Received: 20 May 2021 | Accepted: 17 June 2021

DOI: 10.1111/rda.13980

ORIGINAL ARTICLE

Reproduction in Domestic Animals WILEY

Combined cryopreservation of canine ejaculates collected at a one-hour interval increases semen doses for artificial insemination without negative effects on post-thaw sperm characteristics

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Abstract

A limiting factor in canine artificial insemination (AI) is the low number of insemination doses obtained per ejaculate. In this study, semen was collected from dogs ($n = 28$) either once and frozen directly after collection or the same dogs were submitted to a dual semen collection with a 1-hr interval and the two ejaculates were combined for cryopreservation. We hypothesized that combining two ejaculates increases semen doses per cryopreservation process without negative effects on semen characteristics. Total sperm count was lower in semen from a single semen collection in comparison with the combination of the first and second ejaculate of a dual semen collection ($p < .001$). The percentage of motile and membrane-intact spermatozoa determined by computer-assisted sperm analysis (CASA) in raw semen did not differ between single and combined dual ejaculates and was reduced ($p < .001$) by cryopreservation to the same extent in single (motility 73.7 ± 1.8%, membrane integrity 65.6 ± 2.2%) and combined dual ejaculates (motility 72.7 ± 2.3%, membrane integrity 64.6 ± 2.5%). The percentage of spermatozoa with morphological defects increased after cryopreservation ($p < .001$) but was similar in single and combined dual ejaculates. The CASA sperm velocity parameters decreased with cryopreservation ($p < .001$) but did not differ between single and combined dual ejaculates. The number of insemination doses increased from 2.7 ± 0.4 for single to 4.7 ± 0.8 for combined dual ejaculates ($p < .01$), based on 100 million motile spermatozoa per frozen-thawed semen dose. In conclusion, combining two ejaculates collected at short interval for one cryopreservation process increases the number of AI doses without compromising semen quality.

KEYWORDS

collection frequency, cryopreservation, dog, semen

1 | INTRODUCTION

Although the dog is the first mammalian species were a pregnancy from artificial insemination (AI) has been reported (Spallanzani, 1785), only during the last decades AI with cooled-shipped or cryopreserved semen is increasingly applied in in this species (Farstad, 2000). A limiting factor in canine AI is the low number of insemination doses obtained per single ejaculate. This is even more true for cryopreserved than for cooled-shipped or fresh semen (Nöthling & Shuttleworth, 2005). While one ejaculate may yield between 300 and 400 cryopreserved semen doses in cattle (Humblot et al., 1993) and 10 to 15 doses in horses (Aurich et al., 2020), usually only one to three cryopreserved semen doses per ejaculate can be collected in dogs (Farstad, 2000). With the laboratory effort associated with freezing one ejaculate increasing only marginally with the number of straws or AI doses per freezing process, semen cryopreservation in dogs is, thus, far less economic than in cattle and horses. In order to obtain the number of AI doses desired by dog breeders, male dogs often have to travel repeatedly to the veterinary centre where semen collection and freezing is performed. Both for economic and animal welfare reasons (Herbel et al., 2020), it would be desirable to collect more than one ejaculate within a shorter time window before freezing is done. On the other hand, semen quality decreases during storage at room temperature and semen should, thus, be frozen within a reasonable time after collection. When two ejaculates were collected from dogs at a 1-hr interval, there was no difference in sperm motility and percentage of morphologically normal spermatozoa (England, 1999), but both ejaculates were assessed shortly after collection and no attempt was made for liquid storage or cryopreservation.

In the present study, semen was collected from dogs either once and frozen directly after collection or the same dogs were submitted to a dual semen collection at 1-hr interval and the two ejaculates were frozen directly after the second ejaculate had been obtained. We hypothesized that combining two ejaculates collected 1 hr apart for cryopreservation is without negative effects on post-thaw semen parameters but results in more insemination doses per dog in one cryopreservation process.

2 | MATERIALS AND METHODS

The study was approved by the Ethics and Animal Welfare Committee of Vetmeduni Vienna (study number ETK-014/01/2020). Informed consent was obtained from all dog owners before their animals participated in the study.

2.1 | Animals

Dogs to be included into the study were recruited from breeders who are regular clients of Vetmeduni Vienna or members of a local kennel club. Out of an initial number of 46 dogs, 11 were excluded for azoospermia ($n = 3$), general health problems ($n = 1$), pre-pubertal age ($n = 1$) or because semen collection was not possible ($n = 6$). The remaining 35 dogs were 5.1 ± 3.3 (mean ± *SD*) years old (range 1.2 - 13.2 years) and belonged to the following 19 breeds: Beagle ($n = 9$), English Cocker Spaniel, Border Collie, Australian Shepherd ($n = 3$ each), Miniature Schnauzer, Poodle, Terrier Brasileiro ($n = 2$ each), Keeshond, White Swiss Shepherd Dog, Weimaraner, Czechoslovakian Wolfdog, Tervuren, Bernese Mountain Dog, Rhodesian Ridgeback, Labrador Retriever, Flat Coated Retriever, Cavalier King Charles Spaniel and American Staffordshire Terrier ($n = 1$ each). Before entered into the study, dogs had at least one week of sexual rest.

2.2 | Experimental design

All dogs were submitted for two semen collection sessions 1 week apart. At one occasion, only one ejaculate was collected, processed immediately after collection and the sperm-rich fraction cryopreserved within 1 hr (protocol 'single', see Figure 1). On the other occasion, two ejaculates were collected at an approximate 1-hr interval (57 ± 6 min, range 45 - 73 min), the two sperm-rich fractions were processed for cryopreservation and combined before filling the processed semen into straws (protocol 'dual'). Ejaculates were thawed after storage for at least 24 hr in liquid nitrogen (−196°C). Semen analysis (see 2.3) was thus performed in raw semen and in frozen-thawed semen.

2.3 | Semen collection and semen analysis

Fractionated semen collection was performed by digital manipulation as described previously (Seager et al., 1975). Swabs with vaginal secretions from an oestrous female dog were used to stimulate mating behaviour and, in addition, a non-oestrous female Beagle was brought into the examination room as an additional stimulus if needed.

Immediately after collection, the sperm-rich fraction of all collected ejaculates was analysed for volume, pH and sperm concentration as described previously (Koderle et al., 2009). In both, raw semen and in semen processed for cryopreservation, the sperm concentration was determined by NucleoCounter (ChemoMetec) and the total sperm count was calculated. Semen motility was estimated in raw semen at 40 × magnification under a phase-contrast microscope. In processed semen (i.e. after addition of Uppsala 2 extender, Figure 1) and in frozen-thawed semen, after dilution with a TRIS-fructose-citric acid buffer (Linde-Forsberg, 2001; Schäfer-Somi & Aurich, 2007) to a concentration of 25 million/ml, the percentage of motile spermatozoa, and sperm velocity parameters curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), amplitude of lateral head displacement (ALH) and straightness (STR) were determined by computer-assisted sperm analysis (CASA;

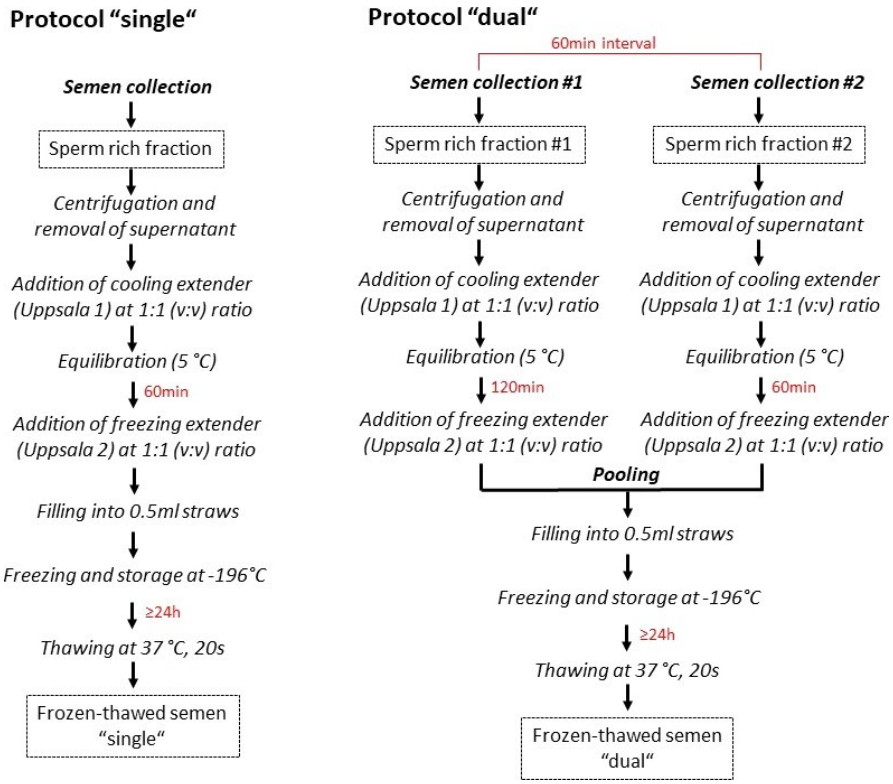

FIGURE 1 Experimental design for semen collection, processing and analysis

SpermVision, Minitube) as described previously (Schäfer-Somi & Aurich, 2007; Schäfer-Somi et al., 2006). At the same steps of semen processing, sperm morphology and membrane integrity were analysed. For the assessment of membrane integrity, SYBR-14/PI (Minitube) was used (Schäfer-Somi et al., 2006). The percentage of morphologically normal sperm was analysed in unstained samples fixed in Hancock's solution. Per sample, 200 spermatozoa were evaluated under a phase-contrast microscope at 1,000 × magnification with oil immersion as described (Koderle et al., 2009).

2.4 | Cryopreservation

The sperm-rich fraction of the ejaculate was cryopreserved with a computer-controlled rate freezing machine (Ice Cube 14 M, Sylab) as described previously (Koderle et al., 2009; Schäfer-Somi et al., 2006). In brief, ejaculates were centrifuged at 800 × g for 8 min and the supernatant was removed until approximately 5 mm above the sperm pellet. Subsequently, the centrifuged semen was diluted at a 1:1 ratio with cooling extender (Uppsala 1, TRIS-citric acid-fructose-egg yolk-glycerol; Linde-Forsberg, 2001) and after equilibration at 5°C for 1 hr or after 2 hr in case of the second ejaculate collected in the 'dual' protocol was further diluted (1:1) with freezing extender (Uppsala 2, glycerol concentration 5%, TRIS-citric acid-fructose-egg yolk-glycerol with Equex paste; Linde-Forsberg, 2001). Semen was filled in 0.5 ml straws (Minitube), frozen immediately thereafter (−17°C/min from +5 to −80°C, −7°C/min from −80 to −130°C) and finally plunged into liquid nitrogen. After storage for at least 24 hr, the straws were thawed at 37°C for 20 s in a water bath. After thawing, the same characteristics as described for fresh semen were analysed. The number of straws representing one AI dose was calculated taking into account post-thaw progressive motility and sperm concentration with at least 100×10^6 motile spermatozoa per AI dose. The number of 0.5 ml straws per AI dose thus ranged from two to four.

2.5 | Statistical analysis

Statistical comparisons were made with the SPSS statistics software (version 26; IBM-SPSS). Because not all data were normally distributed (Kolmogorov Smirnov test), non-parametrical test were used throughout. Taking into account that the same animals were studied repeatedly, differences were analysed by Friedman's test for overall significance followed by pairwise Wilcoxon's test comparisons in case of an overall significant effect. A p-value $< .05$ was considered significant. Results are presented as scatterplots with individual values and mean ± standard error (*SEM*) or, for sperm velocity parameters, as mean ± *SEM* in table format.

3 | RESULTS

Dual semen collection was successful in 28 out of 35 dogs and seven dogs refused to ejaculate a second time (Beagle $n = 2$, Australian Shepherd, Miniature Schnauzer, Terrier Brasileiro, White Swiss Shepherd dog and Rhodesian Ridgeback $n = 1$ each). All further

results are presented only for the 28 dogs were both the single and dual semen collections were successfully performed.

Neither ejaculate volume nor total sperm count differed between the sperm-rich fraction of a single semen collection and the sperm-rich fractions of the ejaculates of a dual semen collection, respectively. As predetermined by the experimental design, volume (single 1.3 ± 0.3, dual 5.3 ± 0.4 ml, $p < .001$) and total sperm count (single 0.37 ± 0.05, dual 0.63 ± 0.10 × 10^9 spermatozoa, $p < .001$) in the combined sperm-rich fraction after dual semen collection was higher than when only a single ejaculate had been collected (Figure 2a,b).

The percentage of motile spermatozoa and the percentage of membrane-intact spermatozoa before freezing did not differ in semen from a single (total motility 92.3 ± 0.8%, membrane integrity 91.2 ± 1.0%) and a dual semen collection (total motility 91.6 ± 1.1%, membrane integrity 90.7 ± 1.1%). Both, the percentage of motile spermatozoa and the percentage of membrane-intact spermatozoa was reduced ($p < .001$) after cryopreservation but again did not differ between cryopreserved semen from a single (total motility 72.3 ± 2.1%, membrane integrity 63.8 ± 2.6%) and dual semen collection (total motility 70.9 ± 2.6%, membrane integrity 62.8 ± 2.9%; Figure 3a,b).

The percentage of spermatozoa with morphological defects was close to identical before cryopreservation in semen from a single and a dual semen collection (40.8 ± 4.1 and 40.4 ± 3.8%) and increased ($p < .001$) in an identical way in frozen-thawed semen from a single and dual semen collection (58.7 ± 3.4 and 60.0 ± 3.5%; Figure 4a). The percentage of spermatozoa with detached acrosomes was below 10% before cryopreservation (single 7.4 ± 0.7, dual 7.9 ± 0.7%; Figure 4b). Freezing-thawing increased ($p < .001$) the percentage of spermatozoa with detached acrosomes to 33.6 ± 2.4% (single) and 33.9 ± 2.4% (double), but there was no significant difference between semen collection-cryopreservation procedures.

The sperm velocity characteristics VCL, VAP, VSL, ALH and STR decreased with semen cryopreservation (all $p < .001$) but both for processed semen before and after cryopreservation were close to identical in semen from a single and a dual combined semen collection (Table 1).

The number of insemination doses with cryopreserved semen increased when dual semen collection at a 1-hr interval was performed (4.7 ± 0.8) compared to freezing a single ejaculate (2.7 ± 0.4, $p < .01$; Figure 5).

4 | DISCUSSION

The results from our study suggest an easy procedure to increase the number of cryopreserved semen doses obtained with one semen freezing procedure from the same male dog. At the same time, no detrimental effects on semen characteristics were determined. The protocol is, therefore, interesting for semen cryopreservation in dogs especially when they have a long journey from their home to the clinic or laboratory where semen collection and freezing is performed.

A drawback in semen cryopreservation for the present study is the failure of a second semen collections or semen collection at all in several otherwise healthy and fertile dogs. Semen collection twice at a short interval is without problems for example in bulls (Seidel and Foote, 1969), stallions (Pickett et al., 1975) and also in men (Check & Chase, 1985). In contrast, individual alpaca stallions from which semen was collected three times per day, ejaculated only seminal plasma during repeated semen collections (Bravo et al., 1997). It is, however, well-possible that training of dogs for the dual semen collection protocol or extension of the interval between collections may help to perform a dual semen collection protocol for semen cryopreservation in almost all males. This emphasizes that semen collection in dogs requires systematic preparation of the animals. Experience of the personal performing semen collections and optimized environment should also not be underestimated. Dog owners planning to produce frozen semen from their animals should understand that this often cannot be achieved with just a single visit to a veterinary practice. There was no breed or size prevalent among dogs were a dual semen collection was not possible.

Optimisation of the semen collection schedule with the aim to improve the reproductive capacity of a semen donor has been addressed in different species (e.g. men: Check & Chase, 1985; dog: England, 1999; bull: Seidel & Foote, 1969, Everett et al., 1978; stallion: Pickett et al., 1975; alpaca: Bravo et al., 1997). With regard to ejaculate volume and pre-freeze semen characteristics, our results are in agreement with data published by England (1999) who demonstrated only a slightly lower total sperm count in the second ejaculate and no difference in the sperm motility and percentage of morphologically normal spermatozoa in semen collected from dogs twice at a 1-hr interval. Similar sperm characteristics in two ejaculates collected from male dogs 30 to 60 min apart were reported (Gunay et al., 2003; Yonezawa et al., 1991) but in these studies, total

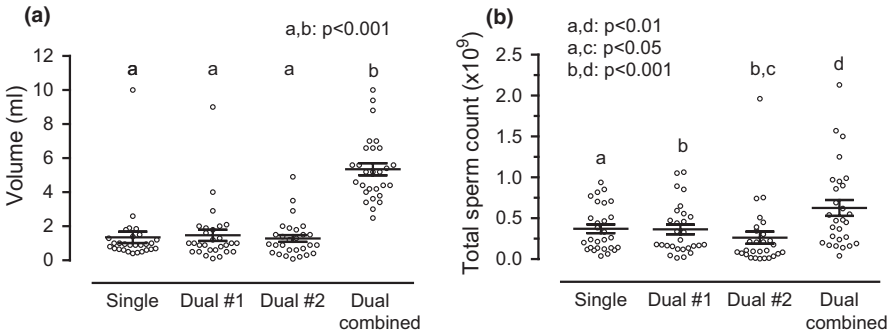

FIGURE 2 (a) Volume and (b) total sperm count in the sperm-rich fraction of a single ejaculate, two ejaculates collected at a 1-hr interval (dual #1 and dual #2) and the combination of the two ejaculates collected at a 1-hr interval (dual combined) in dogs ($n = 28$), values are means ± *SEM*, results of statistical analysis are indicated in the figure

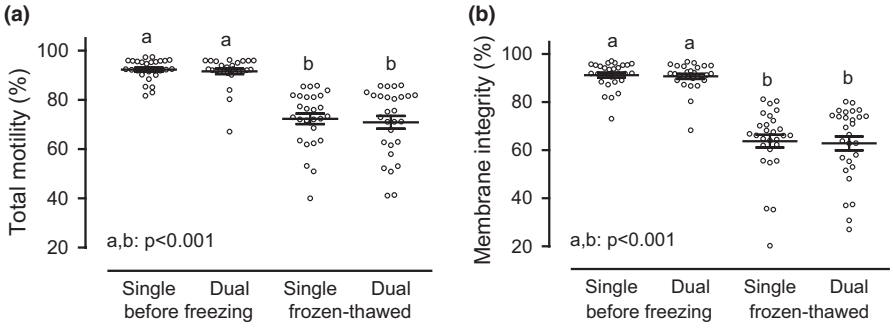

FIGURE 3 (a) Total motility and (b) membrane integrity in raw semen after dilution (25 million/ml) and in cryopreserved semen (single and combined dual ejaculates) in dogs (n = 28), values are means ± *SEM*, results of statistical analysis are indicated in the figure

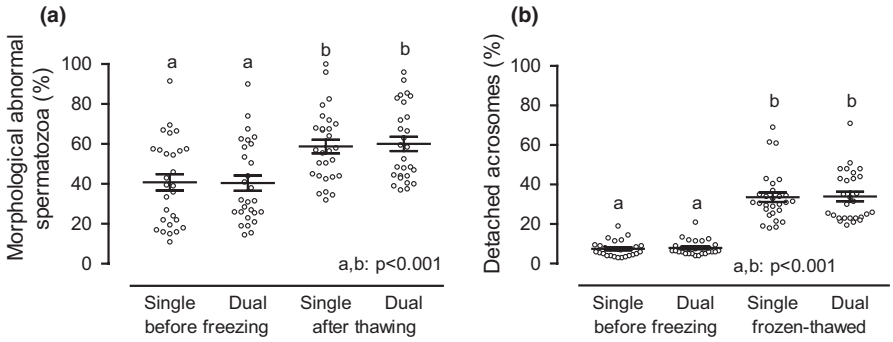

FIGURE 4 (a) Total percentage of spermatozoa with abnormal morphology and (b) percentage of spermatozoa with detached acrosomes in in raw semen after dilution (25 million/ml) and in cryopreserved semen (single and combined dual ejaculates) in dogs (n = 28), values are means ± *SEM*, results of statistical analysis are indicated in the figure

TABLE 1 Sperm velocity characteristics in processed semen directly before cryopreservation and after freezing-thawing of the sperm-rich fraction from a single or a dual combined semen collection in dogs (n = 28), values are means ± *SEM*

	Before cryopreservation		After cryopreservation	
Parameter	Single collection	Combined dual collection	Single collection	Combined dual collection
Curvilinear velocity (VCL; μm/s)	160.0 ± 5.9^{a,b}	158.9 ± 4.4^{a,b}	121.2 ± 3.6^{a,b}	120.9 ± 3.6^{a,b}
Average path velocity (VAP; μm/s)	104.2 ± 3.3^{a,b}	104.8 ± 3.3^{a,b}	75.6 ± 2.2^{a,b}	77.1 ± 2.2^{a,b}
Straight line velocity (VSL; μm/s)	94.6 ± 3.2^{a,b}	95.6 ± 3.4^{a,b}	67.1 ± 2.2^{a,b}	68.5 ± 2.1^{a,b}
Amplitude of lateral head displacement (ALH; μm)	4.2 ± 0.1^{a,b}	4.3 ± 0.1^{a,b}	3.4 ± 0.1^{a,b}	3.5 ± 0.1^{a,b}
Straightness (STR; VSL/VAP × 100)	0.902 ± 0.009^{a,b}	0.904 ± 0.007^{a,b}	0.880 ± 0.006^{a,b}	0.882 ± 0.005^{a,b}

^{a,b}Different superscript letters indicate significant differences ($p < .001$).

sperm count in the second ejaculate was reduced to 20% to 40% of the first ejaculate. In contrast to our data, the increase in AI doses would thus have been only of limited economic interest. The differences between studies with regard to the total sperm count of dual semen collections may be due to sexual activity of the dogs before the respective studies, semen collection techniques or breed and size. The ability to produce a second ejaculate at a short interval after a first semen collection may also depend on the species but apparently, this procedure often results in a decreased sperm output in the second ejaculate. When semen was collected from cattle bulls twice at 40 min intervals, sperm concentration decreased by 40% and total sperm count by 35%. When both ejaculates were frozen, post-thaw semen motility in the first and second ejaculate were, however, close to identical (Seidel & Foote, 1969), which is similar to our findings in dogs. Considerable reductions in sperm count were also reported from stallions undergoing two semen collections per day (Pickett et al., 1975) and from alpacas submitted to three daily semen collections (Bravo et al., 1997). In contrast to these reports, in the present investigation the dual semen collection was only performed once and it cannot be excluded that detrimental effects on total sperm count and semen characteristics would occur if the dual semen collection protocol would be performed repeatedly. Movement of spermatozoa through the epididymal tract of the caput and corpus epididymidis depends on continuous peristaltic contractions of its smooth muscular wall and is not hastened by ejaculation. It requires approximately 4.1 days in a stallion. In contrast, the smooth muscular wall of the cauda epididymis is usually inactive and the time spermatozoa spend in this part of the epididymal tract is influenced by ejaculation and ranges from 2 to 3 days in a sexually active stallion to 10 days in a sexually rested stallion (Amann, 2011).

With regard to overall changes in sperm characteristics caused by cryopreservation, results of our study are largely in agreement with previous reports. The percentages of motile and membrane-intact spermatozoa after cryopreservation obtained in our present

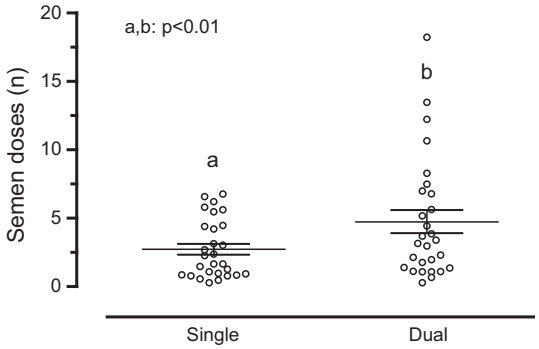

FIGURE 5 Number of insemination doses (with 100 million motile spermatozoa per dose) with cryopreserved semen after processing the sperm-rich fraction from a single or dual semen collection (combined after dual collection at a 1-hr interval) in dogs ($n = 28$), values are means ± *SEM*, results of statistical analysis are indicated in the figure

study are in agreement with some previous reports (Hay et al., 1997; Pena & Linde-Forsberg, 2000a,b) but higher than in some more recent studies (Lucio et al., 2016; Nöthling & Shuttleworth, 2005; Pezo et al., 2017). The studies employed different one-step freezing procedures. Some of these differences among studies may be due to non-optimal freezing curves in experiments were computer-controlled freezing equipment was not available. Besides the percentage of motile spermatozoa, also velocity characteristics determined by CASA, both pre-freeze and after freezing-thawing, in the present study were very similar to previous data from our laboratory (Schäfer-Somi et al., 2006) and other groups (Pena & Linde-Forsberg, 2000a,b). Although absolute values for VCL, VAP and VSL were slightly higher and ALH slightly lower than in another recent study (Lucio et al., 2016), the magnitude of changes induced by freezing and thawing was close to identical.

The dual semen collection protocol of the present study required that processed semen from the initially collected ejaculate had to be equilibrated at 5°C for 2 hr instead of only 1 hr to allow for pooling with the second ejaculate. This increase in the equilibration time was without detrimental effects on semen characteristics which is in agreement with previous studies (Belala et al., 2016; Okano et al., 2004). Beyond that, further extension of the equilibration period during the cryopreservation process of canine semen may even improve characteristics of frozen-thawed semen and depending on the extender is optimal after 6 hr of equilibration (Belala et al., 2016). This suggests that further improvement of a dual semen collection protocol in dogs for cryopreservation is possible with adjusting the interval between semen collections.

4.1 | Conclusions

Studies on the cryopreservation of semen are usually aimed at optimizing the processes of cooling, freezing and thawing in order to minimize sperm damage and maximize the number of insemination doses obtained from an ejaculate. Combining two ejaculates collected at a short interval for one cryopreservation process increases the number of AI doses to be collected without compromising semen quality. Although the ultimate test of success for semen cryopreservation techniques is the evaluation of whelping rate and litter size, this study suggests a practical way to improve the efficiency and profitability of semen freezing in dogs.

ACKNOWLEDGEMENTS

The authors are grateful to Silvia Kluger and Bettina Schreiner for expert technical assistance with semen analysis and cryopreservation and Lisa Kornhoffer for help with the animals.

CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

CA and DL designed the project. DL, JH and SSS performed the experimental animal work and DL and CA the semen analysis. DL and JA compiled and the data and JA, CA and DL wrote and edited the manuscript.

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How to cite this article: Lechner, D., Aurich, J., Schäfer-Somi, S., Herbel, J., & Aurich, C. (2021). Combined cryopreservation of canine ejaculates collected at a one-hour interval increases semen doses for artificial insemination without negative effects on post-thaw sperm characteristics. *Reproduction in Domestic Animals*, 56, 1220–1226. https://doi.org/10.1111/rda.13980