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Effect of Two Freezing Extenders on Characteristic of Fresh and Frozen-Thawed Semen in Endangered Old Kladruber Stallions – A Pilot Study

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ABSTRACT

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The aim of the study was to evaluate the effect of two different extenders on sperm characteristic before equilibration and post-thaw in the endangered Old Kladruber stallions. Also, the response of individual stallions to the extenders used was tested. Semen was collected from six stallions every other day within one week. After centrifugation of the collected sperm-rich fraction, the supernatant was removed and sperm pellets were divided to two aliquots; these were diluted either with Gent (Minitube, Germany) or privately manufactured lactose-EDTA-egg yolk extender (Lact). Three cryopreserved insemination doses (IDs) from each extender (Gent and Lact) were prepared for each stallion from one collection (108 samples from six stallions in total). As a parameter of quality, the motility (computer assisted sperm analysis), viability (fluorescence staining), and morphology (eosin/nigrosine staining) were evaluated after dilution with freezing extenders (fresh) and after thawing (frozen-thawed). The different effects of chosen extenders on the quality of fresh semen were only manifested in higher kinematic parameters of sperm when the Lact extender was used. However, in frozen-thawed samples, the Gent extender yielded significantly better results in all of the evaluated parameters. The representation of sperm subpopulation was significantly influenced by extender in fresh as well as frozen-thawed samples; moreover, we found a significant effect of freezing on the distribution of these subpopulations. The response of individual stallions to chosen extenders was evident in the different quality of fresh as well as frozen-thawed IDs; Gent extender yielded better frozen-thawed IDs. Based on our results, among others describing quality parameters of ejaculate in endangered Old Kladruber stallions, we can recommend using Gent extender for the production of frozen-thawed IDs.

Keywords: reproduction; horse; sperm; cryopreservation; semen extender

The Old Kladruber horse has been bred in the Czech Republic for more than four hundred years. This breed, with unique characteristics and high cultural and historical value (Vostra-Vydrova et al. 2016), is an important gene resource of the Czech Republic and the population has been fully closed since 2002 (Hofmanova et al. 2015). Since the population of this gene resource breed is small, preservation of breeding material (i.e. sperm) is crucial for the preservation and protection of this baroque breed for the future.

Long-term preservation of sperm is possible via cryoconservation. Based on the subjective experi-

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ence of a manager of the only facility in the Czech Republic dealing with cryoconservation of this breed, these stallions are mostly "poor" freezers (Muller, pers. comm.). This might be a consequence of pedigree, as Squires (2013) reported the poorest quality in the Friesian, Andalusian, and Saddlebreds; moreover, quite high inbreeding depression in this breed (Vostra-Vydrova et al. 2016) might negatively interfere with reproduction (Roldan et al. 2006)

Even though the cryopreservation process was introduced many years ago, the possibility of increasing effectiveness has been studied intensively across species since that time (Muchlisin et al. 2015; Zhang et al. 2015). Although our knowledge and techniques for freezing stallion ejaculate have improved within the last 20 years, a considerable proportion of stallions are still not suitable for semen freezing (Katila 2001).

The most likely reason for this is considerable variation among individual stallions in how well their semen retains its fertilizing capacity after freezing and thawing. One of the possible approaches in enhancing the quality of cryopreserved semen is the selection of only males with high quality ejaculate. However, when the ejaculate of stallions belonging to genetic reserves needs to be frozen, the selection of stallions is impossible. Even though some stallions can have problems with cryotolerance, it is only a matter of finding a suitable freezing extender to produce fertile frozen insemination doses (Loomis and Graham 2008). Generally, one would test freeze semen in several extenders and then determine which extender provides the highest post-thaw motility (Squires 2013). That extender would then be used to freeze all of the semen for that particular stallion. However, testing frozen-thawed semen is economically demanding, time-consuming, and laborious. Therefore, it would be more effective to choose the most suitable extender already before the semen is cryopreserved. In the case of the endangered Old Kladruber stallions, which are collected fitfully and where different stallions are introduced every time, the individual response to extender immediately after dilution might be useful knowledge for the subsequent production of cryopreserved semen.

Therefore, the aim of the study was to evaluate the effect of two different extenders on sperm characteristic before equilibration and post-thaw in the endangered Old Kladruber stallions. Moreover, the response of individual stallions to extenders used was also tested.

MATERIAL AND METHODS

Semen collection. The collection of ejaculate was performed in a certified equine reproductive centre (ERC s.r.o., Pardubice-Mnětice, Czech Republic). Before experimental collection, each stallion was collected 3–4 times. Semen (only sperm-rich fraction) used for this experiment was collected using the open type of artificial vagina; six stallions of the Old Kladruber horse breed were collected three times per week (Monday, Wednesday, and Friday) in the presence of a mare in heat.

Semen processing. Immediately after collection, the semen volume (laboratory scale; Minitube, Germany), concentration (Photometer SDM 1; Minitube), and motility of sperm (Eclipse E600; Nikon, Japan) were evaluated. Collected sperm-rich fractions were pre-diluted with a skim milk-based extender and centrifuged at 650 *g* for 15 min. Afterwards, supernatant was removed and sperm pellets were divided into two aliquots which were diluted either with extender Gent (Minitube) or a privately manufactured (in ERC s.r.o.) lactose-EDTA-egg yolk extender (Lact) consisting of lactose, distilled water, glycerol, buffers, antibiotics, EDTA, and 20% (v/v) egg yolk. Extended ejaculates produced in this way were diluted to a final concentration of at least 350×10^6 of progressively motile sperm/insemination dose (ID) and loaded into 0.5 ml straws. Then, the straws were equilibrated at 5°C for 2 h and horizontal freezing was performed in a Styrofoam box (Animal Reproduction Systems, USA). Straws were thawed at 37°C for 30 s. Three ID from each extender (Gent and Lact) were prepared for each stallion from one collection (i.e. 18 samples per stallion during collections each week, amounting to 108 samples for six stallions).

Sperm quality parameters assessment. The qualitative parameters were evaluated in fresh sperms 10–15 min after freezing extenders addition and in frozen-thawed samples after 5 min of incubation at 37°C.

Computer-assisted sperm analysis. The motility of sperm was measured using a CASA module (Nis-Elements, Version 4.30; Laboratory Imaging, Czech Republic). The evaluation was based on the

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analysis of 41 consecutive, digitized images, which were taken at a time lapse of 0.66 s with a camera at a frequency of 60 fps (DMK 23UM021; Imaging Source, Germany). Then, a 3 µl drop of each semen sample was placed in a 37°C pre-warmed Makler chamber (Sefi-Medical Instrument, Israel) and six fields per sample were evaluated using phase contrast microscopy (Eclipse E600; Nikon) equipped with a heating plate pre-warmed at 37°C at 100× magnification. The following motility parameters were evaluated: total motility (TMOT, %), progressive motility (PMOT, %), straightness coefficient (STR, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), and straight line velocity (VSL, µm/s). The threshold values of STR and VAP for progressive motility were 50% and 30 µm/s, respectively. The sperm were considered motile when VAP > 15 µm/s. The distribution of sperm into slow, moderate, and rapid subpopulations was based on mean values of STR, VAP, VCL, and VSL after clustering analysis (see Statistical analysis).

Viability. Overall, 100 µl of sperm suspension was incubated with staining solution consisting of 1 µl of 0.3% formaldehyde, 2.1 µl of carboxyfluorescein diacetate (CFDA) (0.46 mg/ml of dimethyl sulfoxide) (Sigma-Aldrich, USA), and 2.1 µl of propidium iodide (PI) (0.5 mg/ml of physiologic solution) (Sigma-Aldrich). The percentage of live sperm was evaluated under a fluorescent microscope Eclipse E600 (Nikon) counting at least 200 sperm per slide.

Morphology. Sperm morphology was examined by light microscopy (Eclipse E600; Nikon) of smears stained with eosin and nigrosine (Brito et al. 2011). At least 200 sperm per slide were counted to determine the percentage of morphologically normal sperm.

Statistical analysis. Data were statistically evaluated using STATISTICA software (Version 10, StatSoft, CZ). The effect of Gent and Lact extender on TMOT, PMOT, chosen kinematic parameters, viability, and morphology were evaluated with the Student's *t*-test. For motility evaluation, *k*-means cluster analysis was used to classify sperm into subpopulations. The Euclidean distances were computed from four variables – STR, VAP, VCL, and VSL. To determine differences between subpopulations, the χ^2 test was used. Data were evaluated at the $P < 0.05$ level and are presented as Least Squares Means (LSM) ± standard error of the means (SEM).

RESULTS

The aim of this study was to evaluate the effect of two freezing extenders on the functional characteristics of fresh extended and frozen-thawed sperm in the Old Kladruber stallions, also with focus on the response of individual stallions.

The total (TMOT) and progressive (PMOT) motility of sperm in fresh samples extended with Gent and Lact after centrifugation (Table 1) did

Table 1. Parameters of ejaculates extended after centrifugation either with Gent or Lactose extender (mean ± SEM)

	Fresh Gent[1]	Fresh Lact[2]	F-T Gent[1]	F-T Lact[2]
TMOT (%)	70.3 ± 4.1	79.9 ± 3.4	28.9 ± 1.9^{A}	16.9 ± 1.3^{B}
PMOT (%)	53.4 ± 3.7	57.6 ± 3.6	23.1 ± 1.5^{A}	13.1 ± 1.1^{B}
STR (µm/s)	72.8 ± 0.2	73.4 ± 0.2	86.0 ± 0.1^{A}	82.9 ± 0.2^{B}
VAP (µm/s)	98.1 ± 0.3^{a}	99.8 ± 0.3^{b}	75.9 ± 0.3^{A}	67.9 ± 0.4^{B}
VCL (µm/s)	178.4 ± 0.6^{a}	161.4 ± 0.5^{b}	136.6 ± 0.5^{A}	133.2 ± 0.8^{B}
VSL (µm/s)	73.6 ± 0.4^{a}	75.5 ± 0.3^{b}	67.2 ± 0.3^{A}	59.1 ± 0.4^{B}
Live sperm (%)	86.6 ± 1.2	81.9 ± 2.8	48.3 ± 1.7^{A}	41.7 ± 2.0^{B}
Normal sperm (%)	75.7 ± 1.7	80.1 ± 1.8	75.6 ± 1.4	73.8 ± 1.1

TMOT = total motility, PMOT = progressive motility, STR = straightness coefficient, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity

^{a,b}significant differences in a row between fresh samples ($P < 0.05$)

^{A,B}significant differences in a row between frozen-thawed (F-T) samples ($P < 0.05$)

[1]except the percentage of normal sperms, all evaluated parameters significantly differ between Fresh Gent and frozen-thawed (F-T) Gent samples ($P < 0.05$)

[2]all evaluated parameters significantly differ between Fresh Lact and frozen-thawed (F-T) Lact samples ($P < 0.05$)

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not differ (P = 0.07 and P = 0.41, respectively). The cryoconservation significantly decreased the TMOT and PMOT when Fresh Gent and Frozen-Thawed Gent (F-T Gent), Fresh Lact and Frozen-Thawed Lact (F-T Lact) were compared (P < 0.05). Frozen-thawed samples extended with Gent maintained significantly higher TMOT and PMOT compared to Lact samples (P < 0.05).

The STR values were similar in fresh Gent and Lact extender (P = 0.64). The VAP and VSL were significantly lower in Gent compared to Lact (P < 0.05), whereas the VCL was significantly higher in Gent (P < 0.05). In frozen-thawed samples, all kinematic parameters were significantly higher in Gent compared to Lact samples (P < 0.05).

The percentage of live sperms in samples extended either with Gent or Lact did not differ (P = 0.13) (Table 1) and the situation was similar when the percentage of normal sperm was evaluated (P = 0.08). In frozen-thawed samples, all evaluated parameters were significantly higher in Gent compared to Lact extender (P < 0.05), except for the percentage of normal sperm (P > 0.05) (Table 1).

In fresh samples, the percentage of sperm belonging to slow and moderate subpopulation was significantly lower and higher in Gent compared to Lact extender, respectively (P < 0.05) (Figure 1). The proportion of sperm clustered in the rapid subpopulation did not differ between Gent and Lact in fresh samples (P > 0.05). There were significantly less and more sperm in the slow and rapid subpopulations in frozen-thawed samples extended with Gent compared to Lact (P < 0.05). The cryopreservation process significantly increased slow and rapid and decreased the moderate subpopulation in F-T Gent compared to Fresh Gent samples. The situation was similar in Lact samples, except that the proportion of rapid subpopulation did not change between Fresh and F-T samples.

The influence of individual stallion on TMOT, PMOT, live and normal sperm is shown in Supplementary Table S1. In fresh samples, the TMOT was affected by extender in two out of six stallions with significantly higher values in both cases when Lact extender was used (P < 0.05). We did not observe any effect of extender in individual stallions on PMOT in fresh samples. The percentage of live and normal sperm in fresh samples was significantly influenced by extender type in stallion No. 5 and 4, respectively. In frozen-thawed samples the TMOT significantly differed between Gent and Lact extender in three stallions; in all cases, Gent was superior (P < 0.05). The PMOT was significantly higher in Gent samples in three stallions compared to Lact samples (P < 0.05). Similarly to fresh samples, the frozen-thawed samples reached a similar percentage of live sperm, except stallion No. 3, where the ratio increased by 12% in Gent extender.

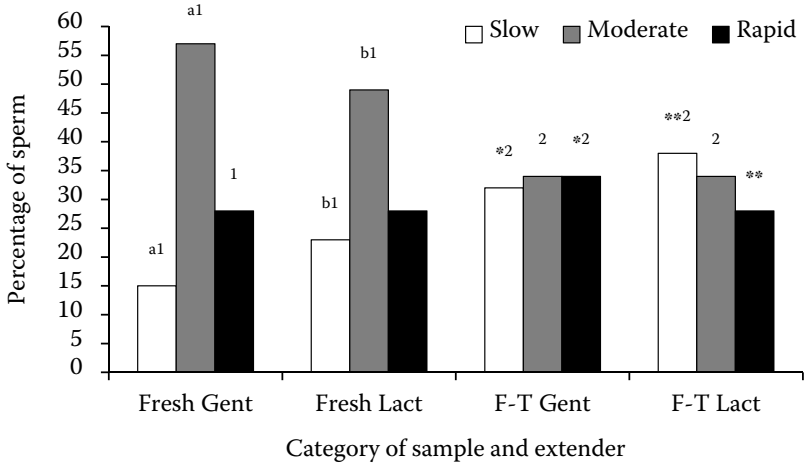

Figure 1. Distribution of sperm subpopulations (slow, moderate, rapid) in fresh and frozen-thawed samples extended with either Gent or Lact extender

[a,b]significant differences within individual subpopulations between Fresh Gent and Fresh Lact samples (P < 0.05)

[*,**]significant differences within individual subpopulations between frozen-thawed (F-T) Gent and Lact samples (P < 0.05)

[1,2]significant differences within individual subpopulations between fresh and frozen-thawed (F-T) Gent and Lact samples (P < 0.05)

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The effect of stallion on the percentage of sperm belonging to slow, moderate, and rapid categories of movement in samples extended with either Gent or Lact is presented in Supplementary Table S2. The proportion of the slow subpopulation in fresh Gent and Lact samples differed significantly in individual stallions. In five out of six and four out of six stallions, we found a lower representation of slow and moderate subpopulations, respectively, in the Gent samples ($P < 0.05$). In two out of five stallions, the rapid subpopulation significantly increased in Gent and in three out of five stallions in Lact extender ($P < 0.05$). When the effects of different extenders on the distribution of sperm in subpopulations in frozen-thawed samples were analyzed, we found very equal patterns. A significantly lower ratio of the slow subpopulation was found in three stallions in Gent and three stallions in Lact ($P < 0.05$). The proportion of the moderate subpopulation differed between extenders in two stallions; one had better results with Gent and one with Lact. In three out of six stallions, the rapid subpopulation was significantly increased in Gent and it was significantly increased in three out of six stallions in Lact extender.

DISCUSSION

The immediate effect of two freezing extenders on total and progressive motility in Old Kladruber stallion fresh sperm samples was not manifested 10–15 min after dilution in the present study. The same results were published by Mantovani et al. (2002) in stallions and Jerez et al. (2016) in rams. Phetudomsinsuk et al. (2009) also did not observe any effect of various freezing extenders on the mentioned parameters in stallions, even after 3 h of storage at 5°C. Although the effect of extenders on total and progressive motility was not as clear when fresh samples were evaluated in frozen-thawed samples, Gent explicitly dominates over Lact extender. Moreover, the quality of frozen-thawed semen in Old Kladruber stallions presented in this study is relatively low, even when the fresh semen fulfilled the criteria for quality ejaculate after centrifugation and dilution (Loomis and Graham 2008). This distinct decrease of quality is probably a consequence of osmotic stress, which could be reduced for example with the addition of low molecular weight amides such as cryoprotectants to extenders (Alvarenga et al. 2005), whose positive effect on cryosurvival was also proven in the highly endangered Przewalski's horse (Pukazhenthi et al. 2014). Moreover, an increased freezing potential of poor freezing stallions when amides are used is expected (Pena et al. 2011).

It is meaningful to also evaluate CASA kinematic parameters when the effect of different types of extenders on sperm quality needs to be evaluated objectively (Folkova et al. 2016). In the ejaculates from the Old Kladruber stallions, the VAP, VCL, and VSL significantly differed between chosen extenders immediately after dilution. Interestingly, the differences in VAP and VSL were higher in Lact by 1.7 and 1.9 μm/s, respectively, but VCL was higher by 17 μm/s in Gent. In full size purebred stallions, only VCL was affected by extender after 3 h of incubation at 5°C (Phetudomsinsuk et al. 2009), suggesting the possibly higher sensitivity to semen extender composition in Old Kladruber stallions. Clearly higher values of VCL in ejaculates where Gent extender was used might provide useful information on better cryotolerance (Ortega-Ferrusola et al. 2009) and fertilizing ability (De Geyter et al. 1998) of sperm diluted with this extender. This was supported in our study because Gent extender maintained post-thaw motility at a significantly higher level than Lact extender.

Even though the immediate influence of extenders used in this study on kinematic parameters of sperm was manifested, we did not see such an effect on the viability and morphology of sperm. Similarly, Phetudomsinsuk et al. (2009) reported that there was no effect of different freezing extenders on viability after 3 h of incubation. Moreover, the situation was similar (no effect on viability and morphology) when different extenders were used for the production of cooled semen (Pugliesi et al. 2012). Similarly to our results, viability was affected by extender in frozen-thawed semen also in the study by Morrillo Rodriguez et al. (2011).

In light of the practical utilization of CASA outputs and the fact that ejaculate consists of heterogeneous populations of sperm whose analyses give us better biological meaning (Simonik et al. 2015), the representation of three subpopulations of sperms (slow, moderate, and rapid) in extended ejaculates was evaluated in the present study. Moreover, for the first time, we published data concerning the effect of different extenders on sperm subpopulation distribution in fresh and subsequently frozen-thawed stallions' ejaculate. In fresh samples where

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Gent extender was used, the percentage of sperm in the slow subpopulation decreased while the proportion of sperm in the moderate subpopulation increased compared to in the Lact extender. Nevertheless, the rapid subpopulation was not affected by the examined extenders, which could imply the similar fertilizing potential of sperm diluted with either Gent or Lact as a positive relationship of the representation of subpopulations with the highest velocity values with fertilizing ability is suggested (Quintero-Moreno et al. 2003). However, this suggestion was not proved in our study. In frozen-thawed samples where Gent extender was used, the proportions of slow, moderate, and rapid subpopulations were lower, the same, and higher, respectively, compared to the Lact extender. In general, changes in sperm subpopulation distribution between Gent and Lact frozen-thawed samples were not as remarkable as marked differences in total and progressive motility, which indicates that the freezing process primarily reduced the number of motile sperm; however, kinematic patterns are more or less sustained.

In the case of males belonging to endangered species or genetic reserves, every ejaculate needs to be processed for the preparation of frozen ID (Roldan et al. 2006). Thus, choosing the most suitable extender for individual stallions is a crucial step (Graham 1996). In the case of Old Kladruber stallions, we found that in four out of six stallions (67%) the fresh sperms maintained similar total motility in both extenders. Progressive motility was not affected and the percentage of live and morphologically normal sperm in individual stallions was in general the same, therefore we are not able to detect any extender preferences in fresh samples. The situation changed after freezing-thawing; the Gent extender maintained significantly higher total and progressive motility in three and three stallions, respectively.

Evaluation of CASA-derived kinematic parameters revealed significant differences between extenders when fresh and frozen-thawed samples were evaluated in individual stallions, which corresponds to individual differences in other breeds (Ortega-Ferrusola et al. 2009; Rezagholizadeh et al. 2015). The determination of slight changes in sperm motility is one of the advantages of the CASA system (Verstegen et al. 2002). Therefore, we highly recommend its use as an equivalent to subjective analysis when highly valuable stallions are tested for extender preference, because we gained better knowledge about the preference in individual stallions in our study when STR, VAP, VCL, and VSL were taken into account.

We found a significant effect of extender in individual stallions regarding the distribution of sperm subpopulations; however, there was no clear pattern in fresh samples, similar to kinematic parameters. In frozen-thawed samples, the distribution of sperms into slow, moderate, and rapid subpopulations was equal for individual stallions between the tested extenders. The effect of stallion on the distribution of sperm subpopulations was also published by Ortega-Ferrusola et al. (2009). The relationship between slow, moderate, and rapid subpopulations of sperm and fertility *in vivo* is unclear; however, Gibb et al. (2014) highlighted a positive relationship of rapidly moving sperm and pregnancy rates in mares. A promising method to decrease the individual response of stallions to semen extenders could be the involvement of Caceres extender in the freezing program of Old Kladruber stallions, which, according to Morillo Rodriguez et al. (2011), diminished stallion-to-stallion variability when they compared this extender to Gent in their study.

In conclusion, this study demonstrates the immediate effect of two extenders on kinematic parameters of fresh sperm in stallions belonging to the Old Kladruber horse breed. The quality of frozen-thawed semen was better when Gent extender was used in general. The preference of stallions for extender when fresh samples were evaluated was not as clear as in frozen-thawed samples where Gent extender showed better results. However, if there is an effort to increase the quality of frozen-thawed semen in Old Kladruber stallions, the techniques for the selection of the best quality sperm from fresh ejaculate could help reach this goal (Morrell 2012).

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