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Effect of cholesterol loaded cyclodextrin on semen cryopreservation of Aksaray Malakli shepherd dogs of different ages

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ABSTRACT

The objective of the study was to determine the effect of cholesterol-loaded cyclodextrin (CLC) on the quality parameters of semen from Aksaray Malakli Shepherd dogs of different age groups. Forty-eight male dogs were divided into 3 groupings according to their ages (young age (Y): ≤3 years, n: 20; middle age (M): 4–6 years, n: 20; old age (O): ≥7 years; n: 8). The sperm-rich portion of the ejaculate from each dog was divided into four aliquots and extended with either tris as a control (C) or tris loaded with 0.5, 1.0, and 1.5 mg/120 × 10^6 CLC as low (L), intermediate (I), and high (H) doses, respectively. Following equilibration for at least half an hour, the straws were frozen in nitrogen vapor and then stored in liquid nitrogen at least for 48 h. Later, the frozen straws were thawed in a water bath for spermatological evaluation. Significant differences were observed between different age groups in terms of the spermatological parameters ($p < 0.05$). The evidence suggests that increasing age is associated with poor in-vitro spermatological parameters and CLC was able to protect the acrosome integrity from cryo-damage during the freeze-thawing process. Better semen freezability characteristics were obtained at young ages, considering the overall parameters.

1. Introduction

Freezing of sperm is crucial for the preservation of genetic material from sires with superior breeding value and endangered species (Schafer-Somi et al., 2006). In dogs, the use of frozen semen reduces time-consuming, dangerous, and costly animal transportation and allows the storage of the spermatozoa of genetically superior dogs whose natural breeding is impossible (Michael et al., 2007). However, the success of cryopreservation depends on the semen quality, which can be affected by many factors, such as the age and physical condition of the animal and cryo-resistance of the sperm. In such situations, age is a very important factor in determining semen quality. Although there are some reports on the relationship between aging and male fertility in humans, very few reports are available on dog fertility. In a human study, the semen fertilization capacity, live spermatozoa rate, testosterone hormone

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level, numbers of sertoli and germ cells, and semen volume were found to decline with age. (Eskenazi et al., 2003; Gunes et al., 2016). After 40–45 years old, the rate of abnormal spermatozoa increases and semen volume declines gradually in men. Further, some studies on different dog breeds demonstrated that age has a significant negative correlation with the proportion of the normal spermatozoa (Rijsselaere et al., 2007; Stone et al., 2013; Rota et al., 2016). However, the effect of aging on the semen quality of Malakli shepherd dogs needs further research for a better understanding of these factors.

Carreira et al. (2017) reported that a spermatozoon has a very peculiar nucleus compared to the somatic cell nucleus. Spermatogenesis is a very complex process, especially when spermiogenesis and the transportation of sperm cells during epididymal passage are considered. Zubkova et al. (2005) reported that the level of disulfide bridging and protamination disturbance decreased in older animals when compared to adult animals. Thus, with aging, motility, DNA and morphological integrity in animals can be affected. Freezing is also known to affect alterations in the phospholipids-cholesterol ratio, which is an important factor in the stabilization of plasma membranes at low temperatures, thus leading to a reduction in the fertility of the sperm (Khan et al., 2017).

Cholesterol plays a major role in protecting the function, structure, and fluidity of cell membranes at the physiological body temperature. It has a complex effect on the properties of the plasma membrane; it decreases membrane permeability and reduces phase changes, provides a proper physical and/or chemical microenvironment for membrane proteins, regulates morphological characteristics, and acts as a membrane antioxidant (Aksoy et al., 2010; Moce et al., 2010a). As for cyclodextrin, it is a cyclic oligosaccharide produced by the degradation of starch. Methyl-β-cyclodextrin, one of the most widely used cyclodextrins, can solubilize hydrophobic molecules, such as cholesterol. Some studies have reported that adding CLC into bull, ram, and stallion semen extenders can double or even triple the sperm cholesterol content (Peters et al., 2000; Moce et al., 2010a; Murphy et al., 2014) as well as inducing an increase in the cholesterol-phospholipid ratio. As a result of this increase, the sperm is protected against damage due to temperature changes during freezing. It is reported that the addition of CLC into semen extenders generally increases the total motility, mitochondrial activity, membrane integrity, and viability of the sperm (Peters et al., 2000; Moce et al., 2010b). In addition, several reports assert that treatment of sperm with CLC decreases acrosome damage, abnormal spermatozoa proportion (Mansour, 2009), and DNA fragmentation (Katanbafzadeh et al., 2014). During the past few decades, researchers have studied the effects of CLC in preventing sperm cell injuries due to freezing in different animal species (Naseer et al., 2015). The Aksaray Malakli dog breed is an important local breed in Turkey and conservation of its genetic material is very crucial, as it might be required to recover lost genetic diversity and decode important genetic information (Inanc et al., 2015). Thus far, there have been no studies on the cryopreservation of Aksaray Malakli semen. Besides, there is no information available on the relationship between the age and quality of frozen semen of this breed and there are few reports on the cryopreservation of dog semen with CLC additives; thus, more research is needed to optimize the conditions and amount of CLC addition.

Therefore, the aim of this study was to determine the effect of CLC on the semen quality parameters after freeze thawing of Aksaray Malakli Shepherd dog semen obtained from dogs of different ages.

2. Materials and methods

2.1. Cyclodextrin preparation

Methyl-β-cyclodextrin was loaded with cholesterol according to the procedure adopted by Purdy and Graham (2004). Initially, 1 g of methyl-β-cyclodextrin was dissolved in 2 mL of methanol. In a separate glass tube, 200 mg of cholesterol was dissolved in 1 mL of chloroform. Later, 450 µL of the cholesterol solution was mixed with the cyclodextrin solution. The solvent was evaporated using nitrogen vapor to obtain a white CLC powder; a CLC solution was then prepared by adding 50 mg of the CLC powder into 1 mL of stock Tris (50 mg/mL) at 37 °C and stirring the solution using a vortex mixer.

2.2. Animals

Forty-eight male Aksaray Malakli Shepherd dogs of different age groupings (young age (Y): ≤3 years; n: 20, middle age (M): 4–6 years; n: 20, and old age (O): ≥7 years; n: 8) were used in this study. Ejaculates were collected by digital manipulation as previously described by Akcay and Tekin (2002). The three fractions were collected separately into different collection tubes that were previously sterilized and pre-warmed. Only the second sperm-rich fraction was processed. Initially, the ejaculates were examined in terms of the fresh spermatological parameters. Fresh semen motility (%) was evaluated using a phase contrast microscope with a warm stage at 37 °C; sperm concentration ($\times 10^6$) was determined by the Hemositometric method (Bearden et al., 2004). Abnormal spermatozoa (%) was examined using a Hancock's solution (Schafer and Holzmann, 2000), while the dead spermatozoa (%) was determined by the eosin-staining method (Akalin et al., 2015) and the sperm pH was examined using a pH strip. The ejaculates containing higher than 250×10^6 spermatozoa/mL with > 70% forward motility were used for further studies. After initial evaluation, the ejaculates were kept in a water bath at 35 °C until further processing. All the experiments were conducted in a humane manner and this study was approved by the Animal Ethics Committee at the Faculty of Veterinary Medicine, Ankara University, Turkey.

2.3. Semen processing

The ejaculates were divided into four groups for each animal; one group without the additive into a tris-based extender (30.0 g tris, 17.0 g citric acid, 12.5 g fructose, 5% v/v glycerol, 20% v/v egg yolk with 1000 mL distilled water at pH 6.8; control group) and

three treatment groups, were firstly diluted with CLC solutions of 0.5, 1.0, or 1.5 mg/120 × 10^6 as low (L), intermediate (I), and high (H) doses, respectively. Following incubation for 15 min at 22 °C, they were extended with a tris-based extender to a final concentration of 50 × 10^6 spermatozoa/mL. The concentration was calculated using a hemocytometer. The diluted samples were filled into 0.25 mL mini straws and equilibrated for 1.5 h at 4 °C. Thereafter, the samples were frozen at −100 °C for 15 min with nitrogen vapor and stored in liquid nitrogen. After at least 48 h, they were thawed in a water bath (37 °C/30 s) for further spermatological examination.

2.4. Post-thawed semen evaluation

2.4.1. CASA motility analysis

A computer-aided sperm analysis system (CASA; SCA, Microptic®, Spain) was used to examine the kinetic parameters and motility of the sperm. Spermatozoa motility was classified as fast (> 80 μm/s), medium (> 50 μm/s), slow (> 20 μm/s), or static. The thawed semen (6 μL) was put onto a slide and mounted with a cover slide for motility analysis at 37 °C with a 10 × objective. Total sperm motility (%), progressive motility (%), Velocity Straight Line (VSL, μm s^{-1}), Velocity Curve Linear (VCL, μm s^{-1}), Wobble (WOB, %), Linearity (LIN, VSL/VCL × 100), Velocity Average Path (VAP, μm s^{-1}), Straightness (STR, VSL/VAP × 100), Amplitude of Lateral Head Displacement (ALH, μm), Beat Cross Frequency (BCF, Hz), and hyperactivity (%) were recorded. For each sample, a minimum of 200 and a maximum of 300 spermatozoa were analyzed in seven different fields.

2.4.2. Abnormal sperm assessment

Abnormal sperm assessment was performed in accordance with the sperm blue staining procedure using a "Sperm Blue®, Microptic®, Spain" kit; the results were evaluated by a CASA system (Van der Horst and Maree, 2010). After sperm smears were prepared for each group, they were allowed to dry at the room temperature. Later, the smear was stained according to the kit manufacturer's instructions. A minimum of 100 spermatozoa were evaluated using the CASA system (SCA, Microptic®, Spain) and the abnormal spermatozoa (%) was determined.

2.4.3. Dead spermatozoa (viability)

SYBR-14/PI (Invitrogen, L-7011) was used to assess the dead spermatozoa as described previously by Garner and Johnson (1995). After staining, at least 200 sperm cells were examined using a fluorescence microscope (Excitation at 450–490 nm, emission at 520 nm; Leica DM 2500) to evaluate semen viability. Sperm cells labeled with a green stain were considered to be live cells with an intact membrane, while those labeled with red or red-green stains were considered to be dead cells with damaged membranes.

2.4.4. Acrosome status

Sperm acrosome integrity was determined using FITC-PNA (Invitrogen, L7381) and a PI staining method, as previously reported by Garner and Johnson (1995). After staining, at least 200 spermatozoa per sample were examined using a fluorescence microscope (Excitation at 450–490 nm, emission at 520 nm; Leica DM 2500). Spermatozoa exhibiting green patches or bright green fluorescence were considered to be defective or damaged acrosomes, while those with no green fluorescence in their acrosome caps were considered to be sperm with an intact acrosome.

2.4.5. Mitochondrial activity

The sperm mitochondrial status was evaluated using a JC-1/PI (Invitrogen, T3168) staining kit, as previously described by Garner et al. (1997). After the staining protocol, at least 200 spermatozoa per sample were evaluated using a fluorescence microscope (Excitation at 450–490 nm, emission at 520 nm; Leica DM 2500). The mitochondrial potential was evaluated based on the total mitochondrial activity in accordance with the JC-1/PI staining method. If a sperm mid-piece displayed green to yellow/orange fluorescence, it was considered to exhibit mitochondrial activity (low mitochondrial activity) and if there was no fluorescence in its mid-piece, it was concluded that the sperm did not display any mitochondrial activity.

2.4.6. DNA integrity

DNA integrity of the spermatozoon was evaluated by the COMET assay method, as previously described by Tasdemir et al (2013). In this analysis, 100 nuclei were examined using a fluorescence microscope (Zeiss, Germany). A DNA nucleotide extends by electrophoresis to organize "comet tails" and the relative intensity of the DNA in the tail reflects the frequency of DNA breakage. As a result, DNA damage could be measured directly in terms of the percentage of total DNA in the comet tails (%). For DNA damage evaluation, a COMET assay III® score program (Perspective Instruments, UK) was used and the analysis was performed blindly using one slide reader. The tail length (μm), tail DNA (%), and tail moment (μm/s) were evaluated (Fig. 1).

2.5. Statistical analysis

Significant differences between the tested groups were analyzed by ANOVA. A Tukey test was used to determine the differences between the groups. For statistical analyses, the significance level was set at 5%. Differences with values of $p < 0.05$ were considered to be statistically significant. The descriptive measurements of the groups are shown as "arithmetic mean ($\bar{X}$) ± standard deviation (SD)". SPSS® for Windows 14.1 (License No: 9869264) was used to analyze the data.

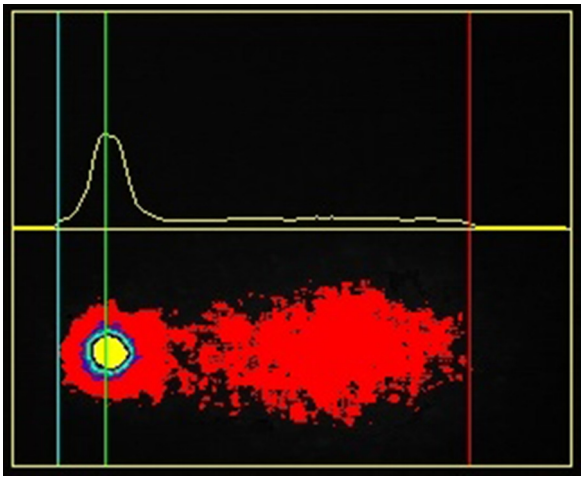

Fig. 1. DNA fragmentation visualized by the COMET assay III® score program (perspective instruments, UK).

3. Results

After thawing the straws, both the relationship of age and CLC were evaluated together for each parameter. As shown in Table 1, the lowest proportion of total abnormal spermatozoa was identified in the young animal group ($p < 0.05$). There were no significant differences in other fresh spermatological parameters ($p > 0.05$). As shown in Table 2, the total motility was greater in the young age group ($p < 0.05$). The highest total motility of 62.7% ± 15.6% was obtained in the Y−L group, while the lowest total motility (33.7% ± 17.4%) was in the O−C group. There were no significant differences in progressive and total motility between different age groups, when each age group was evaluated separately ($p > 0.05$). As shown in Tables 2 and 3, there were significant differences between the treatment groups in terms of the CASA kinetic parameters ($p < 0.05$). VCL and VSL were the parameters most affected by age. In general, the results of the young age group were better than those of the middle and old age groups. An overall analysis of the kinetic parameters indicated a reduction when the dogs were older than 4 years of age. There was no significant relationship between the control and CLC groups regarding the kinetic parameters when the Y, M, and O groups were evaluated separately. Additionally, from Tables 2 and 3, interactions could be detected between age and progressive motility, total motility, VCL, VSL, VAP, LIN, WOB, ALH, and hyperactivity ($p < 0.05$).

The best viability was exhibited by the Y group (67.6% ± 15.0%) when compared to the M and O groups, as shown in Table 4 ($p < 0.05$). Similarly to motility, sperm viability also decreased with an increase in the age of the dogs. Acrosome integrity of the treatment groups (CLC) was superior compared to the C groups except M−C ($p < 0.05$). Mitochondrial activation was greater in group Y than in all other age groups, except for M−L and O−I ($p < 0.05$).

As shown in Table 5, the groups Y and M exhibited a greater percentage of total morphological integrity than group O. The highest total abnormal spermatozoa proportion of 50.7% ± 0.9% was observed in the O−H group, whereas the lowest proportion of 25.5% ± 14.6% was obtained in the M−L group ($p < 0.05$). Further, CLC did not exert a protective effect on the total morphological integrity. From Table 5, it could be inferred that physiological age exerted an influence on the sperm head, tail, and total abnormality ($p < 0.05$). As shown in Table 6, there were significant differences between the treatment groups, with respect to the DNA tail length. It was observed that the tail length increased with aging. According to this parameter, the lowest DNA integrity was determined in the O group, while the Y and M groups exhibited the highest DNA integrity ($p < 0.05$). Furthermore, from Table 6, it can be deduced that there exists a relationship between the age and tail length and tail moment ($p < 0.05$).

DNA fragmentation visualized by the COMET assay III® score program is shown in Fig. 1. In Fig. 2, an interaction between age and CLC level for acrosome integrity can be observed. The lowest acrosome integrity was detected in the control group (0 mg CLC). In Fig. 3, the only interaction that could be detected existed between age and the CLC level for mid-piece abnormality; CLC protected the sperm from such abnormality formation.

Table 1
Mean (± SD) values of fresh spermatological parameters.

Groups	Motility (%)	Dead Spermatozoa (%)	Total Abnormality (%)	pH	Concentration (x10^6)
Y	85.7 ± 5.3	10.8 ± 5.0	13.2 ± 9.5[a]	5.8 ± 0.2	325.1 ± 100.5
M	80.8 ± 10.4	12.8 ± 7.3	27.5 ± 20.2[b]	5.7 ± 0.1	318.0 ± 34.3
O	79.8 ± 14.8	16.3 ± 8.7	23.1 ± 19.0[b]	5.8 ± 0.1	323.7 ± 53.1

[a,b]Different superscripts within the same column demonstrate significant differences ($p < 0.05$).
(young ages (Y) ≤ 3 age; middle ages (M) 4–6 age; old ages (O) ≥7 age).

Table 2
Mean (± SD) values of CASA motility and kinetic parameters after freeze-thawing.

	Groups	Progressive Motility (%)	Total Motility (%)	VCL (μm s^{-1})	VSL (μm s^{-1})	VAP (μm s^{-1})
Y	C	11.4 ± 4.5^{ab}	54.8 ± 11.4^{ab}	80.5 ± 10.6^{a}	32.05 ± 8.5^{ab}	48.7 ± 8.09^{ab}
	L	13.7 ± 5.8^{a}	62.7 ± 15.06^{a}	79.4 ± 12.1^{a}	33.5 ± 11.07^{a}	49.5 ± 10.51^{ab}
	I	14.0 ± 7.5^{a}	54.1 ± 12.7^{ab}	74.8 ± 8.7^{ab}	32.3 ± 9.01^{ab}	46.4 ± 7.97^{ab}
	H	9.06 ± 4.3^{abc}	53.3 ± 11.4^{ab}	69.5 ± 7.9^{abc}	26.7 ± 6.04^{abc}	41.8 ± 5.44^{ab}
Mean		12.05 ± 5.8^{A}	56.2 ± 12.84^{A}	76.06 ± 10.57^{A}	31.16 ± 8.89^{A}	46.66 ± 8.45^{A}
M	C	7.07 ± 5.2^{bc}	40.1 ± 14.4^{bc}	58.9 ± 10.6^{c}	22.8 ± 7.6^{c}	35.7 ± 7.9^{ab}
	L	5.4 ± 2.5^{c}	36.9 ± 11.6^{c}	62.1 ± 8.8^{c}	22.1 ± 4.8^{c}	35.7 ± 5.5^{ab}
	I	7.4 ± 2.2^{cb}	43.6 ± 13.8^{bc}	63.2 ± 8.2^{c}	23.7 ± 4.6^{c}	70.7 ± 10.2^{c}
	H	4.9 ± 3.01^{c}	37.6 ± 16.4^{c}	60.4 ± 11.8^{c}	22.0 ± 5.7^{c}	35.3 ± 7.8^{ab}
Mean		6.23 ± 3.4^{B}	39.5 ± 13.88^{B}	61.17 ± 9.78^{B}	22.73 ± 5.64^{B}	36.02 ± 6.54^{B}
O	C	5.1 ± 4.5^{c}	33.7 ± 17.4^{c}	58.6 ± 12.9^{c}	19.0 ± 6.2^{c}	32.2 ± 5.2^{b}
	L	8.3 ± 6.0^{bc}	40.4 ± 17.7^{bc}	62.9 ± 7.4^{c}	22.4 ± 5.9^{c}	35.3 ± 5.5^{ab}
	I	10.2 ± 6.6^{abc}	45.4 ± 10.9^{bc}	67.1 ± 16.1^{bc}	25.5 ± 8.2^{c}	38.7 ± 9.3^{ab}
	H	5.7 ± 2.3^{c}	35.4 ± 15.2^{c}	57.9 ± 13.1^{c}	19.4 ± 8.1^{c}	31.4 ± 8.4^{b}
Mean		7.38 ± 5.8^{B}	38.7 ± 15.4^{B}	61.6 ± 12.65^{B}	21.62 ± 7.30^{B}	34.45 ± 7.53^{B}
P	**Age**	**< 0.05**	**< 0.05**	**< 0.05**	**< 0.05**	**< 0.05**
	CLC	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
	Age*CLC	0.692	0.596	0.431	0.709	0.509

[a,b,c]Different superscripts within the same column demonstrate significant differences($p < 0.05$).
[A,B]Different superscripts within the same column demonstrate significant differences.
(young ages (Y) ≤ 3 age; middle ages (M) 4–6 age; old ages (O)≥7 age; CLC solutions of 0.5, 1.0 or 1.5 mg/120 × 10^6 as low (L), intermediate (I) and high (H) doses respectively).

Table 3
Mean (± SD) values of CASA kinetic parameters after freeze-thawing.

	Groups	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)	Hyperactivity (%)
Y	**C**	39.4 ± 6.9^{ab}	64.8 ± 7.4^{ab}	60.4 ± 3.7^{abc}	4.4 ± 0.2^{a}	6.3 ± 0.8^{ab}	29.8 ± 10.0^{a}
	L	41.5 ± 8.8^{ab}	66.3 ± 8.3^{ab}	62.06 ± 5.7^{a}	4.3 ± 0.4^{a}	6.6 ± 1.1^{a}	30.9 ± 11.2^{a}
	I	42.7 ± 8.6^{a}	68.3 ± 8.4^{a}	61.8 ± 5.1^{ab}	4.2 ± 0.2^{a}	6.7 ± 0.6^{a}	26.5 ± 9.2^{ab}
	H	38.4 ± 7.7^{ab}	63.3 ± 7.1^{ab}	60.1 ± 4.7^{abc}	4.2 ± 0.2^{a}	6.2 ± 0.6^{ab}	21.3 ± 6.9^{bc}
Mean		40.55 ± 7.97^{A}	65.71 ± 7.80	61.10 ± 4.79^{A}	4.34 ± 0.30^{A}	6.50 ± 0.84	27.1 ± 9.86^{A}
M	**C**	38.2 ± 7.7^{ab}	62.7 ± 8.3^{ab}	60.3 ± 4.8^{abc}	3.9 ± 0.3^{ab}	5.3 ± 2.0^{ab}	13.7 ± 9.6^{bc}
	L	35.6 ± 5.0^{ab}	61.6 ± 5.0^{ab}	57.6 ± 3.3^{abcd}	4.3 ± 0.1^{a}	6.1 ± 0.8^{ab}	13.2 ± 4.2^{bc}
	I	37.6 ± 5.7^{a}	63.4 ± 5.7^{a}	58.9 ± 3.6^{abcd}	4.1 ± 0.3^{a}	6.6 ± 0.7^{a}	15.9 ± 6.1^{cd}
	H	36.3 ± 5.3^{ab}	62.4 ± 5.5^{ab}	58.0 ± 3.8^{abcd}	4.2 ± 0.3^{a}	6.2 ± 0.6^{ab}	12.3 ± 7.1^{bc}
Mean		36.96 ± 5.91^{AB}	64.24 ± 7.31	58.75 ± 3.96^{AB}	4.16 ± 0.32^{A}	6.09 ± 1.27	13.80 ± 6.93^{B}
O	**C**	33.6 ± 3.9^{b}	58.3 ± 13.9^{b}	56.3 ± 8.6^{bcd}	3.2 ± 2.0^{c}	4.9 ± 3.4^{a}	11.0 ± 8.7^{d}
	L	35.2 ± 7.1^{ab}	62.4 ± 7.8^{ab}	55.9 ± 4.2^{cd}	4.0 ± 0.1^{a}	6.5 ± 1.2^{a}	14.2 ± 8.1^{cd}
	I	37.0 ± 4.3^{ab}	64.1 ± 6.0^{ab}	57.6 ± 2.5^{abcd}	4.1 ± 0.4^{a}	6.2 ± 1.0^{ab}	18.7 ± 9.2^{bcd}
	H	33.2 ± 3.7^{b}	59.7 ± 10.9^{ab}	54.3 ± 7.7^{d}	3.3 ± 1.5^{bc}	5.4 ± 2.5^{ab}	10.9 ± 8.2^{bd}
Mean		34.80 ± 8.00^{B}	61.15 ± 9.86	56.09 ± 6.08^{B}	3.63 ± 1.32^{B}	5.79 ± 2.28	13.76 ± 8.76^{B}
P	**Age**	> 0.05	> 0.05	**< 0.05**	**< 0.05**	**< 0.05**	**< 0.05**
	CLC	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
	Age*CLC	0.946	0.865	0.872	0.119	0.781	0.391

[a,b,c,d]Different superscripts within the same column demonstrate significant differences ($p < 0.05$).
[A, B]Different superscripts within the same column demonstrate significant differences.
(young ages (Y) ≤ 3 age; middle ages (M) 4–6 age; old ages (O)≥7 age; CLC solutions of 0.5, 1.0 or 1.5 mg/120 × 10^6 as low (L), intermediate (I) and high (H) doses respectively).

4. Discussion

Today, pedigree breeding is receiving increased attention and there is a growing need for reproductive biotechnology techniques for use by small-animal clinicians (Pena et al., 2006). In the present study, the lowest total abnormal spermatozoa rate (13.2% ± 9.5%) was observed in young animals ($p < 0.05$). There were no significant differences in other fresh spermatological parameters (motility, dead spermatozoa rate, pH, or concentration; $p > 0.05$). In fresh spermatological evaluation, motility, concentration, dead spermatozoa rate, and pH were detected within normal limits. These results are in accordance with the report by Gunay et al. (2003) on German shepherd dog semen. Further, the lowest total abnormal spermatozoa rate was also detected in young animals. With an increase in the age of the animals, there might be an increase in the abnormal spermatozoa rate, which can be caused by either primary (disruptions of spermatozoa production in tubulus seminiferus contortus) or secondary (transport spermatozoa to the epididymis, storage in the epididymis, ejaculation, and genital infections) anomalies in the reproductive tract. However, these parameters were found to be within the normal ranges when compared to previous studies (Baran et al., 2000).

Table 4
Mean (± SD) values of fluorescent staining parameters after freeze-thawing.

	Groups	Sperm Viability (%)	Acrosome Integrity (%)	Mitochondrial Activation (%)
Y	**C**	59.3 ± 11.4^{ab}	35.9 ± 8.4^{c}	49.1 ± 7.8^{ab}
	L	67.6 ± 15.0^{a}	49.2 ± 11.2^{ab}	61.6 ± 10.1^{a}
	I	59.1 ± 12.7^{ab}	49.0 ± 6.7^{ab}	51.3 ± 12.3^{ab}
	H	58.3 ± 11.4^{ab}	51.1 ± 8.7^{ab}	52.3 ± 9.3^{ab}
Mean		61.10 ± 12.83^{A}	46.33 ± 10.57	53.63 ± 10.78^{A}
M	**C**	43.6 ± 17.5^{bc}	43.5 ± 14.9^{abc}	47.5 ± 14.9^{b}
	L	46.2 ± 12.1^{bc}	48.4 ± 15.0^{ab}	48.5 ± 13.5^{ab}
	I	52.8 ± 12.4^{bc}	50.0 ± 16.5^{ab}	47.6 ± 10.6^{b}
	H	45.5 ± 15.9^{bc}	55.8 ± 10.2^{a}	43.3 ± 15.6^{b}
Mean		47.05 ± 14.54^{B}	49.47 ± 14.53	46.77 ± 13.47^{AB}
O	**C**	39.4 ± 17.4^{c}	38.7 ± 12.1^{bc}	40.3 ± 17.2^{b}
	L	45.9 ± 17.5^{bc}	48.0 ± 2.3^{ab}	45.1 ± 14.9^{b}
	I	51.5 ± 10.0^{bc}	52.6 ± 9.7^{a}	51.2 ± 5.5^{ab}
	H	41.1 ± 14.9^{c}	54.9 ± 11.5^{a}	38.3 ± 15.1^{b}
Mean		44.51 ± 15.21^{B}	48.57 ± 11.15	43.77 ± 14.10^{B}
P	**Age**	< **0.05**	> 0.05	0.05
	CLC	> 0.05	< **0.05**	> 0.05
	Age*CLC	0.697	0.915	0.480

$^{a, b, c}$Different superscripts within the same column demonstrate significant differences ($p < 0.05$).
$^{A, B}$Different superscripts within the same column demonstrate significant differences.
(young ages (Y) ≤ 3 age; middle ages (M) 4–6 age; old ages (O)≥7 age; CLC solutions of 0.5, 1.0 or 1.5 mg/120 × 10^6 as low (L), intermediate (I) and high (H) doses respectively).

Table 5
Mean (± SD) abnormal spermatozoa rate after freeze-thawing.

	Groups	Head (%)	Mid- piece (%)	Tail (%)	Total Abnormality (%)
Y	**C**	6.4 ± 3.2^{abc}	1.4 ± 0.4^{cd}	23.9 ± 13.3^{bc}	32.7 ± 11.3^{bc}
	L	5.0 ± 3.5^{abc}	2.6 ± 0.6^{abcd}	20.2 ± 6.7^{c}	27.9 ± 7.9^{c}
	I	9.9 ± 4.4^{a}	1.7 ± 0.4^{cd}	23.8 ± 8.8^{bc}	31.6 ± 9.5^{bc}
	H	7.3 ± 3.3^{ab}	2.3 ± 0.5^{bcd}	24.2 ± 9.3^{bc}	33.9 ± 8.5^{bc}
Mean		7.18 ± 1.04^{A}	2.07 ± 0.25	23.07 ± 9.61^{A}	31.56 ± 9.35^{A}
M	**C**	8.6 ± 3.8^{a}	1.1 ± 0.6^{d}	24.8 ± 11.6^{bc}	33.9 ± 10.2^{bc}
	L	6.2 ± 3.9^{abc}	1.8 ± 0.4^{cd}	18.9 ± 15.8^{c}	25.5 ± 14.6^{c}
	I	8.0 ± 3.0^{ab}	3.7 ± 0.7^{ab}	23.7 ± 12.0^{bc}	35.3 ± 10.0^{bc}
	H	5.4 ± 3.4^{abc}	3.1 ± 0.5^{abc}	23.5 ± 10.7^{bc}	32.3 ± 12.9^{bc}
Mean		8.02 ± 1.04^{A}	2.42 ± 0.25	22.80 ± 12.44^{A}	31.80 ± 13.10^{A}
O	**C**	1.1 ± 0.1^{c}	1.4 ± 0.1^{d}	44.1 ± 1.3^{a}	46.6 ± 1.5^{a}
	L	4.5 ± 0.2^{abc}	3.6 ± 0.2^{ab}	33.6 ± 0.2^{b}	41.7 ± 0.5^{ab}
	I	5.1 ± 0.1^{abc}	4.2 ± 0.7^{a}	31.2 ± 0.6^{b}	40.7 ± 0.7^{ab}
	H	2.6 ± 0.7^{bc}	3.8 ± 0.8^{ab}	44.0 ± 0.5^{a}	50.7 ± 0.9^{a}
Mean		3.37 ± 1.2^{B}	3.27 ± 0.30	38.26 ± 6.02^{B}	44.97 ± 4.21^{B}
P	**Age**	> 0.05	> 0.05	< **0.05**	< **0.05**
	CLC	> 0.05	< **0.05**	> 0.05	> 0.05
	Age*CLC	0.599	0.100	0.460	0.635

^{a,b,c}Different superscripts within the same column demonstrate significant differences ($p < 0.05$).
^{A,B}Different superscripts within the same column demonstrate significant differences.
(young ages (Y) ≤ 3 age; middle ages (M) 4–6 age; old ages (O)≥7 age; CLC solutions of 0.5, 1.0 or 1.5 mg/120 × 10^6 as low (L), intermediate (I) and high (H) doses respectively).

In the present study, the lowest percentage of post-thawed total motility (33.7% ± 17.4%) was observed in the O—C group when compared to the young animal treatment groups ($p < 0.05$). In accordance with the present study, Gungor and Bucak (2016) used 1–3 year old animals to optimize the cryopreservation of Kangal semen (Turkish Shepherd breed). Akcay and Tekin (2002) put forward the effect of different extenders on the cryopreservation of Kangal semen (around 4 years old). In that study, the effect of tris and lactose extenders on post-thawed sperm motility was compared; motilities of 42%–61% and 27%–38%, respectively, were obtained with the tris and lactose extenders.

Similar to our findings, some researchers have reported that the quality of semen decreases as the animal grows older (Eskenazi et al., 2003; Rijsselaere et al., 2007). Indeed, it has been revealed that due to aging, a smaller litter size and decrease in fertility are quite probable after 7 years of age in dogs (Johnston et al., 2001). In contrast, there are several studies in which no significant relationship could be found between age and motility (Berling and Wölner-Hanssen, 1997; Van Der Westerlaken et al., 1998). Although there were no significant differences between the age groups in terms of motility, it was determined that the sperm collected from younger dogs was more resistant to damage induced by the cryopreservation process than the sperm obtained from

Table 6
Mean (± SD) values of DNA damage after freeze-thawing.

	Groups	Tail Length (μm)	DNA (%)	Tail Moment (μm/s)
Y	**C**	81.56 ± 11.5^{abc}	39.0 ± 7.27^{b}	19.0 ± 6.2^{a}
	L	64.9 ± 20.0^{a}	29.8 ± 10.7^{ab}	12.3 ± 7.1^{a}
	I	75.7 ± 10.5^{abc}	33.8 ± 5.9^{ab}	16.3 ± 5.8^{a}
	H	77.9 ± 10.4^{abc}	30.2 ± 6.7^{ab}	15.5 ± 5.5^{a}
Mean		75.16 ± 12.20^{A}	33.40 ± 7.73	15.66 ± 4.80^{AB}
M	**C**	81.1 ± 19.5^{abc}	33.6 ± 8.2^{ab}	14.3 ± 8.0^{a}
	L	77.9 ± 10.4^{abc}	30.7 ± 10.6^{ab}	12.2 ± 6.7^{a}
	I	70.9 ± 25.1^{ab}	26.9 ± 8.46^{a}	17.0 ± 6.5^{a}
	H	80.8 ± 22.2^{abc}	31.6 ± 9.0^{ab}	15.6 ± 7.5^{a}
Mean		76.34 ± 15.31^{A}	30.16 ± 7.66	13.87 ± 5.98^{A}
O	**C**	91.3 ± 12.3^{bc}	33.1 ± 7.8^{bc}	17.5 ± 5.0^{a}
	L	90.9 ± 17.5^{bc}	33.7 ± 10.0^{ab}	19.0 ± 8.1^{a}
	I	93.0 ± 8.7^{c}	31.5 ± 7.0^{ab}	18.1 ± 7.7^{a}
	H	93.4 ± 16.4^{c}	35.0 ± 5.8^{ab}	20.0 ± 6.6^{a}
Mean		92.16 ± 16.51^{B}	33.24 ± 7.52	18.70 ± 6.53^{B}
P	**Age**	< **0.05**	> 0.05	< **0.05**
	CLC	> 0.05	> 0.05	> 0.05
	Age*CLC	0.595	0.225	0.274

^{a,b,c}Different superscripts within the same column demonstrate significant differences ($p < 0.05$).
^{A,B}Different superscripts within the same column demonstrate significant differences.
(young ages (Y) ≤ 3 age; middle ages (M) 4–6 age; old ages (O)≥7 age; CLC solutions of 0.5, 1.0 or 1.5 mg/120 × 10^6 as low (L), intermediate (I) and high (H) doses respectively).

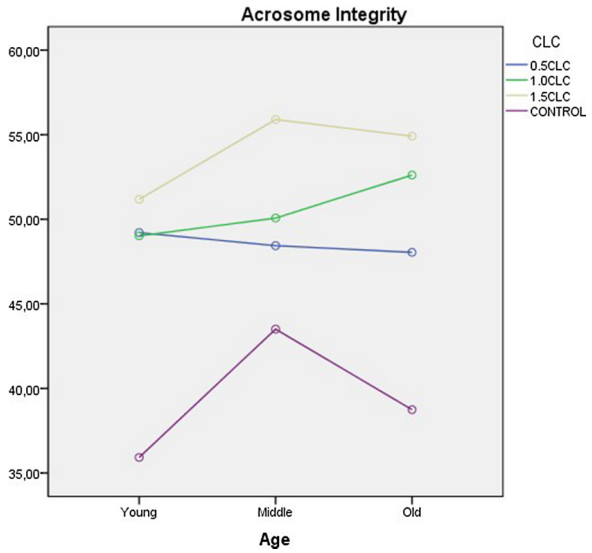

Fig. 2. The interaction between age and CLC level for acrosome integrity.

older dogs. In their study on dog semen cryopreservation, Khan et al. (2017) observed a protective effect of CLC on sperm motility, while Belala et al. (2016) reported that the addition of CLC (a level of 5 mg/120 × 10^6) led to a decrease in progressive motility but increased the total motility. In the present study, we did not observe any positive effect of CLC on the motility. Our results were comparable to other studies conducted with semen from different animal breeds (Purdy and Graham, 2004; Moraes et al., 2010; Oliveira et al., 2014; Inanc et al., 2018).

In terms of kinetic parameters, there were significant differences between different age groups. We could observe that VCL and VSL were particularly affected by age. These parameters decreased when the dogs were more than 4 years old. Some studies reported that VSL and VCL are related directly to fertility in humans (Krause, 1995; Garrett et al., 2003). The importance of VSL for fertility assessment has also been observed in bulls (Gillan et al., 2008). VSL, VAP, and VCL are measures of sperm velocity over specific paths; these values indicate that sperm with high mobility swim faster than those with lower mobility (King et al., 2000). On the other hand, there was no significant effect of CLC on the control groups with respect to the kinetic parameters, when the age groups were evaluated separately.

The viability results were significantly different between different age groups. Similar to fresh and post-thawed motility results, viability decreased when the age of the dog increased ($p < 0.05$). During the evaluation of post-thawed quality parameters, the best

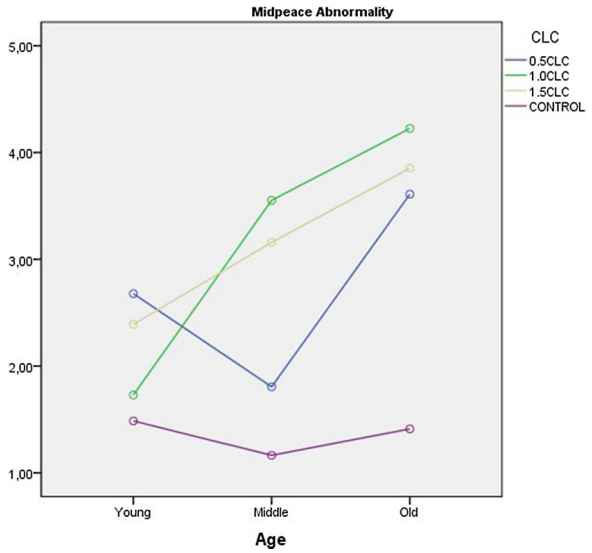

Fig. 3. The interaction between age and CLC level for mid-piece abnormalities.

motility and viability were found to be exhibited by the young age group. Thus, it was concluded that the freezability of the semen obtained from younger animals was better than that of the semen obtained from older Aksaray Malakli shepherd dogs. In contrast to our study, Khan et al. (2017) found a positive effect of CLC on the viability of post-thawed dog sperm. CLC has been shown to improve the viability of bull (Purdy and Graham, 2004) and ram sperm (Moce et al., 2010b). CLC treatments increase the cholesterol content and thus enhance the resistance of sperm to cold shock. The acrosome integrities of the CLC groups were superior to those of the C groups when the age groups were evaluated separately. There was no significant effect of age on this parameter; however, similar to our study, Khan et al. (2017) found beneficial effects of CLC on acrosome integrity with respect to age. This improvement can be associated with the effect of cholesterol on membrane fluidity. The motility and mitochondrial activation outputs were parallel with the neutralization of oxygen-deficient radicals and inhibition of cell damage (De Lamirande and Gagnon et al., 1992; Cummins et al., 1994). A positive interaction has been reported between mitochondrial activation and motility (Kasai et al., 2002; Martinez-Pastor et al., 2004). Similar to these reports, in the present study, motility was better in the young groups than in the middle-aged groups. Moreover, mitochondrial activation in young animals was greater than that in other groups except for the M—L and O—I groups. Despite the methodology differences between these parameters, parallel results were obtained in the present study.

In many studies on canine species, poor semen morphology was shown to be an important indicator of infertility (Oettle, 1993). In the present study, total abnormal spermatozoa rates were lower in the groups Y and M than in the group O, both in the fresh and post-thawed semen samples ($p < 0.05$). Likewise, Auger et al. (1995) stated that the percentage of normal sperm decreased by 0.9% with each year of aging. Previously, it had been assessed that while there was no effect of age on motility, normal spermatozoa rate was changed by aging, with a lower rate observed in dogs older than 9 years (Hendrikse and Antonisse, 1984). Furthermore, Schwartz et al. (1983) noted that coiled tails and microcephalic heads were affected with age. The decrease in normal spermatozoa rate with aging might be caused by disturbed or incomplete spermatogenesis in the tubules of seminiferous contortus (Losweth et al., 1990), testicular degeneration, or lack of mature spermatids (James and Heywood, 1979). According to our results, CLC did not have a protective effect on the total morphological integrity. Similar to these results, Khan et al. (2017) could not find any significant relationship between CLC and the abnormal spermatozoa rate. The differences in abnormal spermatozoa determined in several studies (Moce et al., 2010a, 2010b) may be due to genotypic and breed differences as well as differences in the content of semen diluents and the techniques used for evaluation after freezing and thawing.

It is known that sperm DNA integrity is necessary for proper embryo development and fetal growth. The COMET method is a widely used assay for analyzing DNA breakage in individual cells (Ostling and Johanson, 1984). It is a ideal method to evaluate cells, while maintaining their genetic material integrity in biological studies (Novotna et al., 2007). In the present study, according to the tail length, the highest DNA integrity was obtained in Y and M groups, while the lowest DNA integrity was found in the O group.

In consensus with our study, human (Nie et al., 2012; Moskovtsev et al., 2007) and bovine (Carreira et al., 2017) semen studies revealed that DNA integrity is affected adversely by increasing age. Thus, age-related results can affect both fertility and spermatological parameters during the cryopreservation process. Although Peters et al. (2000) conducted a histopathological examination on the testicles of 74 dogs, they did not detect any decrease in spermatogenesis. However, the occurrence of testicular tumors increased with age. On the other hand, Khan et al. (2017) studied the effects of CLC on DNA integrity using an acridine orange stain method; the stains were observed using a fluorescence microscope and there were no significant differences between the control and CLC groups. In contrast to the current study, Katanbafzadeh et al. (2014) suggested that CLC has a positive effect on DNA integrity. The difference between these results may due the selection of different methods for assessing DNA damage.

5. Conclusions

In conclusion, the evidence suggests that increasing age in dogs is associated with a decline *in vitro* spermatological parameters, such as motility, viability, mitochondrial activation, normal morphology, and DNA integrity. CLC addition has a protective effect on the acrosome integrity of sperm and protects them from cryo-damage during freezing and thawing. Better freezability characteristics were observed when the Aksaray Malakli Shepherd dogs were young.

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