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Chemically defined caseinate extenders and their effect on the cooled semen collected from stallions with long sexual rest

Ředidla s obsahem chemicky definovaných kaseinátů a jejich vliv na chlazené sperma odebrané od hřebců po dlouhé sexuální inaktivitě

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ABSTRACT

The ability of three milk-based extenders to preserve the motility and viability of cooled shipped semen produced by stallions after a long sexual rest was evaluated. In total, 21 ejaculates from 11 stallions were collected after 5–6 months of sexual inactivity and diluted with Kenney, INRA 96, and EquiPlus. Seven parameters were evaluated – spermatozoa viability (VIT, %), total motility (MOT, %), progressive motility (PMOT, %), number of progressive spermatozoa per ml (prog M/ml, million/ml), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s) and straight-line velocity of spermatozoa (VSP, µm/s). These parameters were determined by CASA 2 hours after semen processing and then in 24, 48 and 72-hour intervals of storage at 5 °C. The effect of storage time was highly significant (P<0,001) for all evaluated parameters. The effect of the extender was significant (P<0.05) for VIT, MOT, PMOT, VCL, VSL and VAP. The ejaculates extended with INRA 96 reached significantly (P<0.01) higher values for all evaluated parameters compared to Kenney extender. The INRA 96 extender significantly increased the values of PMOT, VCL and VAP compared to EquiPlus (P<0.05). In all observed extenders, MOT and VIT decreased linearly whereas PMOT decreased exponentially. The fastest decrease of PMOT was observed within 24 hours of storage. The results obtained from using INRA 96 showed that quality of cooled semen for stallions collected after a long period of sexual inactivity can be sufficiently maintained during at least 24 hours of storage.

Keywords: cooled semen, ejaculate, extender, sperm motility, stallion

ABSTRAKT

Cílem této práce bylo porovnat ochranný účinek tří mléčných ředidel na chlazené sperma získané od hřebců po delší sexuální pauze (5 až 6 měsíců). Celkem bylo odebráno 21 ejakulátů od 11 hřebců. K ředění byla využita ředidla Kenney, EquiPLus a INRA 96. Hodnoceno bylo sedm parametrů – vitalita spermií (VIT, %), celková motilita (MOT, %), progresivní motilita (PMOT), počet progresivních spermií v mililitru (prog M/ml, million/ml), průměrná rychlost (VAP, µm/s), křivočará rychlost (VCL, µm/s) a přímočará rychost spermií (VSL, µm/s). Tyto parametry byly stanoveny metodou CASA 2 hodiny po zpracování spermatu a poté po 24, 48 a 72 hodinách skladování při 5 °C. Byl prokázán významný vliv doby skladování spermatu (P<0,001) na všechny hodnocené parametry. Vliv ředidla byl singnifikantní (P<0,05) v případě VIT, MOT, PMOT,

VCL, VSL a VAP. Průměrný ochranný účinek ředidla INRA 96 byl významně lepší po celou dobu skladování u všech sledovaných parametrů než u ředidla Kenney (P<0,01) a než u EquiPlus (P<0,05) u hodnot PMOT, VCL a VAP. U všech sledovaných ředidel hodnoty MOT a VIT klesaly lineárně, zatímco PMOT exponenciálně. Nejrychlejší pokles PMOT byl pozorován v průběhu prvních 24 hodin skladování. Výsledky dosažené s ředidlem INRA 96 ukázaly, že kvalita chlazeného spermatu u hřebců odebraných po delší sexuální inaktivitě může být dostatečně udržena nejméně během prvních 24 hodin skladování.

Klíčová slova: chlazené sperma, ejakulát, ředidlo, motilita spermií, hřebec

INTRODUCTION

The success of equine artificial insemination (AI) depends on maintaining the fertilizing ability of spermatozoa during cold storage. To achieve this goal, several basic and modified extenders have been developed (Ijaz and Ducharme, 1995). Most extenders for equine semen cold storage are based either on skimmed milk or egg yolk (Jasko et al., 1992; Moran et al., 1992; Bergeron and Manjunath, 2006). Milk and milk-based extenders are known to be practical and efficient in protecting equine spermatozoa during cold storage before AI (Michajilov, 1950; Kenney et al., 1975; Palmer, 1984). However, milk is a biological fluid with a complex composition and contains components that could be beneficial or detrimental to sperm survival (Batellier et al., 2001). Some commercial extenders substitute protein-defined milk fraction for milk (Lagares et al., 2012). Fractionating milk has allowed the preparation of purified protein fractions. Among these, native phosphocaseinate (NPPC), composed of total micellar caseins, and β-lactoglobulin were found to be the most effective in supporting the longevity of cold-stored spermatozoa (Batellier, 1997; Batellier et al., 1997). Pellicer-Rubio and Combarnous (1998) state that NPPC is the most efficient component for preserving motility (MOT) and maintaining the fertility of equine spermatozoa stored for 3 days compared to standard skim-milk extenders. Batellier et al. (1997) developed the INRA 96 extender which is a chemically defined medium with NPPC substituting for skimmed milk.

In a healthy stallion, the period of sexual rest is probably the most important aspect of determining the quality of semen (Thompson et al., 2004). It is a common practice for many sport horse stallions to breed a very limited book of mares each breeding season. Therefore, the interval between successive semen collections may be too long to maintain optimal semen quality for a cooled semen shipment program (Samper et al., 2008). The lack of studies performed without stabilizing the extragonadal spermatozoa reserves raises the following questions: what are the semen parameters of the ejaculates of stallions after a long sexual rest? Can the semen parameters in stallions after a long sexual rest be influenced by the choice of an appropriate extender?

The aim of the present work was to evaluate the effect of extenders containing defined milk components with a widely established skimmed milk extender on the motility parameters and viability of stallion spermatozoa obtained after a long sexual rest.

MATERIALS AND METHODS

Stallions

Semen samples were obtained from 11 clinically healthy and fertile stallions (age 3–21 years). Stallions were all involved in a commercial AI program at the National Stud Písek, Czech Republic. The stallions were stabled in the same conditions, i.e. boxes sized at least 5 x 5 m. The stallions' feed dose contained 11 kg hay, 1 kg straw, 3 kg oats, and mineral and vitamin supplements—PREMIN for breeding stallions (VVS Verměřovice, s. r. o., Czech Republic) and STARFIT granulated feed (FIDES AGRO, spol. s. r. o., Czech Republic). Exercise was also an integral part of management, 1–4 hours a day, 6 days a week. It was provided in a combination of turn-out in the paddock, riding, and mechanical walker.

Semen Collection

Twenty-one ejaculates were collected before the beginning of the 2018 breeding season from 11 stallions

(n=10 in January, n=11 in February). The sexual rest of the stallions was approximately five to six months.

Semen collection was performed on a phantom using a Colorado model artificial vagina in the presence of a mare. Immediately after collection, the gel fraction was removed, the semen was filtered through sterile gauze and the volume of ejaculate was recorded. Each ejaculate was split into 3 aliquots and one of the 3 extenders, warmed to 37 °C, was added to each aliquot at a 1:1 dilution. After dilution, the semen samples were equilibrated for 20 minutes at room temperature in the dark. After incubation, the semen was cooled to 5 °C at an approximately 0.3 °C/min cooling rate and stored at 5 °C in a refrigerator. The semen samples were then transferred to the laboratory of ZF JU in České Budějovice. Samples were transported for approximately 60 min in a Styrofoam box with cooling elements.

Semen Extenders

Two commercially available extenders (INRA 96 and EquiPlus) and one privately prepared extender (Kenney) were tested.

INRA 96 is patented and available as a ready-to-use solution (Ref. 016441, IMV Technologies, Saint-Ouen-Sur-Iton, France). This chemically defined extender contains Hanks' salts, glucose (67 mM) and lactose (126 mM) supplemented with a purified milk fraction, and native phosphocaseinate (27 g/l).

EquiPlus (Ref. 13570/0202, Minitüb, Tiefenbach, Germany) contains defined caseinates, glucose, sucrose, buffer and antibiotics (lincomycin and spectinomycin). It was used in the powdered form and dissolved in purified water (Double Distilled Water, pyrogen-free and sterile, Ref. 13570/1000, Minitüb, Tiefenbach, Germany) according to the manufacturer's instructions.

The Kenney extender is based on glucose and non-fat milk and contains 2.4 g non-fat dry skimmed milk, 4.9 g glucose monohydrate, 100 ml redistilled water and antibiotics (0.15 g dihydrostreptomycin and 0.15 penicillin). This extender was created in the Písek, Nový Dvůr laboratory especially for National Stud Písek.

Semen Evaluation

Semen samples were evaluated after transporting the cooled semen. Thus, the semen motility was evaluated at the time of insemination rather than directly after collection at the time of processing.

Spermatozoa motility and kinematic parameters were determined objectively using a Sperm Class Analyzer (SCA, MICROPTIC SL, Barcelona, Spain). This apparatus was equipped with a microscope (Nikon ECLIPSE E200 LED MV Series) with phase-contrast,a heated plate (37 C) and a built-in digital camera (Basler Color) to capture images and transmit them to a computer (Genuine Intel Core i5).

Motility was evaluated using the Sperm Class Analyzer motility module. This module was established by analysing 16 consecutive, digitized photographic images obtained from a single field. These 16 consecutive photographs were taken with a 0.64 s time-lapse, which implied an image capturing velocity of one photograph every 40 ms. Sperm cells were detected automatically. Motility was analyzed using negative phase-contrast (Ph-), 100x magnification and a green filter. Semen samples were opened to remove an aliquot for each time and then resealed. For motility analysis, aliquots were prewarmed to 37 °C for 10 minutes. A prewarmed Leja slide (20 microns in depth; Leja Amsterdam, The Netherlands) at 37 °C was loaded with extended semen (3 µL) and 10 microscopic fields were evaluated. The final semen concentrations for analysis were 30–50 x 10^6 spermatozoa cells/ml. Approximately 100–200 spermatozoa cells were analysed in one microscopic field. Parameters evaluated using the Sperm Class Analyzer were total motility (MOT, %), progressive motility (PMOT, %), number of progressive spermatozoa per ml (prog M/ml, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s) and average path velocity (VAP, µm/s). Spermatozoa were classified according to curvilinear velocity (VCL) as rapid (> 90 µm/s), medium (45–90 µm/s), slow (10–45 µm/s) and static (< 10 µm/s). Spermatozoa were considered progressive with at least 75% straightness (STR).

The viability of spermatozoa was evaluated using the Sperm Class Analyzer vitality module and fluorescent staining FluoVit (MICROPTIC SL). FluoVit is a fluorescent staining solution that permits differentiating the living spermatozoa from the dead spermatozoa using fluorescent dyes (Hoechst and Trihydrochloride Trihydrate, Propidium Iodide). Semen sample (10 µl) was incubated with 1 µl BLUE eppendorf stain (Hoechst 33342, Trihydrochloride Trihydrate 330/380) at 37 °C for 5 minutes and then with 1 µl RED eppendorf stain (Propidium Iodide 536/617) for 5 minutes. After incubation, one drop (4 µl) of stained sample was placed on a glass slide and covered with a glass coverslip. For the analysis of viability, fluorescence, magnification 200x and DAPI filter (EX 330-380, DM 400, BA 420, standard filter for UV) were configured. Heads of membrane-intact (viable) spermatozoa showed bright blue colour, spermatozoa with damaged membranes (dead) were stained red. At least 200 spermatozoa were evaluated per sample and the mean calculated by software provided by SCA. Results are given as percentages of live spermatozoa in the samples.

The chosen parameters were measured for each sample of extended semen immediately after cooling to 5 °C (2 h), and after storage at this temperature for 24, 48 and 72-hour time periods for all extenders.

Statistical Analysis

MS Office and Statistica.12 (StatSoft®, Prague, Czech Republic) were used for data processing. The effect of extender on cold-stored semen characteristics was assessed using analysis of variance with repeated measurements followed by the Tukey test for multiple comparisons. Statistical significance was set at P<0.001 (***), P<0.01 (**) and P<0.05 (*) and tendencies were defined as 0.05<P<0.10 (+). Relationships among time and dependent variables were estimated using linear regression. Regression analyses were used to evaluate how time was related to the dependent variables (parameters), and to explore the forms of these relationships among all studied extenders. The results, in the form of an equation, are shown together with the coefficient of determination (R^2). The velocity of decrease for the obtained values was evaluated using the first derivative, which can be interpreted as an instantaneous rate of change. Data are presented as means ± standard error of the mean (SEM) unless otherwise indicated.

RESULTS

Table 1 shows the significant effect of extender on VIT, MOT, PMOT, VCL, VSL and VAP (P<0.05). As expected, highly significant effect of storage time on all evaluated parameters was manifested (P<0.001). The interaction of time and extender was significant for PMOT and VCL (P<0.05).

Table 1. The effect of extender and storage time on cold-stored semen motility and vitality assessed by analysis of variance with repeated measurements

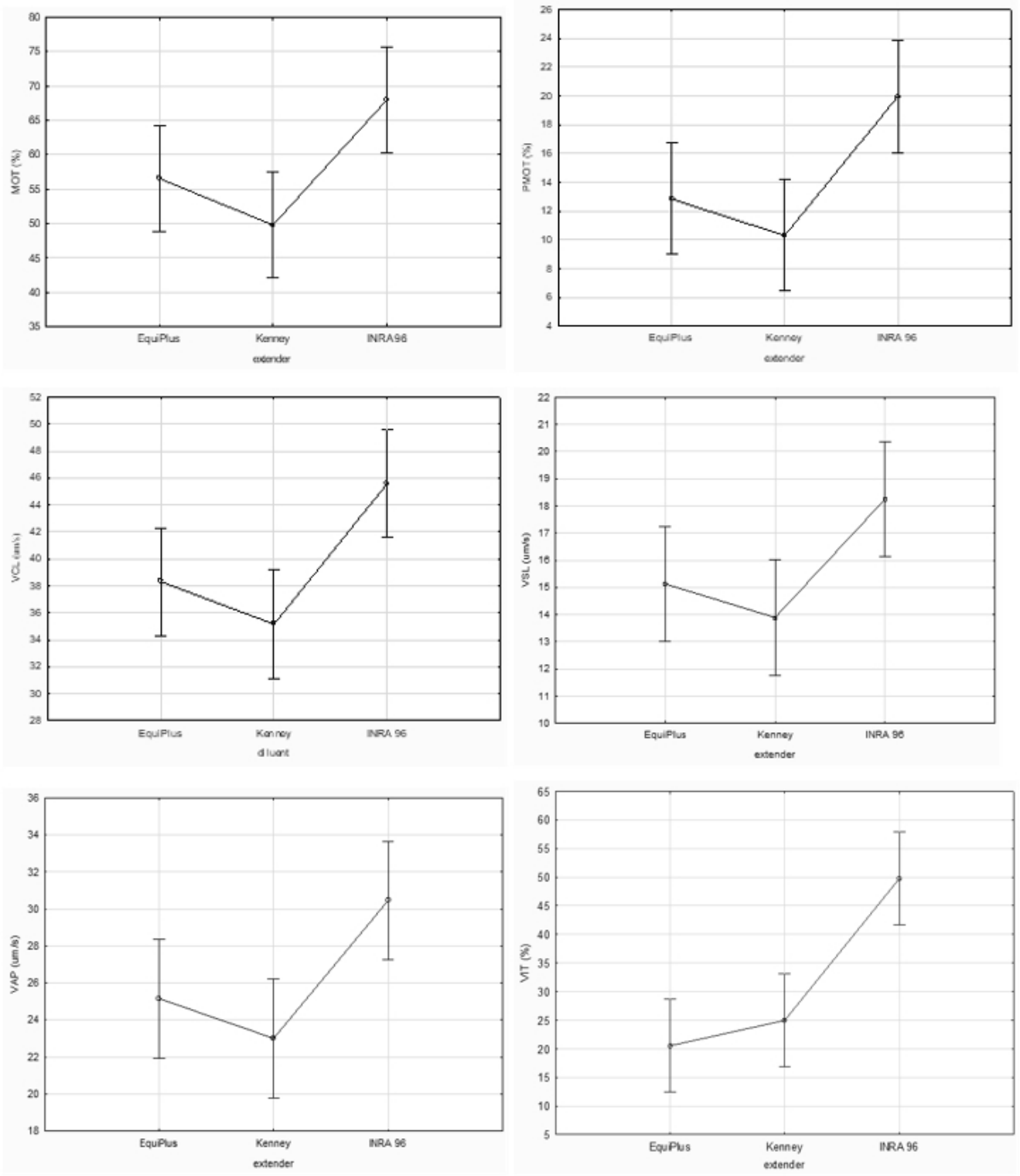
Parameter	extender	time	time*extender
MOT (%)	**	***	-
PMOT (%)	**	***	**
prog M/ml	+	***	-
VCL (µm/s)	**	***	*
VSL (µm/s)	*	***	-
VAP (µm/s)	**	***	-
VIT (%)	***	***	-

Abbreviations: MOT, total motility; PMOT, progressive motility; prog M/mL, million progressive sperm per mL; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; VIT, viability.

Data are presented as statistical significance *P*<0.001 (***), *P*<0.01 (**), *P*<0.05 (*), tendencies were declared at *P*<0.10 (+), *P*>0.10 (-).

The results of multiple comparisons using the Tukey HSD test (Figure 1) demonstrate that the overall protective effect of INRA 96 extender was significantly better than for semen diluted with Kenney (P<0.01). The VIT, PMOT, VCL and VAP were higher for ejaculates extended with INRA 96 (P<0.05) than those extended with EquiPlus. No difference was found between Kenney and EquiPlus extender.

To illustrate the effect of three different semen extenders in detail, the cooled semen parameters (motility characteristics) were evaluated at 2, 24, 48 and 72-hours (Table 2). Our results show that spermatozoa motility parameters differed significantly among extenders.



Abbreviations: MOT, total motility; PMOT, progressive motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; VIT, viability

Figure 1. Graphs of multiple comparisons of INRA 96, EquiPlus and Kenney using the Tukey HSD test within evaluated parameters

Table 2. Effect of extender on stallion semen motility parameters (mean ± SEM) for ejaculates (n=21) stored at 5 °C up to 72 hours

Parameter	2 hours			24 hours		
	EquiPlus	Kenney	INRA 96	EquiPlus	Kenney	INRA 96
MOT (%)	86.86 ± 3.39[1]	82.92 ± 4.12[1]	94.25 ± 1.48[1]	65.79 ± 5.14[a,b,2]	59.45 ± 5.64[a,2]	79.09 ± 4.19[b,2]
PMOT (%)	32.40 ± 3.78[a,1]	27.49 ± 3.95[a,1]	47.53 ± 3.68[b,1]	12.27 ± 2.74[2]	9.94 ± 2.97[2]	19.93 ± 3.26[2]
prog M/mL	76.13 ± 12.29[a,b,1]	65.83 ± 11.15[a,1]	103.68 ± 16.06[b,1]	30.89 ± 7.54[2]	22.79 ± 6.15[2]	46.50 ± 9.64[2]
VCL (µm/s)	60.72 ± 3.61[a,1]	55.05 ± 3.87[a,1]	74.28 ± 3.62[b,1]	39.45 ± 2.69[2]	35.98 ± 2.85[2]	46.47 ± 3.17[2]
VSL (µm/s)	28.99 ± 2.20[a,b,1]	26.74 ± 2.24[a,1]	35.01 ± 2.13[b,1]	15.05 ± 1.55[2]	13.85 ± 1.66[2]	18.38 ± 1.73[2]
VAP (µm/s)	45.45 ± 2.99[a,1]	41.34 ± 3.16[a,1]	56.33 ± 2.89[b,1]	25.51 ± 2.27[2]	23.35 ± 2.49[2]	30.85 ± 2.64[2]
Parameter	48 hours			72 hours		
	EquiPlus	Kenney	INRA 96	EquiPlus	Kenney	INRA 96
MOT (%)	48.64 ± 5.83[a,b,3]	34.78 ± 4.42[a,3]	59.64 ± 5.02[b,3]	28.42 ± 5.01[4]	22.04 ± 3.70[3]	38.90 ± 5.55[4]
PMOT (%)	4.80 ± 1.50[a,b,2,3]	2.72 ± 0.72[a,2]	8.39 ± 2.04[b,3]	1.87 ± 0.60[a,b,3]	1.05 ± 0.29[a,2]	3.92 ± 0.88[b,3]
prog M/mL	11.28 ± 3.46[2]	5.73 ± 1.34[2]	20.98 ± 6.46[2,3]	4.38 ± 1.47[a,b,2]	2.69 ± 0.75[a,2]	9.07 ± 2.73[b,3]
VCL (µm/s)	28.92 ± 2.03[3]	26.44 ± 1.24[3]	33.03 ± 2.54[3]	24.18 ± 1.43[3]	23.16 ± 1.12[3]	28.55 ± 1.66[3]
VSL (µm/s)	9.58 ± 0.82[3]	8.61 ± 0.57[2,3]	11.15 ± 1.05[3]	6.91 ± 0.59[3]	6.37 ± 0.38[3]	8.49 ± 0.62[3]
VAP (µm/s)	16.63 ± 1.48[3]	15.16 ± 0.90[3]	19.15 ± 1.90[3]	13.02 ± 1.07[3]	12.19 ± 0.61[3]	15.50 ± 1.06[3]

Abbreviations: MOT, total motility; PMOT, progressive motility; prog M/mL, million progressive sperm per mL; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity

[a,b] Different superscripts in a row within individual time periods indicate significant differences among extenders ($P<0.05$)

[1,2,3,4] Different superscripts within a row indicate significant differences among time periods within individual extender ($P<0.05$)

Generally, the values of all spermatozoa motility parameters were the highest in the INRA 96 extender over the monitored time. At the 2-hour interval, PMOT, VCL and VAP were significantly higher in INRA 96 compared to the other extenders. The prog M/mL and VSL were significantly higher in INRA 96 compared to Kenney. At 24-hours, MOT was significantly higher in INRA 96 compared to Kenney. PMOT tended toward a significant difference (P=0.06) between these two extenders. Twenty-four hours later (48-hours), only MOT was significantly higher in INRA 96 compared to Kenney and PMOT and prog M/mL tended toward a significant-difference (P=0.05 and 0.07, respectively). At the end of the experimental period (72-hours), MOT did not differ between INRA 96 and Kenney (P=0.07), while PMOT and prog M/mL significantly differed between these two extenders.

Figure 2 illustrates that spermatozoa viability differed significantly among extenders over time. In all four periods of storage, spermatozoa viability was significantly higher in INRA 96 than in Kenney and EquiPlus. At 2 hours, viability in INRA 96 was significantly higher than in Kenney (by 27.82%) and EquiPlus (by 22.06%). After 24, 48 and 72 hours of storage at 5 °C, the percentage of membrane-intact spermatozoa was significantly lower in semen diluted with Kenney (by 30.9, 29.3 and 28.9%, respectively) and EquiPlus (by 27.3, 24.8 and 25.0%, respectively) than in semen diluted with INRA 96.

Finally, the differences in the decrease of VIT, MOT and PMOT can be seen in Table 3. For all evaluated parameters, values of R^2 ranged from 0.15 to 0.64. In the cases of MOT and VIT, a linear regression was observed.

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Table 3. The dependence of semen motility and viability on time evaluated by regression (velocity of decrease in % per hour, prog M/mL per hour and µm/s per hour)

Parameter	Extender	R^2	First Derivative	Velocity of Decreasing in				Average velocity of Decreasing in			Absolute values of decreasing in		
				2 h	24 h	48 h	72 h	2-24 h	24-48 h	48-72 h	0-24 h	24-48 h	48-72 h
VIT	Kenney	0.27	y = -0.27	-0.27	-0.27	-0.27	-0.28	-0.27	-0.27	-0.27	-6.41	-6.41	-6.41
	EquiPlus	0.21	y = -0.23	-0.23	-0.23	-0.23	-0.23	-0.23	-0.23	-0.23	-5.56	-5.56	-5.56
	INRA96	0.15	y = -0.23	-0.23	-0.23	-0.23	-0.23	-0.23	-0.23	-0.23	-5.41	-5.41	-5.41
MOT	Kenney	0.56	y = - 0.88	-0.88	-0.88	-0.88	-0.88	-0.88	-0.88	-0.88	-21.01	-21.01	-21.01
	EquiPlus	0.48	y = - 0.82	-0.82	-0.82	-0.82	-0.82	-0.82	-0.82	-0.82	-19.66	-19.66	-19.66
	INRA96	0.53	y = - 0.79	-0.79	-0.79	-0.79	-0.79	-0.79	-0.79	-0.79	-19.05	-19.05	-19.05
PMOT	Kenney	0.64	$y = -1{,}35e^{-0{,}056x}$	-1.35	-0.35	-0.09	-0.74	-0.74	-0.19	-0.05	-17.78	-4.64	-1.21
	EquiPlus	0.52	$y = -1{,}66e^{-0{,}054x}$	-1.66	-0.45	-0.12	-0.93	-0.93	-0.25	-0.07	-22.33	-6.11	-1.67
	INRA96	0.60	$y = -2{,}15e^{-0{,}046x}$	-2.15	-0.71	-0.24	-1.30	-1.00	-0.43	-0.14	-31.20	-10.34	-3.43

Abbreviations: MOT, total motility; PMOT, progressive motility; VIT, viability

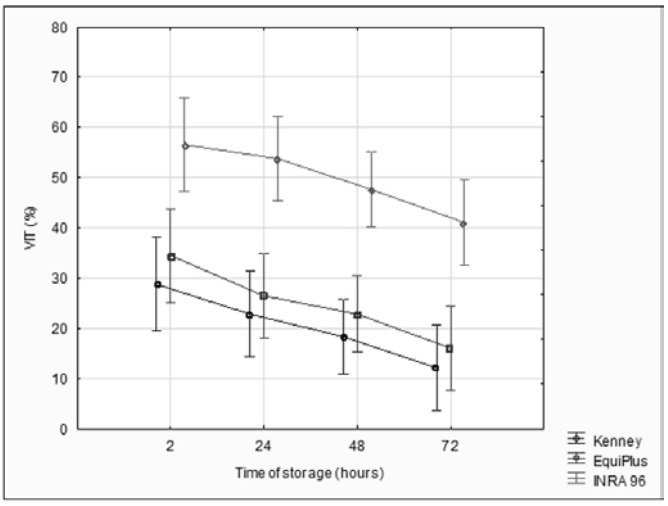

Figure 2. Percentage of vital spermatozoa in semen diluted in three different extenders during storage at 5 °C for 72 hours

In addition, an exponential regression was observed for PMOT, where the velocity of decrease was the fastest in the first 24 hours of storage. In the case of VIT and MOT, the slowest decrease was recorded for the INRA 96 extender (0.23% per hour and 0.79% per hour, respectively). The fastest decrease was observed in the Kenney extender (VIT 0.27% per hour and MOT 0.88% per hour). The opposite trend was observed in PMOT. The fastest decrease was found in the INRA 96 extender. The mean decrease in the first 24 hours in the INRA 96 extender was 1.30% per hour, 0.93% per hour in EquiPlus and 0.74% per hour in Kenney.

DISCUSSION

This study is the first study describing the quality of cooled semen extended with three milk-based extenders in breeding stallions after long sexual rest.

We have demonstrated that the semen characteristics of cold-stored semen were affected by the extender used and the storage time elapsed. Our findings related to ejaculate characteristics of stallions with along sexual rest are consistent with previous studies demonstrating the effect of extender (Webb et al., 2009; LeFrapper et al., 2010) and storage time on stallion semen parameters (Aurich et al., 2007; Janett et al., 2012) when collecting experimental semen samples preceded by repeated semen collection.

In the present study, the differences observed in semen motility among the extenders used agree with the findings of other authors for stallions without sexual rest (Pagl et al., 2006; LeFrapper et al., 2010; Janett et al., 2012; Neuhauser et al., 2018;). The good results for semen motility obtained when INRA 96 extender was used correspond with the special composition of this extender (Pillet et al., 2008). It is advantageous to use native phosphocaseinate as a protective agent in INRA 96 extender (da Silva et al., 2012). Moreover, other substances contained in this extender help to protect spermatozoa obtained after a long sexual rest against the harmful effects of prolonged storage at 5 °C during the 72-hour interval.

The different effects of extenders on spermatozoa motility parameters during prolonged preservation (Table 2) in our study correspond with previously published data (Pagl et al., 2006; Aurich et al., 2007; LeFrapper et al., 2010). The main objective of semen processing in preparation of a cooled insemination dose is to find the best extender that will maintain satisfactory quality during shipping until insemination (Heckenbichler et al., 2011). According to the motility parameters evaluated in our study, the best choice, from the extenders tested, would be INRA 96 when shipping takes about 24 hours in the case of irregularly collected stallions. Our results are in agreement with other studies, although performed with semen from regularly collected stallions, where semen extended with INRA 96 maintained appropriate quality for 24 hours after dilution (LeFrapper et al., 2010; Ortega-Ferrusola et al., 2011; Janett et al., 2012; Bolaños et al., 2014). Nevertheless, in subsequent periods (after 48 and 72 hours of storage at 5 °C), the EquiPlus and INRA 96 equalized. These results are inconsistent with LeFrapper et al. (2010) but the potential weakness of their study is that CASA thresholds are lacking and only two stallions were included. This may be an insufficient number for observation due to individual stallion variability, which was observed in previous studies (Webb et al., 2009; Janett et al., 2012). An insufficient number of observations could have caused a fluctuating effect on the observed parameters of stallion spermatozoa. In this

study semen samples were obtained from 11 clinically healthy and fertile stallions and the effect of extender on cold-stored semen characteristics was assessed using analysis of variance with repeated measurements. The benefit of Repeated Measures Design is that of reduced variance and bias of the model's error by controlling for factors that cause variability among subjects. The error term contains only variability within subjects and not the variability among subjects. By controlling for differences among subjects, this type of design can show much more statistical power.

As regards the detailed motility parameter results, it seems that compared to caseinate extenders, Kenney is the less favourable choice. This result is in agreement with previous studies where semen extended with Kenney reached lower quality in vitro compared to EquiPro (Pagl et al., 2006) or INRA 82, which is a previous version of INRA 96 (Ijaz and Ducharme, 1995). These results were supported by an in vivo study (Batellier et al., 2001). The Kenney extender was developed in 1975 (Kenney et al., 1975) and it represents a basic and cheap option for breeders or equine reproductive centres; however, the composition is very simple. Even when the milk in Kenney extender was substituted by 0.6% sodium caseinate, the total and progressive motility, kinematic parameters and percentage of spermatozoa with intact plasma membrane did not differ between Kenney and Kenney + 0.6% caseinate after 24-hour storage (Martins et al., 2016).

Only spermatozoa both motile and with intact plasma membrane can fertilise an oocyte in vivo (Yanagimachi, 1994) and thus, it is of interest to assess changes in these parameters over time during storage in different semen extenders (Love, 2012).

The sperm motility is highly correlated with sperm viability in stallions. Thus, despite individual variation among stallions, 70 - 80% of the sperm in a fresh ejaculate from a normal stallion are expectedly viable (Sieme, 2009). Theoretically, every motile cell is viable but not all viable cells are motile (Nagy et al., 1999). While the integrity of the plasma membrane covering the principal pieces of the sperm can be assessed by analysis of sperm motility, the integrity of the plasma membrane covering the acrosome is focused on the integrity of the outer acrosomal membrane using microscopy, fluorometers and flow cytometry. Fluorescent stains bind to and stain the DNA of sperm possessing defects of post-acrosomal plasma membrane of the sperm head. Non-viable cells can be determined using membrane-impermeable nucleic acid stains, which penetrate through a damaged plasma membrane and result in fluorescent signals of nuclei of dead spermatozoa. Propidium iodide cannot cross intact cell membranes, it gains access to nuclear DNA only when cell membranes are damaged and non-viable. Bound to nucleic DNA, PI fluoresces red in response to excitation (Sieme, 2009). The fluorochromes used here label the DNA in the sperm head and therefore indicate the sperm head membrane status only. Sperm membrane damage usually starts over the flagellar subdomain, therefore there are sperm cells which have intact head membrane (so they are classified as viable) but are immotile due to the damaged tail membrane (Nagy et al., 1999). The reason for the lower values of sperm viability found in this study is not clear (the procedure of preparation of the preparation for viability assessment in the SCA module was followed exactly and was the same for all evaluated samples, as well as the analysis of samples). Based on lower percentage of viable sperm compared to the percentage of motile sperm, poor calibration of SCA vitality module for viability analysis may be a possible explanation, leading to underestimation of percentage of sperm viability in the sample. Dhurvey et al., (2012) confirm that the disadvantages of CASA are related to the extreme need of validation, quality control and standardization of the measures realized. It is vital to use appropriate optics and the best illumination possible to enhance the contrast of the spermatozoa heads, which in turn facilitate the manual selection of thresholds. Nevertheless, these results are significant in the relative comparison of the values in terms of the effect of time and extenders used.

In our study, spermatozoa viability differed significantly among extenders over time. Our results related to plasma membrane status are different compared to

previously published data where no significant effect of milk caseinates was found in regularly collected stallions (Pagl et al., 2006; Janett et al., 2012). The situation changed when semen was centrifuged before cooling (Pagl et al., 2006). After 24 and 72 hours of storage, the extender containing defined milk protein preserved the plasma membrane status at a significantly higher level (Pagl et al., 2006). The positive effect of INRA 96 on spermatozoa plasma membrane during storage could be a consequence of NPPC action where this part of the extender decreases efflux of cholesterol and thus preserves the phospholipid:cholesterol ratio important for maintenance of proper fluidity of spermatozoa membrane (Bergeron and Manjunath, 2006). This positive effect is quite important when semen needs to be stored for a long time period in 5 °C in order to retain its fertilizing capacity. This positive effect of NPPC is also supported by high concentration of sugars present in INRA 96 which create physical protection and energy source for the spermatozoa cells during preservation (Pillet et al., 2008). Therefore, semen diluted with INRA 96 extender has the best predispositions for maintaining its viability and for subsequent use for insemination from among the tested extenders in our study.

Prolonged cold storage of ejaculated sperm usually causes decreased motility and kinematic parameters (Rigby et al., 2001; Pagl et al., 2006; Bolaños et al., 2014; Love et al., 2015;). Also, Price et al. (2008) and Webb et al. (2009) suggested that the motility and velocity of equine semen are often impaired progressively during storage; this has been confirmed in extenders such as INRA 96 and equivalent (Webb et al., 2009). These findings agree with our results in which a decrease in the values of all evaluated parameters can be seen. Halo et al.'s (2018) results for stallion spermatozoa motility and progressive motility showed a decreasing trend in each time interval. However, after six hours their results showed a rapid percentage drop-off in semen parameters. In our study, INRA 96 kept the highest PMOT in comparison with the other two extenders during the whole storage period. Batellier et al. (2001) stated that INRA 96 is an efficient extender for preserving fertility potential of spermatozoa for at least 24 h. It also seems to protect spermatozoa cells during long-term storage with more efficiency than traditional milk extenders.

CONCLUSIONS

In conclusion, a defined milk protein extender results in comparable or better semen parameters during cold storage than an extender based on skimmed milk when the semen is from stallions after a long sexual rest. The extenders with defined caseinate components (INRA 96 and EquiPlus) showed a slower decline of PMOT at 48 and 72 hours of storage in comparison with skimmed milk-based extender. Moreover, when diluted with INRA 96, the extender containing NPPC, the quality is prolonged in comparison to other extenders (EquiPlus, Kenney) for at least to 24 hours post- collection and dilution. The best results obtained with extender INRA 96 showed that the semen collected after a long period of sexual abstinence could be used for producing insemination doses of cooled semen during the first 24 hours of storage.

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