Original scientific paper
DOI: /10.5513/JCEA01/25.4.4071
Journal of Central European Agriculture, 2024, 25(4), p.884-891

The effect of the amount of fat content in milk extender on ram sperm motility parameters during the storage of 5 °C for 72 h and comparison with the other semen extenders

Vliv obsahu tuku v mléčných ředidlech na parametry motility spermatu beranů během skladování při 5 °C po dobu 72 h a porovnání s ostatními ředidly

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Received: August 17, 2023; accepted: August 5, 2024

ABSTRACT

The objective of this study was to evaluate the effect of the amount of fat content in milk extender and comparison with other semen extender and evaluate their effect on ram sperm motility and kinematic parameters during the liquid storage of 5 °C for an evaluation period of 72 hours. The semen samples were collected from three Šumava sheep rams (4 years old) using an artificial vagina and were diluted by physiological solution, 0.5% fat milk, 1.5% fat milk and AndroMed® extenders with a dilution 1:100. Motility and kinematic parameters were evaluated using the CASA (Computer-assisted sperm analysis) at regular time intervals of 0, 12, 24, 36, 48 and 72 hours after sperm collection. The effect of extenders on motility and kinematic parameters varied significantly among each other. The temporal decrease rate in sperm quality differed between extenders. Physiological solution can be used on the farm for insemination within 24 hours of collection when the sperm quality is still very good. Milk diluent with 0.5% fat has better sperm motility parameters than milk diluent with 1.5% fat, but of all the extenders tested, the milk extenders had the worst quality. The best diluent tested was AndroMed®, which showed good values of motility parameters even after 72 hours of storage at 5 °C.

Keywords: ram ejaculate quality, CASA, motility, kinematic parameters, artificial insemination

ABSTRAKT

Cílem této studie bylo vyhodnotit vliv množství obsahu tuku v mléčném ředidle, zhodnotit vliv ředidla na motilitu a kinematické parametry motility spermií a porovnání s ostatními ředidly v průběhu skladování při 5 °C po dobu 72 hodin. Vzorky spermatu byly odebírány od tří beranů plemene šumavská ovce (4letí) pomocí umělé vagíny a byly naředěny fyziologickým roztokem, mléčným ředidlem s obsahem tuku 0,5 %, mléčným ředidlem s obsahem tuku 1,5 % a ředidlem AndroMed® s ředěním 1:100. Motilita a její kinematické parametry byly hodnoceny pomocí počítačové metody CASA (Computer-assisted sperm analysis) v pravidelných časových intervalech 0, 12, 24, 36, 48 a 72 hodin po odběru spermatu. Velmi výrazně se lišil vliv ředidel na motilitu a kinematické parametry. Lišila se míra dočasného poklesu kvality spermií mezi ředidly. Fyziologický roztok lze použít na farmě k inseminaci do 24 hodin po odběru, kdy je stále velmi dobrá kvalita spermatu. Mléčné ředidlo s 0,5 % tuku má lepší parametry motility spermií než mléčné ředidlo s 1,5 % tuku, ale ze všech testovaných ředidel, měla mléčná ředidla nejhorší kvalitu. Nejlepším testovaným ředidlem byl AndroMed®, který vykazoval dobré hodnoty parametrů motility i po 72 hodinách skladování při 5 °C.

Klíčová slova: kvalita beraního spermatu, CASA, motilita, kinematické parametry, umělá inseminace

INTRODUCTION

Artificial insemination in sheep is important to increase the rate of genetic improvement. The ram sperm is characterized by a small volume, high sperm concentration and the total number of sperm (Romano et al., 2009). Artificial insemination in sheep breeding is performed by using fresh or cooled dilutes semen (O'Hara et al., 2010). The use of cooled semen has practical and economic advantages: it is cheap, easy to handle and can be used for cervical insemination (Mara et al., 2005).

For better storage of the semen, we can dilute it by extender to longer quality from 0 to 168 hours. The basic extenders for dilution and cooled storage of sperm are usually based on either skimmed milk or egg yolk (Allai et al., 2017). Diluents can be composed of whole or skim milk, egg yolk, Tris, Tes, fructose, glucose, and soy or we can use commercial media such as INRA-96® extender (INRA) or phospholipids AndroMed® (Minitube) (Kasimanickam et al., 2011; Yániz et al., 2015; Rather et al., 2016; Bandeira et al., 2017; Allai et al., 2017).

Milk fat primarily consists of triacylglycerols (62%) and phospholipids (26-31%) (Štolcová, 2020). The amount of fat in the extender, particularly glycerol, is important as a protection of the sperm plasma membrane against cold shock (Fiser and Fairfull, 1986). Glycerol can be applied to diluents in various concentrations, but unfortunately, a new study shows that more glycerol reduces the ability to fertilize and no longer contributes to sperm quality (Lin et al., 2023).

Other various special additives such as for example astaxanthin (Fang et al., 2015), nonylphenol (Uguz et al., 2015) or gelatin (Gheller et al., 2018) and others can also be added to the diluent to improve sperm quality (Allai et al., 2018). These extenders allow the storage and preservation of semen quality at 4 °C for up to 72 hours (to 96 hours) (Falchi et al., 2018a). Nowadays, cooling semen at 4 °C for longer times would represent a great help in simplifying field management of reproduction in large flocks but is generally used within 24 hours from collection to obtain acceptable pregnancy rates (O'Hara et al., 2010).

The study aimed to evaluate the ability of two milk-based extenders, phospholipids AndroMed®, and physiological solution to preserve the quality of cooled semen. The motility and kinematic parameters were evaluated by the CASA (computer-assisted sperm analysis) of semen stored at 5 °C for periods up to 72 hours (0, 12, 24, 36, 48 and 72 hours). The study compares the effect of these extenders on concentration, motility, and kinematic parameters.

MATERIAL AND METHODS

Animals

Three rams of the Šumava sheep breed were used, each 4 years old. Their average weight was approximately 70 kg. The rams had access to water, hay, and mineral supplements *ad libitum*. The rams were stabled in the purpose-built facility Čtyři Dvory which is a part of the Department of Animal Husbandry Sciences of the Faculty of Agriculture and Technology, University of South Bohemia in České Budějovice (48.9787925N, 14.4715064E).

Semen collection

The semen was collected using an artificial vagina (pre-warmed at 42 °C) once a week for 5 weeks during January and February of 2019. Semen collection was performed using a phantom ram. The ejaculates were transferred in the polystyrene box (37 °C) immediately after collection to the laboratory of the Department. Each ejaculate was split into 4 aliquots one of the 4 extenders, warmed at 37 °C, was added to each aliquot to make a dilution of 1:100. After equilibration, the semen samples were placed in a refrigerator, and they were cooled to 5 °C.

Semen dilution and extenders

Used extenders were selected based on normal availability for farmers. The first extender was the physiological saline solution (0.9% solution NaCl) (David et al., 2015). The second was skimmed milk-based extender, 0.5 % fat milk (11 g ultra-heat-treated skimmed milk with 0.5 % fat, in 100 ml distilled water heated for 10 min to 95 °C) (Allai et al., 2017).

The other extender was a semi-skimmed milk-based extender, 1.5 % fat milk (11 g ultra-heat-treated milk with 1.5 % fat, in 100 ml distilled water heated for 10 min to 95 °C). The fourth semen sample was diluted by commercial extender AndroMed® (Minitübe GmbH, Tiefenbach, Germany), an egg yolk-free concentrated extender medium for freezing of bull semen and semen of other ruminants from Minitube. AndroMed® has no ingredients of animal origin and contains phospholipids, TRIS, citric acid, sugars, antioxidants, buffers, glycerol, and purest water.

Semen analysis

Semen samples were evaluated after cooled-shipped semen. A total of 60 diluted samples were evaluated. The sperm motility and kinematic parameters were assessed at the samples 0, 12, 24, 36, 48 and 72 hours after collection. Sperm motility, kinematic parameters and sperm concentration were determined objectively using the Sperm Class Analyzer CASA System (SCA, MICROPTIC S.L., Barcelona, Spain). The technology was equipped with an optical microscope (Nikon ECLIPSE E200LED MV Series) with phase-contrast and heated plate, a built in a digital camera (Basler Color) capturing images and transmitting them to a computer (Genuine Intel Core i5).

Parameters evaluated by CASA were: total motility (MOT, %), progressive motility (PMOT, %), number of progressive sperm per ml (prog M/ml, %), rapid spermatozoa (RAP_MOT, %), rapid progressive spermatozoa (RAP_PMOT, %), and kinematic parameters curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity coefficient (LIN, calculated by dividing VSL to VCL, %), straightness coefficient (STR, calculated by dividing VSL to VAP, %), wobble coefficient (WOB, calculated by dividing VAP to VCL, %), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz).

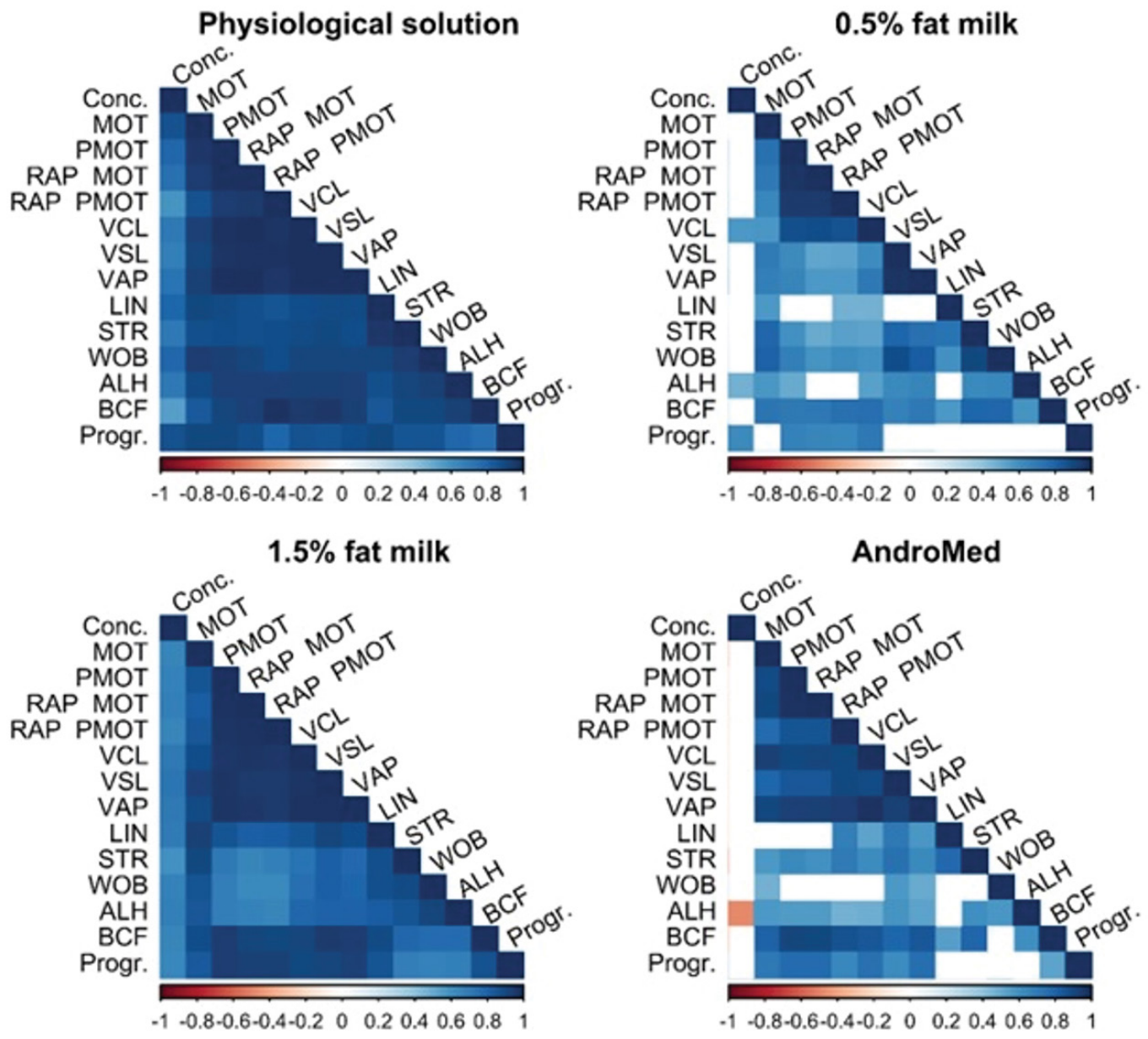
Statistical analysis

All pairwise combinations of motility and kinematic parameters of ram semen quality were related using Pearson's correlation coefficients separately for each semen quality extender. Furthermore, selected parameters of semen motility (MOT, PMOT) and kinematic parameters (VCL, VSL, VAP) were used as a response variable and storage time, extender type and their interaction as predictors. These parameters were chosen on the base their predictive capacity on fertility after artificial insemination (Santolaria et al., 2015). The relationship for VCL, VSL, and VAP was assessed by Generalized Linear Mixed-effect Models (GLMM) in package *lme4* (Bates et al., 2015) with Gamma error distribution, logarithmic link function and testing significance by chi-squared test. MOT and PMOT were analysed also by GLMM in the package *glmmTMB* (Brooks et al., 2017) allowing to use of beta error distribution with logit link function of the response variable, which is suitable for data-constrained from both ends (percentage in our case). In all GLMM analyses, the identity of the ram was used as a random factor. All the analyses were performed in the R software version 4.0.2 (R Core Team, 2020) and visualized using package *corrplot* for the correlation matrix (Wei and Simko 2021) and package *ggplot2* (Wickham 2016) for the remaining graphical outputs.

RESULTS

The effect of physiological solution, milk-based extender and AndroMed® on sperm quality

Concentration, all motility and kinematic parameters were relatively strongly and positively correlated with each other in physiological solution and 1.5% fat milk extender. Furthermore, there was either no or very weak linear relationship between the parameters of concentration, motility and kinematic parameters in the milk supplement with 0.5% fat and AndroMed®. Pairwise relationships between parameters 0.5% fat milk extender and AndroMed® are more variable and poorly correlated with concentration. A negative correlation was found for the relationship between conc and ALH in AndroMed®. The correlation matrix can be found in Figure 1.



Abbreviations: Conc., concentration M/ml; MOT, total motility %; PMOT, progressive motility %; RAP_MOT, rapid spermatozoa %; RAP_PMOT, rapid progressive spermatozoa %; VCL, curvilinear velocity µm/s; VSL, straight-line velocity µm/s; VAP, average path velocity µm/s; LIN, linearity %; STR, straightness %; WOB, wobble coefficient %; ALH, amplitude of lateral head displacement µm; BCF, beat cross frequency Hz; progr., million progressive sperm per ml M/ml.

Figure 1. Pairwise comparisons between sperm motility and kinematic parameters diluted with different extenders – Physiological solution, 0.5% fat milk, 1.5% fat milk and AndroMed® cold at 5 °C. Coloured boxes correspond with the correlation strength expressed by significant Pearson's correlation coefficients while blank boxes show insignificant correlations.

The effect of storage time and type of extender

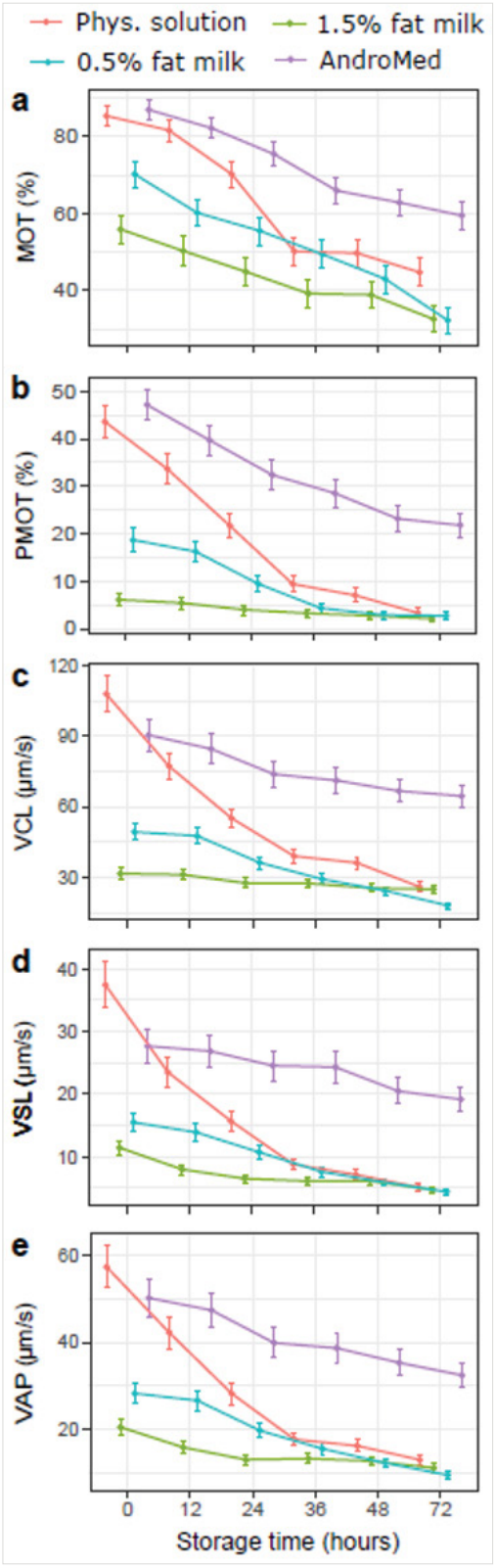
Table 1 shows a strong significant effect of storage time and type of the extender used on all the selected parameters MOT, PMOT, VCL, VSL and VAP. Furthermore, significant interactions between storage time and extender type for all parameters highlight that the speed of sperm quality decreases varies across extenders.

Table 1. The effect of storage time extender type and their interaction on semen motility and kinematic parameters

	Storage time		Extender		Storage time * Extender	
	X^2_5	*P*	X^2_5	*P*	X^2_{15}	*P*
MOT	42.65	< 0.001	85.80	< 0.001	27.64	0.024
PMOT	21.96	< 0.001	119.90	< 0.001	53.34	< 0.001
VCL	21.70	< 0.001	111.70	< 0.001	78.41	< 0.001
VSL	25.27	< 0.001	105.07	< 0.001	78.39	< 0.001
VAP	27.09	< 0.001	109.73	< 0.001	62.29	< 0.001

Abbreviations: MOT, total motility %; PMOT, progressive motility %; VCL, curvilinear velocity µm/s; VSL, straight-line velocity µm/s; VAP, average path velocity µm/s.

In this study, the physiological solution was the best diluent for VCL, VSL and VAP parameters after semen collection (Figure 2; c, d, e). The physiological solution showed high values within 36 hours after collection. The best diluent was AndroMed®, which showed good values of motility parameters even after 72 hours of storage at 5 °C (Figure 2; a, b), while quality parameters of semen in physiological solution dropped dramatically. Milk extenders had low motility and kinematic parameters. The 0.5% fat milk was better than the 1.5% fat milk extender.



Abbreviations: MOT, total motility %; PMOT, progressive motility %; VCL, curvilinear velocity µm/s; VSL, straight-line velocity µm/s; VAP, average path velocity µm/s

Figure 2. The effect of different semen extenders on motility and kinematic parameters in storage time of 0 – 72 hours. Error bars indicate standard error of the mean

DISCUSSION

In this study, extenders were compared: physiological solution, milk extenders with 0.5% fat and 1.5% fat, and commercial AndroMed® extenders. Diluted semen samples were stored in a refrigerator at 5 °C and subsequently evaluated 0, 12, 24, 36, 48 and 72 hours after collection. Concentration, motility and kinematic parameters were assessed by CASA. The study was also designed to investigate the possible better quality of a higher-fat milk diluent. The average sperm parameters used in this study were similar according to most studies (Martí et al., 2011; Zhao et al., 2017; Flores-Gil et al., 2021).

Results in all studies indicate deterioration of sperm quality due to long-time storage of sperm (Allai et al., 2017). The quality of sperm stored for more than 3 days at 4 °C significantly decreases (Gundogan et al., 2010; O´Hara et al., 2010; Falchi et al., 2018b). This is caused by the metabolic activity of the sperm, which changes the pH of the sperm and there is a loss of the integrity of the sperm membrane, followed by their intoxication, reduced motility and loss of fertility (Yániz et al., 2008). To delay this process of vital sperm degradation, you can use suitable supplements for the diluent, which was already known in the 70s when experiments with the use of milk fat in sperm extenders began, for example, Almquist (1962) and the results were very positive, the sperm had a higher quality for a longer period.

It is the fat in the extenders that contributes to the improvement of plasma membrane integrity and motility of ram spermatozoa during liquid storage (Gil et al., 2011). In our study, lower fat extenders showed overall lower motility and kinematic parameters, but higher fat extenders showed a higher positive correlation in most motility parameters. Santolaria et al. (2015) used a UHT milk extender with 0.7% fat and their motility values were higher than in this study. Although Olivera-Muzante et al. (2011) compare UHT skimmed milk to commercial thinners, the results could be better after using higher-fat milk. Better results are also obtained by Yániz et al. (2005) with a fat content of 0.7% milk. Milk extenders made using UHT skim milk are popular because of their ease of production, they maintain good fertilization ability and, according to Galarza et al. (2019), even after 48 h of storage, they retain sufficient kinetic force to overcome the cervical barrier after artificial insemination. Hameed et al. (2024) used a completely fat-free skimmed milk extender for their work and for 24h the results were comparable, but when stored longer than 48h the motility was better with a tris-based extender than with milk extender, but kinematic parameters were similar for all extenders throughout the storage period.

Fat can also be implemented in the diluent in other ways than milk, egg yolk or soy lecithin can be used (Beran, 2014). Comparing the milk extender with the egg extender, Paulenz et al. (2003) reported that the milk extender showed 10% higher fertilization ability compared to the egg yolk extender, although the egg yolk extender showed higher motility values when stored at 5 °C for 30 h. This could mean that the environmental changes that occur on the farm are better tolerated by the sperm in the milk diluent, even though the sperm motility parameters are lower (Paulenz et al., 2002). Akhert et al. (2011) compared all types of extenders mentioned and during the first 3 days of storage the motility values were identical, but after that the diluent with soya lecithin performed better.

Similar results are presented by Kasimanickam et al. (2011) according to which the egg yolk and soy based extenders provide better sperm protection compared to the skim milk extender. Gil et al. (2003) recommend the addition of 5-10% egg yolk to the milk diluent as it increased sperm quality parameters in their study.

Although in this work, physiological solution and milk diluent with 1.5% fat had a higher correlation between individual parameters, AndroMed® had higher parameters of motility and speed after 72 hours, so it is more suitable for longer-term storage of insemination doses. However, the final fertilizing ability of sperm is influenced by a combination of all qualitative parameters: motility, kinematic parameters, viability, acrosome reaction and capacitation ability (Martí et al., 2003). According to

Santolario et al. (2015), the VCL parameter has limited predictive capacity for field fertility, while other kinematic parameters do not.

CONCLUSION

In conclusion, the physiological solution can be used for on-farm use to insemination until 24 hours after collection with very good semen quality results. The 0.5% fat milk was better in the motility and kinematic parameters than the 1.5% fat milk extender. But milk extenders had the worst sperm quality of all extenders tested. The best diluent was AndroMed®, which showed good values of motility parameters even after 72 hours of storage at 5 °C.

ACKNOWLEDGEMENT

This study was funded by grant from the Grant Agency of the University of South Bohemia (project No. GAJU028/2019/Z).

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