Ann. Anim. Sci., Vol. 18, No. 1 (2018) 113–124 *DOI: 10.1515/aoas-2017-0032*

DE GRUYTER
OPEN

EFFECT OF OSTRICH EGG LIPOPROTEINS AND HEN EGG YOLK ON THE QUALITY OF DOG SPERM DURING LIQUID STORAGE AT 5°C*

Anna Dziekońska♦, Dorota Behrendt, Rafał Strzeżek, Władysław Kordan

Department of Animal Biochemistry and Biotechnology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 5, 10-718 Olsztyn, Poland
♦Corresponding author: a.dziekonska@uwm.edu.pl

Abstract

The objective of this study was a comparative analysis of the impact of the addition of lipoproteins from ostrich egg yolk (LPFo) and hen egg yolk (HEY) to a Tris-citric acid-fructose (TCF) extender on the quality of dog sperm preserved at 5°C for 7 days. The sperm-rich fraction of the ejaculate was manually collected and extended using the following extenders: control (TCF), TCF+ 5% addition of LPFo (TCF-LPFo) and TCF+ 20% of addition HEY (TCF-HEY). The analysis of quality included: sperm motility parameters (TMOT, PMOT, VAP, VSL, VCL, ALH, BCF, STR, LIN), normal apical ridge acrosome (NAR), plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), and ATP content. The addition of LPFo and HEY to the TCF extender had a beneficial effect on all quality parameters of the stored sperm compared with the TCF extender. However, the analysis of sperm motility parameters revealed differences (P≤0.05) between TCF-LPFo and TCF-HEY extenders as affected by storage day. Semen extended in TCF-LPFo was characterized by lower values of TMOT and VAP on Day 5 and by lower values of TMOT and VCL on Day 7, compared to that extended in TCF-HEY. The analysis of PMOT, VSL, ALH, BCF, STR, LIN, PMI, MMP and ATP content showed no differences (P≥0.05) between TCF-LPFo and TCF-HEY extenders. The results suggest that TCF extender supplementation with LPFo or HEY improves the quality of dog sperm stored at 5°C. Both LPFo and HEY protect motility, membrane integrity and can contribute to the improvement of the energy status of stored dog sperm. LPFo can be alternatively used instead of HEY for dog semen preservation in the liquid state.

Key words: dog, sperm, quality, egg yolk, lipoproteins

A significant increase has recently been observed in the number of artificial insemination (AI) procedures applied in dog breeding that involve the use of chilled and frozen semen (Linde-Forsberg, 1995; Farstad, 2000). The chilling of semen is usually applied in the case of short-term storage as the quality of chilled semen

*Work financed by the University of Warmia and Mazury in Olsztyn (project no. 0103.0803).

is superior to that of the frozen-thawed one (England and Ponzio, 1996). The use of chilled canine semen extends many advantages, most of all the process of its preparation is itself simpler, cheaper and obviates the need for special equipment as in the case of frozen semen preparation. In addition, insemination of bitches with chilled semen results in a higher pregnancy rate and a higher number of pups being born than that with frozen-thawed semen (Linde-Forsberg, 1995), which weighs in favour of this preservation method to be commonly applied in dog reproduction.

The longevity of chilled spermatozoa is curbed and dependent on the diluent applied. Ample works have demonstrated that the storage of dog spermatozoa in citrate- or Tris-egg yolk extenders allows preserving their high quality for many days (Goericke-Pesch et al., 2012; Verstegen et al., 2005). Hen egg yolk (HEY) is commonly applied in the preservation technology of semen of many animal species, including dog (Beccaglia et al., 2009). Yolk components, in particular its low-density lipoproteins (LDL), protect spermatozoa against cold and osmotic shock (Bencharif et al., 2008) and counteract the adverse effect of seminal plasma proteins (BSPs) on sperm plasmalemma during storage (Plante et al., 2016).

The use of HEY as a component of extenders may, however, be limited as it has been demonstrated that the granules of egg yolk exert a negative effect on sperm respiration and motility (Watson and Martin, 1975; Strzeżek et al., 2014). The zinc ion-dependent proteins present in boar seminal plasma, protecting plasma membranes of stored spermatozoa, were also found to be capable of precipitating yolk components, which substantially deteriorates the protective role of HEY over plasmalemma of spermatozoa subjected to a cold shock treatment (Strzeżek and Hopfer, 1987). Dog seminal plasma was also found to contain zinc ion-binding proteins that display similar properties against membranes of cold-stored spermatozoa (Mogielnicka-Brzozowska et al., 2014). Furthermore, HEY was also demonstrated to be at high risk of microbiological contaminations with, e.g., *Salmonella*, *Staphylococcus* or even viral ones, e.g. with an avian influenza virus (Strzeżek et al., 2014). Hence the search for a substitute component of the extender that would serve similar functions to HEY, and for the solution that would ensure a significant reduction in microbiological contaminations of the stored semen seems advisable.

The technology of boar semen preservation involved the addition of lipoproteins isolated from ostrich egg yolk (LPFo) in a lyophilized form, which had a beneficial effect upon the quality of stored spermatozoa (Dziekońska et al., 2009, 2017; Fraser et al., 2007; Strzeżek et al., 2014). In addition, the LPFo were shown to positively affect the quality and antioxidative properties of cryopreserved dog sperm (Strzeżek et al., 2012). The experimental results achieved have become a premise to apply LPFo addition to canine sperm during storage in a liquid state.

The objective of this study was, therefore, a comparative analysis of the effect of LPFo and HEY addition to a TCF extender on the quality of dog sperm stored at a temperature of 5°C for 7 days. The quality of sperm was assessed based on the analysis of its motility parameters, acrosome integrity, membrane integrity, mitochondrial activity (transmembrane mitochondrial potential), and ATP content.

Material and methods

Animals and preparation of samples

Four sexually mature dogs of mixed breeds, aged from 2 to 10 years, were used in this study. The pre-sperm and sperm-rich fractions of ejaculate (3 times per dog) were collected by digital manipulation as described by Kutzler (2005) at weekly intervals.

The dogs were housed in individual pens and fed with commercial canine food. Water was available *ad libitum*. All animal procedures were conducted in accordance with accepted standards of the Local Ethical Review Committee in Olsztyn.

The semen was processed using the method proposed by Kasimanickam et al. (2012) with some modifications. The fractions of ejaculate were collected into a calibrated polypropylene plastic vial, taking care to separate the fractions. The volume of each fraction was determined, and the sperm concentration was measured with a Bürker counting chamber (Equimed-Medical Instruments, Kraków, Poland) under a light microscope. The semen (sperm-rich fraction of ejaculate) was centrifuged (720 × g, 5 min at room temperature). After the seminal plasma was removed, the obtained pellet of sperm was divided into 3 parts and each part was diluted with each of the following extenders: Tris-citric acid-fructose extender (TCF), TCF+5% of lyophilized lipoprotein from the ostrich egg yolk (TCF-LPFo), and TCF+20% of hen egg yolk extender (TCF-HEY). The TCF and TCF-HEY extenders were prepared in accordance with the method proposed by Rota et al. (1995). The LPFo preparation procedure was described by Strzeżek et al. (2005). In brief, the ostrich egg yolk was diluted with sterile water and intensively stirred prior to centrifugation at 7800 x g for 30 min at room temperature. The supernatant (water dissolved fractions) was dispersed using an ultrasound disintegrator and was recentrifuged for the complete removal of granules. The supernatants obtained from the LPFo samples were lyophilized and stored at −80°C until required for liquid storage.

The volume of the samples of diluted semen was 5 mL. The sperm concentration in the samples was again measured and it remained at approximately 30×10^6 spermatozoa/mL. All steps for the above procedures were conducted at room temperature. Next, the samples were gradually cooled to 5°C (Kasimanickam et al., 2012) and stored at the same temperature for seven days (Day 1–Day 7).

Evaluation of sperm quality

Parameters of sperm quality (sperm motility parameters, normal apical ridge acrosome, plasma membrane integrity, mitochondrial membrane potential, and ATP content) were analyzed in extended semen on Day 1, 3, 5 and 7.

Sperm motility parameters

Sperm motility characteristics in fresh or chilled semen were evaluated using the Hamilton-Thorne Sperm Analyzer IVOS version 12.3 (Hamilton-Thorne Biosciences, Beverly, MA, USA). A droplet (approximately 5 µL) of semen was placed in a Makler counting chamber (Sefi-Medical Instruments Ltd., Israel) at 37ºC, and six fields were examined per sample. Fluorescent dye IDENT (Hamilton-Thorne Bio-

sciences, Beverly, MA, USA) dissolved in a medium for embryos holding (EHM, IMV Technologies, L'Aigle, France) was used in order to identify sperm cells and to discriminate between spermatozoa and other cells, debris and egg yolk particles (Nizanski et al., 2009). The software settings for the semen analyzer were those recommended by the manufacturer for the analysis of dog sperm: frame acquired 30, frame rate 60 Hz, minimum cell contrast 75, minimum cell size 6 pixels, straightness threshold 75%, path velocity threshold 100 μm/s, low VAP cut-off 9.9 μm/s, low VSL cut-off 20 μm/s, static size gates 0.80–4.93, static intensity gates 0.49–1.68, and static elongation gates 22–84. The motility parameters determined by the IVOS analyzer were: total motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear line velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %).

Normal apical ridge acrosome (NAR)

The percentage of spermatozoa with NAR acrosomes was evaluated in accordance with a previously described method (Fraser et al., 2007). Briefly, boar spermatozoa with NAR acrosomes exhibited a uniform Giemsa staining pattern overlying the acrosomal region, whereas spermatozoa with damaged acrosomes displayed a clear patchy staining pattern, with damaged apical ridges or loose acrosomal caps. Aliquots of the stained cells were examined under a bright light microscope (Olympus CH 30, Tokyo, Japan), equipped with oil-immersion lens, at 1000 × magnification. A minimum of 200 cells per slide were examined in each aliquot and classified as spermatozoa with NAR acrosome or damaged apical ridge acrosome.

Plasma membrane integrity (PMI)

Sperm plasma membrane integrity was assessed with the dual fluorescent probes, SYBR-14 and propidium iodide, PI (Live/Dead Sperm Viability Kit; Molecular Probes, Eugene, OR, USA) according to a previously described method (Garner and Johnson, 1995) with some modifications. Briefly, sperm samples (200 μL, 30×10^6 spermatozoa/mL) were extended in 200 μL of the HEPES saline medium (130 mM NaCI, 4 mM KCI, 14 mM fructose, 10 mM HEPES, 1 mM $CaC1_2$, 0.5 mM $MgC1_2$, 0.1% BSA) and incubated with 4 μL of 1 mM SYBR-14 solution in a HEPES-BSA solution (pH 7.4) and 4 μL of PI (2.4 μM in Tyrode's salt solution) at 37°C for 10 min. Aliquots of the stained sperm cells were examined at 600 × magnification under a fluorescence microscope (Olympus CH 30, Tokyo, Japan). Approximately 200 cells per slide were examined in each aliquot and classified as membrane-intact (%) and membrane-damaged spermatozoa (%).

Mitochondrial membrane potential (MMP)

Sperm MMP was assessed using the JC-1 with PI fluorescent probes (Molecular Probes, Eugene, USA) in accordance with a previously described method (Dziekońska et al., 2009), with some modifications. Briefly, sperm samples (200 μL, 30×10^6 spermatozoa/mL) were extended in 200 μL of the HEPES saline medium. One microliter of a JC-1 solution (1 mg JC-1/mL anhydrous dimethyl sulphoxide)

was added to the sperm samples that were next incubated at 37°C for 15 min. Aliquots of the stained sperm samples were counterstained with 4 μL of the PI solution (0.5 mg PI/mL phosphate-buffered solution, PBS), prior to examination at 600 × magnification under a fluorescence microscope (Olympus CH 30, Tokyo, Japan), equipped with dichroic mirrors for blue (DMB) and green excitations (DMG) for JC-1 and PI, respectively. The percentages of sperm cells displaying only orange-red fluorescence at the mid-piece region were considered as viable spermatozoa with high MMP, whereas non-viable spermatozoa with low MMP fluoresced red in the head and green in the mid-piece region. A minimum of 200 cells per slide were examined in each aliquot.

ATP content

ATP content was determined with the ATP Bioluminescence Assay Kit CLSII (Roche Diagnostic GmbH, Germany). Prior to testing, 100 μL of sperm suspensions (30×10^6 spermatozoa/mL) were added to 900 μL of 100 mM Tris buffer containing 4 mM EDTA (pH 7.75) and boiled for 5 min. After boiling and cooling, the samples were centrifuged at 1000 × g for 60 s and the supernatants were transferred to the fresh tube. Subsequently, 100 μL of supernatant (sperm extract) were added to 100 μL luciferase reagent. Measurements of luminescence were performed using a Junior Bioluminometer (Berthold Technologies, GmbH & Co. KG, Germany) in accordance with the assay kit protocol. The ATP content of a sample was calculated from a standard ATP curve and expressed as nmol/10^8 spermatozoa.

Statistical analysis

The data were subjected to ANOVA using the general linear model (GLM) procedure from Statistica software package, version 10.5 (StatSoft, Tulsa, OK, USA). All results were expressed as means ± standard error of the mean (SEM). Significant main effects were compared with the Duncan's *post hoc* test and were considered significant at $P \leq 0.05$.

Results

Results of the evaluation of motility parameters of dog sperm preserved in a liquid state were presented in Table 1. The analysis of results showed higher ($P \leq 0.05$) values of TMOT for the TCF-HEY extender compared with the TCF extender during seven days of storage (Day1–Day7). The higher TMOT values were also demonstrated for TCF-LPFo compared with TCF extender, but only on the first day of storage. Significant differences ($P \leq 0.05$) were found between the TCF-LPFo and TCF-HEY extenders in TMOT values on Day5 and on Day7 of storage. In the PMOT evaluation, higher ($P \leq 0.05$) values were reported for both TCF-HEY and TCF-LPFo compared to TCF regardless of storage days. Similar correlations were observed in results of VSL, ALH, and LIN analyses. Higher values of VAP were noted for TCF-LPFo in comparison to TCF on Day1 and Day3 of storage, and for TCF-HEY compared with TCF on Day1, Day5 and Day7 of storage. The VCL analysis showed lower values

for TCF-HEY compared to TCF during the entire storage period of extended semen (Day1–Day7) and for TCF-LPFo compared to TCF from Day3 of semen storage. In addition, the VCL analysis revealed significant differences between TCF-LPFo and TCF-HEY on Day7 of storage. In turn, the BCF analysis showed lower values for TCF-LPFo and TCF-HEY compared with TCF on Day1 and Day7. The percentage of sperm showing straightness (STR) was higher (P≤0.05) for TCF-LPFo and TCF-HEY compared to TCF during storage from Day3 (Day3–Day7).

Table 1. CASA motility parameters of dog sperm after chilling with different extenders: Tris-citric acid-fructose extender (control, TCF); TCF with the addition of lipoprotein fractions isolated from ostrich egg yolk (TCF-LPFo), and TCF with the addition of hen egg yolk (TCF-HEY) over a storage period of 7 days at 5ºC

Parameters of motility	Extender	Day 1	Day 3	Day 5	Day 7
TMOT (%)	TCF	89.3±1.2 aA	91.3±0.8 aA	88.6±1.4 aA	80.5±2.2 aB
	TCF-LPFo	95.1±0.3 bA	92.9±0.7 abAB	89.1±1.4 aB	85.1±1.8 aB
	TCF-HEY	94.1±0.6 bA	95.7±0.5 bA	95.2±0.7 bA	90.5±1.2 b A
PMOT (%)	TCF	69.0±2.7 aA	63.8±2.7 aAB	51.6±2.6 aB	42.5±2.5 aB
	TCF-LPFo	75.2±1.3 bA	73.8±1.2 bA	62.3 ±1.9 bB	58.7±2.1 bB
	TCF-HEY	76.6±1.0 bA	75.2±1,1 bA	68.9±1.6 b A	58.5±2.0 b B
VAP (µm/s)	TCF	131.0±1.8 aA	131.2±2.0 aA	126.6±2.2 aA	120.1±3.3 aB
	TCF-LPFo	143.9±1.8 bA	137.6±1.8 bAB	124.9±2.2 aB	122.2±2.7 abB
	TCF-HEY	141.9±1.7 bA	136.8±1.4 abAB	134.0±2.2 bB	129.5±2.0 bB
VSL (µm/s)	TCF	115.5±2.2 aA	111.6±2.3 aAB	101.8±2.2 aB	95.6±2.5 aB
	TCF-LPFo	127.1±1.3 bA	122.5±1.6 bAB	107.8±2.1 bB	104.9±2.0 bB
	TCF-HEY	127.8±1.4 bA	120.4±1.1 bB	115.0±2.2 bB	108.4±1.9 bB
VCL (µm/s)	TCF	196.2±2.9 aA	204.6±3.3 a AB	212.4±3.3 a B	217.8±4.2 a B
	TCF-LPFo	191.9±3.0 ab	188.9±3.3 b	188.4±3.9 b	188.6±7.4 c
	TCF-HEY	184.6±3.6 bA	185.9±2.2 bAB	194.6±3.3 bAB	200.4±3.2 bB
ALH (µm)	TCF	7.1±0.2 aA	7.7±0.2 a AB	8.4±0.2 a AB	8.8±0.3 aB
	TCF-LPFo	6.3±0.1 bA	6.3±0.1 bA	7.1±0.2 bB	7.2±0.4 bB
	TCF-HEY	5.9±0,1 bA	6.3±0.1 bA	7.1±0.1 bB	7.6±0.2 bB
BCF (Hz)	TCF	20.8±1.2 a	16.8±1.2	15.3±0.6	19.6±1.2 a
	TCF-LPFo	14.3±1.2 b	15.5±0.8	16.0±1.2	16.6±0.8 b
	TCF-HEY	15.5±1.1 b	14.4±0.9	15.7±1.1	14.6±0.6 b
STR (%)	TCF	87.5±0.7 A	84.5±1.0 aAB	80.2±0.9 a	79.6±1.2 aB
	TCF-LPFo	88.8±0.6	88.8±1.0 b	85.4±1.0 b	85.4±1.3 b
	TCF-HEY	89.2±0.8 A	87.8±0.6 bA	85.5±0.8 bAB	83.3±0.9 bB
LIN (%)	TCF	60.7±1.7 aA	57.4±1.9 aAB	50.4±1.2 aB	45.9±1.5 aB
	TCF-LPFo	69.0±0.8 bA	67.2±1.4 b AB	59.4±1.7 bB	56.8±2.6 bB
	TCF-HEY	71.6±1.1 bA	67.5±0.8 b AB	61.6±1.3 bB	58.6±1.4 bB

a, b, c – differences (P≤0.05) between extenders (vertical).

A, B – differences (P≤0.05) between days of storage (horizontal).

Parameters: TMOT, total motility; PMOT, progressive motility; VAP, velocity average path; VSL, velocity straight line; VCL, curvilinear velocity; ALH, mean amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness (VSL/VAP × 100%); LIN, linearity (VSL/VCL × 100%).

Table 2. Acrosome and plasma membrane integrity, mitochondrial membrane potential and ATP content of dog sperm after chilling with different extenders: Tris-citric acid-fructose extender (TCF); TCF with addition of lipoprotein fractions from ostrich egg yolk (TCF-LPFo), and TCF with addition of hen yolk egg (TCF-HEY) over a storage period of 7 days at 5ºC

Parameters	Extender	Day 1	Day 3	Day 5	Day 7
NAR (%)	TCF	88.9±0.9 A	87.5±1.3 AB	86.1±1.1 AB	84.2±2.7 B
	TCF-LPFo	88.2±0.9 A	87.6±1.0 AB	85.9±1.0 AB	84.2±1.5 B
	TCF-HEY	88.5±1.1 A	88.3±1.1 AB	87.8±0.6 AB	86.4±1.1 B
PMI (%)	TCF	93.1±0.7 A	88.2±1.9 aB	85.2±0.2 aB	80.5 ±0.2 aC
	TCF-LPFo	93.2±0.4 A	93.1±0.3 bAB	92.9±0.6 bAB	90.1±1.5 bB
	TCF-HEY	94.2 ±0.6 A	94.1±0.8 bA	92.8±0.7 bAB	91.0±0.7 bB
MMP (%)	TCF	93.7±0.8 aA	88.6±0.8 aB	86.9±1.3 aBC	84.9 ±1.6 aC
	TCF-LPFo	93.1±0.5 aA	92.4±1.3 abA	90.9 ±1.6 bAB	88.2±1.7 abB
	TCF-HEY	94.5±0.7 aA	93.2±1.0 bAB	92.9±1.7 bAB	91.4±1.1 bB
ATP (nmol/10^8sperm)	TCF	4.6±2.5 A	3.5±2.0 A	3.1±1.7 AB	0.5±0.3 aB
	TCF-LPFo	4.1±1.8 A	4.6±1.9 A	2.6±0.8 AB	1.1±0.3 abB
	TCF-HEY	4.9±2.3 A	4.9±2.1 A	4.8±2.2 AB	1.5±0.4 bB

a, b, c – differences (P≤0.05) between extenders (vertical).

A, B, C – differences (P≤0.05) between days of storage (horizontal).

Parameters: NAR, normal apical ridge acrosome; PMI, plasma membrane integrity; MMP, mitochondrial membrane potential; ATP, ATP content.

With storage time there was a slow decline in values of the majority of sperm motility parameters (TMOT, PMOT, VAP, VSL, BCF, STR, LIN) regardless of the extender used. A significant decrease in the value of most sperm motility parameters was observed on Day5 or Day7, as affected by the extender applied.

No significant differences (P≥0.05) in the percentage of normal apical acrosome were observed between extenders regardless of the day of storage (Table 2). In the PMI analysis, higher (P≤0.05) values were found for TCF-LPFo and TCF-HEY in comparison to TCF during storage from Day3 (Day3–Day7). Similar relationships were observed in MMP evaluation for the TCF-HEY extender. In turn, in the case of the TCF-LPFo extender, higher MMP values compared with TCF were found on Day5 of storage. There were no significant differences between TCF-LPFo and TCF-HEY extenders regardless of the day of storage in results of PMI, MMP and ATP content. On Day7 of storage, significant differences were noted in ATP content between TCF-HEY and TCF.

Over storage time, there was a successive decrease in NAR, PMI and MMP values, which depended on the extender and storage day. In the NAR analysis, a significant decrease (P≤0.05) was noted in the values of this parameter on Day7 compared to Day1 of storage, irrespective of the extender. In the PMI and MMP analysis, a significant decrease was observed in their values from Day3 of storage for TCF and on Day7 of storage for TCF-LPFo and TCF-HEY extenders. In the ATP analysis, there was even a slight increase in ATP values for TCF-LPFo and TCF-HEY on Day3, which was followed by a significant decrease in ATP value on Day7 of storage.

Discussion

This study was aimed at comparing the effects of an addition of a lyophilized lipoprotein fraction isolated from ostrich egg yolk (LPFo) and of hen egg yolk (HEY) to a Tris-citric acid-fructose (TCF) extender on the quality of dog sperm stored at a temperature of 5°C for 7 days.

Sperm motility is the key parameter indicative of the quality of stored semen and is expressed by its energy status and potential fertilization capability (Volpe et al., 2009; Nizanski et al., 2009). In this study, a slow successive decrease was observed in values of all sperm motility parameters over the entire storage period, as evaluated with the CASA system. The extent of these decreases was, however, different as affected by the extender applied. Values of the majority of the motility parameters, notably the TMOT, PMOT, VSL, BCF, STR and LIN, were observed to increase in the case of sperm extended in TCF-LPFo and TCF-HEY in comparison with the control extender TCF. The addition of both LPFo and HEY to the TCF extender was demonstrated to positively affect the analyzed motility parameters of dog sperm stored in a liquid state. The PMOT and VSL, ALH, BCF, STR and LIN percentages did not differ significantly between the LPFo-TCF and HEY-TCF extenders over the cold storage period. This study confirms findings from multiple previous studies indicating that egg yolk addition to an extender has a positive effect on motility preservation in stored dog sperm (Rota et al., 1995; Iguer-Ouada and Verstegen, 2001). Similar effects were reported for LPFo which improved sperm motility when added to boar semen stored in both liquid and cryopreserved state (Dziekońska et al., 2009; Fraser et al., 2007). The application of both HEY and LPFo in cryopreservation of dog semen was also found to positively affect post-thawing sperm motility parameters (Strzeżek et al., 2012).

The preservation of an appropriate sperm motility is inseparably linked with the integrity of acrosomal and plasma membranes of the stored sperm (Dziekońska et al., 2013; Volpe et al., 2009). Earlier research has demonstrated that egg yolk addition to stored semen had a positive effect upon integrity of acrosomes and plasma membranes of sperm exposed to cold shock (Watson, 1981), which was also shown in analysis of PMI in this study. This positive effect of egg yolk addition on sperm membranes is due to its properties. The main constituents of egg yolk include lipoproteins which show affinity to various sperm structures; by interacting with them, they protect spermatozoa against the cold shock (Demianowicz and Strzeżek, 1996). Many research works have indicated that the egg yolk lipoproteins, low-density lipoproteins (LDL) in particular, interact with the seminal plasma proteins (BSPs) that are responsible for modifying membrane lipids, thus protecting the membranes (Plante et al., 2016). Some BSPs exert an adverse effect on stored sperm as they cause cholesterol and phospholipids efflux from sperm membranes. The effects of BSPs on plasma membranes of spermatozoa result in their destabilization and their increased susceptibility to the cold shock (Plante et al., 2016; Watson, 1981).

The LPFo also contains lipoproteins and significant quantities of triacylglycerols, phospholipids, and cholesterol (Strzeżek et al., 2005, 2014). Earlier investigations have confirmed that LPFo, likewise HEY, protects the plasma membranes of boar

sperm against cold shock (Fraser et al., 2007; Strzeżek et al., 2005, 2014). Fraser et al. (2010) have demonstrated that LPFo lipoproteins interact with plasmalemma of boar spermatozoa mainly in the region of the head and the mid-piece, which may be of great significance to the preservation of their metabolic functions during storage (Dziekońska et al., 2017). It may be hypothesized that in this study the presence of both HEY and LPFo in the TCF extender served a similar protective role over dog sperm membranes, which was indicated by better PMI and MMP results obtained upon the use of these extenders compared to the TCF extender over the examined storage period.

In this study, we did not notice any significant effect of storage time on acrosome integrity in a time span of Day1–Day5, independent of extender, which was – to some extent – correlated by the results concerning sperm motility parameters. Study results may indicate that dog sperm acrosomes are characterized by significant resistance to the cold shock, which ensures their high survivability, as there is much evidence that dog sperm stored for many days preserves the appropriate motility as well as integrity of acrosome and plasma membranes (Nizanski et al., 2009; Verstegen et al., 2005).

Preservation of the better membrane integrity of sperm is indispensable for the apt course of metabolic processes, including glycolysis and oxidative phosphorylation, which are the main pathways producing energy in the form of ATP for the spermatozoa (Du Plessis et al., 2015; Mukai and Okuno, 2004). Energy produced in these processes (ca. 60%) fuels mainly spermatozoa motility. This study demonstrated the positive effect of the addition of egg yolk to the TCF extender on the ATP content in spermatozoa, which could also contribute to ensure better motility of sperm during liquid storage. The glycolytic pathway is acknowledged to be the main source of energy for spermatozoa motility preservation in boar, mouse, or dog (Marin et al., 2003; Mukai and Okuno, 2004; Rigau et al., 2001). However, the mitochondrial ATP production is also significant for motility and many other sperm functions. Some reports have demonstrated that the energy produced by mitochondria is indispensable for proper sperm motility, maintenance of the appropriate level of intracellular ROS and NADH, and for such processes as the acrosome reaction and oocyte penetration (Rodríguez-Gil and Bonet, 2016; Ruiz-Pesini et al., 2007). Abnormalities in the functioning of sperm mitochondria may be manifested by changes in MMP level that may, in turn, affect changes in sperm motility (Gączarzewicz et al., 2015; Guo et al., 2017; Volpe et al., 2009), which was also observed in this study. The TCF-LPFo and TCF-HEY extenders were better at protecting MMP compared to the TCF extender, which could also affect the higher ATP content determined in the sperm, that was produced in the process of oxidative phosphorylation.

In addition, the higher metabolic activity of sperm stored in TCF-LPFo and TCF-HEY in comparison with TCF extender expressed by ATP content could be attributed to the presence of additional energy substrates used in sperm metabolism. Spermatozoa use various energetic substrates including mainly monosaccharides but also lactate, citrate, glycerol, fatty acids, and even triacylglycerols and phospholipids (Jones and Bubb, 2000; Medrano et al., 2006; Rodríguez-Gil and Bonet, 2016). They metabolize mostly exogenous substrates, whereas the metabolism of endogenous

substrates is limited to ca. 10% production of ATP (Hammmerstedt, 1988). The TCF-LPFo and TCF-HEY extenders used in this study also contained, apart from fructose, energetic substrates derived from egg yolk, i.e. phospholipids, triacylglycerols and even small amounts of glucose. The presence of these substrates could, thus, affect the increased ATP content in stored sperm, as there are some reports showing that phospholipids are the important energy source in dog sperm (Kmenta et al., 2011). It is, therefore, likely that their addition to the TCF extender could have also contributed to a better energetic efficiency of dog sperm stored under cold shock temperature.

Conclusions

The lyophilized lipoprotein fraction isolated from ostrich egg yolk (LPFo) may be an alternative form used instead of hen egg yolk (HEY) to a Tris-citric acid-fructose (TCF) extender in dog sperm preservation. We demonstrated that the 5% addition of LPFo to TCF extender was capable of preserving spermatozoa that have been stored in a refrigerator at 5°C for at least 5 days. This means that the spermatozoa possessed good quality: motility, membrane integrity, mitochondrial function (MMP) and ATP content comparable with the spermatozoa preserved in the TCF-HEY extender. The presence of LPFo and HEY in the TCF extender protected the membrane integrity of sperm and can contribute to the improvement of the energetic status of dog sperm stored at cold shock temperature.

Author's contributions

Anna Dziekońska designed the experiment and conducted 'Effect of ostrich egg lipoproteins and hen egg yolk on the quality of dog sperm during liquid storage at 5°C'. Dorota Behrendt and Rafał Strzeżek collaborated in the experimental part, Władysław Kordan reviewed the manuscript.

References

Beccaglia M., Anastasi P., Chigioni S., Luvoni G.C. (2009). TRIS-lecithin extender supplemented with antioxidant catalase for chilling of canine semen. Reprod. Domest. Anim., 44: 345–349.

Bencharif D., Amirat L., Anton M., Schmitt E., Desherces S., Delhomme G., Langlois M.L., Barrie`re P., Larrat M., Tainturier D. (2008). The advantages of LDL (Low Density Lipoproteins) in the cryopreservation of canine semen. Theriogenology, 70: 1478–1488.

Demianowicz W., Strzeżek J. (1996). The effect of lipoprotein fraction from egg yolk on some of the biological properties of boar spermatozoa during storage of the semen in liquid state. Reprod. Domest. Anim., 31: 279–280.

Du Plessis S.S., Agarwal A., Mohanty G., van der Linde M. (2015). Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use? Asian J. Androl., 17: 230–235.

Dziekońska A., Fraser L., Strzeżek J. (2009). Effect of different storage temperatures on the metabolic activity of spermatozoa following liquid storage of boar semen. J. Anim. Feed. Sci., 18: 638–649.

Dziekońska A., Fraser L., Majewska A., Lecewicz, M., Zasiadczyk Ł., Kordan W. (2013). Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17 degrees C. Pol. J. Vet. Sci., 16: 517–525.

Dziekońska A., Kinder M., Fraser L., Strzeżek J., Kordan W. (2017). Metabolic activity of boar semen stored in different extenders supplemented with ostrich egg yolk lipoproteins. J. Vet. Res., 61: 127–133.

England G.C.W., Ponzio P. (1996). Comparison of the quality of frozen-thawed and cooled-rewarmed dog semen. Theriogenology, 46: 165–171.

Farstad W. (2000). Current state in biotechnology in canine and feline reproduction. Anim. Reprod. Sci., 60–61: 375–387

Fraser L., Dziekońska A., Strzeżek R., Strzeżek J. (2007). Dialysis of boar semen prior to freezing-thawing: Its effects on post-thaw sperm characteristics. Theriogenology, 67: 994–1003.

Fraser L., Zasiadczyk Ł., Strzeżek J. (2010). Interactions of egg yolk lipoprotein fraction with boar spermatozoa assessed with a fluorescent membrane probe. Folia Histochem. Cytobiol., 48: 292–298.

Garner D.L., Johnson L.A. (1995). Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod., 53: 276–284.

Gączarzewicz D., Udała J., Piasecka M., Błaszczyk B., Stankiewicz T. (2015). Storage temperature of boar semen and its relationship to changes in sperm plasma membrane integrity, mitochondrial membrane potential, and oxidoreductive capability. Turk. J. Biol., 39: 582–594.

Goericke-Pesch S., Klaus D., Failing K., Wehrend A. (2012). Longevity of chilled canine semen comparing different extenders. Anim Reprod Sci., 135: 97–105.

Guo H., Gong Y., He B., Zhao R. (2017). Relationships between mitochondrial DNA content, mitochondrial activity, and boar sperm motility. Theriogenology, 1: 276–283.

Hammerstedt R.H., Volonte C., Racker E. (1988). Motility, heat and lactate production in ejaculated bovine sperm. Arch. Biochem. Biophys., 266: 111–123.

Iguer-Ouada M., Verstegen J.P. (2001). Long-term preservation of chilled canine semen: effect of commercial and laboratory prepared extenders. Theriogenology, 55: 671–684.

Jones A.R., Bubb W.A. (2000). Substrates for endogenous metabolism by mature boar spermatozoa. J. Reprod. Fertil., 119: 129–135.

Kasimanickam V.R., Kasimanickam R.K., Memon M.A., Rogers H.A. (2012). Effect of extenders on sperm mitochondrial membrane, plasma membrane and sperm kinetics during liquid storage of canine semen at 5°C. Anim. Reprod. Sci., 136: 139–145.

Kmenta I., Strohmayer C., Müller-Schlösser F., Schäfer-Somi S. (2011). Effects of a lecithin and catalase containing semen extender and a second dilution with different enhancing buffers on the quality of cold-stored canine spermatozoa. Theriogenology, 75: 1095–1103.

Kutzler M.A. (2005). Semen collection in the dog. Theriogenology, 64: 747–754.

Linde-Forsberg C. (1995). Artificial insemination with fresh, chilled extended, and frozen-thawed semen in the dog. Semin. Vet. Med. Surg. (Small Anim.), 10: 48–58.

Marin S., Chiang K., Bassilian S., Lee W.N., Boros L.G., Fernandez-Novell J.M., Centelles J.J., Medrano A., Rodriguez-Gil J.E., Cascante M. (2003). Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. FEBS Lett., 554: 342–346.

Medrano A., Fernández-Novell J.M., Ramió L., Álvarez J., Goldberg E., Rivera M.M., Guinovart J.J., Rigau T., Rodríguez-Gil J.E. (2006). Utilization of citrate and lactate through a lactate deydrogenase and ATP-regulated pathway in boar spermatozoa. Mol. Reprod. Dev.,73: 369–378.

Mogielnicka-Brzozowska M., Dziekońska A., Strzeżek R., Załęcki M., Majewska A., Tołścik K., Kordan W. (2014). Effect of seminal plasma zinc-binding proteins on motility and membrane integrity of canine spermatozoa stored at 5°C. Bull. Vet. Inst. Pulawy, 58: 163–168.

Mukai C., Okuno M. (2004). Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol. Reprod., 71: 540–547.

Nizanski W., Klimowicz M., Partyka A., Savic M., Dubiel A. (2009). Effects of the inclusion of Equex STM into Tris-based extender on the motility of dog spermatozoa incubated at 5°C. Reprod. Domest. Anim., 44: 363–365.

Plante G., Prud'homme B., Fan J., Lafleur M., Manjunath P. (2016). Evolution and function of mammalian binder of sperm proteins. Cell Tissue Res., 363: 105–127.

Rigau T., Farre M., Ballester J., Mogas T., Pena A., Rodriguez-Gil J.E. (2001). Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology, 56: 801–815.

Rodríguez-Gil J.E., Bonet S. (2016). Current knowledge on boar sperm metabolism: Comparison with other mammalian species. Theriogenology, 85: 4–11.

Rota A., Strom B., Linde-Forsberg C. (1995). Effects of seminal plasma and three extenders on canine semen stored at 4 °C. Theriogenology, 44: 885–900.

Ruiz-Pesini E., Díez-Sánchez C., López-Pérez M.J., Enríquez J.A. (2007). The role of the mitochondrion in sperm function: Is there a place for oxidative phosphorylation or is this a purely glycolytic process? Curr. Top Dev. Biol., 77: 3–19.

Strzeżek J., Hopfer E. (1987). Zinc ion-dependent protein in boar semen. I. Egg yolk precipitating activity and some biochemical properties. Anim. Reprod. Sci., 13: 117–131.

Strzeżek J., Lecewicz L., Dziekońska A., Fraser L. (2005). A simple method of extraction of lipoprotein fractions from avian egg yolk – protective effect on cooled boar semen. Theriogenology, 63: 496–497.

Strzeżek R., Koziorowska-Gilun M., Stawiszyńska M. (2012). Cryopreservation of canine semen: the effect of two extender variants on the quality and antioxidant properties of spermatozoa. Pol. J. Vet. Sci., 15: 721–226.

Strzeżek J., Fraser L., Lecewicz M., Strzeżek R., Dziekońska A. (2014). Preparation for the preservation of animal semen, particularly boar (in Polish). Patent Number: PL217869-B1.

Verstegen J.P., Onclin K., Iguer-Ouada M. (2005). Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris-glucose extender: *in vitro* and *in vivo* studies. Theriogenology, 64: 720–733.

Volpe S., Leoci R., Aiudi G., Lacalandra G.M. (2009). Relationship between motility and mitochondrial functional status in canine spermatozoa. Reprod. Domest. Anim., 44: 275–278.

Watson P.F. (1981). The roles of lipid and protein in the protection of ram spermatozoa at 5 degrees C by egg-yolk lipoprotein. J. Reprod. Fertil., 62: 483–492.

Watson P.F., Martin C.A. (1975). The influence of some fractions of egg yolk on the survival of ram spermatozoa at 5°C. Aust. J. Biol. Sci., 28: 145–152.

Received: 9 V 2017
Accepted: 3 X 2017