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Identification of Sperm Subpopulations in Water Buffalo Ejaculates: Changes in Cryopreservation Stages and Bull Variation

Excel Rio Santos Maylem[1, 2], Maria Elizabeth Dela Cruz Leoveras[1], Emma Villanueva Venturina[3], Eufrocina Dela Peña Atabay[2] and Edwin Curading Atabay[2, 3]

1. Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija 3120, Philippines

2. Reproductive Biotechnology Unit, Philippine Carabao Center National Headquarters and Genepool, Science City of Muñoz, Nueva Ecija 3120, Philippines

3. Philippine Carabao Center at Central Luzon State University, Science City of Muñoz, Nueva Ecija 3120, Philippines

Abstract: This study aimed to identify and characterize the sperm subpopulations existing in water buffalo semen using a computer assisted sperm analyzer (CASA), as well as assess the effects of cryopreservation on the sperm subpopulation structure and evaluate bull variability. The semen of eight Bulgarian Murrah bulls was collected by 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 (P_1 and P_2). Subpopulation P_1 represents those spermatozoa that moved most rapidly and progressively (46.29%), and subpopulation P_2 includes spermatozoa with relatively low velocity or poorly motile but with high progressiveness (53.41%). There was a decline on the population of P_1 sperms from fresh (52.52%), pre-freeze (45.73%) to post-thaw (35.17%) stages and significant difference on the sperm kinematics between P_1 and P_2. A significant decline in the values of distance, velocity and amplitude of lateral head (ALH) parameters were observed at post-thaw stage, while an increase was observed on trajectory and beat cross frequency (BCF) kinematics. Values of sperm kinematics were also significantly different ($P < 0.05$) among all bulls. The frequency distribution of spermatozoa on both subpopulations P_1 and P_2 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 P_1 sperms are denoted as good freezer bulls. In sum, kinematic characterization of water buffalo sperm and clustering into subpopulation enable to identify bulls that are more resistant to cryopreservation and production of quality semen for genetic propagation.

Key words: Sperm subpopulations, buffalo semen, sperm kinematics, cryopreservation, computer assisted sperm analyzer.

1. Introduction

The intensified use of artificial insemination (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 the 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 (CASA) has brought an advantage in determining the semen quality. Currently, it is the most objective and detailed method, with improved repeatability and 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 define the movement of the sperm based on its distance travelled, velocity, head

Corresponding author: Excel Rio Santos Maylem, M.Sc., research field: biology.

movement and trajectory [2, 3].

Sperm subpopulations, 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 both *in vitro* and *in vivo* [4-6]. The cryopreservation process had also been found out to significantly modify the distribution of spermatozoa within subpopulations, and 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 [5, 7-9]. However, very few efforts are being given to water buffaloes.

The objective of this research was to identify and characterize the different sperm subpopulations in water buffalo semen, also assess the effects of cryopreservation on the kinematic parameters and frequency distribution within the different sperm subpopulations, and 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 age ranging from 6 to 10 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 Cryopreservation Processing of Semen

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 CASA evaluation of fresh semen. The remaining semen was processed for cryopreservation, following the methods described by Mamuad and Venturina [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 h to one week of processing, frozen semen was thawed in 37 °C water for 15 s for the post-thaw quality assessment using CASA.

2.3 Sperm Motility Evaluation Using CASA

The CASA (Hamilton Thorne IVOS II ver. 14) 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 s, which is equivalent to 60 Hz. The evaluation procedure was done using 3 μL of the diluted semen sample with an adjusted sperm concentration (25×10^6/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 11 defaults perm 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)—the average velocity measured over the actual point to point followed by the cell, straight line velocity (VSL)—the average velocity measured in a straight line from the beginning to the end of the track, and average path velocity (VAP)—the average velocity of the smoothed cell path. It also includes the different head movements, which are the amplitude of lateral head (ALH) movement—the mean width of the head oscillation as the sperms swim, and beat cross frequency (BCF)—the frequency of sperm head crossing the average path on either direction. Finally, The trajectory includes linearity (LIN)—the ratio of straight line velocity over curvilinear velocity, straightness (STR)—the ratio of straight line velocity over path velocity, and wobble (WOB)—the ratio of average path velocity over curvilinear velocity. Progressive sperm motility is defined by 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 [11], Ward's method in hierarchical clustering, separate dendrograms for all the parameters was made. A multivariate *k*-means cluster analysis was followed to classify the number of sperms into a reduced number of subpopulations according to their patterns of movement. The clusters made were based on the dendrograms 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 software 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 (P_1 and P_2) defined with a significantly large cluster 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.

Subpopulation P_1 represents those spermatozoa that moved most rapidly and progressively, as indicated by high VAP (153.80 ± 33.10 μm/s), VSL (128.22 ± 39.67 μm/s) and VCL (271.62 ± 64.61 μm/s) together with a high STR (83.13% ± 18.25%), BCF (32.08 ± 9.46 Hz), LIN (49.02% ± 16.06%), WOB (57.92% ± 10.66%) and moderate ALH (10.32 ± 3.29 μm). It also has longer distances travelled, as shown by high DAP, DSL and DCL (54.29 ± 21.48, 45.72 ± 21.76 and 96.15 ± 39.87 μm, respectively).

Subpopulation P_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 shorter distances travelled as indicated by low DAP, DSL and DCL.

The sperm subpopulations formed were significantly different ($P < 0.05$) from each other on the distance and velocity parameters, with subpopulation P_1 being the highest. The values for head movement were also

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

Kinematic parameters	Sperm subpopulations	
	P_1	P_2
No. of sperms	38,162 (46.29%)	44,033 (53.41%)
Distance travelled		
DAP (μm)	54.29 ± 21.48^a	24.37 ± 14.41^b
DSL (μm)	45.72 ± 21.76^a	20.31 ± 14.12^b
DCL (μm)	96.15 ± 39.87^a	41.39 ± 22.80^b
Velocity		
VAP (μm/s)	153.80 ± 33.10^a	60.15 ± 32.04^b
VSL (μm/s)	128.22 ± 39.67^a	50.31 ± 32.10^b
VCL (μm/s)	271.62 ± 64.61^a	101.20 ± 48.94^b
Head movements		
ALH (μm)	10.32 ± 3.29^a	4.79 ± 2.85^b
BCF (Hz)	32.08 ± 9.46^b	32.37 ± 11.51^a
Trajectory		
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 having different superscripts on each row are significantly different from each other at 0.05 significance level.

significantly different ($P < 0.05$) with each other, however, there was a higher BCF recorded in subpopulation P_2. In terms of trajectory, LIN and WOB were significantly higher in subpopulation P_2, while STR was significantly higher in subpopulation P_1.

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% belonged to subpopulation P_1 (most rapid and progressive), and P_1 was seen to be significantly decreasing ($P < 0.05$) from 45.73% at pre-freeze to 35.17% at post-thaw. This in general denotes the proportions of spermatozoa that are moving most rapidly and progressively (P_1) during cryopreservation in a declining manner. Conversely, an increasing percentage of low velocity or poorly motile but with high progressiveness spermatozoa was observed in subpopulation P_2 across the cryopreservation stages. A total of 17.35% of the sperms in the subpopulation P_1 was lost or reassigned to subpopulation P_2 after cryopreservation.

Significant differences ($P < 0.05$) on the sperm kinematics between subpopulations across the cryopreservation stages were observed. A decrease on the distance travelled (DAP and DCL) by the sperms on both subpopulations from fresh to post-thaw stages was also observed. However, DSL values were also decreasing at pre-freeze stage, but subpopulation P_1 increased at post-thaw stage. A decreasing observation in both subpopulations was recorded on the velocity parameters (VAP, VCL and VSL) from fresh to post-thaw stage. Meanwhile, an increase in the value of ALH was 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 μm and 4.15 vs. 4.67 μm for P_1 and P_2, respectively). 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 (31.99 vs. 35.00 Hz and 33.35 vs. 33.96 Hz for P_1 and P_2, respectively). In

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

Kinematic parameters	Sperm subpopulations					
	Fresh stage		Pre-freeze stage		Post-thaw stage	
	P_1	P_2	P_1	P_2	P_1	P_2
No. of sperms	15,114 (52.52%)	13,664 (47.48%)	16,378 (45.73%)	19,435 (54.27%)	6,191 (35.17%)	11,413 (64.83%)
Distance travelled						
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
Velocity						
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
Head movements						
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
Trajectory						
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 having different superscripts on each row are significantly different from each other at 0.05 significance level.

terms of trajectory, STR, LIN and WOB decreased at pre-freeze and eventually increased at post-thaw for both subpopulations. It was also observed that the values for LIN and WOB were significantly higher in subpopulation P_2, unlike for the other sperm kinematic parameters wherein the values of subpopulation P_1 were 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 P_1 (most rapid and progressive) and subpopulation P_2 (poorly motile or low velocity and high progressiveness) sperms was found to be significantly different ($P < 0.05$) among bulls, as presented in Fig. 1. In subpopulation P_1, bull 2 had the significantly highest frequency, while bull 7 was the lowest. Conversely, bull 7 had the significantly highest frequency of subpopulation P_2 sperms and bull 2 had the least.

During the pre-freeze stage, a decline in the percentage of subpopulation P_1 sperms was observed among all the bulls with no significant difference ($P > 0.05$) from each other. On the other hand, subpopulation P_2 was 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 P_1 sperms and there was significant difference among all bulls. Overall, bull 8 had the highest frequency in subpopulation P_1, while bull 7 had the least. Meanwhile for the subpopulation P_2, all the bulls were also significantly comparable with each other with bulls 7 and 2 with the highest frequency.

The decline percentage at post-thaw of subpopulation P_1 sperms was observed to be highest in 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

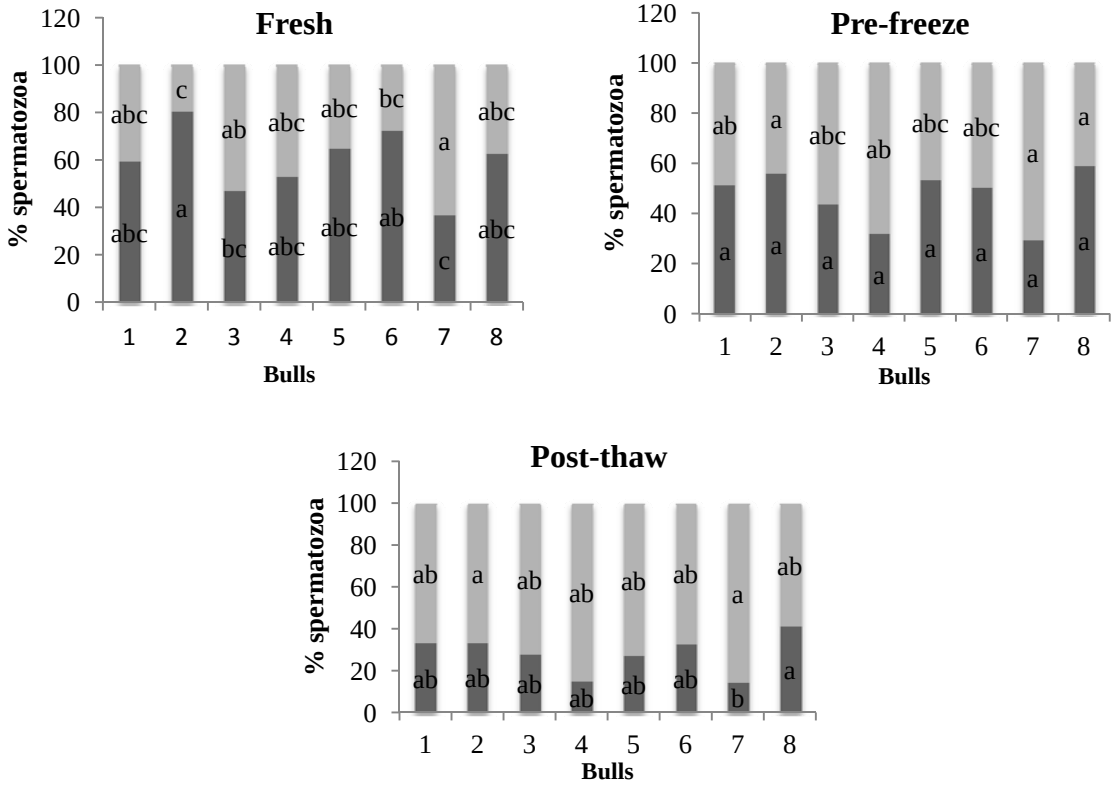

Fig. 1 Relative frequency distribution of motile spermatozoa in mean percentages (n = 8) within subpopulations P_1 (gray) and P_2 (light gray) between bulls in fresh, pre-freeze and post-thaw semen.
Different letters inside the columns indicate significant differences within subpopulations between bulls at 0.05 significance level.

with the lowest decline on the percentage of sperms on subpopulation P_1 of 17.57% and 21.21%, respectively.

3.4 Effect of Individual Variability on the Sperm Kinematic Parameters within the Sperm Subpopulations

There was a significant difference observed on the sperm kinematics of the two subpopulations among all bulls as seen in Fig. 1. In subpopulation P_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 (P < 0.05) least distance (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) and velocity (VAP: 162.21 ± 29.62 vs. 147.87 ± 29.64 μm/s; VSL: 145.75 ± 29.62 vs. 131.36 ± 28.35 μm/s; VCL: 283.04 ± 64.07 vs. 255.14 ± 63.38 μm/s). Meanwhile for the ALH, bull 1 had the highest (10.18 ± 3.33 μm), while bull 4 had the least (8.81 ± 3.37 μm). For the head movement, bull 8 (34.37 ± 7.90 Hz) and bull 4 (33.82 ± 9.21 Hz) had the highest BCF, while bull 2 (30.81 ± 8.02 Hz) and bull 1 (31.29 ± 8.52 Hz) had the significantly least. For the trajectory, bull 4 had the significantly highest STR of 90.74% ± 9.07%, while bull 2 had the least STR of 88.70% ± 8.56%. For the LIN and WOB, bull 4 had the highest, while bull 5 had the least (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 P_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 (P_1 and P_2). 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 progressive moving sperms (subpopulation P_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 [5, 9, 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 has 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 P_1 to P_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 P_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. [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 P_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]. In this study, the results of an average of 35.17% of the most rapid and progressive sperms subpopulation at post-thaw is higher than that reported by Muiño et al. [9], who reported only 25.3% kept 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, 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, 8, 15]. 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 artificial insemination program, or maximize its consumption by lowering the insemination doses so as to cater more dams to impregnate and spread its genetics.

5. Conclusions

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 rapidly and progressively or slowly and progressively. The effect of cryopreservation was seen in the decline on the frequency of rapidly and progressively 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 rapidly and progressively moving sperms maintained at post-thaw, indicating a good freezability of the bull. Further studies are needed in determining the importance of identification of sperm subpopulations in water buffalo and its use in predicting fertility.

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