KINEMATIC ANALYSIS OF FRESH BULL SPERM UNDER DIFFERENT DILUTION RATIOS OF BIOXCELL® EXTENDER

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ABSTRACT. This study aims to evaluate the effects of different dilution ratios of BioXcell® extender (1:10, 1:100, 1:1000) on kinematic parameters of fresh bull sperm using a computer-assisted sperm analysis (CASA) system, to identify an optimal dilution ratio for laboratory assessments that maintains bull sperm quality during the evaluation process. A total of 36 bulls' ejaculations were used and underwent analysis using computer-assisted sperm analysis (CASA). The data was analysed using SPSS software. Sperm progressive motility (PM) slightly declined with higher dilution ratios, with the control group showing the highest PM percentage (66.88±1.13%) and the 1:1000 dilution group showing the lowest percentage (60.24±2.80%), but the difference was not statistically significant. Curvilinear velocity (VCL) significantly increased with higher dilutions, with the control group at 194.64±5.50 µm/s and the 1:1000 dilution group at 234.61±5.21 µm/s. Linearity (LIN) decreased significantly with higher dilution, from 68.50±1.21% in the control group to 57.04±1.31% in the 1:1000 dilution group. The amplitude of lateral head displacement (ALH) also increased significantly with higher dilutions. Overall, the best dilution ratios appear to be 1:10 (for both control and treatment group). It provides a balance between maintaining high progressive motility and preventing excessive lateral head displacement while ensuring other velocity parameters are within an optimal range.

Keywords: CASA, dilution factors, semen extender, sperm quality, kinematic parameters

INTRODUCTION

The preservation of sperm quality has been observed to be significantly influenced by the dilution factors of extenders. The osmotic balance, essential for sperm integrity, is determined by the type and concentration of extenders used. Studies have demonstrated that acrosome integrity and membrane functionality are better preserved under optimal dilution conditions, which directly affect motility and overall viability post-thaw (Yendraliza *et al.,* 2022; Pardede *et al.,* 2020; Qadeer *et al.,* 2022). For instance, marked variations in post-thaw motility and fertility outcomes across species have been reported when specific sperm concentrations were combined with extenders such as Triladyl® or Tris egg yolk (Yendraliza *et al.,* 2022; Pardede *et al.,* 2020). Improvements in sperm kinematics and morphology have also been linked to the use of appropriate diluent dosing (Steele *et al.,* 2020; Sultan *et al.,* 2024). Furthermore, oxidative stress has been mitigated through the addition of antioxidants to extenders, enhancing post-thaw viability (De Albuquerque Lagares *et al.,* 2021). As a result, the significance of extender dilution in optimising artificial insemination outcomes through effective sperm preservation techniques has been highlighted (Zhu *et al.,* 2022; Jia *et al.,* 2024).

Semen extenders have been acknowledged as essential for maintaining sperm viability during storage and transport (Mokhtassi-Bidgoli *et al.,* 2023; Shepherd *et al.,* 2024). These solutions have been reported to preserve motility while

reducing metabolic activity, thereby extending spermatozoa's lifespan (Fernandez-Novo *et al.,* 2023). Significant improvements in sperm quality have been attributed to extenders such as egg yolk and milk-based solutions (Arif *et al.,* 2022; Murphy *et al.,* 2018). Additionally, oxidative stress during cryopreservation has been reduced by antioxidants and protective agents, improving sperm quality (Shokry *et al.,* 2021; El-Nagar, 2017). Fertility outcomes have been shown to vary with the choice of extender, further emphasising the importance of extender optimisation (Crespilho *et al.,* 2012; Murphy *et al.,* 2018).

Conventional methods have been surpassed by computer-assisted sperm analysis (CASA) in assessing male fertility, particularly in evaluating motility and kinematics (Diansyah *et al.,* 2022; Chaturvedi *et al.,* 2021). However, the decline in quality of cryopreserved sperm post-thawing has been recognised as a limitation, underlining the necessity for advanced preservation techniques (Bintara *et al.,* 2022). Optimisation of sperm kinematic parameters is therefore crucial for enhancing AI efficiency in cattle breeding.

Artificial insemination (AI) has been identified as a key factor in advancing genetic diversity and productivity in cattle breeding (Waberski, 2018; Gianegitz *et al.,* 2024). Its success has been primarily linked to bull sperm quality, which is evaluated through kinematic parameters such as velocity, motility, and morphology (Piddubna *et al.,* 2022). Positive correlations between fertility outcomes and parameters like curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) have been established through research (S *et al.,* 2021; Hossain *et al.,* 2022).

The study aimed to evaluate the effects of different dilution ratios of BioXcell® extender (1:10, 1:100, 1:1000) on kinematic parameters of fresh bull sperm using a computer-assisted sperm analysis (CASA) system and thus to identify the optimal dilution ratio for laboratory assessments on fresh sperm quality using the IVOS II (Hamilton-Thorne, USA) machine. Parameters such as VCL, VSL, VAP, straightness (STR), and linearity (LIN) are being assessed.

MATERIALS AND METHOD

Sample Collection

The semen used in this study was collected at the National Institute of Veterinary Biodiversity (NIVB) in Jerantut, Pahang, which operates under the Department of Veterinary Services (DVS), Malaysia. The collection process involved a total of 36 ejaculations from selected superior bulls such as Belgian Blue, Holstein-Friesian, Mafriwal, and Kedah-Kelantan breeds. These procedures were carried out under controlled conditions by skilled operators. It's important to note that all the bulls were in good health and ranged in age from 2 to 6 years.

Sample Preparation

Fresh bull semen must first meet the criteria established by the Department of Veterinary Services (DVS) before further assessment. Specifically, these criteria require a minimum volume of 2 mL and a sperm concentration exceeding 600×10^6 sperm/mL. Following the qualification, three independent sets of samples are prepared for each specified dilution factor. Dilutions are performed using the BioXcell extender (New Hampshire, USA) at ratios of 1:10 (control), 1:10, 1:100, and 1:1000. The control dilution is prepared by authorised personnel following standardised procedures. In contrast, the remaining dilutions are conducted by researchers while adhering to the same protocols.

To assess the effect of sperm concentration on motility and kinetic parameters in fresh bull

semen, samples were diluted at ratios of 1:10, 1:100, and 1:1000. The 1:10 dilution was used as a standard reference, while the higher dilutions were selected to reduce cell-to-cell interactions, thereby enabling more accurate evaluation of individual sperm movement using computer-assisted sperm analysis (CASA). Previous research suggests that moderate dilution levels (between 1:10 and 1:20) tend to maintain or slightly improve motility, whereas more extensive dilution may alter kinetic profiles and reveal the upper limits of sperm velocity (Jiang *et al.,* 2023; Lone *et al.,* 2022). The inclusion of 1:100 and 1:1000 dilutions in this study was intended to explore how sperm motility responds under conditions of minimal crowding. This approach enhances the precision of CASA measurements and addresses a notable gap in the literature, as most previous studies have focused on lower dilution ranges. Finally, to ensure reliable analysis, all samples are maintained in a 37°C water bath for no longer than 30 minutes before evaluation.

Evaluation of fresh sperm quality

In each experiment, a 30 µl sample was transferred to a cuvette for sperm count. The cuvette was then placed in a photometer device (Minitube, Germany) to ascertain the sperm concentration. This process was repeated three times to ensure the reliability of the results. The sperm was evaluated using a device called IVOS II (Hamilton-Thorne, USA), a Computer-Assisted Sperm Analysis (CASA) system, across four different dilution ratios of BioXcell® extender. This involved microscopic examination of a 5 µl sample droplet placed on a slide and covered with a coverslip. Estimations of motility and other kinetic parameters were made from observations across five distinct microscopic fields for each sample, all maintained at a temperature of 37°C. The parameters studied included motility (%), progressive motility, PM (%), curvilinear velocity, VCL (µm/s), average path velocity, VAP (µm/s), straight-line velocity, VSL (µm/s), linearity, LIN (%), straightness, STR (%), and amplitude of lateral head displacement, ALH (µm). Sperm quality was assessed at 10 different locations to obtain an average reading of the acquired parameters.

Data Analysis

All data were analysed using Statistical Product and Service Solutions (SPSS 25.0 for Windows; SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to assess the normality of data distribution. Although the sample size was large, the data were assumed to follow a normal distribution. Descriptive statistics were reported as mean±standard error of the mean (SEM), presented as (mean±SEM).

Before applying one-way analysis of variance (ANOVA), Levene's test was conducted to evaluate the assumption of homogeneity of variances across groups. This step ensured that variability between groups did not compromise the reliability of the ANOVA results. As the assumption was violated, a post-hoc test was performed to identify specific group differences. A p-value of less than 0.05 was considered statistically significant for all analyses.

RESULTS

In this study, four different dilution factors were evaluated: 1:10 (control), 1:10, 1:100, and 1:1000, as shown in Table 1. There is a slight decline in PM with increasing dilution ratios. The control group (1:10) showed the highest percentage of PM at 66.88+1.13, while the 1:1000 dilution had the lowest PM percentage at 60.24+2.8. Although the change was not statistically significant according to the ANOVA results ($F=1.706$, $p=0.174$), higher dilution was associated with a negative impact on sperm motility.

VCL significantly increased with higher dilution ratios. The control group had a VCL of

194.64±5.50 µm/s, while the 1:1000 dilution reached 234.61±5.21 µm/s. ANOVA indicated significant differences between groups (F-value = 15.754, $p < 0.001$). Although VAP increased with higher dilutions, the difference was not statistically significant (ANOVA F-value = 1.685, $p = 0.173$). An increase in VSL was observed across dilutions, with no significant differences (ANOVA F-value = 0.680, $p = 0.566$).

On the other hand, LIN decreased significantly with higher dilutions. The control group had LIN at 68.50±1.21%, while the 1:1000 dilution showed 57.04±1.31%. ANOVA confirmed significant differences (F-value = 21.988, $p<0.001$). STR also showed a slight decrease at higher dilution levels, with statistically significant differences (ANOVA: $F = 7.373$, $p<0.001$). ALH increased significantly with higher dilutions. The control group had ALH at 6.82±0.22 µm, while the 1:1000 dilution reached 8.08±0.26 µm. ANOVA showed significant differences (F-value = 8.187, $p<0.001$).

DISCUSSION

The assessment of sperm motility parameters in bulls is crucial for evaluating fertility potential. Different studies have established available ranges for a few motility parameters, including progressive motility (PM), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), and amplitude of lateral head displacement (ALH) (Fernandez-Novo *et al.,* 2021; Hidalgo *et al.,* 2021; Pernas *et al.,* 2023; Rossi *et al.,* 2022).

Progressive motility (PM) is a key indicator of semen quality, with acceptable values in bulls typically ranging from 50% to 70%, depending on the study (Nagy *et al.,* 2015; Perumal *et al.,* 2016; Miraz *et al.,* 2022). PM levels below 50% are generally considered suboptimal and may be associated with reduced fertility outcomes (Rai *et al.,* 2017; Hossain, 2023). In the present findings, PM declined slightly with increasing dilution ratios, showing the highest value in

Table 1. Effect of different dilution ratios of diluent under the conditions 1:10 (control), 1:10, 1:100, and 1:1000 on spermatozoa progressive motility and kinetic parameters during fresh bull spermatozoa assessment.

Parameter	Control (1:10)	1:10	1:100	1:1000	ANOVA F-value	p-value
Progressive Motility (PM) (%)	66.88 ± 1.13	65.17 ± 1.46	61.57 ± 2.54	60.24 ± 2.80	1.706	0.174
Curvilinear Velocity (VCL) (µm/s)	194.64 ± 5.50^{a}	210.48 ± 5.25^{a}	222.16 ± 5.84^{b}	234.61 ± 5.21^{bc}	15.754	<0.001
Average Path Velocity (VAP) (µm/s)	92.20 ± 2.49	100.10 ± 2.56	103.70 ± 2.87	110.20 ± 2.79	1.685	0.173
Straight-line Velocity (VSL) (µm/s)	60.98 ± 2.34	65.28 ± 2.52	67.74 ± 2.59	71.90 ± 2.71	0.680	0.566
Linearity (LIN) (%)	68.50 ± 1.21^{a}	63.93 ± 1.38^{a}	60.23 ± 1.58^{b}	57.04 ± 1.31^{bc}	21.988	<0.001
Straightness (STR) (%)	79.12 ± 1.18^{a}	76.11 ± 1.27^{a}	72.83 ± 1.36^{b}	70.22 ± 1.43^{bc}	7.373	<0.001
Amplitude of Lateral Head Displacement (ALH) (µm)	6.82 ± 0.22^{a}	7.02 ± 0.22^{a}	7.44 ± 0.24^{b}	8.08 ± 0.26^{bc}	8.187	<0.001

Note: a, b, c: Different superscripts in the same column indicate significant differences ($p\leq0.05$).

the control group (1:10) at 66.88±1.13%, and the lowest at 1:1000 dilution with 60.24±2.80%. Although the differences were not statistically significant, a negative trend in motility was evident at higher dilutions. This decline may be linked to compromised sperm membrane integrity and reduced mitochondrial activity, which increases apoptotic cell rates (Arif *et al.,* 2022; Hussaini *et al.,* 2017). Additionally, excessive dilution can disrupt osmotic balance during cryopreservation, further affecting sperm viability (Arif *et al.,* 2022; Abdellatif *et al.,* 2022).

Velocity parameters such as curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) are critical indicators of sperm motility and fertility. VCL reflects the sperm's speed along its actual trajectory, with acceptable values for bull sperm typically ranging from 100 to 200 µm/s (Nagy *et al.,* 2015; Perumal *et al.,* 2016; Miraz *et al.,* 2022). Recent studies have reported VCL values exceeding this range under varying dilution levels. Nagy *et al.* (2015) identified VCL as a strong predictor of fertility, while Perumal *et al.* (2016) and Miraz *et al.* (2022) emphasised its role in reproductive success. VAP, which measures the average velocity along a smoothed path, generally falls between 50 and 150 µm/s (Nagy *et al.,* 2015; Miraz *et al.,* 2022) and has shown consistent improvement across dilution levels. Shao *et al.* (2024) confirmed these trends, reinforcing VAP's importance in motility evaluation.

Advanced tools such as computer-assisted sperm analysis (CASA) have further enhanced the precision of VAP measurements (Zheng *et al.,* 2023; Barakat *et al.,* 2015). VSL, indicating the straight-line speed from start to end, is typically acceptable between 30 and 100 µm/s (Nagy *et al.,* 2015; Perumal *et al.,* 2016; Miraz *et al.,* 2022), and also showed incremental changes across dilutions. However, increasing dilution ratios can negatively affect sperm quality due to reduced seminal plasma, which contains motility-supporting proteins (Hussaini *et al.,* 2017; Jiang *et al.,* 2024). Higher concentrations (C100) have been shown to yield better motility and viability than C50 or C25 (Hussaini *et al.,* 2017). While dilution often lowers VCL, VAP, and VSL, some evidence suggests that reduced sperm density may lessen oxidative stress during cryopreservation, potentially enhancing motility (Hussaini *et al.,* 2017). Notably, a selective rise in VCL at certain dilutions may result from reduced cell-to-cell interference, allowing more erratic movement (Hayden *et al.,* 2014; Cheng *et al.,* 2022), possibly as a compensatory response to mitochondrial dysfunction and apoptosis (Arif *et al.,* 2022). Thus, the impact of dilution on velocity parameters is multifaceted and influenced by extender composition and cryopreservation stress. In this study, VCL was found to increase at higher dilution levels, highlighting the need for further investigation into the effects of BioXcell® extender composition.

Other kinematic parameters, such as linearity (LIN), straightness (STR), and amplitude of lateral head displacement (ALH), play a vital role in evaluating bull sperm motility and fertility. LIN, which measures the straightness of the sperm's path, is generally expected to exceed 40%, while STR, indicating directional consistency, should be above 50% for optimal fertility (Perumal *et al.,* 2016; Afriani *et al.,* 2023; Nagy *et al.,* 2015; Miraz *et al.,* 2022). Although both parameters tend to decline with increasing dilution, the values remain within acceptable ranges. ALH, typically ranging from 3 to 6 µm, reflects the lateral movement of the sperm head and is essential for assessing swimming patterns that influence fertilisation success (Miraz *et al.,* 2022; Perumal *et al.,* 2022). Factors such as age and breed can significantly affect ALH and related motility traits, with older bulls often showing reduced motility and altered parameters (Setiawan *et al.,* 2020; Kurniawan *et al.,* 2020; Isnaini *et al.,* 2022).

Dilution ratios further influence these parameters. Higher dilution levels often reduce velocity (VAP, VCL, ALH) while increasing LIN and STR, as observed in C100 compared to C50 and C25 concentrations (Hussaini *et al.,* 2017). These changes are primarily attributed to reduced seminal plasma, which contains proteins essential for motility and viability (Hussaini *et al.,* 2017). Additionally, dilution and cold storage may suppress metabolic activity but also trigger oxidative stress, damaging sperm membranes and mitochondria (Jiang *et al.,* 2024). Behavioural adaptations, such as sperm adhesion to tube walls due to weakened stimulants like albumin, and increased energy expenditure under excessive dilution, further compromise motility (Jiang *et al.,* 2024). While moderate dilution may reduce oxidative damage, careful management is essential to maintain sperm quality for artificial insemination (Hussaini *et al.,* 2017; Jiang *et al.,* 2024).

Finally, excessive dilution has been shown to impair sperm motility, viability, and functional integrity, ultimately reducing fertility rates. Over-dilution with tris extenders negatively affected motility parameters such as VAP, VSL, VCL, and ALH, leading to increased dead sperm populations (Lone *et al.,* 2022). Similarly, Jiang *et al*. (2024) reported diminished viability and acrosome integrity at dilution ratios exceeding 5 million sperm per dose. Karan *et al.* (2018) emphasised that high dilution rates compromised post-thaw semen quality, while oxidative stress during prolonged dilution periods further impaired motility and fertility (Abdel-Latif *et al.,* 2022). Therefore, optimising dilution ratios is critical for maintaining sperm quality and ensuring successful fertilisation outcomes (Lone *et al.,* 2022; Yadav *et al.,* 2024; Hussaini *et al.,* 2017).

CONCLUSION

This study demonstrates that a 1:10 dilution ratio, whether applied as a control or treatment, provides an optimal balance for preserving bull sperm quality. At this ratio, progressive motility remains high, lateral head displacement is minimised, and velocity parameters such as VCL, VAP, and VSL fall within acceptable ranges. Additionally, determining the ideal volume of semen extender dilution reduces the risk of overuse and associated costs. These findings underscore the importance of carefully regulating dilution ratios during sperm evaluation, as excessive dilution can impair motility and compromise overall viability. Although the adverse effects of higher dilution are evident, further research is warranted to elucidate the underlying physiological mechanisms, identify the optimal dilution range, and assess the role of extender composition. Such investigations will be critical for optimising semen processing protocols and improving the success of artificial insemination in bovine reproduction.

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