.07% while bull 2 had the least 88.70±8.56%). For the LIN and WOB bull 4 had the highest while bull 5 had the least were LIN: 58.45±15.08 vs 52.05±10.79 and WOB: 63.70±12.46 vs 57.90±9.18 respectively. Significant differences were also observed among bulls on the kinematic parameters defining the distance, velocity, head movement and trajectory of Subpopulation 2 sperms.

4. Discussion

The results of the present study indicate that the semen of water buffalo can be characterized based on their sperm kinematic parameters into two subpopulations. This number of sperm subpopulations formed is consistent among all eight bulls, either in fresh, pre freeze or post thaw stages. The differences among bulls are established by the proportion of sperms assigned to the subpopulation of rapid and progressively moving sperms (Subpopulation 1) at all stages. The number of sperm subpopulations defined by this study is different from the subpopulations formed in cattle bulls, stallion and other mammals with three to four subpopulations [9][5][12]. Although the formed subpopulations in this study does not fit with the common finding of three or four well-defined sperm populations among mammalian ejaculates, to our knowledge, the characterization of sperm subpopulations in fresh or frozen thawed buffalo semen has not been previously investigated.

The cryopreservation process had significant effects on the frequency distribution of spermatozoa within subpopulations. The movement of one spermatozoon to another subpopulation is due to the change in its motility pattern which is affected by the entire process from equilibration to freezing and thawing. The shift from Subpopulation 1 to 2 describes the loss in ability of the sperm to control its semi permeability experiencing a sort of false hyper activation [9]. It can be seen that the results on the frequency of spermatozoa belonging to Subpopulation 1 were significantly reduced from pre-freeze to post thaw. During freezing, the sperm plasma membrane undergoes a phase transition from the liquid crystalline phase to the gel phase due to a decrease in temperature. This irreversible changes induced by lipid phase transitions during cooling warming may possibly affect the movement characteristics of spermatozoa during semen processing [13]. According to Muiño et al. (2008) [9], ejaculates with the highest populations of rapid and progressive sperm were also the most resistant to cryopreservation and showed the best post-thaw sperm longevity. The declining percentage of most rapid and progressive (Subpopulation 1) sperms in the study is seemingly acceptable. Although it has been known that a substantial number (50%) of sperm are damaged during cryopreservation [14]. Our results of having an average of 35.17% of the subpopulation's most rapid and progressive sperms at post thaw is higher than Muiño et al. (2008) [9] who has kept only 25.3% in cattle bull semen. It can be then assumed that the frozen thawed water buffalo semen can contain a good number of most rapid and progressive sperms capable for fertilization.

In this study, individual variation was seen among bulls especially on the subpopulation of spermatozoa with the most rapid movement and progressiveness. The change in the distribution between the two subpopulations is consistent among all bulls as affected by the cryopreservation process. There had been a decreasing trend on the frequency of the sperms with most rapid and progressiveness characteristics from fresh to pre freeze to post thaw stage among all bulls, in which conversely an increase in the population of poorly motile or low velocity but high progressiveness sperms. Bulls in this study with greater proportion of most rapid and progressiveness sperm subpopulation at the onset still contained higher proportions of it at post thaw. This characteristic of the sperms of a bull can be attributed to its higher cryosurvivability as compared to others. Several researchers suggested that the sperms belonging to the highest velocity and progressiveness can be considered as the sperm with the highest fertilizing potential [5][15][8]. Thus, it is of utmost importance to determine the different subpopulations of motile spermatozoa existing in bull semen sample for a better projection of the movement of the sperms and its possible fertilizing ability. Similar results for the differences on the sperm subpopulation distribution were found in human[16], dog [8] and Holstein bulls [9] ejaculates.

The study on determining the individual sperm kinematics could be useful in improving the buffalo semen quality assessment by detecting subtle changes on the sperm movement across cryopreservation, in which, eventually may affect its fertilizing function. However, to strengthen justification on the fertilizing potential of the most rapid and progressive sperms, bulls having higher proportion of this, may be used in-vitro or in-vivo to see an existing correlation. The findings of this can be used as a predicting formula for the fertilizing rate of a bull based on the movements of its sperm. This information could also be used to select bulls for continuous use in the AI program, or maximize its consumption by lowering the insemination doses so as to cater more dams to impregnate and spread its genetics.

In conclusion, the study on water buffalo sperm kinematics indicates a two well-defined sperm subpopulations existing in fresh, pre-freeze and post thaw semen stages. Water buffalo sperm generally moves either rapid and progressive or slow and progressive. The effect of cryopreservation was seen in the decline on the frequency of rapid and progressive moving sperms of all bulls. Differences among bulls are recognized on the distribution of sperm subpopulation with most rapid and progressive sperms. The greater percentage of most rapid and progressive moving sperms maintained at post thaw indicates a good freezability of the bull. Further studies are needed in determining importance of identification of sperm subpopulations in water buffalo and its use in predicting fertility.

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