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Research Article

The Effect of Concentration and Storage Time on Short-Term Storage of Ram Sperm

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Abstract: The present study was conducted to observe the effect of different dilution rates on ram sperm motion characters (motility, progressivity, velocity and average of speed), head diameter, mitochondrial membrane potential (MMP) and live intact acrosome rate during liquid storage for 96 h at 5°C. Normospermic semen samples that obtained from 4 adult rams were pooled. Pooled semen was split into two aliquots; Low Dilution Rate (LDR) (400 million) and High Dilution Rate (HDR) (20 million) motile spermatozoa per insemination dose/0.25 mL straw and were diluted with a skim milk-egg yolk-based extender. It was observed that the Total Motility (TM) and total progressivity of the LDR group decreased more rapidly than HDR group over time, in general. From the 12th h to the 96th h; the rapid progressivity ($P<0.01$), MMP ($P<0.05$), live sperm rates with intact acrosome ($P<0.01$) and straightness (STR) ($P<0.001$) of the HDR group results were statistically better than LDR. It was concluded that the spermatological properties examined during storage were damaged at different times and were affected by sperm concentration and also storage time.

Keywords: *Acrosome, Concentration, Kinematics, MMP, Motility, Ram, Semen, Extender, Storage*

Koç Spermasının Kısa Süreli Saklanmasına Konsantrasyonun ve Muhafaza Süresinin Etkisi

Öz: Bu çalışma, koç spermasının 5°C'de 96 saat süre ile saklanmasında farklı sulandırma oranlarının sperm hareketleri ile ilişkili (motilite, progressivite ve ortalama hız) parametreleri, baş çapı, mitokondriyal membran potansiyeli ve canlı sağlam akrozoma sahip spermatozoa oranlarına etkilerinin ortaya konulması amacıyla gerçekleştirildi. Dört baş erişkin koçtan elde edilen normospermik özelliğe sahip ejekulatlar birleştirildi. Birleştirilen ejekulatlar ikiye bölünerek; Düşük Dilüsyon Oranı (LDR) (400 milyon) ve Yüksek Dilüsyon Oranı (20 milyon), motil spermatozoa tohumlama dozunda/0.25 mL payet olacak şekilde yağsız süt tozu yumurta sarısı bazlı sulandırıcı ile sulandırıldı. Genel olarak Düşük sulandırma oranı (LDR) grubunda TM ve progressivitenin Yüksek sulandırma oranı (HDR) grubuna göre daha hızlı azaldığı gözlemlendi. On ikinci saatten 96. saate kadar ki saklama süresinde, HDR grubunda elde edilen hızlı progresif motilitenin ($P<0.01$), mitokondriyal membran potansiyelinin ($P<0.05$) ve sağlam akrozoma sahip canlı spermatozoa oranının ($P<0.01$), doğrusallığın (STR) ($P<0.001$) LDR grubuna göre istatistiksel olarak daha yüksek olduğu gözlemlendi. Saklama süresince incelenen spermatolojik özelliklerin farklı zamanlarda zarar gördüğü, ayrıca sperm konsantrasyonu ve saklama süresinden de etkilendiği sonucuna varıldı.

Anahtar sözcükler: *Akrozom, Koç, Konsantrasyon, Kinematikler, MMP, Motilite, Saklama, Sperma, Sulandırıcı*

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Introduction

Artificial insemination (AI) is an assisted reproductive technique performed by transplanting a certain amount of fresh, diluted, cooled, or frozen sperm into the female reproductive channels. In livestock, AI is a helpful technique for genetic improvement and management of reproduction [1]. With the application of sperm storage (short-term or long-term) techniques, it is possible to benefit more from the genetically superior males in breeding programs [2].

The damaging effects of cryopreservation on ram sperm morphology, and function, and the high cost of intrauterine insemination; makes chilled, liquid preservation the most realistic current option for semen storage and cervical AI. Compared to frozen-thawed semen, liquid-stored semen possesses the advantages of convenient easier handling, higher fertility results, and economy [3]. Moreover, cooled semen insemination doses require fewer spermatozoa per dose, which means more AI doses per ejaculate. The main disadvantage of diluted, chilled, or cooled semen is its short fertile life compared to frozen semen, which makes it unfeasible for long storage periods or transportation over long distances [4,5]. For that reason, fresh, diluted or cooled ram semen AI is only an alternative to frozen semen when the insemination is done within a short period of time after collection [6-8].

There are many studies about liquid storage of mammalian semen published recently [9-16]. Lowering mammalian semen temperatures (5-12°C) results in a reduction of cellular metabolism and reactive oxygen species, thus prolonging sperm survival [17]. Furthermore, hypothermia destabilizes the activity of the sodium-potassium pump by causing intracellular sodium levels to rise to cytotoxic levels. Accordingly, it causes membrane damage [17,18].

During the dilution, cooling, storage and freeze-thawing process, the semen extenders protect spermatozoa metabolism in many ways, including stabilizing the plasma membrane and maintaining intracellular and intramembrane ionic concentrations, thus reducing cold shock damage and osmotic shock. The control of pH of the medium and bacterial growth provides extra support to reduce oxidative damages [17,19-22]. Sperm dilution rate affects the buffering capacity of the extender and its ability to remove metabolic residues [23]. Reducing the sperm concentration could facilitate not only the increased usage of selected sires but also the more prolonged storage period of liquid semen [24]. Lipid peroxidation is a metabolic process where reactive oxygen species (ROS) are formed by the oxidative degeneration of poly-unsaturated fatty acids [25]. Increasing sperm concentrations would increase ROS levels, also the presence of nonviable or poor-quality spermatozoa and cell debris in the milieu escalate ROS level. ROS overproduction negatively affects sperm function and morphology. The reduction of sperm concentration and lowering temperature decrease both the ROS production and acidification of the extender through the accumulation of lactic acid and CO_2 from glycolysis and oxidative phosphorylation, respectively [9,22,25,26]. Although high sperm concentration supports fertility, it can also shorten overall lifespan of spermatozoa by exhausting energy resources [21].

The sperm concentration and days of storage were associated with the deterioration in the structural, functional and sperm motility parameters [10,27-30]. A reduction in the quality of stored semen when the sperm dilution rate decreased was reported in ram [29,31], stallion [32], alpaca [30], and bulls [24,33,34]. There are relatively few studies on the effect of semen concentration on ram semen quality during liquid storage for 96 h at 5°C [29,35]. For intra-uterine insemination, the minimum effective dose of both fresh and frozen semen is $20x10^6$ motile spermatozoa [36-39] and for intravaginal insemination it is 400 x 10^6 motile spermatozoa [8,36,39]. Therefore, the objective of this study was to determine time-related changes in motility and kinetics parameters, MMP and acrosome integrity of ram semen stored at two different concentrations (High dilution rates (HDR): 20 x 10^6 and Low dilution rates (LDR): 400 x 10^6 motile spermatozoa/insemination dose) at 5°C for 96 h in skim milk-egg yolk-based extender.

Material and Methods

Ethical Statement

Semen collection procedures of the present study were approved by the local ethics committee of Sheep Breeding Research Institute, Ethics Committee on Animal Research (2021/048).

Animal Management and Semen Collection

This study was carried out during the breeding season, using 2-3 elderly fertility proven 2 Kivircik (Local Breed) and 2 Ramlic (Rambouillet*Daglic (Local Breed)) rams, which were raised in the Sheep Breeding Research Institute, which is located at 40.32 latitude, 27.91 longitude coordinates. During the study, the lowest and the highest temperatures were 17-26°C and relative humidity averaged 70%. During the study, rams and sheep were kept closed; 1 kg of cut feed (Barley, SSM, Salt and Marble powder) was given to the rams. In addition, 100 grams of raisins and 1 egg per day were given to the rams as supplementary nutrients.

Ram semen was collected by an artificial vagina and then quickly transferred to a 30°C water bath. Ejaculates, which have normospermic properties; (volume >0.9 mL, concentrations >1800 x 10^6/mL, mass movement >4 (1-

5) and total motility ≥70%) were used [23]. Initially neat sperm motility and mass activity were examined using a microscope (Olympus Corporation, CX31, Japan). Sperm concentration was determined using a sperm density meter (IMV Technologies, Ovine Accucell®, France). The experiment was repeated until 8 replicas were completed.

Semen Processing

Ejaculates which have normospermic characteristics were pooled to avoid the individual effect. Each pooled ejaculates were divided into 2 groups (High Dilution Rate (HDR) and Low Dilution Rate (LDR). The HDR group and LDR groups were diluted at 30°C with skim milk-based extender (10 g Skim milk powder (Sigma® 70166), 1 g of glucose (Sigma® G7021), 5% egg yolk) [16] at a final concentration 80 x 10^6 spermatozoa/mL and 1600 x 10^6 spermatozoa/mL, respectively. The extender osmolality was 380 mOsm.

Diluted semen temperatures were gradually reduced from 30°C to 4°C (at -0.3°C/min) within 2 h and stored for 4 days (until 96 h after collection) for further evaluation. Spermatological examinations; motility and kinematic parameters, live/intact acrosome, MMP were evaluated after dilution at 30°C (0^{th} h), after cooling to the 4°C (2^{nd} h), and at 12^{th}, 24^{th}, 48^{th}, 72^{nd} and 96^{th} hours during the storage period.

Semen Evaluation

- Sperm Motion Characteristic

A computer-assisted sperm analyzer (CASA) (SCA®, Microoptics, Spain) was used for the sperm motion characteristic [40]. For each evaluation semen was re-diluted with Tris-based egg yolk free extender at 16 x 10^6 spermatozoa/ml concentration to avoid over concentration failure. The 3 µL of re-diluted semen were placed in a specific slide (Leja®, Ref. 025107, IMV Technologies, France) for CASA. The CASA system provides progressive motility and total motility values with curvilinear velocity (VCL), rectilinear velocity (VSL), average path velocity (VAP), linearity (LIN) and straightness (STR), also; rapid, medium, slow sperm were examined. When evaluating spermatozoon motion properties, field settings; at least-up to 15-70 µm², speed settings; static <10 µm/s, slow to medium >45 µm/s, fast >75 µm/s, progressive (STR>80). Additionally, sperm head area was also analyzed. The analysis continued until at least 7 areas or at least 500 spermatozoa were analyzed.

- Flow Cytometry Analyses

All flow cytometer analyzes were performed on the Guava easyCyte® microcapillary flow cytometry device, which includes the CytoSoft program. (Guava Technologies Inc., Hayward, CA, USA; distributed by IMV Technologies). The device has a single blue laser (488 nm), two photodiodes (forward scatter, side scatter), three photo multiplicators (green: 525/30 nm, yellow: 583/26 nm, and red: 655/50 nm) and appropriate optical filters and brackets. Each analysis was carried out until 5000 sperm cells with scatter and fluorescent properties were counted. The performance of the device is checked daily with the Guava Check kit (Guava Technologies®, Inc., Millipore, Billerica, MA, USA).

- Plasma and Acrosome Integrity

Plasma and acrosome integrity of dead and living spermatozoa were analyzed by Easykit 5 kit (ref. 025293; IMV Technologies) that measures the integrity of the acrosome and cytoplasmic membrane simultaneously according to the manufacturer's protocol. For this purpose, 200 µL EasyBuffer B (Ref. 023862; IMV Technologies) and 5 µL of tris diluted semen (8000 spermatozoa/µL) were added to ready to use 96 well plate. The sperm was incubated at 37°C for 45 min. It was kept in a dark environment until the analysis was carried out. The analysis continued until 5000 sperm and the ratio of live/intact acrosome sperm were calculated by the program (EasySoft, ref. 024842; IMV Technologies) [41].

- Mitochondrial Membrane Potential (MMP)

Sperm MMP, (polarized/ depolarized) were analyzed by EasyKit 2 (ref. 024864; IMV Technologies) according to the manufacturer's protocol. Sperm with high concentrations of fluorochromes match as polarized mitochondria (with Δψm high) whereas mitochondria accumulating lower concentrations of fluorochromes were calculated as depolarized (and have a low Δψm). For this purpose, 10 µL of pure ethanol has been added to ready to use 96 well plate for the purpose of dissolving fluorochrome. Then 50000 sperm in total with 190 µL Easybuffer B and 5 µL of Tris diluted semen (8000 spermatozoa/µL) were added to the well plate. The sperm was incubated at 37°C for 30 min and kept in the dark until the analysis was performed. The analysis continued until 5000 sperm and mitochondrial potential density/ratio was calculated in the program (EasySoft, ref. 024842; IMV Technologies) [41].

Statistical Analysis

Obtained semen parameters were expressed as Mean ± Standard Error. Homogeneity of spermatological and kinematic parameters was controlled by Levene's test, one-way variance (One Way ANOVA) in comparing the difference between the groups; GLM procedure and Bonferroni multiple comparison test was applied in comparison of data obtained at different times in groups. All statistical analyses were performed using the SPSS package program (IBM® SPSS Statistics for Windows, Version 23).

Results

The changes of cooled sperm motilities (total, progressive, rapid, medium, slow and static), MMP and live sperm with intact acrosome in the function of concentration and storage period for 96 h were given in *(Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 7)*. The dilution rates had no effect on the semen characteristic except rapid and medium progressivity, Head diameters and STR at the dilution stage.

For the HDR group, the sperm total motility (P<0.001), progressive motility (P<0.001), rapid progressive motility (P<0.05), medium progressive motility (P>0.05) and MMP (P<0.05) were significantly decreased as storage time increased. For the velocity parameters; while sperm with rapid motion rate was decreased (P<0.001); the medium (P>0.05), slow (P<0.01) and static (P<0.001) sperm rates were increased. Sperm speed-related parameters VCL, VAP, VSL, ALH, and BCF decreased over time (P<0.001). For the LDR group, total motility (P<0.001), progressivity (total (P<0.001), rapid progressive motility (P<0.01) and medium progressive motility (P<0.001), MMP (P<0.01) and live sperm with intact acrosome (P<0.001) were significantly decreased as storage time increased. For the velocity parameters; while sperm with rapid motion rate was decreased (P<0.001); the medium (P>0.05), slow (P<0.001) and static (P<0.001) sperm rates were increased. The speed-related values VSL (P<0.001), STR (P<0.001), LIN (P<0.001), and BCF (P<0.01) were decreased with increased duration of storage (P<0.001).

After cooling at 5°C within 2nd h, while the sperm samples with HDR revealed higher progressive motility (P<0.01),

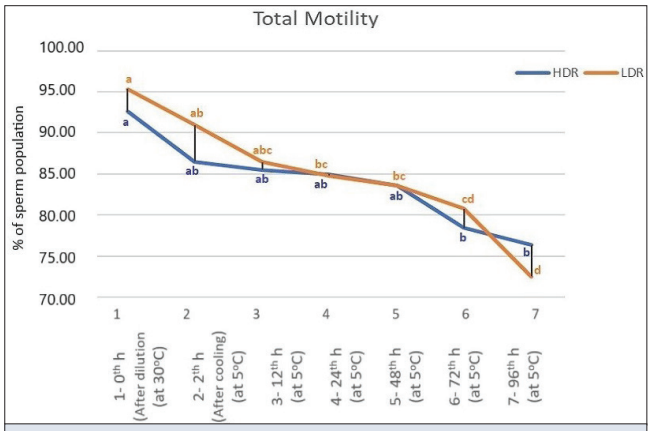

Fig 1. Total motility parameters in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,d:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05)

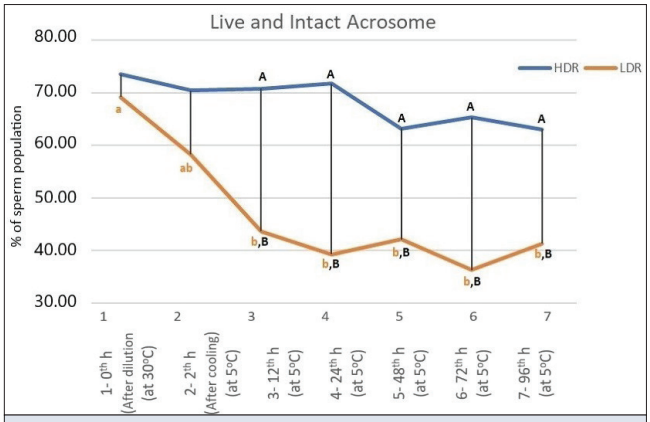

Fig 3. The sperm with live and intact acrosome parameters in HDR and LDR groups for 96 h storage

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,b:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05), **A,B:** The difference between the different letter between the HDR, LDR groups is statistically significant at the same time zone (P<0.05)

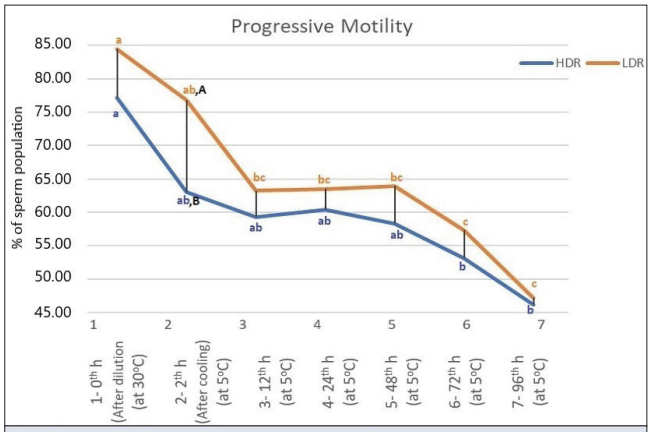

Fig 2. Progressive motility parameters in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,c:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05), **A,B:** The difference between the different letter between the HDR, LDR groups is statistically significant at the same time zone (P<0.05)

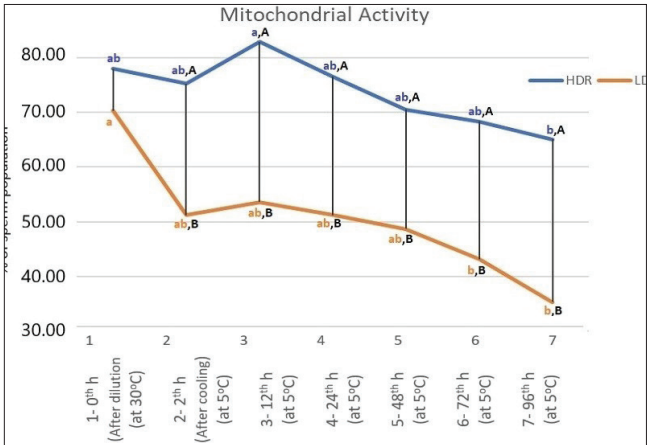

Fig 4. MMP parameters in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,b:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05), **A,B:** The difference between the different letter between the HDR, LDR groups is statistically significant at the same time zone (P<0.05)

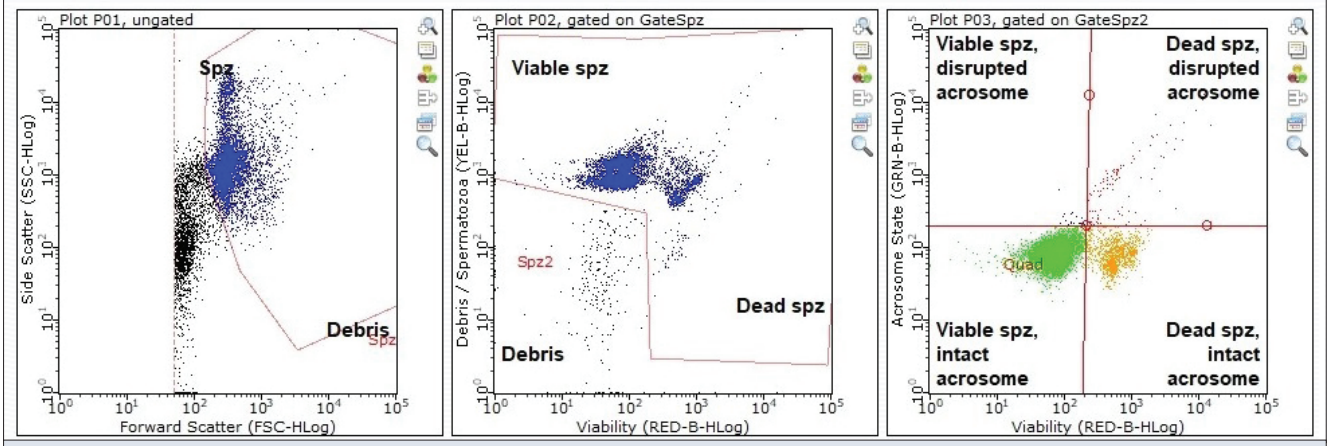

Fig 5. The obtained dotplots from flow cytometer analysis viability and acrosome ıntegrity at the rightest histogram. Green population: Viable and intact acrosome, Orange population: Dead and intact acrosome, Red points: Dead and damaged acrosome, Blue points: Viable but damage acrosome

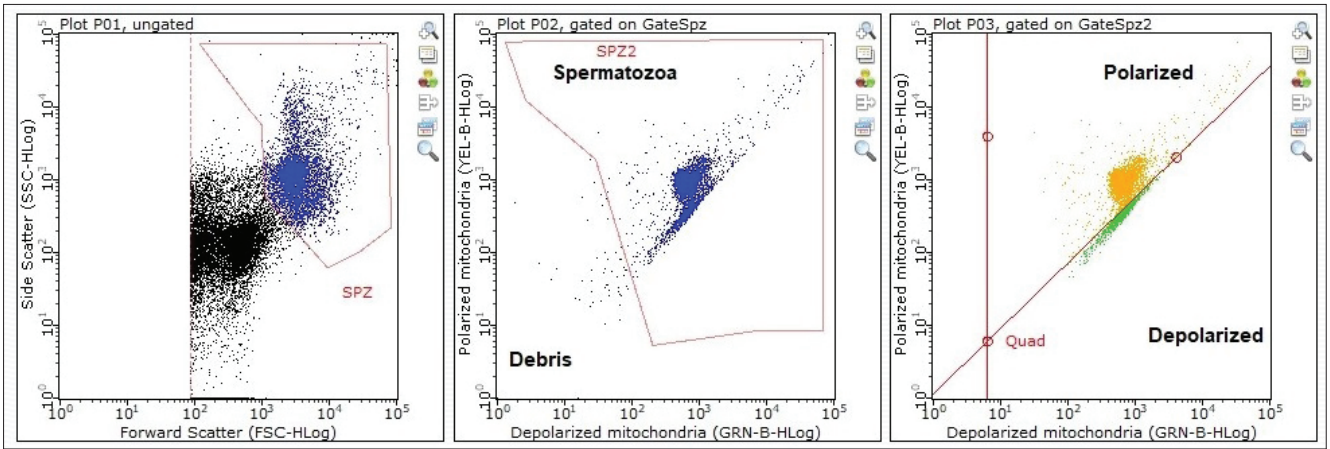

Fig 6. The obtained dotplots from flow cytometer analysis (Mitochondrial membrane potential)

At the rightest histogram (Orange population; Polarized mitochondria/High Mitochondrial Potential), (Green population; Depolarized mitochondria/Low Mitochondrial Potential)

rapid progressive motility (P<0.01), MMP (P<0.001) and slow sperm ratio (P<0.01), the sperm samples with LDR revealed higher progressive motility (P<0.05), medium (P<0.001) progressive motility and head diameters (P<0.05). From 12th h to 96th h; the rapid progressivity (P<0.01), MMP (P<0.05), live sperm rates with intact acrosome (P<0.01) and STR (P<0.001) of the HDR group results were better than LDR. The LIN (P<0.01) was better until 72nd h. The LDR group medium progressivity (P<0.01) and VAP (P<0.05) were better than HDR from the 12th to the 72nd h.

It was observed that the TM and PM of the LDR group decreased more rapidly than HDR group over time. Statistically, while both TM and PM of the HDR group decreased at the 72th h, TM and PM in the LDR group decreased at the 24th h and 12th h; respectively (P<0.05). The rapid progressive motility rates of the HDR were close to LDR during storage. While the medium progressive motility rate of the HDR group was similar to the diluted semen until 96th h, the LDR group differed at 72nd h of the storage. The MMP of HDR group was similar to the diluted semen until 72nd h but in the LDR group it was similar until 48th h (P<0.05). For the sperm rates with intact acrosome, in the HDR group, the increasing dilution rates protected sperm rates with intact acrosome until 96th h, but in the LDR group this protection was only until 2nd h (P<0.05). The velocity parameters were similar except rapid and slow spermatozoa rates at 2nd h in general.

However, in the both groups time-related head diameter differences at the different time zones were similar, the LDR group head diameter was slightly higher than HDR group in general.

The kinematic parameters in HDR and LDR groups for 96th h were given in *Table 1*. While VCL, VAP and ALH of LDR group were similar to the diluted semen until 96th

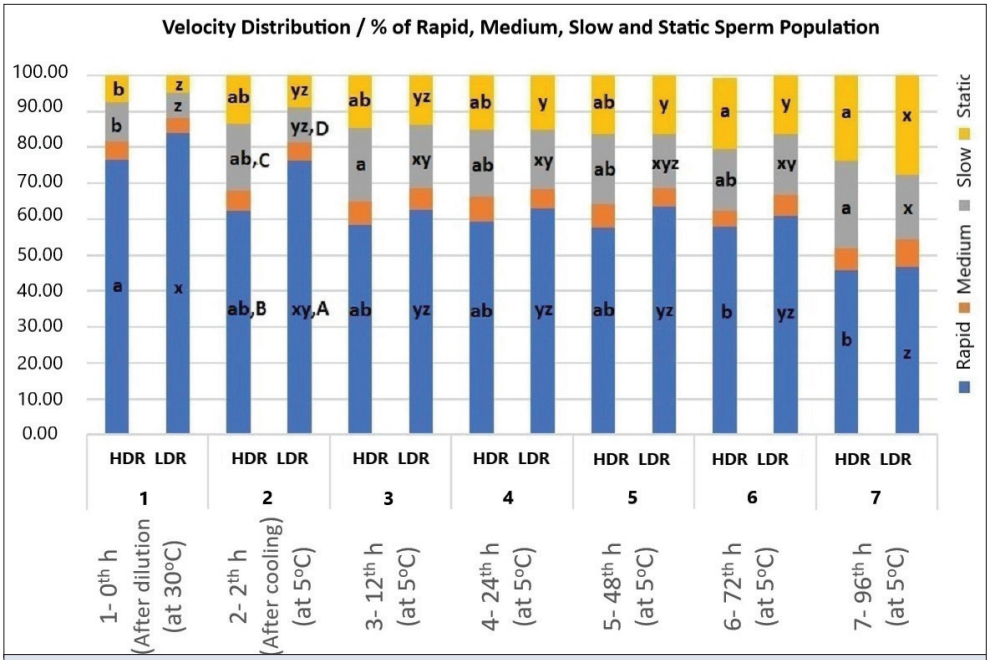

Fig 7. Velocity releated sperm ratios in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate (Laparoscopic insemination dose 20x10^6 sp/straw), **LDR:** Low Dilution Rate (Cervical insemination dose 400x10^6 sp/straw), **a,b:** The difference between different letters for the same characteristic (rapid, medium, slow or static) is statistically significant in HDR group. (P<0.05), **x,y,z:** The difference among different letters for the same characteristic (rapid, medium, slow or static) is statistically significant in LDR group (P<0.05), **A,B:** The difference between different letters for the same characteristic (rapid) is statistically significant in the same time zone (P<0.05), **C,D:** The difference between different letters for the same characteristic (slow) is statistically significant in the same time zone (P<0.05)

h, these parameters in the HDR group were until 2nd h, 2nd h, and 12nd h, respectively (P<0.05). The VSL of both groups were similar to the diluted semen until 72nd h. The cooling, storage period, and dilution rates did not affect the LIN and STR of the HDR group until 96th h but in the LDR group this similarity was until 24th h. In addition, the WOB and BCF of both groups were similar to the diluted semen until 96th h.

Discussion

In this study, we evaluated the effects of different dilution rates on ram sperm motion related (motility, progressivity, velocity and average of speed) parameters, head diameter, MMP and live intact acrosome rate during liquid storage for 96 h at 5°C. Fresh ram semen volume, concentration and motility ranges were 0.5 to 2.0 mL, 3.5 x 10^9 to 6.0 x 10^9 spermatozoa/mL and greater than 85%, respectively. The characteristics of fresh semen were in agreement with the data on ram semen reported in the literature [42-45].

Semen dilution removes adsorbed proteins, natural antioxidants, and other beneficial components in seminal plasma that are required for the maintenance of the membrane integrity and function of spermatozoa [33]. There have been considerable studies on the changes that occur when semen is diluted with different media which is known as dilution effect [33]. Dilution effect may be minimized by appropriate dilution rate and by extender with balanced salt, energy and buffering capacity [33]. The higher volume of seminal plasma and its contents may be one of the reasons for slightly better preservation of functional parameters at the low diluted group at 0th h. The increasing dilution rates increased rapid progressive motility and STR but not medium progressive motility and head diameters (P<0.05). The loss of intracellular water affects protein structure and function, which alter cell enzyme activity [46]. In this study, sperm head diameters of the LDR group were bigger than the HDR group (P>0.05).

Mainly, liquid sperm preservation involves slowing metabolic and biochemical function of spermatozoa by declining temperatures to 0-15°C [23,32,35]. Sperm cooling to +5°C has a controversial effect on semen parameters [47]. It was observed that cooling to +5°C within 2 h negatively affected observed semen parameters. Reducing sperm temperatures for cold storage to 5°C inflicts sub-lethal damages to spermatozoa [21].

Storage temperature and duration, concentration, and extender ingredients can influence sperm quality and survivability during liquid preservation [21]. At the 2nd h of the storage, the HDR revealed higher rapid progressivity (P<0.01), MMP (P<0.001), and slow velocity (P<0.01), and lower total (P<0.05) and medium (P<0.001) progressivity, medium velocity and head diameters (P<0.05), compared to LDR group, in the study.

Table 1. *The average values of kinematic parameters and head areas in HDR and LDR groups for 96 h storage*

Kinematic Parameters		Group	n	0th h	2nd h	12th h	24th h	48th h	72nd h	96th h	*P Value*
				Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	
Average Values of Speed	Head Area (μm²)	HDR	8	42.06±0.34^{B}	41.58±0.45^{B}	41.68±0.26^{B}	41.87±0.41	41.73±0.42^{B}	40.93±0.20^{B}	40.94±0.34	0.204
		LDR	8	43.08±0.22^{A}	43.13±0.36^{A}	42.87±0.32^{A}	42.57±0.46	42.97±0.36^{A}	42.57±0.31^{A}	42.11±0.64	0.573
			P Value	0.025	0.019	0.014	0.281	0.043	0.001	0.136	
	VCL (μm/s)	HDR	8	250.80±11.50^{a}	211.50±14.80^{ab}	198.98±8.08$^{bc.B}$	191.19±8.37$^{bc.B}$	189.30±8.29^{bc}	194.14±9.79^{bc}	157.86±9.35^{c}	0.001
		LDR	8	256.40±29.60	237.50±28.50	242.60±16.50^{A}	263.20±19.80^{A}	245.70±27.60	230.50±16.20	187.70±23.80	0.390
			P Value	0.862	0.430	0.033	0.005	0.070	0.075	0.263	
	VAP (μm/s)	HDR	8	152.14±7.55^{a}	125.96±8.44^{ab}	116.47±5.72$^{bc.B}$	114.99±4.98$^{bc.B}$	113.71±5.40$^{bc.B}$	116.29±6.18$^{bc.B}$	91.84±6.47^{c}	0.001
		LDR	8	159.10±15.50	147.30±14.50	145.07±8.95^{A}	158.50±11.00^{A}	146.30±13.40^{A}	145.00±8.49^{A}	118.40±12.00	0.313
			P Value	0.692	0.223	0.018	0.003	0.040	0.016	0.072	
	VSL (μm/s)	HDR	8	95.01±6.08^{a}	76.35±7.35^{ab}	75.68±4.17^{ab}	74.50±4.14^{ab}	70.12±5.78^{b}	71.96±4.29$^{ab.A}$	55.01±4.83^{b}	0.001
		LDR	8	85.55±8.37^{a}	80.47±7.98^{a}	75.74±5.59^{a}	77.95±5.91^{a}	66.74±5.34^{ab}	59.94±3.33$^{ab.B}$	47.66±4.58^{b}	0.001
			P Value	0.376	0.710	0.993	0.639	0.674	0.044	0.288	
	STR (%)	HDR	8	59.40±2.42^{A}	56.39±2.74	60.49±1.42^{A}	60.00±2.00^{A}	56.89±2.54^{A}	56.87±1.20^{A}	52.68±2.02^{A}	0.157
		LDR	8	52.37±1.10$^{a.B}$	52.80±1.38^{a}	50.65±1.49$^{a.B}$	48.80±1.65$^{ab.B}$	44.34±1.24$^{bc.B}$	40.85±1.62$^{c.B}$	39.68±0.78$^{c.B}$	0.001
			P Value	0.019	0.260	0.001	0.001	0.001	0.001	0.001	
	LIN (%)	HDR	8	35.31±1.37	32.41±1.47	34.32±1.32^{A}	34.97±1.34^{A}	33.16±2.06^{A}	33.36±1.25^{A}	28.72±1.83	0.075
		LDR	8	33.37±1.31^{a}	33.36±1.21^{a}	29.37±0.61$^{ab.B}$	28.71±0.84$^{abc.B}$	25.81±0.67$^{bc.B}$	24.74±1.15$^{bc.B}$	24.27±1.30^{c}	0.001
			P Value	0.326	0.626	0.004	0.001	0.004	0.001	0.067	
	WOB (%)	HDR	8	60.15±1.89	58.38±1.38	56.85±1.83	58.20±0.84	57.81±1.37	57.96±1.24	53.99±1.83	0.192
		LDR	8	64.11±2.45	63.60±2.02	59.13±1.00	59.35±0.81	58.04±1.81	60.34±0.72	60.33±2.36	0.129
			P Value	0.221	0.051	0.292	0.344	0.921	0.119	0.052	
	ALH (μm)	HDR	8	2.20±0.09^{a}	1.92±0.12^{ab}	1.84±0.06^{abc}	1.77±0.07$^{bc.B}$	1.75±0.06^{bc}	1.79±0.07^{bc}	1.54±0.07^{c}	0.001
		LDR	8	2.21±0.23	2.07±0.22	2.12±0.13	2.27±0.15^{A}	2.14±0.23	1.98±0.13	1.68±0.19	0.407
			P Value	0.969	0.562	0.093	0.013	0.125	0.258	0.501	
	BCF (Hz)	HDR	8	30.27±1.85^{a}	24.57±1.96^{ab}	23.90±1.34^{ab}	24.26±1.29^{ab}	23.27±1.98^{ab}	23.50±1.51^{ab}	18.03±1.36^{b}	0.001
		LDR	8	26.58±1.71^{a}	24.84±1.44^{ab}	21.94±1.51^{ab}	21.61±1.40^{ab}	22.07±1.57^{ab}	20.40±0.85^{ab}	18.73±1.13^{b}	0.005
			P Value	0.165	0.913	0.348	0.189	0.644	0.097	0.699	

HDR: *High Dilution Rate (Laparoscopic insemination dose. 20x10⁶ sp/straw);* ***LDR:*** *Low Dilution Rate (Cervical insemination dose. 400x10⁶ sp/straw);* ^{a,d} *The difference between different letters in the same row is statistically significant (P<0.05)*
0th h *(After dilution. at +30°C);* ***2th h*** *(After cooling. at +5°C. -0.3°C/min);* ***12th h, 24th h, 48th h, 72th h, 96th h*** *(at +5°C)*
^{A,B} *The difference between different letters in the same column is statistically significant (P<0.05)*

Ram sperm concentration significantly affect the sperm structural and functional parameters during the storage period at 4°C [29]. Both sperm concentrations and storage duration significantly affected the motility, morphology and membrane integrity [35]. For the HDR and LDR group; the sperm total motility (P<0.001 and P<0.001), progressive motility (P<0.001 and P<0.001), rapid progressive motility (P<0.05 and P<0.01), medium progressive motility (P>0.05 and P<0.001), MMP (P>0.05 and P<0.01) and live sperm with intact acrosome (P>0.05 and P<0.001) were significantly decreased as storage time increased, respectively. The declines in these sperm parameters during storage period may be originated from the metabolic activity, metabolic products, and pH changes [19,21]. For the velocity-related parameters of both HDR and LDR groups, it was observed that while there was a time-dependent decrease with sperm rapid motion (P<0.001); there was an increase with medium (P>0.05), slow (P<0.01) and static (P<0.001) motion. For the HDR group the VCL, VAP, VSL, ALH (P<0.001) and BCF and for the LDR group the VSL, STR

and LIN (P<0.001), and BCF (P<0.01) values of speed were decreased with increased duration time.

The decrease of MMP over time may occur due to the metabolic activities at 4°C and the changes in the pH due to metabolic products [29]. At low sperm concentrations, reduced ROS production persists throughout the storage period [24]. The MMP of HDR group was similar to the diluted semen until 72nd h but the LDR group was similar until 48th h (P<0.05). The lower numbers of sperm consume less glucose from media than with higher concentrations so that more energy source remains in the environment. The group with lower sperm numbers retained higher viability from 6 h to 5 d compared with treatments with higher concentrations [24]. Also as expected; the rapid progressivity (P<0.01), MMP (P<0.05), live sperm rates with intact acrosome (P<0.01), STR (P<0.001) and LIN (P<0.01) in the HDR group retained better than that of in LDR group; from the 12th h to the 96th h, in our study.

It was observed that different spermatological motion features are changed at different times and it was affected by the sperm concentration. The possible physiological reasons for lower motility in LDR group may be due to the effects of endogenous free radical production.

The semen extenders reduce the protective effects of the beneficial components of seminal plasma that are required for the maintenance of membrane integrity and functions of spermatozoa [48]. The VCL, VAP and ALH were similar to the diluted semen until 96th h in LDR group but until 2nd h, 2nd h, and 12nd h in the HDR group were, respectively (P<0.05). The VSL of both groups was similar to the diluted semen until 72nd h. The higher volume of seminal plasma and its contents in LDR group may be one of the reasons for better preservation of functional parameters.

The sperm diluted to 10 million spermatozoa/mL and stored at ambient temperature retained higher viability, and lower osmotic and ROS stress compared with the sperm stored at 60 million spermatozoa/mL [24,34]. A decline in acrosome integrity of alpaca sperm stored at 4°C is not observed until 72 h after the onset of liquid storage [30]. In the HDR group, the increasing dilution rates protected spermatozoa acrosomes until 96th h, but in LDR group it was until only 2nd h (P<0.05). This difference could be explained by differences of the osmotic stress and ROS level of both groups.

A low sperm number in a high osmotic environment causes more water to be released into the environment compared to the high number group. The LDR group head diameter was slightly higher than HDR in general. However, time-related head diameter differences of both groups were similar. The larger mean values of sperm head area are associated with larger mean values of ALH, a parameter which is related to sperm hyperactivation [49]. The ALH was slightly higher in LDR group that have larger head diameters.

In summary, we conclude that different spermatological motion features, MMP and live/intact acrosome rates changed at different times and it was affected by the sperm concentration and storage time. Following storage for 96 h, better sperm characteristics were achieved in the HDR group. While important sperm characteristics for fertility (such as; total motility, progressive motility, rapid progressive motility, medium progressive motility, MMP, live and intact acrosome, VSL, STR and LIN) did not change dramatically until 72th h in HDR group, however, these semen characteristics of the LDR group changed statistically earlier (at the 2nd h).

Availability of Data and Materials

The authors declare that the data that support the findings of this study are available from the corresponding author (C. Dayanıklı), upon reasonable request.

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Conflict of Interest

None of the authors have any conflict of interest to declare.

Ethical Statement

Semen collection procedures of the present study were approved by the local ethics committee of Sheep Breeding Research Institute, Ethics Committee on Animal Research (2021/048).

Author Contributions

CD: experimental design, methodology and investigation, semen collection, spermatological analysis, formal analysis, wrote manuscript. ES: semen collection, spermatological analysis. BB: contribution to experimental design, methodology and investigation, formal analysis, editing manuscript. BU: editing manuscript. ZN: experimental design, writing manuscript & editing, visualization, supervision. All authors read, revised, and approved the final manuscript.

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