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The combination of CASA kinetic parameters and fluorescein staining as a fertility tool in cryopreserved bull semen

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Abstract: Prediction of male fertility with in vitro semen quality parameters remains a challenge for the bull industry. Fluorescein staining and computer-assisted semen analysis (CASA) ensure kinetically proper and functionally objective results to improve spermatological parameter control. Therefore, we aimed to examine the CASA kinetic parameters and fluorescein staining of cryopreserved bull semen and its relationship with fertility. A total of 90 frozen straws from 5 Simmental bulls were evaluated. After semen was thawed, the motility, kinetic parameters, and insemination doses were evaluated by CASA; fluorescein stainings were evaluated using a fluorescein microscope. The 60th-day nonreturn rates (NRRs) were recorded for determination of pregnancy rates. There were significant differences in motility and kinetic parameters among bulls. Bull 3 had the lowest percentage of NRR and curvilinear velocity, straight line velocity, beat-cross frequency, and lateral head displacement parameters ($P < 0.05$). A positive correlation was detected between the pregnancy rate and some kinetic parameters. The correlation between the CASA parameters was determined as well. We concluded that various kinetic parameters obtained with CASA software system algorithms and fluorescein stainings can be linked to fertility. However, further research is needed to acquire a more precise link with fertility.

Key words: Computer-assisted semen analysis, motility, bull semen, kinetic parameters, fluorescein staining, fertility

1. Introduction

Fertility is becoming a more substantial issue for bovine reproduction, particularly following the introduction of artificial insemination, since a single bull is used for breeding numerous cows (1). Conventionally, sperm characteristics have been assessed using a spectrophotometer and microscope. Morphologic abnormalities occurring during semen production may have contrary effects on fertility and sperm function (2). Sperm motility can indicate the viability of spermatozoa and expression of structural integrity. It is one of the most important parameters related to fertilization. During the last two decades, computer-assisted semen analysis (CASA) has been introduced for acquiring objective information on different sperm qualifications, progressivity, and sperm kinetic parameters. It ensures valuable information on several parameters such as progressive motility, average path velocity (VAP), curvilinear velocity (VCL), lateral head displacement (ALH), straight line velocity (VSL), and beat-cross frequency (BCF) (3). In recent years, the effectiveness of detecting sperm damage has been enhanced through technological developments. Fluorescent staining technology involves the use of fluorescent dyes, either indirectly or directly binding them with part of the spermatozoa, or evaluating damage in the function or structure of the sperm, e.g., the membrane, mitochondria, acrosome, or DNA (4). Various fluorescent staining techniques have also been used to examine semen viability, acrosome status, and mitochondrial activity. These measurements are considered useful for both in vitro examination and for predicting field fertility (5).

This experimental design contains three main in vitro sperm assessment techniques performed in steps and compares them with in vivo field results. Therefore, the objectivity of this study verifies the individual impact of the evaluated parameters, aiming to investigate the relationship among field fertility, CASA motility, kinetic parameters, and fluorescein staining results of cryopreserved bull semen.

2. Materials and methods

Frozen straws were brought to the Ankara University Reproduction and Artificial Insemination Spermatology Laboratory for in vitro semen analysis to validate the

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import license from Germany. Semen samples were obtained from 5 different Simmental bulls. For each bull, 18 differently dated ejaculates were evaluated.

2.1. Evaluation of semen parameters

2.1.1. Analysis of CASA motility

A Sperm Class Analyzer (Microptic S.L., Barcelona, Spain) was used to analyze various kinematic parameters and sperm motility. The spermatozoa motility properties were set as static, slow (>50 μm/s), medium (>75 μm/s), and fast (>100 μm/s). Thawed semen (6 μL) was placed onto the slide, covered with a coverslip, and analyzed with a 10× objective on a preheated microscope stage (37 °C). Progressive motility (%), total motility (%), VSL (straight line velocity, μm s^{-1}), VAP (average path velocity, μm s^{-1}), VCL (curvilinear velocity, μm s^{-1}), linearity (LIN, [VSL/VCL] × 100), ALH (amplitude of lateral head displacement, μm), STR (straightness, [VSL/VAP] × 100), WOB (wobble, [VAP/VCL] × 100), and BCF (beat-cross frequency, Hz) were determined. A total of 200–400 spermatozoa per sample in 6 microscopic fields were evaluated.

2.1.2. Fluorescein staining

For the evaluation of viability, acrosome integrity, and mitochondrial activity, various staining procedures were followed. Afterward, at least 200 sperm in every specimen were analyzed using a fluorescent microscope (Leica DM 2500, Germany).

2.1.2.1. Dead spermatozoa (viability)

Dead spermatozoa rate was determined using a sperm LIVE/DEAD kit (SYBR-14/PI, Invitrogen, L-7011). For assessment of the live/dead spermatozoa rate (%), Bucak et al.'s (6) method was used.

2.1.3. Acrosome integrity

Acrosome status of each sperm cell was determined with FITC-PNA (L-7381, Invitrogen) and the PI staining method (6).

2.1.4. Mitochondrial activity

The mitochondrial activation status was determined using the JC-1 (Invitrogen, T-3168)/PI staining method as previously described by Garner et al. (7).

2.2. Evaluation of in vivo fertility

The selection of cattle for the experimental design was made based on reproductive records, clinical examination, and rectal palpation. Detailed reproductive records were obtained from the owner. After the initial clinical inspection, body condition score (BCS) was determined; animals having a BCS lower than 2.5 or higher than 3.5 were discarded. Finally, all animals were examined for possible reproductive tract pathologies by rectal palpation. The selected 1265 Simmental primiparous cows, all less than 5 years of age, were randomly assigned as 253 cattle for each of the 5 bulls. Animals were kept in uniform conditions in Kavak, Samsun, Turkey.

Prior to insemination, estrus was detected by visual standing heat and confirmed by cervical mucus discharge. For clinical confirmation, rectal palpation was performed on the genital tract for uterine contractility and high myometrial tone. In addition to that, the presence of Graafian follicles was determined to support the confirmation of estrus with ovarian status. After visual and clinical confirmation of estrus, animals were inseminated at least 12 h after the onset of estrus. Straws were thawed in a water bath (37 °C) for 30 s, and inseminations were carried out rectovaginally by depositing a 0.25-mL straw into the corpus uteri.

Nonreturn rate (NRR) is defined as the proportion of cows not seen to come back into estrus within a 60-day period after breeding; the cows are thus considered to be pregnant. In this study, NRR was selected as a fertility result since the data can be quickly obtained with a reasonable cost, and it can be accepted as an indirect sign of fertility. Compared to other fertility traits, NRR is less biased by selection, and if the causes that influence it can be controlled, it may be used as a reliable indicator of the fertility of the bull (8).

All of the experimental work on the animals was conducted according to the standard veterinary application by the same expert and certified veterinarian in line with the laws and regulations of the local ethics committee.

2.3. Statistical analysis

The significant differences between the bulls were analyzed by ANOVA. To determine the differences between groups, Tukey's test was used. Statistical analyses were done with a minimum 5% margin of error. The descriptive measurements of the bulls are given in the tables as arithmetic mean ($\overline{X}$) ± standard deviation (SD). SPSS for Windows 14.1 (License No. 9869264) was used for analysis of the data. NRRs were evaluated with the chi-square test. Differences with values of $P < 0.05$ were considered to be statistically significant. A regression analysis was performed on the CASA parameters with pregnancy rates, and fluorescent stainings with pregnancy rates with Minitab 16 (License No: 16.1.1.1).

3. Results

In Figure 1, NRRs are 81.8%, 84.9%, 70.0%, 84.2%, and 89.4%, respectively; there was no statistically significant difference between the bulls in the first insemination ($P > 0.05$).

As shown in Figures 2–6, there were positive correlations between pregnancy rate and VCL, VAP, VSL, ALH, and BCF, respectively.

In Table 1, there is a statistically significant difference between the bulls for CASA kinetic parameters and fluorescein staining, except for acrosome integrity. The highest viability (64.29 ± 3.40) and mitochondrial activity

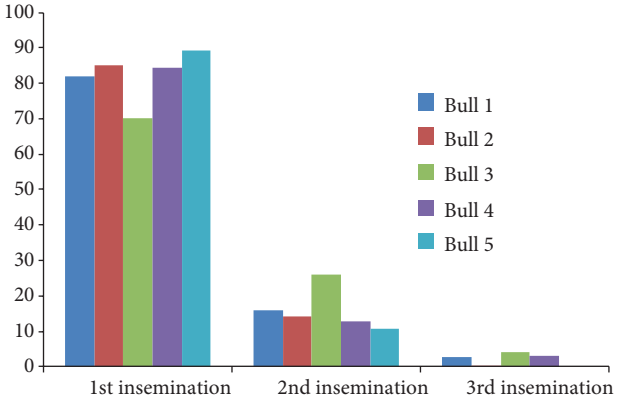

Figure 1. In vivo fertility results (NRR %) of bulls. There was no statistically significant difference among the bulls for NRR (P > 0.05).

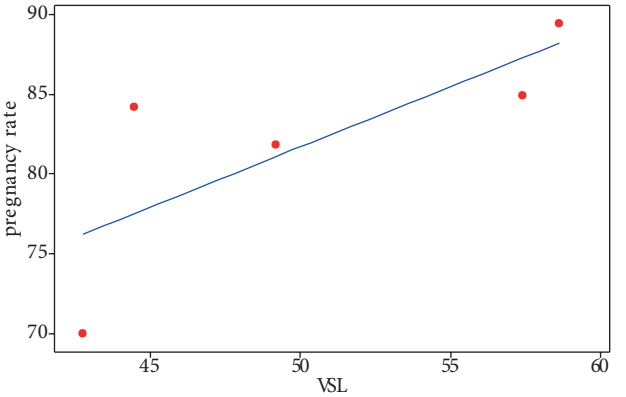

Figure 4. Relationship between VSL (µm/s) and pregnancy rate (%) after artificial insemination. r = 0.755.

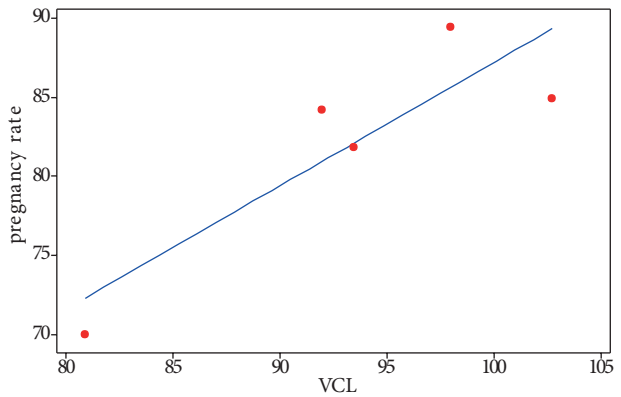

Figure 2. Relationship between VCL (µm/s) and pregnancy rate (%) after artificial insemination. r = 0.874.

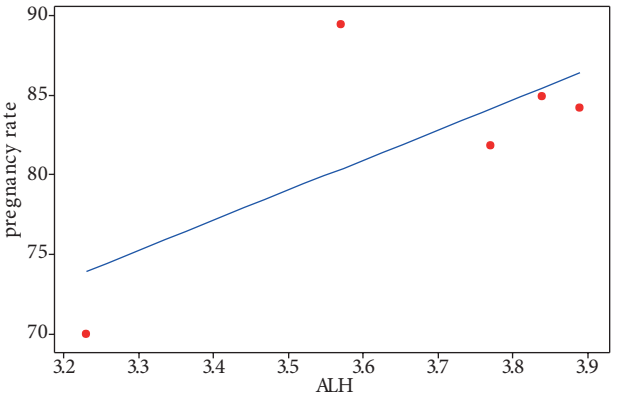

Figure 5. Relationship between ALH (µm/s) and pregnancy rate (%) after artificial insemination. r = 0.701.

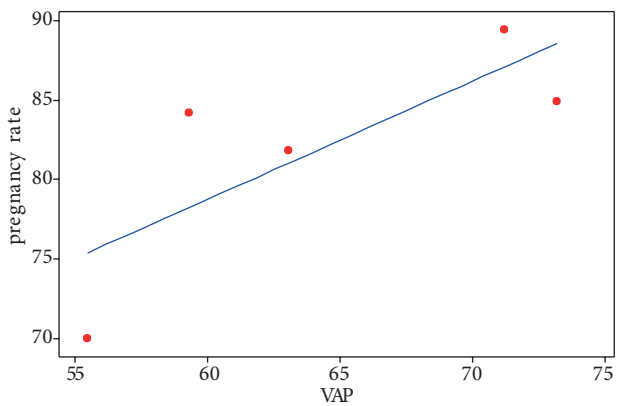

Figure 3. Relationship between VAP (µm/s) and pregnancy rate (%) after artificial insemination. r = 0.778.

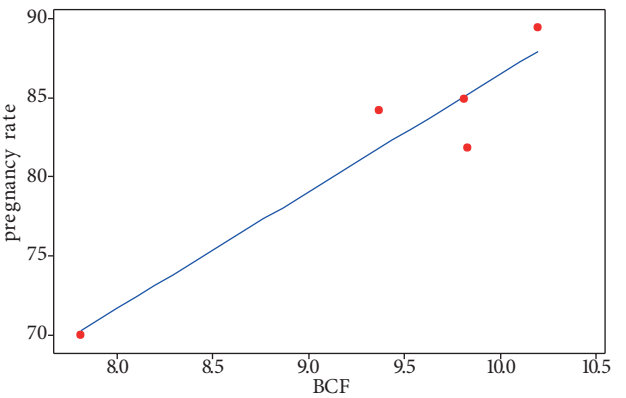

Figure 6. Relationship between BCF (Hz) and pregnancy rate (%) after artificial insemination. r = 0.953.

Table 1. Mean (±SD) CASA motility, kinetic parameters, and fluorescent staining values after thawing the semen.

Bull	1	2	3	4	5	P
Progressive motility (%)	22.26 ± 6.66^{b}	33.31 ± 13.37^{a}	21.10 ± 6.51^{b}	19.87 ± 9.24^{b}	25.98 ± 10.77^{b}	*
Total motility (%)	46.93 ± 9.85^{b}	63.88 ± 15.16^{a}	47.15 ± 6.82^{b}	51.04 ± 14.34^{b}	45.44 ± 13.77^{b}	*
VCL (μm/s)	93.44 ± 7.92^{b}	102.74 ± 9.80^{a}	80.87 ± 15.76^{c}	91.95 ± 11.28^{b}	97.98 ± 6.02^{b}	*
VSL (μm/s)	49.19 ± 7.45^{a}	57.42 ± 12.28^{a}	42.74 ± 10.65^{b}	44.05 ± 12.16^{a}	58.64 ± 7.08^{a}	*
VAP (μm/s)	63.07 ± 6.81^{bc}	73.20 ± 11.74^{a}	55.44 ± 11.42^{c}	59.28 ± 11.02^{c}	71.23 ± 6.5^{b}	*
LIN (%)	52.64 ± 6.32^{bc}	55.34 ± 7.86^{ab}	52.82 ± 7.50^{bc}	47.21 ± 7.97^{c}	60.02 ± 6.19^{a}	*
STR (%)	77.72 ± 4.28^{ab}	77.72 ± 5.47^{ab}	76.57 ± 4.94^{ab}	73.21 ± 7.25^{b}	80.67 ± 6.90^{a}	*
WOB (%)	67.51 ± 4.53^{bc}	70.88 ± 5.68^{ab}	68.67 ± 5.51^{ab}	64.09 ± 4.80^{c}	72.93 ± 5.22^{a}	*
ALH (μm/s)	3.77 ± 0.42^{a}	3.84 ± 0.28^{a}	3.23 ± 0.72^{b}	3.89 ± 0.31^{a}	3.57 ± 0.38^{a}	*
BCF (Hz)	9.83 ± 0.96^{a}	9.81 ± 0.63^{a}	7.81 ± 1.68^{b}	9.37 ± 1.62^{a}	10.20 ± 0.88^{a}	*
Hyperactivity (%)	27.48 ± 8.72^{b}	35.28 ± 10.01^{a}	28.85 ± 7.82^{b}	25.26 ± 9.24^{b}	24.64 ± 7.28^{b}	*
Conc. (mil./straw)	13.86 ± 4.50^{b}	17.38 ± 6.53^{ab}	13.43 ± 3.58^{b}	20.14 ± 8.80^{a}	15.31 ± 3.59^{ab}	*
Viability (%)	48.65 ± 2.4^{c}	64.29 ± 3.4^{a}	46.98 ± 1.9^{c}	55.30 ± 2.01^{b}	49.15 ± 1.9^{c}	*
M. Activity (%)	50.04 ± 8.37^{b}	56.5 ± 1.03^{a}	54.8 ± 6.56^{b}	49.7 ± 4.6^{b}	54.2 ± 6.4^{b}	*
A. Integrity (%)	44.8 ± 10.5	49.6 ± 3.5	42.7 ± 9.35	47.6 ± 10.9	44.5 ± 5.2	-

^{a,b,c} Different superscripts within the same row demonstrate a significant difference ($P < 0.05$). *Statistically significant.

(56.5 ± 1.03) were seen in Bull 2 ($P < 0.05$). In addition, Bull 2 had the highest total motility, progressive motility, VAP, and VCL; the highest concentration was obtained from Bull 4, with 20.14 ± 8.8 (million spermatozoa/straw) ($P < 0.05$).

As presented in Table 2, correlations between the CASA parameters were obtained. The highest positive correlation was detected between the VCL and VAP parameters, followed by the correlation between LIN and WOB.

4. Discussion

The CASA instruments have been available since the mid-1980s (9); there are now numerous research studies to define the role of CASA in field fertility outcomes. Over the past decades, during the fertilization process, motility of sperm has drawn remarkable interest (10); in an evaluation of fertilizing ability, motility is accepted as one of the most crucial parameters (11).

In the present study, there were significant differences among bulls in terms of motility and kinetic parameters. High positive correlations were detected between VCL, VSL, BCF, and ALH kinetic parameters and pregnancy rate. Some studies reported that ALH, VSL, VCL, and LIN are correlated with fertility in humans (12). Other scientists have described significant interactions between different kinetic parameters for bull spermatozoa and in vivo fertility (13). The importance of VSL in the fertilizing capacity of spermatozoa has been observed in both bulls (14) and humans (15), and it was confirmed in the present study as both the lowest VSL and NRR values were obtained from Bull 3, in addition to the positive correlation acquired between these parameters. VSL, VAP, and VCL are all measurements of sperm velocity over specific paths, so these values indicate that sperm classified as having high mobility swim faster than those classified with lower mobility (16). Thus, the role of VSL in sperm transport may be during the passage through the female reproductive tract and penetration of the oocyte vestments (14). In predicting the achievement of pregnancy, ALH values were found to be more reliable than the conventional criteria of semen quality (9). Our study demonstrated that ALH also has a relationship with fertilization rates. In vitro fertilization rates were also observed to be significantly correlated with STR (17). Moreover, LIN values have been determined to interact with fertility (12). However, in this study, we did not find any interaction between LIN, STR, and fertilization. This may be caused by the usage of different animal genotypes and species, semen extenders, different CASA system (IVOS, SCA), or fertilization type (in vivo or in vitro).

In the present study, Table 2 shows the correlation between various sperm kinetic parameters evaluated by CASA. The highest positive interaction was detected between the VCL and VAP kinetic parameters, followed by the interaction between WOB and LIN. In rabbit and bull semen, in accordance with the present study,

Table 2. Correlations between the CASA parameters.

	Progressive motility (%)	Total motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm/s)	BCF (Hz)	Hyperactivity (%)
Total motility (%)	0.764***										
VCL (μm/s)	0.480***	0.216*									
VSL (μm/s)	0.684***	0.211*	0.812***								
VAP (μm/s)	0.678***	0.277**	0.896***	0.979***							
LIN (%)	0.639***	0.115	0.360***	0.830***	0.716***						
STR (%)	0.547***	0.042	0.404***	0.772***	0.669***	0.876***					
WOB (%)	0.672***	0.218*	0.286**	0.777***	0.676***	0.975***	0.812***				
ALH (μm/s)	–0.061	0.054	0.320**	–0.008	0.088	–0.300**	–0.237*	–0.328**			
BCF (Hz)	0.191	–0.029	0.605***	0.466***	0.496***	0.21*	0.251*	0.112	0.559***		
Hyperactivity (%)	0.560***	0.559***	0.238*	0.258*	0.284**	0.181	0.191	0.211*	–0.208*	0.07	
Concentration (million/straw)	–0.135	0.138	–0.112	–0.24*	–0.169	–0.314**	–0.416***	–0.223*	0.376***	–0.135	0.31**

*P < 0.05, **P < 0.01, ***P < 0.001.

velocity parameters strongly correlated with progressive motility (13–18), indicating that spermatozoa with forward motility and a straight linear path may cover more distance in a short period of time. In addition to that, a strong and highly significant correlation was found between straightness and linearity in bulls (13). In a study conducted on rabbits, strong correlations between WOB and LIN parameters were observed as well (18).

In recent years, examination of plasma membrane or whole organelles of spermatozoa with fluorescent staining for viability, acrosome integrity, and mitochondrial activity has been introduced to sperm quality assessments (19). Plasma membrane status (viability) is crucial for the boundary between oocyte and spermatozoa in the female genital tract epithelium. Although in the present study, the relationship between the mentioned sperm quality parameters and NRR was found to be lower than expected, novel spermatological quality parameters that are relative to fertilization should be investigated. In many studies, significant relationships between sperm quality parameters were observed (20). Although some researchers have found a statistically important correlation between NRR and motility (21), there are contrary reports, as well (22). A significant interaction was indicated between viability and fertilization in fluorescent staining (20–23). Some studies reported that viability in fluorescence studies is essential for estimating fertilization capacity (23). In our study, sperm progressive motility, total motility, and viability parameters were in positive relation with NRR.

Mitochondria are accessible energy-producing organelles located in the midpiece of spermatozoa, and they ensure energy to the tail filaments. Hence, the mitochondria facilitate an efficient impulse so that the sperm can both reach the oocyte and be able to penetrate the zona pellucida (24). There are dense fibers located on the axoneme in the midpiece of the sperm cell. These fibers provide potential (mitochondrial activity) to produce ATP energy and motility of spermatozoa (25). In the present study, the highest mitochondrial activity and motility results were seen in the same animal: Bull 2 ($P < 0.05$). The lowest mitochondrial activity results were observed in Bulls 1 and 4. Thus, these results confirm that potential changes of the mitochondrial membrane could be an accurate sign of physiological disruption (26).

Acrosomal integrity is essential for fertility, as the acrosomal contents do not exit the cell until the spermatozoon comes into contact with the zona pellucida. Hence, penetration of sperm into the zona pellucida can be aided with the released proteases (27). Intact sperm with proper stimulation and induction of the acrosome reaction are necessary for successful fertilization (28). Anilkumar et al. (29) found that there was a positive correlation among the motility, acrosome integrity, and distance traveled by bovine spermatozoa in the artificial mucus or cervical canal. In this study, FITC-PNA was used as the staining method to identify in vitro acrosome integrity in the bulls; however, no statistically significant difference was found among the bulls ($P > 0.05$). Similar to our results, Kjaestad et al. (30) found a significant interaction between velocity and motility, but not between acrosome integrity and motility.

In conclusion, in vitro evaluation of bull sperm using the combination of various fluorescent staining methods and CASA kinetic parameters, which have positive correlations with pregnancy rate, can be a useful tool for predicting the fertility of semen samples for artificial insemination. This result may provide a reliable estimation of fertilization ability in bull semen.

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