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Spermatological Parameters *In Vitro* of Holstein Bulls and Relationship with Male Fertility*

Halil Dönmez[1] & Muhammed Enes İnanç[2]

ABSTRACT

***Background*:** The main purpose of evaluating spermatological parameters is to assess the relationship between semen quality and fertility. Extensive research is currently being conducted on freezing, storage, and thawing of bovine semen. Therefore, it is crucial to assess the quality of frozen semen. The freezing of semen causes different degrees of damage to the sperm cell. Damages reduce the ability of the spermatozoon to fertilize the oocyte. Damages reduce the ability of sperm to fertilize oocytes. This study aims to determine the relationship between fertility and spermatological parameters of frozen-thawed bull semen routinely used in artificial insemination (AI).

***Materials, Methods & Results*:** Frozen semen samples from 5 different imported Holstein bulls (5 different collection dates) and 175 cows 2-4 years of age were used. These bulls are commonly used by AI organizations in breeding. Frozen-thawed bull semen straws were analyzed for motility using a phase-contrast microscope with a heated stage, plasma membrane and acrosome integrity (PMAI), viability, lipid peroxidation level, and mitochondrial oxidative stress were performed with a flow cytometer. PMAI was evaluated by The FITC/PNA-PI double staining protocol, lipid peroxidation level were detected by BODIPY/SYBR 14 double staining protocol; sperm viability was determined using SYBR-14/PI double staining protocol, and sperm mitochondrial oxidative stress was evaluated by MITOSOX/PI double staining protocol. The flow cytometric tests were performed using a CytoFLEX flow cytometer Beckman Coulter equipped with emission filters at 610 ± 20 nm, 585 ± 42 nm, 525 ± 40 nm, and 50 mW (488 nm) laser output. An average of 10.000 spermatozoa were analyzed for each evaluation. Pseudocolor plots were used to compare the side scatter area (SSC-A) with the forward scatter area (FSC-A) of sperm cells to facilitate the selection process. For the AI, cows estrus was confirmed by detecting the graff follicle by rectal palpation and assessing uterine tone. Once clinical and observational signs confirmed estrus, AI was performed. Pregnancy rates were determined 60 days after AI using ultrasound. No significant correlations were found between in vitro spermatological parameters and pregnancy rates ($P > 0.05$). In one bull, motility scores correlated with pregnancy rate ($P < 0.05$). There were significant differences in PMAI and mitochondrial oxidative stress parameters ($P < 0.05$). Positive correlations were found between motility, PMAI, and viability. Furthermore, PMAI was positively correlated with viability and lipid peroxidation levels. Conversely, mitochondrial oxidative stress is negatively correlated with viability and lipid peroxidation. A positive correlation was observed between viability and lipid peroxidation levels. ($P < 0.05$). No statistically significant difference was observed between the bulls with regard to pregnancy rates.

***Discussion*:** Different authors showed a positive correlation between motility and fertility results. Also, flow cytometric results correlated with non-return rate and other spermatological parameters. This study concluded that different organelles and structures within a cell work closely together to carry out metabolic processes. For the accurate prediction and correlation of fertility, it is necessary to create multiple combinations of spermatological parameters. It is also essential to study the associations and correlations between high- and low-fertility bulls and to investigate the pathways leading to their differences.

Keywords: artificial insemination, bull, fertility, flow cytometry, spermatological parameters.

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[1]Private Veterinary Practitioner, Burdur, Turkey. [2]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University (MAKU), Burdur. CORRESPONDENCE: M.E. Inanç [enesinanc@hotmail.com]. Department of Reproduction and Artificial Insemination, Veterinary Medicine, Burdur Mehmet Akif Ersoy University - MAKU. 15030 Burdur, Turkey.

INTRODUCTION

The semen freezing processes began as biodiversity measures and are now widely used commercially. Freezing semen causes different degrees of damage to sperm cells [21]. Thus, it would be useful to have a fertility prediction system based on the evaluation of several spermatological parameters after freezing and thawing to remove animals with low fertility potential from breeding. In this manner, considerable time savings and economic gains can be achieved. Several parameters have been used to estimate semen fertility [14].

In the last decade, the assessment of fertilization ability has given great importance to examining motility in spermatological evaluations [12,25]. Additionally, fluorescent staining techniques have been used to determine parameters, such as viability [22], acrosome status [31], mitochondrial activity [8], lipid peroxidation levels [6], and DNA integrity [11,22]. Research has shown that these measurements are critical for assessing fertility potential in both *in vitro* and *in vivo* studies [1,31]. Several studies have demonstrated a correlation between fertility and spermatological parameters [2,9,18,24]. Research recommends analyzing multiple parameters together when conducting a regression analysis [14]. Specialized equipment, such as microscopes with fluorescent attachments, computer-aided sperm analysis systems (CASA), and flow cytometry devices, are now available owing to technological advancements in analyzing and evaluating spermatological parameters.

The primary objective of this study was to investigate the fertility capabilities of frozen-thawed bull semen under controlled laboratory conditions and explore any potential correlations between its fertility and its routine use in AI procedures.

MATERIALS AND METHODS

Animals and study design

In the study, frozen-thawed semen samples from 5 Holstein bulls and 175 cows 2-4 years of age were used. These bulls are commonly used by AI organizations in breeding. Each bull's semen was collected on 5 different dates, providing a diverse dataset to analyze their fertility abilities and correlations. Thirty-five AI (7 AI per different collection date) were performed for each bull. The Local Ethics Committee on Animal Research at Burdur Mehmet Akif Ersoy University approved the protocol for animal use in this study (approval number: 17.11.2021/821).

In vitro *spermatological parameters*

Five different collection dates of each bull semen sample were analyzed using phase-contrast microscopy and flow cytometry. Before evaluation, the 2 frozen straws were thawed in a 37°C water bath for 30 s.

Motility

Semen motility was assessed using a phase-contrast microscope with a heated stage set at 37°C and a magnification of 400× (20).

Evaluations of flow cytometric analysis

Tests were performed using a CytoFLEX flow cytometer Beckman Coulter[1] equipped with emission filters at 610 ± 20 nm, 585 ± 42 nm, 525 ± 40 nm, and 50 mW (488 nm) laser output. An average of 10,000 spermatozoa were analyzed for each evaluation. Pseudocolor plots were used to compare the side scatter area (SSC-A) with the forward scatter area (FSC-A) of sperm cells to facilitate the selection process. Forward scatter height (FSC-H) and forward scatter area (FSC-A) were used to exclude doublets from the analysis [9]. Stain stock solutions were prepared using DMSO and stored at -20°C, from which 50 µL aliquots were prepared. Flow cytometric analysis was performed using the CytExpert 2.3 software[1].

The FITC/PNA-PI staining protocol was used to detect sperm plasma membrane integrity (PMAI) [19]. Staining was performed using 100 µg/mL FITC-PNA (with an emission wavelength of 521 nm, Sigma, L7381)[2] and 2.99 mM propodium iodide (PI, Invitrogen, L7011)[3] [with an emission wavelength of 629 nm]. Frozen-thawed semen samples were adjusted to a sperm concentration of $5x10^6$ (10 µL) by dilution with 492 µL PBS. Subsequently, 5 µL FITC and 3 µL PI were added to the mixture and incubated at 37°C for 15 min in the dark. After incubation, the samples were analyzed and the FITC- and PI- populations were recorded as PMAI levels (%).

Sperm viability was determined using SYBR-14 and PI protocols [19]. A mixture of 1:10 SYBR-143 (Invitrogen, L7011) (with an emission wavelength of 519 nm) and 2.99 mM PI was used. The frozen-thawed semen samples were adjusted to a sperm concentration of $5x10^6$ (10 µL) by dilution with 492 µL PBS. Next, 5 µL of SYBR-14 and 3 µL of PI were added, and the mixture was incubated at 37°C for 15 min in the dark. After the incubation period, the samples were analyzed, and the SYBR 14+ and PI- populations (%) were recorded as viability levels.

Semen lipid peroxidation levels were detected by 5 μM BODIPY (emission wavelength of 519 nm, D38611, Invitrogen Molecular Probes)[3] /SYBR 14 double staining using the modified protocol [27]. For staining, frozen-thawed semen samples were adjusted to a sperm concentration of $5x10^6$ (10 μL) by dilution with 496 μL PBS. Subsequently, 3 μL BODIPY and 3 μL SYBR-14 were added to the mixture, which was incubated at 37°C for 15 min in the dark. After the incubation period, the samples were analyzed and BODIPY+ and SYBR 14+ populations (events) were recorded as indicators of lipid peroxidation levels.

Mitochondrial oxidative stress in semen was evaluated by MITOSOX/PI (Invitrogen) staining using the double staining method [5]. The frozen-thawed semen samples were adjusted to a sperm concentration of $5x10^6$ (10 μL) by dilution with 492 μL PBS. Subsequently, 5 μL MITOSOX (with an emission wavelength of 582 nm (5 μM), M36008, Molecular Probes)[3] and 3 μL PI were added to the mixture, which was then incubated at 37°C for 15 min in the dark. After the incubation period, the samples were analyzed and MITOSOX+ and PI+ populations (%) were recorded as indicators of mitochondrial oxidative stress levels.

Artificial insemination and pregnancy examination in cows

For the AI, cows from the Burdur in Turkiye were carefully selected based on their reproductive history and thorough clinical examinations. The study included animals that had calved at least once, had no reproductive problems and were 45-60 days postpartum. Only animals in their 1st postpartum heat were selected for this study. In addition, the selected cows had body condition score between 2.5 and 3.5. prior to AI, external clinical signs and discharge were evaluated. Clinically, estrus was confirmed by detecting the graff follicle by rectal palpation and assessing uterine tone. Once estrus was confirmed by clinical and observational signs, AI was performed at least 12 h after the onset of estrus based on the information provided by the owner. AI was performed via the rectovaginal method into the corpus uteri using thawed semen at 37°C for 30 s. Pregnancy rates were determined 60 days after AI using Bonway (Bw560v)[4] ultrasound.

Statistical analysis

Before performing the significance tests, the data were assessed for normality using the Shapiro-Wilks test, which is based on the assumptions of parametric testing. Levene's test was used to check the homogeneity of variances. ANOVA was used to control for differences between bulls statistically. Duncan's test was then used for the evaluation of differences between groups. Descriptive statistics were calculated for each variable and presented as mean ± standard error of the mean (mean ± SE). The pregnancy rates were analyzed using the chi-squared test. Additionally, regression analysis was utilized to determine the relationship between spermatological parameters and fertility.

All statistical analyses were performed using the SPSS 22.0 software package[5], with a maximum error rate of 5%. A significance level of 0.05 was used to determine statistical significance.

RESULTS

The mean spermatological parameters of the AI bulls are presented in Table 1. There was no statistical difference between the bulls in terms of motility, viability, and lipid peroxidation levels ($P > 0.05$). The lowest PMAI was found in bull 2 ($P < 0.05$); the highest mitochondrial oxidative stress was found in bulls 2 and 4 ($P > 0.05$).

The correlations between spermatological parameters and pregnancy rates were shown in Table 2. No statistically significant relationship was found between the pregnancy rates and spermatological parameters ($P > 0.05$). When the spermatological parameters were evaluated, a positive correlation was found among motility and PMAI and viability, a positive correlation among PMAI with viability and BODIPY+, a negative correlation with MITOSOX+, a negative correlation between MITOSOX+ viability and BODIPY+, and a positive correlation between viability and BODIPY+ ($P < 0.05$).

Correlations between individual pregnancy rates and spermatological parameters were shown in Table 3. In the individual evaluation, a statistically significant correlation ($P < 0.05$) was found only between Bull 2 and motility.

The pregnancy rates of semen used for AI are shown in Table 4. No statistically significant difference was observed between the bulls with regard to pregnancy rates.

Table 1. Mean (± SEM) spermatological parameters of bulls subjected to AI.

Bulls	Motility (%)	PMAI (%)	MITOSOX + (%)	Viability (%)	BODIPY + (events)
1	54.50 ± 4.56	71.49 ± 1.77^{b}	23.86 ± 3.59^{a}	65.03 ± 3.03	725.40 ± 145.57
2	51.50 ± 1.27	68.30 ± 2.20^{b}	33.84 ± 4.33^{ab}	48.92 ± 6.21	1330.40 ± 559.70
3	59.37 ± 4.60	67.31 ± 4.48^{b}	22.70 ± 2.58^{a}	63.89 ± 3.54	1752.68 ± 555.62
4	54.75 ± 5.57	48.57 ± 3.66^{a}	47.17 ± 6.06^{b}	52.44 ± 12.66	704.50 ± 206.55
5	60.00 ± 3.70	70.41 ± 4.80^{b}	29.02 ± 6.58^{a}	64.73 ± 4.57	1108.60 ± 210.43
Total	55.90 ± 1.77	65.91 ± 2.20	31.00 ± 2.68	59.94 ± 2.73	1141.80 ± 180.28
P		*	*		

* ^{a,b}Different superscripts within the same column demonstrate significant differences ($P < 0.05$). PMAI: Plasma membrane and acrosome integrity rate; MITOSOX +: Mitochondrial oxidative stress rate; Viability: A live spermatozoa rate; BODIPY: Lipit peroxidation rate.

Table 2. Correlation results between spermatological parameters and pregnancy.

	Motility	PMAI	MITOSOX +	Viability	BODIPY+	Pregnancy
Motility						
PMAI	,262**					
MITOSOX +	-,121	-,649**				
Viability	,221**	,313**	-,675**			
BODIPY +	,039	,256**	-,271**	,272**		
Pregnancy	,021	,027	-,058	,063	,078	

**$P < 0.01$. PMAI: Plasma membrane and acrosome integrity rate; MITOSOX+: Mitochondrial oxidative stress rate; Viability: A live spermatozoa rate; BODIPY+: Lipit peroxidation rate.

Table 3. Correlations between individual pregnancy & spermatological parameters in artificially inseminated bulls semen.

	Bulls	Motility	PMAI	MITOSOX+	Viability	BODIPY +
PREGNANCY	1	-,089	-,224	-,075	,101	,279
	2	,416*	-,050	,019	,333	,240
	3	,099	-,086	,69	,097	,68
	4	-,035	-,198	,63	,003	,088
	5	,220	,206	-,217	,234	,151

*$P < 0.05$. PMAI: Plasma membrane and acrosome integrity rate; MITOSOX+: Mitochondrial oxidative stress rate; Viability: A live spermatozoa rate; BODIPY+: Lipit peroxidation rate.

Table 4. Pregnancy rates of bull semen used in AI.

Bulls	Pregnancy (%)	
1	85	29/35
2	82	28/35
3	74	25/35
4	74	25/35
5	74	25/35

DISCUSSION

The effect of bulls used in AI on fertility has been evaluated by *in vitro* spermatological parameters because of the widespread use of frozen bull semen in the field. These parameters provide simple, accurate, and reliable benefits to the cattle industry through in vitro semen examination of bull fertility [1,15,30]. Motility has been considered to be the most important indicator of fertility [25]. Januskauskas *et al*. [23] reported that there is a relationship between the non-return rate (NRR) at day 56 and progressive motility. Ibanescu et al. [18] used the CASA system to study the motility of ejaculates before freezing and at 0 and 3 h after thawing (after incubation at 38°C). Kinetic parameters were also analyzed with the non-estrus return rate (NERR56) at 56 days after the 1st AI at 1 year. The results showed a positive correlation between NERR56 and high-velocity and low-linearity values before freezing. However, no significant correlation was found between NERR56 and total motility or progressive motility at any of the time points studied. Furthermore, the present study did not reveal any statistically significant differences in bull motility after thawing. According to Hopkins *et al*. [17], bull semen should have a minimum motility of at least 30% to be considered suitable for AI, whereas motility results of 70% and above are considered highly favorable. In addition, according to regulations on the minimum sperm quality tests of imported and domestic bulls in Türkiye, those found to be within normal limits were allowed to be imported. Table 2 shows the analysis of parameters and field fertility results, revealing no significant correlation between pregnancy rates and spermatological parameters in all bulls ($P > 0.05$). Moreover, semen analyses of individual bulls yielded results that were consistent with those of previous studies. As shown in Table 3, a positive correlation between motility and pregnancy results was observed specifically in bull 2. This finding suggests that this relationship may be related to sperm transport within the female genital tract and oocyte penetration. Supporting the results of this study, previous research by Rodriguez-Martinez [32] also found a correlation between subjective motility and field fertility. However, Stalhammar *et al*. [34] reported that strong correlations were not observed with motility scores ≥ 50% in different breeds [3].

Successful fertilization depends on appropriate stimulation and induction of the acrosome reaction in spermatozoa with intact acrosomes. Inanc *et al*. [20] found a positive correlation between progressive motility, total motility, viability parameters and NRR. The lowest motility and mitochondrial activity were observed in frozen-thawed bull semen from the same animal. Motility and acrosome integrity are positively correlated with the distance traveled by spermatozoa in artificial bovine mucus or the cervical canal [4,28]. Bollwein *et al*. [7] reported that plasma membrane integrity (viability) and high mitochondrial membrane potential values were not associated with the 56-day NRR. This discrepancy may be because these parameters are only important for fertilization and not for embryo development [35].

The lack of a correlation between pregnancy rate and viability, as well as mitochondrial oxidative stress values, supports this interpretation. Therefore, it is suggested that the number of spermatozoa present in the straw [10] can offset these parameters. In this study, the use of proven fertile animals and a minimum concentration of 5 million motile spermatozoa per straw, in accordance with import criteria, was consistent with this finding.

Maintaining a balance between the production of reactive oxygen species (ROS) and antioxidants is crucial for preventing oxidative stress and the resulting damage caused by ROS. This study found no significant correlation ($P > 0.05$) between fertilization and oxidative stress parameters, including those measured by BODIPY and MITOSOX, as well as acrosome integrity. Gliozzi *et al*. [14] reported similar findings, with no direct relationship between oxidative stress parameters and fertility. Interestingly, Sellem *et al*. [33] observed a negative correlation between oxidation in living cells but found contrasting results concerning sperm cells and fertility. Specifically, they found that an increase *in vitro* oxidation induced by hydrogen peroxide was positively correlated with sperm fertility. These results highlight that oxidation has a dual nature, with both positive and negative effects. An increase in intracellular oxidation levels can negatively impact the lipid structure, leading to a reduction in cell integrity and impaired fertilization. Nevertheless, if oxidation is adequately controlled, it may aid capacitation and enhance fertilization competence. Our investigation concluded that there was no link between lipid peroxidation and fertility outcomes, implying that oxidation occurs within the usual and desired range.

The plasma membrane potential with high mitochondrial activation is positively correlated with viability, low intracellular calcium, and acrosome integrity [9]. Bollwein *et al*. [7] also reported a strong correlation between structural changes in the plasma membrane and mitochondrial membrane potential, which is consistent with the correlations found in the present study. These results indicate that a high mitochondrial membrane potential corresponds to a live sperm population with intact acrosomes, suggesting that mitochondria play an important role in the intracellular apoptotic pathway in mammalian spermatozoa, in addition to their involvement in motility and sperm metabolism [13,16,26,29]. A negative correlation was observed between mitochondrial oxidative stress and cell viability, supporting the notion that apoptosis is a planned cell death process. Increased cell death could affect cell viability, supporting the hypothesis of previous studies. However, in the present study, we found a positive correlation between the population level of lipid peroxidation and viability, probably because of the detection of lipid peroxidation in the living population. These results illustrate the interrelationships and close metabolic activities of organelles and structures within the cell.

CONCLUSIONS

In conclusion, to establish the correlation between field fertility results and spermatological parameters examined in the laboratory, it is essential not only to consider individual parameter associations but also to create multiple spermatological evaluation parameters. This approach allows for a better prediction and understanding of fertility-related correlations. Furthermore, associations and correlations should also be made in bulls with high and low fertility, and studies should be conducted on how and by which pathways these relationships and differences between bulls are formed.

MANUFACTURES

[1]Beckman Coulter. Brea, CA, USA.

[2]Sigma. Saint Louis, MI, USA.

[3]Invitrogen. Waltham, MA, USA.

[4]Bonway. Shenzhen, Guangdong, China

[5]SPSS Inc. Chicago, IL, USA.

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***Ethical approval*.** This study was carried out after the animal experiment was approved by The Local Ethics Committee on Animal Research at Burdur Mehmet Akif Ersoy University approved the protocol for animal use in this study (approval number: 17.11.2021/821).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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