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Effect of different extenders on motility and some sperm kinematics parameters in Norduz goat semen

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Abstract: Use of computer-assisted sperm analyzers (CASAs) to evaluate sperm fertilizing ability has great importance for the artificial insemination industry. For motility evaluation, different CASA systems and diluents are being used. These assays are poor in predicting sperm fertility, because only the samples with markedly poor quality can be detected. However, there is not a unitary extender worldwide. Therefore, we aimed to evaluate the postthaw kinetic parameters of curvilinear velocity (VCL), straight-line velocity (VSL), and average-path velocity (VAP) with two different extenders (skim milk, SM; Tris, TS). Semen was collected twice a week with an artificial vagina from four adult Norduz bucks. The ejaculates having normospermic quality were split into two equal aliquots and frozen. Sperm motility (%), VCL, VSL, VAP, viability (%), and abnormal spermatozoa (%) were evaluated. Regarding the abnormal spermatozoa rates and viability, percentages of motility were significantly higher in SM extender ($P < 0.05$), and VCL, VSL, and VAP rates were found higher with TS. In conclusion, according to the in vitro findings of this study, motility rates are more favorable in SM extenders than TS, and spermatozoa swim significantly faster in TS extender compared to SM.

Key words: Computer-assisted sperm analyzer, cryopreservation, motility, Norduz goat, velocity

1. Introduction

The first studies on buck sperm freezing were carried out by Polge and Smith (1). Afterwards, Baker (2) stated that frozen-thawed goat semen cannot be used practically, which was assumed to be the starting point of several studies about buck semen freezing (3).

Semen cryopreservation consists of common stages for any protocol, such as collection and extension of semen, addition of cryoprotectant, equilibrium, storage, and thawing procedure (4). However, motility could be affected at each stage and there are noticeable variations even between individuals. This leads to the need of assessment of specific sperm motility modifications at each step, as well as assessment of sperm motility (5).

The most commonly used extenders for freezing goat semen are Tris-egg yolk (TS) and skim milk (SM). Cryopreservation of goat sperm, and especially freezing, may cause structural, biochemical, and functional damage within the spermatozoon, resulting in losses in motility and viability and therefore in fertility.

Extenders and their components are shown to be the primary cause of damage in semen. Egg yolk is the mutual component in both extenders, which is used as a cryoprotectant protein. Egg yolk (lysolecithin) and milk-based diluents (SBUIII) interact with the seminal plasma lipase a content of the bulbourethral secretion and this interaction is known to be toxic for the semen (6,7). There are no worldwide unitary extenders for semen cryopreservation and each one consists of different ingredients and particle sizes.

Motility, viability, and morphology of the spermatozoa are the most important parameters for evaluating the fertilization capacity and fertility itself. A study that employed a computer-assisted sperm analyzer (CASA) showed that the semen samples with higher values of curvilinear velocity (VCL), straight-line velocity (VSL), and average-path velocity (VAP) parameters provided higher rates of pregnancy (8). Among these parameters, the VAP value indicated the highest correlation with the pregnancy rate. The straightness and linearity values were also parallel with the fertilization capability. It is known that, with the faster progression of the goat semen migration to the oviduct with the aid of seminal plasma and female genital secretions, a higher rate of semen colonization in the oviduct will occur (9).

In this study, Norduz goat semen frozen by using two different types of diluent (TS and SM) was assessed in terms of semen motility and velocity (VAP, VCL, and

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VSL) in order to evaluate the in vitro effects of diluents on semen motility and diluent effectiveness on the semen freezing process.

2. Materials and methods

2.1. Housing

In this study, 4 adult Norduz bucks (aged 2–3 years) with proven fertility were used. The bucks were maintained under a constant nutritional regime at the Ankara University Veterinary Faculty Research Farm. The study was approved by the Ankara University Animal Experiment Committee (2011-114-440), and each buck was fed a daily diet of 1 kg of concentrate, dried grass, salt lick, and water ad libitum

2.2. Semen collection and initial evaluation

A total of 32 ejaculates were collected twice a week from each buck in the nonbreeding season via artificial vagina using estrogenized teasers (April and May). The ejaculates were evaluated and accepted for freezing if the following criteria were met: volume varying between 0.75 and 2 mL, sperm concentration of 3×10^9 sperm/mL, motility percentage higher than 70%, and less than 10% abnormal sperm in total.

2.3. Freezing and thawing the semen

The ejaculates collected from each buck were divided into two aliquots and diluted with two different extenders (TS and SM). The semen was adjusted to a final concentration of 100 million spermatozoon in straws of 0.25 mL in volume. After 2 h of equilibration, the straws were placed horizontally 4 cm above the surface of nitrogen vapor at –120 °C for 12 min, and then plunged into liquid nitrogen and stored at –196 °C. The straws were thawed in a water bath of 37 °C for 30 s and motility, abnormal spermatozoa rate, and viability were assessed (10).

Tris extender: Tris-egg yolk extender was prepared using 3.63 g of tris-(hydroxymethyl)-aminomethane, 1.8 g of citric acid, 0.5 g of glucose, and 10% egg yolk and 5% glycerol in 85 mL of distilled water (11).

Skim milk extender: Milk extender was prepared by using 10 g of skim milk powder and 0.9 g of glucose in 100 mL of distilled water, heated to 95 °C for 10 min and then cooled to room temperature before the addition of 10% egg yolk (11).

2.4. Motility assessment

Motility was expressed as percentage; soon after dilution and equilibration processes, with a phase-contrast microscope, at least three different fields with 10× magnification were evaluated. Sperm motility, VAP, VCL, and VSL parameters were assessed with a CASA (Sperm Class Analyzer, (SCA), Version 3.2.0, Microptic S.L., Barcelona, Spain), using existing species-specific evaluation parameters for bucks. Preset values for the instrument were as follows: for the Basler camera, which can take 60 frames per second, image brightness of 60, contrast of 750, and light of 1000 were adjusted; minimum average path at 50 μm/s and >50% progressive motility were accepted; motility parameters of static, slow (>40 μm/s), medium (>70 μm/s), and rapid (>100 μm/s) were set; and kinetic parameters of VCL (>80 μm/s), VSL (>50 μm/s), and VAP (>25 μm/s) were set. Five microliters of each sample was evaluated in Leja motility analysis slides. For each sample, from 200 to 300 spermatozoa in 7 different areas were analyzed to evaluate the motility. Total motility was taken as the sum of progressive and nonprogressive motility. VCL (μm/s; curvilinear velocity, time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions with the microscope), VSL (μm/s; straight-line (rectilinear) velocity, time-averaged velocity of a sperm head along the straight line between its first detected position and its last), and VAP (μm/s; average path velocity, time-averaged velocity of a sperm head along its average path computed by smoothing the curvilinear trajectory according to algorithms in the CASA instrument) were indicated (12).

2.5. Abnormal spermatozoa rate and viability assessment

The evaluation of viability was carried out with eosin-nigrosin staining. The smears were prepared routinely by mixing one drop of semen sample with two drops of the stain on a warm slide and immediately spreading the stain with one edge of a second slide. The viability was assessed by counting 200 spermatozoa under a phase-contrast microscope (400× magnification) (13).

For the sperm morphology assessment, at least two drops of semen were added to Eppendorf tubes, containing 0.5 mL of Hancock's solution. One drop of this mixture was placed on a microscope slide and then covered with a cover slip. The percentages of abnormal sperm (detached heads, acrosomal aberrations, abnormal midpieces, and tail defects) were recorded by counting a total of 200 spermatozoa under the phase-contrast microscope (magnification 1000×; oil immersion) (13).

2.6. Statistical analyses

The Student t-test and Mann–Whitney U test were used to determine the statistical difference between SM and TS extenders for each variable, after evaluating the data for normality by using the Shapiro–Wilks test as a parametric test assumption. $P < 0.05$ was considered statistically significant. SPSS 14.01 was used for statistical analysis.

3. Results

Spermatological characteristics of fresh semen are shown in Table 1. Buck 4 was found to have the highest motility; however, the differences between bucks were found to be statistically nonsignificant ($P > 0.05$). Since the concentration of Buck 2 was found to be the lowest ($2.7 \times$

Table 1. Spermatological characteristics of fresh semen.

Animal	N	Volume	pH	Motility	Concentration
Buck 1	8	0.8 ± 0.2	6.9 ± 0.4	78.2 ± 4.6	3.4×10^9
Buck 2	8	1.2 ± 0.1	6.9 ± 0.2	75.5 ± 7.4	2.7×10^9
Buck 3	8	1.1 ± 0.1	7.1 ± 0.2	81.2 ± 4.7	3.4×10^9
Buck 4	8	1.1 ± 0.2	6.6 ± 0.1	82.4 ± 5.1	3.3×10^9
Total (X ± Sx)	32	1.0 ± 0.2	6.8 ± 0.2	79.3 ± 5.5	3.2×10^9

10^9), the volume was found to be the highest and yet had the lowest motility.

3.1. Motility parameters: VCL, VSL, and VAP

Postthawed spermatological parameters of both extenders are indicated in Table 2. For individual differences between animals, Buck 4 had the highest motility rates with SM diluent and was statistically significant. However, there were no significant differences in kinetic parameters. SM diluent had significantly higher results than TS extender (Table 3), whereas the kinetic parameters of SM diluent had better VCL values than SM extender (Table 4).

3.2. Abnormal and viability

Sperm viability and morphologically abnormal spermatozoa rates during freezing with SM and TS are shown in Table 5. The statistical analysis indicated significant differences only between Buck 4 and the rest.

4. Discussion

Today, motility is accepted as the most commonly used parameter for evaluating the postthawed viability of semen (fertilization capability). However, motility alone is not enough to evaluate the fertilization capacity. Therefore, semen motility results should be supported by other parameters such as viability, morphology, oviduct adhesion test, hypoosmotic swelling test, and zona pellucida binding test. Many factors play a role in evaluating the postthawed viability, and semen possessing individual differences within their natural circumstances make it more complicated (14). As motility parameters are estimated subjectively in a semiquantitative way, various results ranging from 30% to 60% of the same ejaculates have been reported for both humans and animals. Using a CASA provides an objective and correct approach for the evaluation of semen motility. However, to increase the reliability with accurate results as well as to define comparison criteria, it is important to identify the effective factors and provide necessary standards.

Within this approach, to evaluate the diluent factor in this study, TS and SM diluents were used and postthawed motility, VCL, VSL, and VAP values were determined by CASA while the individual differences were recorded. Overall, CASA values were found to be affected by the different extenders (Table 6). Motility values obtained with CASA by other researchers varied from 40.3% to 78.5% with TS (15–22) and from 52.2% to 67.2% with SM (15,21). While evaluating the differences between these obtained results, in vivo results should also be taken into consideration, since the presence of all different somatic and environmental criteria that were mentioned above proves the various natures of in vitro outcomes. CASA systems can provide the objectivity for the results of in vitro sperm evaluations as well as eliminating the bias conditions.

The best spermatozoa viability was seen when freezing with SM extender. This can be due to the ion and water exchanges between intracellular and extracellular environments and ice crystallization for the cytoplasm being denser than the medium. The difference in dead

Table 2. The postthawed individual motility, VCL, VSL, and VAP values in SM extender.

SM	N	Motility	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)
Buck 1	8	54.9 ± 4.6 [a]	84.8 ± 10.5 [a]	46.4 ± 13.3 [a]	61.5 ± 11.9 [a]
Buck 2	8	51.1 ± 1.2 [a]	84.5 ± 1.3 [a]	45.4 ± 2.0 [a]	61.8 ± 2.3 [a]
Buck 3	8	59.2 ± 3.9 [a]	96 ± 1.5 [b]	59.5 ± 1.7 [b]	74 ± 1.3 [b]
Buck 4	8	75.8 ± 2.2 [b]	85.3 ± 4.1 [a]	53.7 ± 3.4 [a]	66.7 ± 3.2 [a]

a–b: Means within the same column that have different superscripts are significantly different ($P < 0.05$).

Table 3. The postthawed individual motility, VCL, VSL, and VAP values with TS extender.

TS	N	Motility	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)
Buck 1	8	47.9 ± 1.8 [a]	110 ± 1.3 [a]	67.7 ± 2 [a]	83 ± 2.3 [a]
Buck 2	8	46.8 ± 3.2 [a]	105.3 ±1.9 [a]	65.3 ± 3.8 [a]	78.8 ± 2.7 [a]
Buck 3	8	51.4 ± 0.9 [a]	79.4 ± 2.4 [b]	32.6 ± 3.3 [b]	49.9 ± 1.8 [b]
Buck 4	8	46.4 ± 1.6 [a]	113.5 ± 4.4 [a]	70.9 ± 5.8 [a]	85.9 ± 5.5 [a]

a–b: Means within the same column that have different superscripts are significantly different ($P < 0.05$).

Table 4. Average of total motility, VCL, VSL, and VAP values for two different diluents.

Parameter	N	SM	TS	Significance
Total motility	32	60.1 ± 2.8	48.1 ± 1.1	*
VCL (μm/s)	32	87.6 ± 3.0	102.1 ± 3.6	*
VSL (μm/s)	32	51.2 ± 3.6	59.1 ± 4.3	-
VAP (μm/s)	32	66 ± 3.3	74.4 ± 3.3	-

*: $P < 0.05$.

spermatozoa rates among individuals might be due to different values in the osmotic pressures of the semen and the extender depending on its components (23,24).

Abnormal spermatozoa ratios with SM extender were found to be lower than those with TS. The variations between the percentages of abnormal spermatozoa may occur due to the differences in the evaluation methodology of abnormal sperm, the content of extenders, osmotic pressure differences, pH, age of the bucks, or even genotypic and individual differences (18,21,23–25).

It should be taken into account that the significant statistical difference between the postthaw motility values obtained with SM and those obtained with TS could be affected by various factors. Among these factors, TS extender contains fewer and smaller particles and has lower viscosity than SM extender, and its viscosity being lower makes it easier to identify the spermatozoa that are immobile; this, therefore, may result in lower values of motility (Table 6).

When individual differences are ignored, total motility and VCL, VSL, and VAP values are higher for spermatozoa moving faster with TS extender, yet this supports the same hypothesis. By the CASA method, spermatozoa motility and morphometric characteristics of different freezing methods in different animal species can be evaluated objectively, under in vitro conditions. Furthermore, studies on animals can provide a comparative model for humans in order to solve problems like infertility, as recommended by the European Society of Human Reproduction and Embryology (24). In a previous study, each sample was diluted with physiologic saline (PS) that contained bovine serum albumin, thyroid pyruvate lactate albumin (TALP), and potassium-thyroid pyruvate lactate albumin (K-TALP) with highly concentrated potassium medium to a final concentration of 25×10^6 spermatozoa and then left for incubation for 2 h before the CASA evaluation. The results showed that there were significant differences due to the used mediums. The motility results for TALP, K-TALP, and PS were 76%, 42%, and 29%, respectively (26). In addition, the absence of a standard extender composition in evaluations made for CASA systems causes variations in results. Different sizes of the component particles in extenders can be detected by the optic system during in vitro evaluations and can cause the system to fail in most cases. Therefore, the solutions and dilution ratios are critical for conducting semen analysis with CASA systems. Therefore, standardizations of the equipment and software used in CASA systems should be checked regularly.

In conclusion, in this study, SM extender usage for freezing Norduz buck semen provided higher results, whereas for the assessment of CASA parameters, TS extender revealed higher kinematic parameters. The

Table 5. Postthawed individual abnormal and viability percentages of spermatozoon.

Buck	n	TS dead (%)	SM dead (%)	TS abnormal (%)	SM abnormal (%)
1	8	40.0 ± 8.9 [a]	36.5 ± 6.5 [a]	31.5 ± 4.4 [a]	25.25 ± 6.6 [a]
2	8	38.7 ± 7.0 [a]	33.7 ± 8.0 [a]	31.7 ± 3.5 [a]	29.2 ± 3.3 [a]
3	8	37.0 ± 5.0 [a]	30.7 ± 7.0 [a]	34.7 ± 6.0 [a]	22.7 ± 4.0 [a]
4	8	41.2 ± 4.0 [a]	20.7 ± 3.0 [b]	26.7 ± 4.0 [a]	16.7 ± 3.0 [b]
Total	32	39 ± 6.3	29 ± 8.3	31.8 ± 5.2	24.0 ± 6.1

a–b: Means within the same column that have different superscripts are significantly different ($P < 0.05$).

Table 6. Evaluation of kinetic parameters in different studies.

Extender	Kinetic parameters			Study
	(VCL µm/s)	VSL (µm/s)	VAP (µm/s)	
TS	102.1 ± 3.6	59.1 ± 4.3	74.4 ± 3.3	Present study
	83.2 ± 8.9	-	-	(18)
	128.1 ± 3.3	68.2 ± 1.8	84.3 ± 1.7	(20)
	146.4 ± 0.6	104 ± 0.6	117 ± 0.6	(21)
	175.83 ± 11	92.6 ± 1.9	118.7 ± 4.4	(21)
	193.1 ± 24.6	159.6 ± 5.5	-	(22)
SM	87.6 ± 3.0	51.2 ± 3.6	66 ± 3.3	Present study
	119.2 ± 4.7	81.2 ± 1.8	93.1 ± 2.6	(21)

postthawed motility values in general could be misleading since the conventional method is semiquantitative and various parameters could affect the objective CASA method. In addition, it should not be forgotten that these results alone are not sufficient to assess fertilization capability. Studies applying CASA systems have indicated that the range of reference values that were processed for the evaluation system should be provided, since this is the only possible way to make an objective evaluation. Researchers using CASA systems must be experienced and have the knowledge to solve any problems that might occur. Intensive and continuing education should be implemented in laboratories; researchers should be able to describe the influences on the slide, cubicle volume, framing frequency, resolution, lens, magnification rate, spermatozoa number counted in one area, size of the slide that is used, and temperature during analysis.

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