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THE USE OF COMPUTER ASSISTED SPERM ANALYZER IN EVALUATING THE SPERM KINEMATICS OF FRESH AND FROZEN-THAWED BUFFALO SEMEN

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ABSTRACT

The cryopreservation process impairs the kinematic movement of sperm cells which account for a reduced motility at post thaw. The study is made to evaluate the sperm kinematics of fresh and frozen-thawed buffalo semen. Three successive semen collections were made and ejaculates from the donor bulls (n=6) were evaluated using the computer assisted sperm analyzer (CASA). Semen was analyzed based on sperm motility, speed of movement and kinematic parameters which is specific on velocity, head behavior and swimming pattern. Results revealed a significant ($P<0.05$) change on the sperm motility (progressively motile, motile and static) and speed of movement (rapid, medium and slow) from fresh to post-thaw stage. The total population of moving sperm cells were found to have a minimal reduction and still maintaining a relatively high percentage (76.18%) at post-thaw. Meanwhile, based on the sperm kinematics, significant decrease ($P<0.05$) were found on average path velocity, curvilinear velocity, straight line velocity, beat cross frequency, linearity and wobble but not on the amplitude of lateral head movement and straightness. With the slight decline at post- thaw, a good combination of sperm kinematics is still observed making the semen and the donor bulls evaluated acceptable for cryopreservation and artificial insemination.

Keywords: buffalo semen, CASA, cryopreservation, fresh, frozen-thawed

INTRODUCTION

Albeit success of Artificial Insemination is multi-factorial, semen experts agree that it is largely dependent on the use of good quality semen, thus, efforts on improving the production and analysis of semen is becoming essential in the livestock industry.

Sperm cells are very sensitive to alterations in the osmolality of the surrounding solution; if the change is intolerable, it loses their motility (Tasdemir *et al*., 2013). Aside from that, motility indicates active metabolism and integrity of membranes and is of great importance for fertilizing activity as it aids sperm transport within the female reproductive

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tract and for egg penetration (Muiño *et al*., 2008). On the contrary, subjective motility grading yields to 30-60% variation on the scores given by different observers, which may lead to improper assessment of the possible use of the semen (Kathiravan *et al*., 2008), and would practically affect the productivity of the animals in the long run.

Nowadays, the Computer Assisted Sperm Analyzer (CASA) is being tapped as the most efficient method to determine the quality of the semen due to its capacity to determine sperm motility quantitatively based on its velocity, head behavior and swimming pattern (Sundararaman *et al*., 2012; Vincent *et al*., 2012). The different sperm movements formed based on the kinematics data obtained by the CASA system is its ultimate edge from the subjective motility grading.

One of the major concerns of the semen processing units is the effect of cryopreservation, including the possible contribution of variation in bulls (Muiño *et al*., 2008, 2009). Researchers found out that cryopreservation significantly modified the distribution of sperm cell within subpopulations and that the magnitude of it in fresh ejaculates was positively correlated with their resistance to freezing. This then implies that cryopreservation impairs the functionality of the sperm cell based on its loss in viability (Yániz *et al*., 2015).

With the intention of coming up with semen of good quality, assessment of the motility and kinematic characteristics of fresh and frozen-thawed semen of donor bulls using the computer assisted sperm analyzer is the main objective of this study. This effort is significant in enhancing the cryopreservation process through selection of good quality semen for the increase in AI efficiency in water buffaloes.

MATERIALS AND METHODS

Semen from donor water buffalo bulls (n=6) of the Philippine Carabao Center at Central Luzon State University (PCC at CLSU) were collected at 5:00-6:00 in the morning and is used in the study. Three subsequent collections were made. The standard semen collection and freezing protocol of the Center was followed using only the first semen ejaculates of the bulls. Immediately after collection, each ejaculate was processed for freezing. The fresh extended semen that was already placed in straws was transported in 15°C water to the Reproductive Biotechnology laboratory within an hour. The semen samples were then diluted in 1:1 ratio with a normal saline, and analyzed using CASA.

After 24 hours to one week of storage, frozen semen straws from each bull were thawed at 38°C water for 15 seconds and were diluted in 1:1 ratio in normal saline before being analyzed using CASA.

The Hamilton Thorne IVOS II ver. 12.1 computer assisted sperm analyzer (Hamilton Thorne, Inc. 100 Cummings Center, Suite 465E, Beverly, MA, USA) was used to perform this study. The default technical setting for cattle (Table 1) was adopted in evaluating the sperm motility characteristics and motion patterns of fresh and frozen buffalo semen.

For the evaluation procedure, 3 µl semen samples with an adjusted sperm concentration ($40x10^6$/ml) was initially loaded into the four chambers of the specialized glass slide for CASA (Leja® slide, Leja Products B.V., Luzernestraat 10, 2153 GN Niewuw Vennep, The Netherlands). Operation starts by scanning seven randomly allocated fields for each sample, recording at least 100 motile sperm cells. Semen samples were then automatically analyzed by CASA, and being evaluated with default eight kinematic parameters, the details of which are summarized in Table 2.

Table 1. Technical setting of Computer Assisted Sperm Analyzer (Hamilton-Thorne IVOS 12.1) for motility assessment of buffalo sperm.

Parameters	Set Value
Frame rate (Hz)	60
Frames acquired	30
Minimum contrast	80
Minimum cell size (pixels)	5
Cell size (pixels)	5
Cell intensity (pixels)	70
Path velocity (VAP) (um/s)	50
Straightness (STR) (%)	70
VAP cut-off (um/s)	30
VSL cut-off (u/s)	15

Table 2. Selected parameters of sperm motility measured by CASA (Partyka *et al*., 2012).

Kinematic Parameters	Description	Unit
Average Path velocity (VAP)	the average velocity of the smoothed cell path	µm/sec
Straight line velocity (VSL)	the average velocity measured in a straight line from the beginning to the end of the track	µm/sec
Curvilinear velocity (VCL)	average velocity measured over the actual point to point followed by the cell	µm/sec
Amplitude of lateral head (ALH)	mean width of the head oscillation as the sperms swim	µm
Beat cross frequency (BCF)	frequency of sperm head crossing the average path on either direction	Hz
Straightness (STR)	the ratio of straight line velocity over path velocity	%
Linearity (LIN)	the ratio of straight line velocity over curvi-linear velocity	%
Wobble (WOB)	the ratio of average path velocity over curvi-linear velocity	%

Sperm motility values initially given by CASA were for the populations of progressively motile and motile sperm cells, which are normally presented as percentages. Generally, sperm cells with VCL values > 10 µm/sec are considered Motile (MOT) and sperm cells with VAP and STR of >50 µm/sec and >70 µm/sec respectively,

are considered Progressiveley Motile (PMOT). They were further categorized based on their speed of the movement into Rapid, Medium, Slow and Static and herein defined as:

1. Rapid (RAP): if VAP > MVV
2. Medium (MED): if LVV < VAP <MVV
3. Slow (SLOW): if VAP < LVV or VSL < LVS
4. Static (ST): the fraction of sperms not moving during the analysis.

Wherein, MVV (Medium VAP threshold value, 50 μm/sec), LVV (Low VAP threshold value, 30 μm/sec), LVS (Low VSL threshold value, 15 μm/sec) were the set values of the system.

The track details of individual sperm cells were automatically recorded in Microsoft Excel® 2007 software (Microsoft Redmond Campus, Redmond, Washington, US). Accordingly, the data being given by CASA are actual sperm cell counts captured by the system, arranged and recorded in the database and some are converted to algorithms.

The data on motility and movement characteristics were presented as mean percentages, whereas the data in kinematic characteristics were in means. The data were analyzed using analysis of variance (ANOVA) in split plot design and Tukey-Kramer's Test at 0.05 level of significance using Statistical Analysis Software (SAS) (SAS Institute, North Carolina State University, North Carolina, USA).

RESULTS AND DISCUSSION

There are three sperm motility characteristics that are provided by the CASA namely: progressively motile, motile or static. Statistically, the study revealed a significant (P<0.05) decline on the values of sperm motility characteristics between treatments or on the cryopreservation process (pre-freeze and post-thaw) (Figure 1).

Initially, at pre-freeze stage, majority of the sperm cells were PMOT (46.36%), MOT (39.43%), and only few were ST (14.36%). A high percentage of PMOT at pre-freeze are needed so as to expect a higher percentage of its population after freezing. Similarly, a high percentage of PMOT is a marker of good quality semen and can serve as basis for selection of donor bulls. Conversely at post-thaw, the highest distribution was observed in the MOT (44.74%), followed by the PMOT (31.44%), and then ST (23.83%) sperm. As to the effect of the cryopreservation within each sperm cells' population, a significant decrease (P<0.05) was observed in the percentage of PMOT at post-thawing, but was not significantly different (P>0.05) for MOT sperm cells. Meanwhile, there was a significant increase (P<0.05) in the population of ST sperm cells at post-thaw. This notable decline in the population of PMOT resulted to the increase in the percentage distribution of MOT and ST sperm cells at post-thaw indicating that the cryopreservation procedure, indeed, contributed to the changes on the sperm movement, and overall motility.

The same decrease in the sperm motility frequency was observed by Kumar *et al*. (2011) in Toda buffalo after freezing. Meanwhile, Dhurvey *et al*. (2012) strengthened the claim by reporting that injuries on the physiology of the sperm cell due to cryopreservation could be very well reflected in the sperm motility. It is important to note, however, that around 80% of the sperm populations are motile (both PMOT and MOT) at the end of the cryopreservation process, rendering the semen evaluated suitable for AI use. It has been suggested that the high motile and progressive subpopulations are the most suitable and are being associated with the fertilization process (Henning *et al*., 2013).

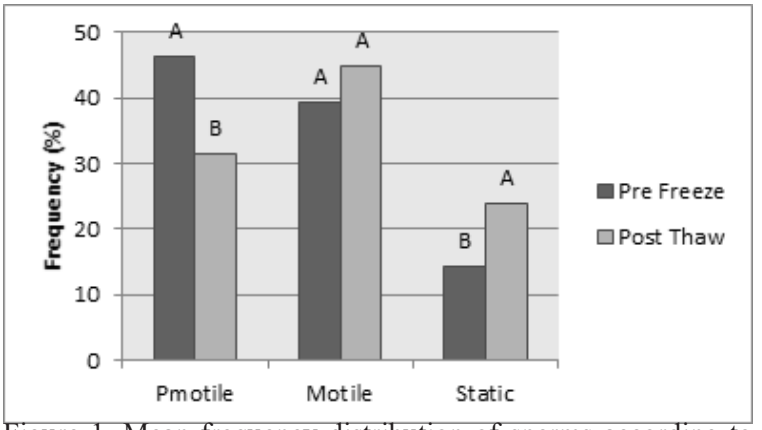

Figure 1. Mean frequency distribution of sperms according to motility populations of bulls at pre-freeze and post-thaw stages. Means having the same subscripts are not significant from each other (P>0.05).

It is having been expressed though, that the mean values of MOT or PMOT sperm cells in a given sample do not reflect the great heterogeneity in movement characteristics of individual sperm within a given sample, indicating that this set of parameters (PMOT, MOT and ST) may not be enough to come up with the desired outcome. In this case, other kinematic parameters were reported to play a great contribution in determining sperm quality (Henning *et al*., 2013), thus was further considered in the succeeding work.

The next level of evaluating the sperm through CASA is the speed of movement which is characterized as Rapid, Medium and Slow. The effect of the treatment (pre-freeze and post-thaw) on the semen revealed that the treatment or cryopreservation has significant effect over the sperm movements.

In terms of the effect of cryopreservation on sperm population (Figure 2), the Rapid sperm cells has the highest percentage (73.03%) at pre-freeze which is greater than those of the Medium (10.33%) and Slow (16.47%) groups. At post-thawing, however, a significant decrease (P<0.05) in Rapid group (56.96%) was observed. In contrast, the population of Medium (13.77%) and Slow (29.36%) sperm cells increased significantly at post-thaw (P<0.05). The observed shift or change in sperm population after freezing explains that cryopreservation contributed to the impaired cell movement thus, sperm cells were re-categorized to Medium and Slow population. As reflected in the graph, the majority of the decrease which is around 15% in the Rapid moving sperm cells was re-categorized mostly to Slow moving since only a few was added to the Medium moving sperm cells.

The shift in percent distribution could be attributed to the cryopreservation process, wherein exposure to the cryoprotective agent, glycerol, is toxic to the sperm cell thereby reducing its movement (Maxwell and Watson 1996). Moreover, Muiño *et al*. (2008) reported that semen samples having more Rapid and Progressive subpopulations were comparatively more cryoresistant than those that are not. Although, there was a significant decrease in the percentage of Rapid moving sperm cells after freezing, the results show that the semen evaluated contains more than 50% of Rapid sperm cells in their frozen samples, which is desirable enough for a good quality semen.

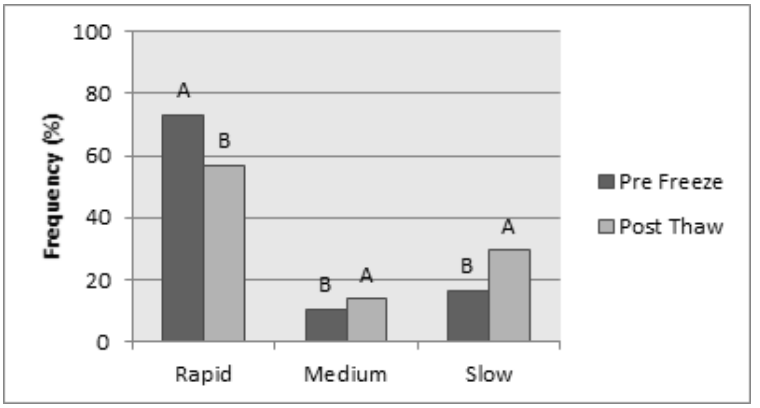

Figure 2. Mean frequency distribution of sperms according to the speed of movement of bulls at pre-freeze and post-thaw stages. Means having the same subscript are not significantly different with each other (P>0.05).

The highlight of use of CASA is its detailed assessment of sperm velocity, head behavior and swimming pattern. These sperm movements as categorized into eight kinematic parameters were assessed at fresh semen and its changes were also observed at post-thaw with changes shown in Table 3.

Among all the parameters, it was found out that there was a significant interaction between the kinematic parameters and stages of cryopreservation, across the three sperm movement populations. Statistically, sperm kinematic characteristics: VAP, VSL, VCL, BCF, LIN and WOB have a significant difference (P<0.05) between pre- and post-freezing. On the other hand, ALH and STR are not affected by the treatment or cryopreservation process.

The results of the present study show that VAP value at pre-freeze in Rapid sperm cells is significantly (P<0.05) higher than post-thaw and the trend is likewise observed in Medium and Slow sperm cells. VSL values follow similar trend. Velocity parameters (VAP, VSL, VCL) are highest in Rapid sperm but with significant (P<0.05) decrease of values at post- thaw. On the other hand, ALH and STR values of 3 sperm populations did not decrease at post-thaw, whereas LIN and WOB values at pre-freezing differ significantly (P<0.05) from post-thaw in Rapid sperm cell group, but not in the Medium and Slow groups which remain the same after freezing. There was a general decrease in the measured values of the kinematic parameters from pre-freeze to post-thaw stages in the three sperm populations.

A growing body of evidence suggests that during freezing and thawing procedures, the eight kinematic parameters are known to be sensitive to both the physical and biochemical changes in the buffalo sperm cell (Henkel *et al*., 2004). In the current study, majority of the sperm cell motion characteristics are decreasing significantly. Of the motility parameters evaluated in this study, VAP, VCL, VSL, ALH, BCF and LIN are considered the most significant factors in determining fertility (Cancel *et al*., 2000). Specifically, VCL and VAP were found to be related to the ability of sperm cell to migrate through the female genital tract, increasing the chance of fertilization (Tsakmakidis, 2010). Furthermore, Cox *et al*. (2006) included VSL in the parameters to have better spermatozoon migration efficiency in

Table 3. Mean values of the sperm kinematics of different sperm populations at pre-freeze and post-thaw stages.

Kinematic Parameters	Rapid		Medium		Slow	
	Pre-Freeze	Post-Thaw	Pre-Freeze	Post-Thaw	Pre-Freeze	Post-Thaw
Average Path Velocity (VAP)	118.32[a]	106.33[b]	36.04[a]	34.96[b]	26.95[a]	24.32[b]
Straight Line Velocity (VSL)	92.79[a]	82.64[b]	27.32[a]	25.89[b]	9.56[a]	9.46[a]
Curvilinear Line Velocity (VCL)	196.54[a]	182.39[b]	72.33[a]	72.66[a]	63.96[a]	59.68[a]
Amplitude of Lateral Head (ALH)	7.62[a]	7.67[a]	3.99[a]	4.05[a]	3.67[a]	3.39[a]
Beat Cross Frequency (BCF)	37.68[a]	32.98[b]	35.49[a]	33.49[b]	38.59[a]	37.01[b]
Straightness (STR)	78.94[a]	77.59[a]	76.03[a]	74.61[a]	52.23[a]	54.07[a]
Linearity (LIN)	50.97[a]	48.27[b]	41.77[a]	39.80[a]	24.60[a]	25.77[a]
Wobble (WOB)	62.57[a]	60.64[b]	53.46[a]	48.76[a]	45.70[a]	44.87[a]

Means having the same superscripts within a row are not significant from each other at $P>0.05$.

homologous cervical mucus. Computer-aided sperm analysis parameters are regarded as related to fertility results both in-vivo and in-vitro situations (Henning *et al*., 2013).

In related development, the parameters VAP, VCL, ALH, BCF and RAP (rapid) are associated with hyperactivated motility and capacitation. Elevated VCL values are characteristic of sperm cells incubated under capacitating conditions and indicative of it undergoing vigorous hyperactivated pattern of activity characterized by high amplitude of flagella binding. VCL are found to be of 105 to 143 µm/sec for capacitated and 114 to 195 µm/sec for hyperactivated sperm cells (Chatiza *et al*., 2012). In case of the positive interaction between treatments of the ALH and STR, it can be observed in the sperm tracks that the smaller the movement of the sperm cell head to its left and right (ALH), the straighter is its movement. In addition, ALH reported increased value due to hyperactivation during the process of capacitation *in vivo* was associated with fertility and reproduction. The significant increase of ALH value of buffalo sperm cells in the present study is primarily attributed to the detrimental effect of cell cryopreservation and thawing processes. The side to side movement of the head or the amplitude lateral movement exhibited early on in both fresh and frozen buffalo semen can adversely affect its motility and overall quality, and probably its reproductive ability thereafter.

Finally, there are three levels of classification and was found out to have significant effects on the sperm motility, speed of movement and kinematic characteristics at fresh and

post-thaw stages. To highlight the result, cryopreservation is said to have an influence on the velocity, but not on amplitude of head movement and straightness of the swimming pattern. With the minimal change observed from fresh to frozen-thawed stage, the semen collected and evaluated is found acceptable for use. It further explains that the bulls used in this study can be denoted as good freezer bulls as they can maintain a good percentage of semen at post- thaw. Therefore, with the use of CASA, a decision can be made objectively whether to discard or keep the semen and the bulls in the breeding line as semen donors for the AI program.

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