CHANGES IN QUALITY OF NATIVE AND FROZEN-THAWED SEMEN IN RELATION TO TWO COLLECTIONS PERFORMED IN A 24-HOUR INTERVAL AND ADDITION OF CLARIFIED EGG YOLK TO EXTENDER*

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The aim of the study was to evaluate the effect of repeated semen collection and the substitution of normal egg yolk with clarified egg yolk to commercially produced semen extender on qualitative parameters of frozen-thawed canine semen. Two semen collections were scheduled in a 24-hour interval and in each of six dogs, three 1st and three 2nd collections were performed. The frozen-thawed sperm samples were prepared either with clarified or normal egg yolk and motility and viability were evaluated. The effect of the sequence of semen collection was demonstrated by significant differences in motility and also in viability of sperms both in native and frozen-thawed ejaculate. The percentage of viable sperms was significantly higher in samples from the 2nd compared to the 1st collection. This trend was the same also in motility except in native ejaculate. The addition of clarified egg yolk was beneficial for higher survival of sperms immediately after thawing and also after 30 min of incubation, compared to samples with normal egg yolk. Sperm motility evaluated after thawing was higher in samples with clarified egg yolk, without an apparent connection with semen collection sequence. The decrease of values of the qualitative parameters of sperms observed in the period of 30 min of incubation was significantly slowed down when clarified egg yolk was used. This was especially obvious in samples from the 2nd collection.

dog; reproduction; cryoconservation; sperm; motility; viability

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INTRODUCTION

Artificial insemination (AI) with frozen semen is increasingly used as a method of reproduction in dogs. It enables more efficient breeding, because from one semen collection it is possible to produce a large amount of insemination doses (IDs) and these can be distributed regardless of the geographic location of the breeding animals. Other advantages of AI are better control of venereal disease spread, also intolerance between a bitch and dog can be prevented (Kutzler, 2005), and the costs connected with the maintenance of a stud dog in a colony can be reduced (Payan-Carreira et al., 2011).

If the owners of a breeding dog want to produce frozen ID, long-distance travel to a specialized workplace is sometimes unavoidable. Considering the economic demands, it could be beneficial to perform repeated semen collections. It was found that the quality of native semen is affected by frequency of semen collection (England, 1999; Gunay et al., 2003; Vágenknechtová et al., 2010). However, the effect of repeated semen collection on the freezability of canine (*Canis lupus f. familiaris*) ejaculate has not been studied to date.

In light of AI effectivity, the quality of collected ejaculate and chosen extender play an important role. There is a huge variability in the freezability of

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ejaculate among dogs (Kosiniak-Kamysz et al., 2007), hence the collected ejaculate should undergo several laboratory tests to evaluate its suitability for cryopreservation (Kutzler, 2005). It is ideal to take advantage of a combination of several tests to evaluate sperm motility, viability, morphology, integrity of plasma membrane, chromatin structure, and DNA content (Peña, 2004). A very important prerequisite for production of fertile frozen ID is the type of semen extender used (Farstad, 2008). For the purpose of effective semen cryopreservation in dogs, many kinds of semen extenders are used. However, the components are more or less constant: TRIS, citric acid, fructose, distilled water, antibiotics, and cryoprotectants (Oliviera et al., 2006; Schäfer-Somi et al., 2006). The latter are fundamental and this is the reason why they are subjected to intensive research (reviewed in Farstad, 2008).

Although different cryoprotectants can be used for producing the frozen-thawed IDs, the most frequently used in dogs is glycerol (Futino et al., 2010) ranking among substances which penetrate into cells (Holt, 2000). There is a second group of cryoprotectants, called non-penetrating. Among these belong skimmed milk, trehalose, and egg yolk (EY) (Tonieto et al., 2010). Non-penetrating cryoprotectants do not penetrate through plasma membrane. They can take effect directly in a membrane or modify the surface of a membrane. They also help decrease the freezing point of medium and prevent the extracellular ice crystal formation (Kundu et al., 2002). Moreover, compounds of EY act preventively against spontaneous capacitation (Witte, Schäfer-Somi, 2007).

The use of EY has some disadvantages, among which the non-standardized composition, risk of microbial contamination, and possible inhibition of cell respiration can be classified (Al Ahmad et al., 2008; Hu et al., 2011; Bencharif et al., 2013; Svobodová, Tůmová, 2014). The negative effect of the whole EY can be eliminated by extraction of different functional elements of EY (e.g. phospholipids, low density lipoproteins). However, these methods are expensive and require special laboratory equipment. Nevertheless, it is possible to use an alternative method, known as clarification (Wall, Foote, 1999). This method, based on high-speed centrifugation of EY and distilled water, is practicable in common laboratory conditions and there is no need for expensive chemicals or special methods. The resulting substance has a decreased content of components negatively influencing sperms (e.g. density lipoproteins). The effect of clarified egg yolk (CY) on the quality of frozen-thawed semen has already been studied in bulls (Christensen et al., 2005), rams (Gil et al., 2000), stallions (Torres-Boggino et al., 1995), and red wolves (Lockyear et al., 2009). Even though the search for the optimal method of canine semen cryopreservation is constant, the influence of CY on the quality of frozen-thawed semen has not to date been studied in dogs.

The aim of the study was to evaluate the effect of repeated semen collection and the substitution of normal with clarified egg yolk to commercially produced semen extender on qualitative parameters of frozen-thawed canine semen.

MATERIAL AND METHODS

Reagents

All the chemicals used in this study were of analytical grade and, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, USA). Fluorescent probe carboxyfluoresceine diacetate was prepared in dimethyl sulfoxide (DMSO) 0.46 mg ml^{-1} and propidium iodide in physiological saline 0.5 mg ml^{-1}. Prepared aliquots were stored at –20°C until their usage. Physiological saline was buffered with Sorenson's buffer to pH 6.8 ± 0.2.

Animals and semen collection

In our study, 6 dogs of the German Shepherd breed were used. All the dogs were healthy, without reproductive abnormalities and their ages ranged from 2 to 6 years. Only the second sperm-rich fraction was collected by digital manipulation into calibrated sterile plastic tubes. In this study, in each of the 6 dogs, two collections were performed within a time interval of 24 h. Each dog was collected 6 times (i.e. three 1^{st} and three 2^{nd} collections), which means that a total of 36 sperm samples were collected from 6 dogs. The dogs were sexually rested for at least 3 months before each double collection (two collections in a 24-hour interval). All collections were performed by the same technique, the same laboratory technician, and under constant conditions.

Initial semen evaluation

Immediately after collection, the semen was subjected to volume and sperm concentration analysis, the latter with the Bürker chamber. Motility was evaluated subjectively under a Nikon Eclipse Ci-L microscope (Nikon, Tokyo, Japan) equipped with a negative phase-contrast lens with ×100 magnification. Only samples with more than 70% of motile sperms and a concentration of more than 3×10^9 sperms in ejaculate were used for cryopreservation.

Semen processing

After the initial evaluation, the ejaculate was centrifuged at 700 *g* for 5 min and supernatant was removed.

Table 1. Viability and motility of sperms in native and frozen-thawed (F-T) samples immediately after (T0), or 30 min (T30) after thawing

Parameter	Native	F-T (T0)	F-T (T30)
Live (%)	59.00 ± 3.19[a]	34.88 ± 1.13[b]	29.29 ± 1.13[c]
MOR (%)	no data	0.98 ± 0.15[a]	0.73 ± 0.15[b]
ALH (μm)	10.52 ± 0.09[a]	3.49 ± 0.01[b]	2.37 ± 0.01[c]
BCF (Hz)	8.44 ± 0.04[b]	11.28 ± 0.02[a]	8.45 ± 0.02[b]
LIN (%)	54.81 ± 0.22[a]	42.31 ± 0.10[b]	31.08 ± 0.11[c]
STR (%)	81.18 ± 0.211[a]	71.73 ± 0.10[b]	62.47 ± 0.11[c]
VAP (μm s^{-1})	98.80 ± 0.47[a]	45.57 ± 0.19[b]	25.01 ± 0.21[c]
VCL (μm s^{-1})	157.49 ± 0.81[a]	70.05 ± 0.25[b]	47.58 ± 0.28[c]
VSL (μm s^{-1})	79.91 ± 0.42[a]	41.29 ± 0.19[b]	20.74 ± 0.21[c]
WOB (%)	66.13 ± 0.15[a]	53.43 ± 0.08[b]	43.54 ± 0.09[c]

Live = live sperms, MOR = moribund sperms, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency, LIN = linearity, STR = straightness, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity, WOB = wobble

[a–c] values with different superscripts in a row significantly differ ($P < 0.05$)

Consequently, extender was added to the centrifuged ejaculate to a final concentration of 200 × 10^6 sperms per ml. The extender used in this study was TRIS-based CaniPlus Freeze (CF) from Minitübe (Tiefenbach, Denmark), with the addition of two different types of egg yolk. Whole egg yolk (EY) was added to the final concentration of 20% v/v in a solution of sperms and extender. Clarified egg yolk (CY) was prepared by the technique described by Holt et al. (1996). Briefly, egg yolk was separated from the albumen and rolled on filter paper. Afterwards, yolk was collected with a sterile syringe and added to the centrifuge tube with ultrapure water (1:3) and centrifuged at 10 000 *g* for 30 min at 5°C. After centrifugation, the pellet at the bottom was discarded and plasma (supernatant) was used as the CY. The CY was added to the final concentration of 20% v/v in a solution of sperms and extender. The extended semen samples were filled into 0.25 ml straws and placed into a refrigerator (5°C) for 2 h for equilibration. After this period, the straws were placed on an iron rack 4 cm above the liquid nitrogen level for at least 15 min and then placed directly in a container with liquid nitrogen.

Sperm motility evaluation

The straws were thawed in a water bath at a temperature of 38°C for 60 s. Motility was evaluated using Computer Assisted Sperm Analysis (CASA) NIS Elements 4.30 (Laboratory Imaging Ltd., Prague, Czech Republic). This software worked with camera Imaging Source DMK 23UM021 with a frequency of 60 frames per s. After thawing, 3 μl of semen sample diluted in physiological saline solution (6.8 pH) to concentration of 30 × 10^6 sperms per ml were placed in the calibrated Leja® counting chamber with 20 μm depth (IMV Technologies, L'Aigle, France). The preheated counting chamber (37°C) was placed on the heating plate with the same temperature and 5 random fields were analyzed at ×100 magnification. The following parameters of sperm motility were analyzed: amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), linearity (LIN, %), straightness (STR, %), average path velocity (VAP, μm s^{-1}), curvilinear velocity (VCL, μm s^{-1}), straight line velocity (VSL, μm s^{-1}), and wobble (WOB, %).

Sperm viability evaluation

For sperm viability assessment, the fluorescent staining according to Harrison, Vickers (1990) was used. Briefly, sperm sample was diluted to volume 950 μl with a final concentration of 1–10 × 10^6 sperms per ml. Afterwards, 20 μl of carboxyfluoresceine diacetate and 20 μl of propidium iodide were added. Sperm suspension was additionally fixed with 10 μl of 0.3% solution of formaldehyde. Incubation proceeded without light access in the incubator at a constant temperature of 37°C for 10 min. After the time period of incubation, 7 μl of sample were transferred to a microscopic slide and mounted under coverslip using nail polish. Evaluation was performed using a fluorescent microscope Nikon Eclipse E6000. Live sperms (Live) emitted green fluorescence, dead sperms were recognized as red, moribund (MOR) sperms emitted green and red fluorescence. In each sample, 200 sperms were counted and categories expressed in percentage.

Statistical analysis

Data were analyzed in STATISTICA software (Version 12, 2013). When two groups of variables were compared, Student's *t*-test was used for data evaluation. Before Student's *t*-test was used, normality and homogeneity were tested. Once normality was af-

Table 2. Viability and motility of sperms in native and frozen-thawed (F-T) samples immediately after (T0), or 30 min (T30) after thawing, depending on the semen collection sequence

Parameter	Native		F-T (T0)		F-T (T30)	
	1st collection	2nd collection	1st collection	2nd collection	1st collection	2nd collection
Live (%)	57.00 ± 3.85^{2a}	61.50 ± 4.3^{1a}	31.30 ± 1.36^{2b}	40.00 ± 1.63^{1b}	25.70 ± 1.28^{2c}	34.45 ± 1.53^{1c}
MOR (%)	no data	no data	0.60 ± 0.20^{2}	1.50 ± 0.25^{1}	0.38 ± 0.15^{2c}	1.22 ± 0.18^{1c}
ALH (μm)	9.47 ± 0.08^{2a}	12.08 ± 0.09^{1a}	3.24 ± 0.02^{2b}	3.90 ± 0.02^{1b}	2.10 ± 0.02^{2c}	2.79 ±0.02^{1c}
BCF (Hz)	9.43 ± 0.10^{1b}	6.9507 ± 0.13^{2b}	10.85 ± 0.03^{2a}	11.96 ± 0.04^{1a}	8.00 ± 0.03^{2c}	9.15 ± 0.04^{1c}
LIN (%)	52.23 ± 0.31^{2a}	58.64 ± 0.37^{1a}	42.00 ± 0.12^{2b}	42.80 ± 0.15^{1b}	29.13 ± 0.14^{2c}	34.17 ± 0.17^{1c}
STR (%)	78.50 ± 0.14^{2a}	85.17 ± 0.16^{1a}	71.80 ± 0.13^{1b}	71.62 ± 0.16^{2b}	61.04 ± 0.14^{2c}	64.80 ± 0.18^{1c}
VAP (μm s^{-1})	104.15 ± 0.81^{1a}	90.85 ± 0.98^{2a}	39.92 ± 0.24^{2b}	54.53 ± 0.30^{1b}	20.48 ± 0.27^{2c}	32.20 ± 0.34^{1c}
VCL (μm s^{-1})	166.76 ± 1.10^{1a}	143.69 ± 1.34^{2a}	62.24 ± 0.32^{2b}	82.43 ± 0.40^{1b}	42.19 ± 0.35^{2c}	56.13 ± 0.44^{1c}
VSL (μm s^{-1})	82.54 ± 0.81^{1a}	75.98 ± 0.99^{2a}	36.02 ± 0.24^{2b}	49.62 ± 0.30^{1b}	16.38 ± 0.27^{2c}	27.66 ± 0.33^{1c}
WOB (%)	65.25 ± 0.24^{2a}	67.43 ± 0.29^{1a}	53.02 ± 0.10^{2b}	54.07 ± 0.13^{1b}	41.63 ± 0.11^{2c}	46.58 ± 0.14^{1c}

Live = live sperms, MOR = moribund sperms, ALH = amplitude of lateral head displacement, BCF = beat-cross frequency, LIN = linearity, STR = straightness, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity, WOB = wobble

^{1,2}values with different superscripts in a row within the Native, F-T (T0), and F-T (T30) group significantly differ ($P < 0.05$)

$^{a-c}$ values with different superscripts in a row within the 1st collection and 2nd collection group significantly differ ($P < 0.05$)

fected, the Mann-Whitney test was used instead. In the case of three or more groups of variables, Cochran's test and Shapiro-Wilk test were used for normality and homogeneity evaluation. The one-way ANOVA with subsequent Fisher's test for multiple comparisons was used. Data are expressed as Least Squares Means ± SEM and were established on a $P < 0.05$ level of significance.

RESULTS

There was a slight increase of ejaculate volume between the 1st and the 2nd semen collections, from 1.38 ± 0.13 ml to 1.45 ± 0.15 ml ($P > 0.05$), respectively. At the same time, the concentration non-significantly decreased from 624.80 ± 163.15 × 10^6 to 495.75 ± 182.40 × 10^6 sperms per ml ($P > 0.05$) when the 1st and the 2nd semen collections were compared, respectively.

The motility parameters and viability of native and frozen-thawed sperm samples are shown in Table 1. Compared to native semen, the freezing-thawing significantly decreased all evaluated parameters in sperm samples and subsequent incubation for 30 min led to a further statistically significant decrease ($P < 0.05$) of all evaluated parameters. During the 30-minute incubation, the proportion of MOR sperms also decreased ($P < 0.05$) (Table 1).

The sequence of semen collection was projected to differences in motility and viability ($P < 0.05$) of native as well as frozen-thawed sperm samples (Table 2). The values of BCF, VAP, VCL, and VSL in native ejaculate were higher in the samples obtained from the 1st compared to the 2nd collection ($P < 0.05$). The percentage of viable sperms and motility (ALH, LIN, STR, and WOB) in native ejaculate was higher in the sperm samples of the 2nd compared to the 1st collection ($P < 0.05$).

The decrease of values in evaluated parameters between native and thawed samples after thawing (at T0) was significantly smaller in parameters of motility ($P < 0.05$) in the case of lower input value from the 1st (ALH, LIN, STR, WOB) and the 2nd (VAP, VCL, VSL) collections (Table 2). However, after 30 min of incubation (T30), the decrease was significantly greater ($P < 0.05$) in these samples. On the other hand, if the input value of evaluated parameters in native ejaculate was higher (VAP, VCL, VSL – the 1st collection; ALH, LIN, STR, WOB – the 2nd collection), an expressive decrease occurred after thawing (T0) ($P < 0.05$), while, after 30 min of incubation, the subsequent decrease was significantly lower ($P < 0.05$). Except STR, the absolute values of all evaluated parameters after thawing (T0) and 30 min of incubation (T30) were significantly higher ($P < 0.05$) in samples from the 2nd compared to the 1st collection (Table 2).

In the case of the 1st collection, in CFCY samples (extender with clarified egg yolk) the ratio of live, moribund sperms and also the STR were significantly higher at T0 compared to CFEY samples (extender with whole egg yolk) ($P < 0.05$, Table 3). The values of other evaluated parameters were significantly higher in CFEY compared to CFCY at T0 ($P < 0.05$). Similar results were also obtained at T0 in samples from the 2nd collection. However, the ratio of live sperms did not differ significantly ($P > 0.05$) when both extenders (CFCY and CFEY) were compared. The values of motility and viability parameters were significantly higher in samples from the 2nd collection compared to the 1st collection ($P < 0.05$, Table 3).

Table 3: The viability and motility of sperms in frozen-thawed (F-T) samples immediately after (T0), or 30 minutes (T30) after thawing, depending on the semen collection sequence (1st vs 2nd collection) and type of extender (CFEY vs. CFCY).

	F-T (T0)				F-T (T30)			
	1st collection		2nd collection		1st collection		2nd collection	
Parameter	CFEY	CFCY	CFEY	CFCY	CFEY	**CFCY**	**CFEY**	**CFCY**
Live (%)	28.67 ± 1.97^{3a}	34.35 ± 1.75^{2a}	39.10 ± 2.36^{1a}	41.00 ± 2.10^{1a}	23.48 ± 1.68^{4b}	28.25 ± 1.88^{3b}	32.75 ± 2.00^{2b}	36.39 ± 2.25^{1b}
MOR (%)	0.52 ± 0.19^{3}	0.68 ± 0.37^{3}	1.03 ± 0.23^{2}	2.11 ± 0.44^{1a}	0.33 ± 0.12^{3}	0.45 ± 0.28^{3}	0.91 ± 0.15^{2}	1.57 ± 0.34^{1b}
ALH (μm)	3.40 ± 0.02^{2a}	3.03 ± 0.03^{4a}	4.22 ± 0.03^{1a}	3.20 ± 0.04^{3a}	2.20 ± 0.02^{3b}	1.96 ± 0.02^{4b}	2.85 ± 0.02^{1b}	2.69 ± 0.02^{2b}
BCF (Hz)	11.06 ± 0.04^{2a}	10.58 ± 0.05^{3a}	12.37 ± 0.05^{1a}	11.05 ± 0.07^{2a}	8.01 ± 0.04^{3b}	8.00 ± 0.04^{3b}	8.73 ± 0.05^{2b}	9.91 ± 0.06^{1b}
LIN (%)	42.83 ± 0.17^{2a}	40.90 ± 0.19^{3a}	44.10 ± 0.20^{1a}	39.93 ± 0.28^{4a}	29.37 ± 0.18^{3b}	28.83 ± 0.20^{4b}	34.30 ± 0.20^{1b}	33.95 ± 0.28^{2b}
STR (%)	71.29 ± 0.17^{3a}	72.47 ± 0.19^{2a}	70.78 ± 0.19^{4a}	73.48 ± 0.28^{1a}	61.28 ± 0.19^{2b}	60.74 ± 0.22^{3b}	64.80 ± 0.23^{1b}	64.67 ± 0.31^{1b}
VAP (μm/s)	42.59 ± 0.40^{2a}	36.36 ± 0.37^{3a}	59.87 ± 0.45^{1a}	42.78 ± 0.55^{2a}	21.15 ± 0.28^{3b}	19.65 ± 0.29^{4b}	31.22 ± 0.33^{2b}	33.93 ± 0.40^{1b}
VCL (μm/s)	63.58 ± 0.50^{3a}	60.46 ± 0.50^{4a}	86.23 ± 0.57^{1a}	74.09 ± 0.74^{2a}	41.97 ± 0.38^{4b}	42.46 ± 0.42^{3b}	53.31 ± 0.44^{2b}	61.12 ± 0.58^{1b}
VSL (μm/s)	38.63 ± 0.39^{2a}	32.55 ± 0.37^{3a}	54.67 ± 0.45^{1a}	38.54 ± 0.54^{2a}	17.06 ± 0.28^{3b}	15.54 ± 0.28^{4b}	26.80 ± 0.33^{2b}	29.17 ± 0.40^{1b}
WOB (%)	54.60 ± 0.13^{2a}	50.92 ± 0.16^{3a}	56.34 ± 0.15^{1a}	49.09 ± 0.23^{4a}	42.13 ± 0.15^{3b}	41.01 ± 0.17^{4b}	47.01 ± 0.17^{1b}	45.82 ± 0.23^{2b}

[1, 2, 3, 4] Values with different superscripts in a row within a F-T (T0) and F-T (T30) group significantly differ ($P < 0.05$).

[a, b] Values with different superscripts in a row among F-T (T0) and F-T (T30) group (1st collection CFEY (T0) vs. 1st collection CFEY (T30) etc.) significantly differ ($P < 0.05$).

It is obvious from Table 3 that 30 min of incubation in all experimental groups led to a statistically significant decrease of evaluated parameter values ($P < 0.05$). However, the influence of incubation on the occurrence of moribund sperms was non-significant ($P > 0.05$). CFCY, in comparison with CFEY, significantly ($P < 0.05$) slowed down the decrease of values in all evaluated parameters during the 30 min of incubation ($P < 0.05$). This effect was the largest in most of the parameters (% of live sperms, ALH, BCF, LIN, VAP, VCL, VSL, WOB), mainly in the 2nd collection.

DISCUSSION

The quality of native ejaculate in dogs is influenced by the time interval between repeated semen collections. The offset between semen collections shorter than 75 min was projected to a significant decrease in the volume of sperms and concentration of sperms. However, the motility and viability of the sperms were almost the same (England, 1999; Gunay et al., 2003). Longer intervals (24 and 48 h) between collections did not affect the concentration or motility of sperms, but the volume of native ejaculate significantly decreased after the 1st up to the 3rd semen collection (Vágenknechtová et al., 2011). In our experiment, the volume and sperm concentration of sperm-rich fraction was the same in the samples from the 1st and 2nd collections performed in a 24-hour interval. The sequence of semen collection chosen in our study was projected to significant differences in viability and motility. The mean velocity of sperms in the samples from the 2nd collection was lower, the pattern of movement was more linear, and we simultaneously observed a significant increase in the proportion of viable sperms. These differences, especially in viability, could be explained by the relatively long sexual abstinence of dogs before the 1st collection (3 months or even longer). No previous experience with semen collection could also play a role. Despite the aforementioned differences in ejaculate quality, the native semen from both collections fulfilled common criteria for cryoconservation.

The cryoconservation process generally deteriorates qualitative parameters of ejaculate, as is obvious from a number of studies (Rota et al., 2007; Neagu et al., 2010; Strzeżek et al., 2012). Therefore the aim of many experiments is to find such a method of cryopreservation that would decrease the losses of fertile sperms to the minimum. One possible method is to optimize technological procedures (Rota et al., 2005; Schäfer-Somi et al., 2006). Another and more common method is to modify the composition of the freezing extender (Bencharif et al., 2010; Strzeżek et al., 2012).

In our experiment, we chose the second option. Hence we studied the influence of the addition of CY. At the same time, the effect of semen collection sequence on the freezability of dogs' sperms was evaluated for the first time. Under the conditions of our experiment, in terms of survival evaluated on the level of viability, the cryoconservation was better tolerated by sperms from the 2nd collection. The influence of the semen collection sequence on motility was ambiguous. The higher value of a single parameter of motility in native ejaculate in samples from the 1st collection was followed by a greater decrease owing

to freezing. However, there was a gradual decrease during subsequent incubation. On the contrary, a lower value led to a more moderate decrease after thawing, followed by a more expressive decrease during the 30-minute incubation. This trend in the decreasing extent of motility parameters was, however, the same for the 1st and the 2nd collections carried out in the interval of 24 h.

The clarification of egg yolk was previously tested in experiments by Fernández-Santos et al. (2006) and Lockyear et al. (2009) on sperms of Iberian red deer (*Cervus elaphus hispanicus)* and red wolf (*Canis rufus*), respectively. In both experiments, 20% of CY was added to extender. The results of the study using Iberian red deer sperms show a significantly higher cryoprotective influence of CY on motility and viability of epididimal sperms in comparison with whole egg yolk (Fernández-Santos et al., 2006). In this study, the use of CY resulted in a significantly higher percentage of viable and motile sperms even after 2 h of incubation. On the other hand, in red wolf the clarification of egg yolk performed by filtration through a 0.8 µm filter did not affect the cryoprotective effect of CY on motility and the status of plasma membrane of sperms (Lockyear et al., 2009). Moreover, in the study by Lockyear et al. (2009), the protective effect did not appear even during subsequent incubation for a period of 1–24 h after thawing.

The sperms obtained for the aforementioned experiment were collected by electroejaculation which, in comparison with the digital manipulation used in our study, is probably the reason for the differences in ejaculate composition caused by the different methods of seminal gland stimulation by electroejaculation (Pintus, Ros-Santaella, 2014). Moreover, the proportion of progressively motile sperms was evaluated subjectively in the experiment by Lockyear et al. (2009). In comparison with Fernández-Santos et al. (2006) and our study, different methods of egg yolk clarification were used.

In our experiment, the use of extender supplemented with clarified egg yolk (CFCY) had a better ability to reduce the negative effects of cryoconservation on the viability of dog sperms. The better effect of CFCY was also manifested in a modulation of the decrease of values of all the monitored parameters after 30 min of incubation.

The mutual effect of the semen collection sequence and the addition of CY to semen extender on the motility and viability of frozen-thawed dogs' sperms has not been published to date. From the results of our study, the improving effect of CY on the viability of frozen-thawed sperms is obvious, especially in the samples from the 2nd collection. Nevertheless, the motility was affected by the cryopreservation process, without any apparent consequence between the method of egg yolk preparation and semen collection sequence. On the other hand, after 30 min of incubation, the positive effect of CY addition was apparent both on motility and viability. This was the most expressive again in the samples from the 2nd semen collection.

CONCLUSION

Repeated semen collections from dogs in the time interval of 24 h yielded quality native ejaculate suitable for cryoconservation from both collections. Even longer sexual abstinence (more than 3 months) before the 1st collection does not need to signify the inferior quality of collected ejaculate.

According to our results, we can recommend the use the clarified instead of whole egg yolk for cryoconservation, in particular with regard to its ability to markedly slow down the decline of motility and viability of sperms. This effect was observed immediately after thawing. After subsequent incubation, it was even more evident. The aforementioned conditions of incubation (30 min, 38°C) mimic the conditions in the reproductive tract of the bitch, advocating the potential ability of clarified egg yolk to improve the fertilizing potential of the insemination dose and to increase the effectivity of the insemination process in dogs.

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