First dose optimization study on freezing Anatolian buffalo semen

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ABSTRACT

The main objective of sperm production centers is to produce as many straws as possible from the obtained ejaculates using the optimal dilution rate. To this end, this study is the first to evaluate the effect of different semen extender rates on Anatolian buffalo semen quality. Ejaculates were collected by artificial vagina from three Anatolian buffalo bulls. These ejaculates were divided into three aliquots and filled into 0.25 ml straws with soy-based extenders at concentrations of 35, 25, and 15 million sperm/straw (n=105). The straw samples of different sperm concentrations were frozen. The quality of sperm was evaluated after thawing (37 °C, 30 sec) and following the thermoresistance test (37 °C, 3 h). The post-thaw total motility and progressive motility values were similar between the groups. However, following the thermoresistance test, there was a significant decrease in total motility in the 35 million sperm/straw group, and the progressive motility was significantly higher in the 25 million sperm/straw group. There was no statistically significant difference between the groups in terms of sperm kinetic parameters, except for VSL after thawing, as well as VAP and LIN values following the thermoresistance test. The overall mean PMAI and STR values were the highest in the 25 million sperm/straw group. In conclusion, it is recommended to dilute the Anatolian buffalo semen at a concentration of 25 million/0.25 ml when freezing it with a soy-based semen extender. In addition, it is considered that soy-based extenders compensate for cryo-damage to sperm motility for a short time, and the thermoresistance test should be applied for objective evaluation in dose optimization studies.

Introduction

The frozen bull semen industry has become a global market share with high economic potential with the advances in the safe transportation of frozen semen in liquid nitrogen and acceptable conception rates with artificial insemination. The main purpose of frozen sperm production centers is to produce as many straws of frozen semen as possible from the obtained ejaculates while maintaining high quality (8, 34).

Since the discovery of the cryoprotectant property of glycerol in 1949 by Polge et al. (25) studies on semen extenders and diluent rate optimization are still ongoing to minimize the biochemical and mechanical stress to which spermatozoa are exposed during the cryopreservation process (32).

Currently, the optimal dilution rate of semen extenders in mammalian semen varies according to breed and species, and even the concentration of sex-sorted sperm of the same breed differs considerably. For instance, the dilution rates to the final concentration of some mammalian species have been reported as 400 million (M) sperm/ml for rams, 400 M sperm/ml for goats, 300 M sperm/ml for boars, 100 M sperm/ml for stallions, and 60 M sperm/ml for bulls. If bull semen is sexed, dilution rates for the final concentration range from 8 M to 20 M sperm/ml (4, 6, 13, 14, 22, 26, 31).

The first buffalo semen was frozen by Roy et al. (27). Previously, many semen extenders and dilution rate optimization studies were carried out for quality semen freezing in river and swamp buffalo breeds (2, 9, 29). However, our study is the first to investigate the optimal dilution rate using soybean-based extenders for the cryopreservation of semen collected from Anatolian buffalo bulls in Türkiye, which originated from the Mediterranean subgroup of river buffaloes (30). To investigate the ideal dilution rate for Anatolian buffaloes, three different dilution rates were considered. These are 15, 25 M sperm/0.25 ml, and 35 M/0.25 ml, which are commonly used in the freezing of bull and buffalo semen. (1, 13, 22). Therefore, the objective of the current study was to compare the spermatozoa total motility and progressive motility, spermatozoa movement parameters, plasma membrane, and acrosome integrity at hours, 0, and 3 after thawing using frozen semen diluted at different rates ranging from 15, 25, and 35 M spermatozoa/straw.

Materials and Methods

This study was conducted at the Artificial Insemination Laboratory, Biotechnology Division, International Center for Livestock Research and Training Institute (located at an altitude de 1,080 meters above the mean sea level at 39.96°N latitude and 33.10°E longitude).

Preparation of artificial vagina (AV): A bovine AV was prepared by filling the water jacket with water hot enough to achieve a final AV internal temperature of 40-42 °C (IMV, France). The internal surface of the AV was coated with a thin layer of sterile and non-spermicidal petroleum jelly to lubricate it. A 15-ml sterile glass cylindrical tube was placed at the end of the AV to collect the sperm samples and carry them to the laboratory. The AV and the collection tube were placed into a protective felt sheath to maintain the optimal temperature during the collection process (2, 5).

Semen collection and dilution: The semen was collected using a teaser female buffalo (Lalahan Model). Each bull came to the semen collection area from its pen without a handler and exhibited mounting. Ejaculates were collected from three Anatolian buffalo bulls (at least 4 years old and housed in individual boxes) using the AV twice per week (5). After sample collection, each tube containing the fresh ejaculate was kept in a water bath at 36 °C. Only ejaculates with more than 80% total motility were used for semen processing and cryopreservation. Each ejaculate was divided into three aliquots to form different sperm concentration groups: 15 M sperm/0.25 ml, 25 M sperm/0.25 ml, and 35 M sperm/0.25 ml (n=105). The sperm concentrations of each group were determined using the Accucell photometer (IMV, L'Aigle, France). A commercial soybean-based semen extender (Andromed, Minitube, Germany) was used to dilute the semen samples. The semen samples of the three groups were diluted with the extender to the final concentrations of 15, 25, and 35 M sperm/0.25 ml, respectively.

Semen freezing process: The extended semen samples were cooled slowly to +4 °C for equilibration in a cold handling cabinet for three hours. After equilibration, the semen samples were loaded into 0.25-ml French mini straws (IMV, L'Aigle, France) using an automatic straw filling and sealing machine (MX4, IMV, L'Aigle, France). Subsequently, the straws were frozen to -140 °C (a programmed rate of -3 °C/min from +4 to -10 °C; -40 °C/min from 10 to 100 °C; and -20 °C/min from -100 to -140 °C) using a digital freezing machine (Digital cool 5300ZB 250, IMV, L'Aigle, France). Following the freezing stage, the straws were plunged into liquid nitrogen for storage at -196 °C (2, 5).

Computer-aided sperm analysis: A computer-aided semen analyzer system (CASA; IVOS I, Hamilton Thorne, USA) was used to examine various sperm motion characteristics after thawing (37 °C, 30 sec) and incubation (thermoresistance test; 37 °C, 3 h). After thawing, two-straw pairs were pooled from each straw batch in a microcentrifuge tube before analysis. Then, a 3-µl sample of semen was placed onto a prewarmed four-chamber slide and inserted into CASA with a 10× objective (IMV, L'Aigle, France), and the analysis was performed at 37 °C. Motile spermatozoa with an average path velocity (VAP) of 50 µm/s and a straight-line velocity (VSL) of 70% were evaluated as progressively motile (%). Other kinematics values, namely VAP (µm/s), VSL (µm/s), curvilinear velocity (VCL, µm/s), linearity [LIN=(VSL/VCL)×100], straightness [STR=(VSL/VAP) ×100], the amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz), were also evaluated and expressed with their units. Approximately 400 spermatozoa per sample were evaluated in five microscopic fields (5, 28).

Flow cytometric analysis: Plasma membrane and acrosome integrity (PMAI) in frozen-thawed semen were assessed with a CytoFlex flow cytometer (Beckman Coulter, USA). The semen samples were analyzed using an argon laser at a 488 nm excitation wavelength and a laser optical output power of 50 mW. Fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA; green fluorescence)/propidium iodide (PI)-combined staining procedures were followed as described by Korkmaz et al. (16). Accordingly, 2.5 µL of FITC-PNA (100 µg/mL) and 1.5 µL of PI (2.99 mM) were added into 5 µL of semen previously diluted in 246 µL of phosphate-buffered saline solution. Then, the diluted semen samples

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containing the dyes and the solution were assessed using flow cytometry after a 15-minute equilibration at 37 °C. The structural integrity of the plasma membrane and acrosome were evaluated in four subgroups: a) FITC-PNA label- and PI label-negative sperms with an intact plasma membrane and acrosome (accepted as viable), b) FITC-PNA label-negative and PI label-positive sperms with an intact acrosome and non-intact plasma membrane, c) FITC-PNA label-positive and PI label-negative sperms with a non-intact acrosome and intact plasma membrane, and d) FITC-PNA label- and PI label-positive sperms with a non-intact acrosome and plasma membrane after except for non-cellular events (Figure 1).

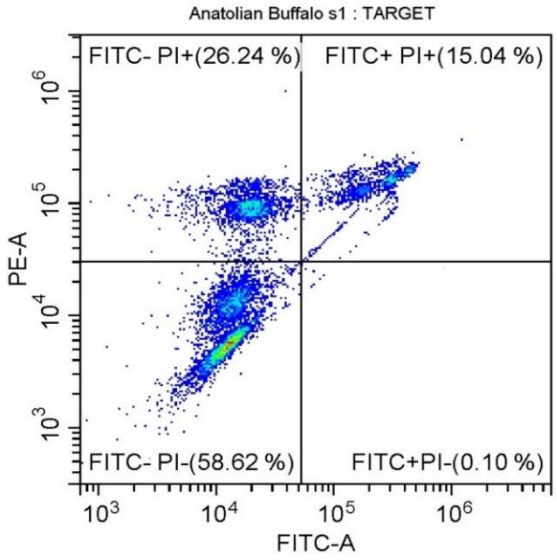

Figure 1. Flow cytometric evaluation of frozen-thawed Anatolian buffalo semen stained with FITC-PNA/PI for the analysis of the plasma membrane and acrosome integrity (FITC-, PI- live sperm with an intact acrosome population (p.); FITC-, PI+=dead sperm with an intact acrosome p.; FITC+, PI-=live sperm with a reacted acrosome p.; FITC+, PI+=dead sperm with a reacted acrosome p.).

Statistical Analysis: Descriptive statistics for the data were calculated and presented as mean ± standard error. A mixed model was utilized to determine the effect of sperm concentration, the effect of incubation time, and their interaction on CASA and flow cytometry parameters. The following repeated measures model was used:

$$Y_{ijk} = \mu + D_i + T_j + (D \times T)_{ij} + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, D_i is the sperm concentration group (i=15, 25, and 35), T_j, is the effect of incubation time (j=hour 0 and hour 3), $(D \times T)_{ij}$ is the interactions between the sperm concentration groups i and the effect of incubation time j, and e_{ijk} is residual error. The intra-group assessment of the animals was undertaken as a random effect, while the sperm concentration group, incubation time effect of sampling, and their interaction were assessed as fixed effects. Any significant difference was compared using simple effect analysis with the Bonferroni adjustment. The criterion of P<0.05 was considered significant in all the analyses. All the data were analyzed using IBM SPSS Statistics for Windows, version 23.0.

Results

Table 1 presents the dose, time, and dose x time interactions of the sperm total motility, progressive motility, and PMAI values of the semen samples after thawing and following the three-hour incubation period.

The total motility value after thawing was statistically similar between the groups, while the total motility values following the thermoresistance test were similar in the 15 M sperm/0.25 ml group and the 25 M sperm/0.25 ml group (41.65±1.73 and 44.14±1.73, respectively), but significantly higher compared to the 35 M sperm/0.25 ml group (P<0.05).

Table 1. Effect of different dilution rates on the post-thaw spermatozoon total motility, progressive motility, and PMAI values of the groups at hours 0 and 3 (mean±pooled SEM).

		Time			P-Value		
	Group	**0. h**	**3. h**	**Overall mean**	**Dose**	**Time**	**D*T**
Total motility (%)	15M sperm/0.25 ml	52.94 ± 1.73^{a}	41.65 ± 1.73^{b,A}	47.30 ± 1.22	<0.001	<0.001	0.048
	25M sperm/0.25 ml	58.71 ± 1.73^{a}	44.14 ± 1.73^{b,A}	51.42 ± 1.22			
	35M sperm/0.25 ml	53.34 ± 1.73^{a}	33.51 ± 1.73^{b,B}	43.42 ± 1.22			
Overall mean		55 ± 1	39.77 ± 1				
Progressive motility (%)	15M sperm/0.25 ml	22.10 ± 0.89^{a}	9.45 ± 0.89^{b,B}	15.77 ± 0.67	<0.001	<0.001	0.042
	25M sperm/0.25 ml	23.87 ± 0.89^{a}	16.30 ± 0.89^{b,A}	18.09 ± 0.67			
	35M sperm/0.25 ml	20.67 ± 0.89^{a}	6.90 ± 0.89^{b,B}	13.79 ± 0.67			
Overall mean		22.22 ± 0.58	9.55 ± 0.58				
PMAI (%)	15M sperm/0.25 ml	41.90 ± 1.46	33.20 ± 1.46	37.55 ± 1.03^{C}	<0.001	<0.001	0.073
	25M sperm/0.25 ml	57.00 ± 1.46	42.10 ± 1.46	49.55 ± 1.03^{A}			
	35M sperm/0.25 ml	52.26 ± 1.46	38.20 ± 1.46	45.23 ± 1.03^{B}			
Overall mean		50.38 ± 0.84^{a}	37.83 ± 0.84^{b}				

SEM: Standard error of the mean, M: million, PMAI: Plasma membrane and acrosome integrity.
^{a,b} Different letters on the same row for each parameter represent statistically significant differences (P<0.05).
$^{A, B}$ Different letters on the same column in different groups for each parameter represent statistically significant differences (P<0.05).

Table 2. Effect of different dilution rates on the post-thaw spermatozoon kinetic values of the groups at hours 0 and 3 (mean±pooled SEM).

		Time			P-Value		
	Group	**0. h**	**3. h**	**Overall mean**	**Dose**	**Time**	**D*T**
VCL (µm/s)	15M sperm/0.25 ml	169.76 ± 4.80	132.80 ± 4.80	151.28 ± 3.39			
	25M sperm/0.25 ml	161.24 ± 4.80	129.64 ± 4.80	145.44 ± 3.39	0.469	<0.001	0.822
	35M sperm/0.25 ml	167.56 ± 4.80	130.90 ± 4.80	149.23 ± 3.39			
Overall mean		166.19 ± 2.77^{a}	131.11 ± 2.77^{b}				
VAP (µm/s)	15M sperm/0.25 ml	101.59 ± 2.29^{a}	69.36 ± 2.29^{b,A}	85,47 ± 1.62			
	25M sperm/0.25 ml	94.68 ± 2.29^{a}	65.19 ± 2.29^{b,AB}	79.93 ± 1.62	0.033	<0.001	0.021
	35M sperm/0.25 ml	101.48 ± 2.29^{a}	59.67 ± 2.29^{b,B}	80.57 ± 1.62			
Overall mean		99.25 ± 1.32	64.74 ± 1.32				
VSL (µm/s)	15M sperm/0.25 ml	79.88 ± 1.54^{a,A}	50.92 ± 1.54^{b}	65.40 ± 1.13			
	25M sperm/0.25 ml	74.32 ± 1.54^{a,B}	48.77 ± 1.54^{b}	61.54 ± 1.13	<0.001	<0.001	0.002
	35M sperm/0.25 ml	68.69 ± 1.54^{a,C}	50.19 ± 1.54^{b}	59.44 ± 1.13			
Overall mean		74.29 ± 0.96	49.96 ± 0.96				
ALH (µm)	15M sperm/0.25 ml	7.25 ± 0.12	6.60 ± 0.12	6.92 ± 0.09			
	25M sperm/0.25 ml	7.21 ± 0.12	6.75 ± 0.12	6.98 ± 0.09	0.316	<0.001	0.681
	35M sperm/0.25 ml	7.34 ± 0.12	6.87 ± 0.12	7.10 ± 0.09			
Overall mean		7.27 ± 0.08^{a}	6.74 ± 0.08^{b}				
STR (%)	15M sperm/0.25 ml	75.63 ± 0.83	73.83 ± 0.83	74.73 ± 0.64^{B}			
	25M sperm/0.25 ml	77.35 ± 0.83	75.83 ± 0.83	76.59 ± 0.64^{A}	0.039	0.012	0.942
	35M sperm/0.25 ml	75.92 ± 0.83	74.63 ± 0.83	75.7 ± 0.64^{AB}			
Overall mean		76.3 ± 0.56^{a}	74.76 ± 0.56^{b}				
LIN (%)	15M sperm/0.25 ml	46.95 ± 0.92^{a}	42.51 ± 0.92^{b,AB}	44.73 ± 0.71			
	25M sperm/0.25 ml	46.36 ± 0.92	44.39 ± 0.92^{A}	45.38 ± 0.71	0.238	<0.001	0.005
	35M sperm/0.25 ml	47.68 ± 0.92^{a}	40.31 ± 0.92^{b,B}	43.99 ± 0.71			
Overall mean		47 ± 0.63	42.40 ± 0.63				
BCF (Hz)	15M sperm/0.25 ml	28.46 ± 0.39	28.31 ± 0.39	28.39 ± 0.28			
	25M sperm/0.25 ml	29.21 ± 0.39	28.50 ± 0.39	28.86 ± 0.28	0.485	0.079	0.643
	35M sperm/0.25 ml	28.97 ± 0.39	28.11 ± 0.39	28.54 ± 0.28			
Overall mean		28.88 ± 0.23	28.31 ± 0.23				

The progressive motility value after thawing was statistically similar between the groups (P>0.05). Following the thermoresistance test, the 15 M sperm/0.25 ml and 35 M sperm/0.25 ml groups had similar progressive motility values, while the 25 M sperm/0.25 ml group had a statistically significantly higher value (16.30±0.89) (P<0.05).

The overall mean PMAI values of the 15, 25, and 35 M sperm/0.25 ml groups were 37.55±1.03, 49.55±1.03, and 45.23±1.03, respectively (P<0.001). The highest PMAI value was observed in the 25 M sperm/0.25 ml group, with the values being determined as 57.00±1.46 at hour 0 and 42.10±1.46 at hour 3 after thawing (P<0.001). However, the dose x time interaction for the PMAI value was statistically non-significant (P>0.05).

The dose, time, and dose x time interaction values of the sperm kinetic parameter variables are shown in Table 2. The changes in the VCL values over time were statistically significant (P<0.001); however, there was no statistically significant difference between the three groups regarding the dose and dose x time interaction values (P>0.05). In contrast, the dose, time, and dose x time interactions statistically significantly differed between the groups for the VAP value (P<0.05). Although the VAP value measured at hour 0 after thawing was similar between the groups, it was significantly higher in the 15 M sperm/0.25 ml group (69.36±2.29) at hour 3 (P<0.05). Concerning the VSL value, the difference between the groups after thawing was statistically significant, and this value was found to be higher in the 15 sperm/0.25 ml group (79.88±1.54) (P<0.05). However, the VSL value obtained following the three-hour incubation period was statistically similar between the groups (P>0.05). When the ALH values measured at hours 0 and 3 were compared, statistically significant differences were observed (P<0.05). However, there was

no significant difference between the groups in terms of dose x time interaction (P>0.05). The statistical evaluation of the STR value was significant for dose and time (P<0.05) and non-significant for the dose x time interaction (P>0.05). While the LIN value was similar between the groups after thawing, it was significantly higher in the 25 M sperm/0.25 ml group after the three-hour incubation period (44.39±0.92) (P<0.05). There was no statistically significant difference in the evaluation of the BCF value in terms of dose, time, and dose x time interaction (P>0.05).

Discussion and Conclusion

In buffalo breeds, the estrus phase varies between 5 and 72 h, and the time of ovulation varies between 26 and 33 h after estrus, which is considerably longer than the estrus and ovulation times observed in cattle breeds (21). Therefore, to determine the fertilization ability of buffalo semen, it is important to repeat sperm quality analyses not only after thawing but also following the thermoresistance test applied at a certain time and temperature or after an incubation period.

In this study, no statistically significant difference was observed in the 15 M, 25 M, and 35 M sperm/0.25 ml groups in terms of total motility after thawing (hour 0), but there was a statistically significant decrease in the total motility value in the 35 M sperm/0.25 ml group after three hours of incubation at 37 °C. Similarly, in terms of the progressive motility value, there was no statistically significant difference between the groups after thawing, but a smaller decrease was detected in the 25 M sperm/0.25 ml group after the three-hour thermoresistance test. In a study conducted on Sahiwal bull semen using the tris egg yolk extender, Lone et al. (17) found that the 20 M and 15 M sperm/0.25 ml groups had similar total and progressive motility values after thawing and it was higher compared to the 10 and 5 M sperm/0.25 ml groups. In another study examining crossbred bull (*Bos Taurus* × *Bos indicus*) semen diluted with the Bioxcell® and Triladyl® extenders, Vera-Munoz et al. (33) formed three groups of 30, 15, and 5 M sperm/0.25 ml and reported that the total motility value was higher in the 30 M sperm/0.25 ml group. Patil et al. (23), who used tris egg-yolk and liposome-based extenders in Murrah breed buffaloes, determined that the total motility and progressive motility values of the 20 and 12 M sperm/0.25 ml groups after thawing were similar and higher than the 2 M sperm/0.25 ml group. The post-thaw total motility and progressive motility values reported in the studies mentioned above are consistent with our findings (30 M, 20 M, 15 M, and 12 M sperm/0.25 ml) except for the high dilution rate. In the literature, it has been observed that high dilution rates (2 M, 5 M, and 10 M sperm/0.25 ml) adversely affected the post-thaw total motility and progressive motility values. This could be because the high dilution of semen reduces the amount of proteins, antioxidants, and other beneficial compounds in the seminal plasma, which are necessary for spermatozoa to maintain membrane integrity and function (3, 10, 12, 17).

In this study, after the three-hour thermoresistance test, statistically significant differences were detected between the groups in terms of total motility and progressive motility values. In terms of the third-hour total motility value, the highest decrease was seen in the 35 M sperm/0.25 ml group, while the highest third-hour progressive motility value belonged to the 25 M sperm/0.25 ml group. According to previous research, the cryotolorence of buffalo sperm is lower than that of cattle breeds due to the low membrane phospholipid content in the sperm membrane structure and seminal plasma (3, 9, 11, 12, 15, 18). Unlike the studies mentioned above, we used a soybean-based extender containing phospholipids. This extender is considered to have compensated for the short-term cryodamage to sperm motility (total and progressive motility) at hour 0 after thawing (22). This hypothesis is supported by the results of another study that examined Murrah buffalo semen frozen with the tris egg egg-yolk-based tender and showed a decrease of approximately 70% in motility after the two-hour thermoresistance test compared to the post-thaw (hour 0) motility value (3).

In this study, the PMAI value was the highest in the 25 M sperm/0.25 ml group at hours 0 and 3 after thawing. In some studies evaluating the plasma membrane of sperm with the hypoosmotic swelling (HOS) test, it has been suggested that a high dilution rate adversely affects the plasma membrane of sperm. Consistent with this idea, Patil et al. (23) found that the plasma membrane integrity of Murrah semen samples at the 20 M sperm/0.25 ml dose was higher than that of the semen samples at the 12 and 2 M sperm/0.25 ml doses. Similarly, Vera-Munoz et al. (33) reported that the plasma membrane integrity of crossbred bull (*Bos taurus* × *Bos indicus*) semen samples at the 30 M sperm/0.25 ml freezing dose was higher than that of the 15 and 5 M sperm/0.25 ml groups. Lone et al. (17) showed that the plasma membrane values of the Sahiwal bulls' semen samples in the 20, 15, and 10 M sperm/0.25 ml groups were similar but higher than the 5 M sperm/0.25 ml group. In another crossbred bull (*Holstein-Friesian x Tharparkar*) study using the same freezing doses, the 10 and 5 M sperm/0.25 ml groups were found to have much lower plasma membrane integrity than the 20 and 15 M sperm/0.25 ml groups (13). In addition, in these studies, sperm acrosome integrity was manually evaluated with the FITC-PNA- and PI-modified staining protocols. Lone et al. (17) determined the sperm acrosome integrity of the 20

and 15 M sperm/0.25 ml groups be similar and higher than the 10 and 5 M sperm/0.25 ml groups, while Karan et al. (13) reported that the acrosome integrity of the 20 M sperm/0.25 ml group was higher compared to the 15, 10, and 5 M sperm/0.25 ml groups. Unlike the studies mentioned above, in the current study, plasma membrane integrity was evaluated using flow cytometry with FITC-PNA and PI staining, along with acrosome integrity, after thawing and following the three-hour incubation period. Although flow cytometry, a more objective method than the HOS test or manual staining methods, allows for the examination of a considerably higher number of sperm populations (10 thousand to 20 thousand spermatozoa), the data we obtained were parallel to the above-mentioned studies.

In this study, no statistically significant difference was found between the groups in terms of sperm kinetic parameters, except for the VSL value at hour 0 after thawing, which was significantly higher in the 15 M sperm/0.25 ml group. These results are generally consistent with those reported by Lone et al. (17) and Patil et al. (23). After the three-hour incubation period, significant differences were determined between the groups in terms of the mean VAP and linearity LIN values. At hour 3, the mean VAP value was higher in the 15 M sperm/0.25 ml group, while the mean LIN value was higher in the 25 M sperm/0.25 ml group. In addition to the total motility value, linear motility is important in the progression of the sperm toward the oocyte and the penetration of the zona pellucida (19, 20). The high LIN value in the 25 M sperm/0.25 ml group indicates that the rate of circularly moving sperms was lower in this group. This is also supported by the progressive motility value obtained from this group at hour 3.

Studies on the optimal dilution rate of semen generally focus on bull (*Bos taurus and Bos indicus*) semen. In this regard, there is limited literature on buffalo semen. Previous studies examining buffalo and bovine semen indicate that an excessive dilution rate (2 to 5 M sperm/0.25 ml) reduces sperm quality after thawing. This is considered to be related to the partial or complete removal of the components in the seminal plasma that are protective of the sperm (3, 10, 12, 13).

In general, the optimal dilution rate for cattle breeds is specified as 10 or 15 M spermatozoa per 0.25-ml straw (13, 22). However, in the current study, in which the optimal dilution rate was investigated for the first time in Anatolian buffalo semen, it was determined that the 25 M sperm/straw dose was more suitable for higher semen quality after thawing. This may be due to the biochemical differences between bovine and buffalo semen (7, 12, 24).

The conception rate is the most important indicator in determining a bull's fertilization ability. However, we were not able to evaluate the conception rate in this study, mainly because Anatolian water buffalo is widely grown in small-scale enterprises with different management and feeding systems. Patil et al. (23) reported a higher conception rate in the 20 M sperm/straw group than in the 15, 10, and 5 M sperm straw groups, which is parallel to their spermatological analysis results. Accordingly, it is predicted that a higher conception rate can be achieved at a dose of 25 M sperm/straw.

In conclusion, this study aimed to determine the optimal dilution rate for the cryopreservation of Anatolian buffalo semen by applying the main spermatological analyses, such as sperm kinematic, morphological, and thermoresistance tests. The findings of this study suggest that dosing the semen at 25 M sperm per straw hence, a final concentration of 100 M sperm/ml is considered effective when a soy-based semen extender is used. However, there is a need for both in vitro and in vivo studies to determine the optimal dilution rate for different cryopreservation protocols.

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Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Animal Experiments Local Ethics Committee of the International Center for Livestock Research and Training Institute, Ankara (Decision Number: 26.12.2018-160).

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

IB, DŞ, FK, SŞ, and MŞ conceived and planned the experiments. IB, DŞ, FK, SŞ performed spermatological experiments. The UK carried out a statistical analysis. IB

took the lead in writing the manuscript. All authors provided critical feedback and helped shape the manuscript.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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