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Effects of Double Layer Centrifugation on the Improvement of Sperm Quality in Dogs: A Comparative Note among Different Breeds

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Abstract

The evaluation of sperm quality in the laboratory is essential to improve efficiency in assisted reproduction. As in other species, for the dog there are reports that prolonged contact of sperm with some components of seminal plasma is associated with decreased motility and sperm viability. Thus, the centrifugation is a technique widely used to concentrate the spermatozoa and eliminate the supernatant. The purpose of this study was to evaluate the effect of double layer centrifugation on the percentages of total sperm motility and progressive sperm motility of the dog's semen submitted to the dilution, single layer centrifugation, cooling and storage at 5 °C for 24 and 48 hours. For this purpose, ejaculates of 30 healthy male dogs were evaluated, by taking into account the comparison among the conventional sperm parameters (ejaculate volume, sperm concentration, total sperm motility and sperm progressive motility). The semen samples were examined in standard baseline condition of fresh semen (FS), after dilution (AD), after dilution and single layer centrifugation (SLC), after double layer centrifugation (DLC). According to the different time points, the semen samples were evaluated in baseline conditions, immediately after their collection at (T0), at 24 h (T24) and at 48 h (T48), to evaluate the effect of different treatments on the semen's quality. Results showed a significant effect of double layer centrifugation on the improvement of total sperm motility and progressive sperm motility percentages of dogs. The use of cooling fresh semen soon after the double layer centrifugation will improve the semen quality up to 48h, with a special emphasis for the percentages of total sperm motility and sperm progressive motility, adding an alternative technical approach to reproductive performance in male breeding dogs.

Keywords: Dog; Progressive Sperm Motility; Sperm Improvement; Total Sperm Motility

Introduction

It is well known that good semen quality is physiologically associated with high fertility, when considering dogs characterized by similar management factors and the best veterinary care and husbandry (Hesser et al., 2017). Poor semen quality, in fact, is often correlated to oxidative stress in both humans and animal species (Ahsan et al., 2014; Aitken et al., 2014; Zubair, 2017), to quantity of components of dietary, especially their deficiency (Domoskawska et al., 2019), or to unknown aetiology (Fontbonne, 2011; Domoskawska et al., 2018).

Semen evaluation and fertility assessment in a large population of purebred dog breeding facility were recently recorded in a longitudinal study, including endpoints of mating, female fecundity and pregnancy (Hesser et al., 2017). Hence, total motility evaluation yielded no significant differences between the fresh and chilled groups or among the age groups (young, middle, senior), ranging from 1 to 10 years. Moreover, progressive motility showed significant differences only within senior dogs, where fresh semen showed the higher progressive motility compared to the chilled samples. What is more, the average path velocity (VAP) was lower in chilled samples than fresh samples, and it was the lowest in senior dogs (Hesser et al., 2017).

It is possible to presume that sperm production, according to testicular function, is influenced by internal and external factors (Fontbonne, 2011). Among the internal factors, it is well known that that the prostatic fluid is unsuitable for the preservation of the dog's semen at 4-8 °C, exerting harmful effects upon the spermatozoa during freezing process (Sieme et al., 2004; Rijsselaere et al., 2007). Different centrifugation's programs were described to improve the sperm quality, according to the low, intermediate or high centrifugation intensities (Farstad and Berg, 1989; Fougner, 1989; Thomas et al., 1993; Pena et al., 1998; Linde-Forsberg et al., 1999; Rota et al., 1999; Verstegen et al., 2005), but no data related to dead spermatozoa percentage are available. Hidalgo et al. (2015) showed that dog semen centrifuged after collection and extended with CaniPRO Freeze can be frozen after 24 hours of cold storage in a Neopor box, obtaining similar results to semen immediately frozen after collection.

Recently, a practical and efficient technique that results in adequate numbers of sperm to be sexed, with a quantity and quality of cells sufficient for breeding, was standardized in the bovine species (Seidel, 2007). To perform an artificial insemination with pregnancy outcomes, the appropriate minimum value of motility is 50% with fresh semen (Concannon and Battista, 1989), and this value was only achieved with use of Ficoll (62.2%) in the study of Mothé (2018). Single layer centrifugation resulted to be the best protocol to improve sperm quality in chilled canine semen in comparison to the double layer centrifugation before and after cooling (Dorado et al., 2015; Gálvez at al., 2015).On the other hand, Mascarenhas and Rego de Paula (2018) concluded that the centrifugation protocol, even at low intensity, significantly reduces sperm motility, the membrane integrity and sperm viability of dogs, without differences in fresh or centrifuged semen of dogs among the different breeds studied. On this basis, the hypothesis of the present research was that the gradient of semen centrifugation could represent a concrete and applied alternative to improve the total sperm motility and the progressive sperm motility percentages. The purpose of this study was to evaluate and compare the effect of single layer and double layer centrifugations at different time points on the improvement of the dog's semen submitted to the dilution, cooling and storage at 5 °C for 24 and 48 hours.

Materials and Methods

The research complied with guidelines of Good Clinical Practices (EMEA, 2000). This study was performed according to the ethical principles that have their origins in the Italian Veterinarians' Ethical Code (Passantino, 2007), and the Italian and European regulations on animal welfare (D.L. 26/2014; Directive 2010/63/EU).

Animals

The study was carried out on 30 male dogs, belonging to three different breeds, represented by 10 Dachshund, 10 English Setter and 10 the Labrador Retriever, aged 7.5 ± 1.5 years. Dogs were individually housed in different family homes, and the same commercial food for large breed was administered two times a day, whilst water was *ad libitum*. The recruitment, enrollment, samples collection, and analysis were carried out between October 2019 and April 2020 at the Veterinary Teaching Hospital of the Messina University, Italy. Animal inclusion criteria were based on animals' history, physical examination, as well as reproductive ultrasound of prostate gland and testicles to exclude pathological conditions, complete blood cell count, biochemistry and hormonal profiles. All dogs had no history of receiving medication during the previous 3 months or nutritional supplements, and management factors such as diet, veterinary care and husbandry were similar for all dogs to minimize the impact of heterogeneous factors. The animals were in good general conditions with normal sexual libido (typical physiological sexual behavior during natural mating and no problems with semen collection if artificial insemination was performed), without any disorders or abnormalities of the genital tract.

Blood Samples and Analysis

Owner consent was obtained to blood sample and semen collections, and owners received a copy of all laboratory test results. Blood samples were collected from the cephalic vein, and approximately 3 mL were placed in a sterile tube with ethylenediamine tetraacetic acid (EDTA) for complete blood cell count, performed with a xt1800 (Sysmex). Another aliquot of about 5 mL was collected in a sterile glass tube to obtain serum sample for chemistry profile, using a Clinic Chemistry: bt 3500 (Biotechnic Instruments).

Sperm Samples and Analysis

Before the assessment of semen quality, all subjects underwent the routine training for the manual seminal collection, using a teaser estrus female as a mount. Semen was collected by manual manipulation as described by Linde-Forsberg (2001) in the presence of a teaser bitch in heat. Ultrasound examinations of the testes and prostate were undertaken once on each dog, using Mindray M9 ultrasound machine (Mindray, Italy) with a linear (testes) and microconvex (prostate) 6.6 to 13.5 MHz transducers, and did not show any pathological condition. The testes were scanned in the sagittal (length), transverse (width), and dorsal (height) planes using the electronic calipers of the machine, and the testicular volume was calculated using the formula for an ellipse: volume = length × width × height × 0.5236 (Paltiel el al., 2002). The total testicle volume (TTV) was calculated by adding up the volume of each testicle. Prostate volume (PV) was calculated using the following formula: [1 / 2.6 (L × W × D)] + 1.8 (Kamolpatana et al., 2000).

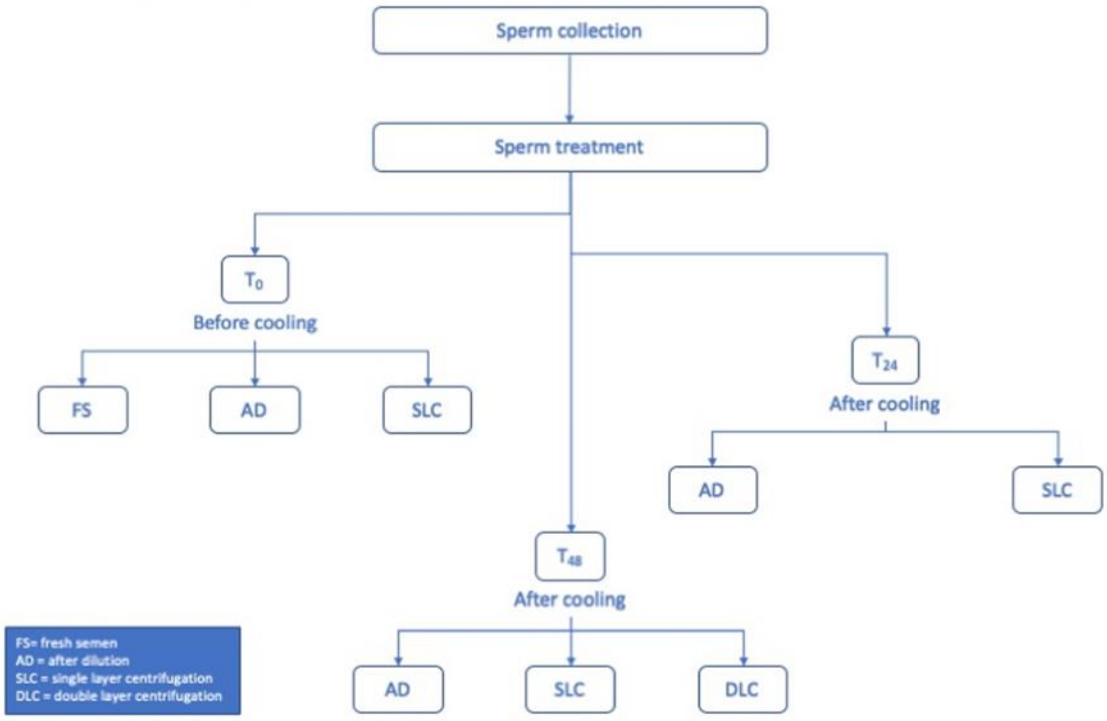
The preliminary collection of semen, on 3 consecutive days, seemed sufficient for both training and minimizing the extragonadal sperm reserves in all dogs, in order to avoid a reduction in motility due to sperm aging and increased debris (Johnston et al., 2001). The semen collection was conducted in a comfortable environment, away from possible sources of distraction for the animal and provided with a flooring that prevented it from sliding.

The semen sample was immediately examined at the laboratory of the University Veterinary Teaching Hospital of the Department of Veterinary Sciences, for macro (volume, color, smell and pH) and microscopic (motility, concentration, morphology and vitality) evaluations. The microscopic examination was performed by placing a 2 µL aliquot of seminal material of the 2nd fraction, using a micropipette (Eppendorf Reference variable 2-20 µL), on a Leja chamber (SC 10-01-04-B, Leja, GN Nieuw-Vennep, NL) and analyzed with the aid of a Nikon Eclipse Ni phase contrast optical microscope, equipped with a heated stage, 10x/0.25 Ph1 phase contrast objective, Blaser Scout sca780-54fc digital

camera (resolution 782x582 pixels; 54 frames per second) and computerized automatic semen analysis system SCA (Sperm Class Analyzer, Microptic Automatic Diagnostic System).

Three ejaculates from each dog (n=30 samples) were collected, with 48-hours interval between collections, into pre-warmed (36-38 °C) sterile graduated conical tubes. Only the second and part of the third fractions were collected. Semen was evaluated before and after centrifugation in discontinuous density gradients for ejaculate volume, sperm concentration, sperm motility, progressive sperm motility. The semen was submitted to dilution with the commercial CaniPRO™Chill10 extender (Minitüb, Germany), cooling and storage at 5 °C for 24 and 48 hours. An aliquot of diluted semen was centrifuged at 1800 rpm (700 x g) for 15 min at a temperature of 20°C. After centrifugation, the pellet (tube bottom) was resuspended with the extender at the final concentration of $50x^{106spz/mL}$ and the values for the same sperm variables were reevaluated. The fresh semen (FS) samples were examined in standard baseline condition, after dilution (AD), after dilution and single layer centrifugation (SLC), and after double layer centrifugation (DLC). According to the different time points, the semen samples were evaluated immediately after their collection at (T0), at 24 h (T24) and at 48 h (T48), to evaluate the effect of different treatments (AD, SLC, DLC) on the semen's quality (Figure 1).

Figure 1: Flow diagram of dogs' semen evaluation submitted to different treatments



After the clinic examination and reproductive parameters evaluations, male dogs were used for natural breeding or their fresh semen was used for artificial insemination (AI). To assess the semen quality monitor and predict the successful of fertilization rates for dogs, a longitudinal evaluation, including the endpoints of female fertility and pregnancy, was added. For this purpose, to monitor and predict the successful of fertilization rates for Labrador Retrievers, a longitudinal evaluation, including the endpoints of female fertility, pregnancy and the number of vital puppies, was done (Liotta and Majolino, 2015; Quartuccio et al., 2020).

Statistical Analysis

To compare fresh semen (FS) samples, after dilution (AD), after dilution and single layer centrifugation (SLC) and

after double layer centrifugation (DLC) treatments and the different time points: immediately after their collection at (T0), at 24 h (T24) and at 48 h (T48), ANOVA-test was applied, by SAS/STAT® software (2017) and differences were considered significant if (P≤0.05). Results were reported as means ± standard deviation of the mean.

Results

The haematological and chemical profiles were assessed to verify the good health status of all dogs used in this study; for this purpose, they are not discussed in the present section. The comparison of haematological and chemical patterns with published ranges were in line with literature data (Niżański et al., 2011; Schäfer-Somi, 2015; Valverde et al., 2019) nevertheless, these profiles were not discussed in the present study, because their values showed only the clinical healthy status of dogs.

Ultrasound Examinations and Semen Parameters

The ultrasound examinations and semen parameters (mean ± SD) in different dog's breeds are shown in Table 1. The testicular volume ranged from 4.08 cm^3 of Dachshund dogs to 12.73 cm^3 of Labrador Retrievers. The prostatic volume ranged from 9.51 cm^3 of Dachshund dogs to 19.60 cm3of Labrador Retrievers. The ejaculate volume produced by each dog ranged from 3.06 mL of Dachshund dogs to 12.46 mL of Labrador Retrievers; the sperm concentration ranged from 119.10 (10^6/mL) of Dachshund dogs to 149.25 (10^6/mL) of Labrador Retrievers. The sperm progressive motility showed a wide range from 28.90 to 82.77 (%).

Table (1): Ultrasound examinations and semen parameters (mean ± SD) in different dog's breeds

	Breeds		
	Dachshund	**English Setter**	**Labrador Retriever**
No. of dogs	10	10	10
Testicular volume (cm^3)	4.72 ± 0.64	9.01 ± 0.86	11.65 ± 1.08
Prostatic volume (cm^3)	10.36 ± 0.85	14.03 ± 1.40	16.37 ± 3.23
Ejaculate volume (mL)	4.20 ± 1.14	6.10 ± 2.33	9.90 ± 2.56
Sperm concentration(10^6/mL)	110.15 ± 8.95	115.36 ± 10.69	131.26 ± 17.99

Total Sperm Motility Percentage

Total sperm motility percentage (mean ± SD) after different treatments in three dog's breeds is shown in Table 2. At T0, Dachshund dogs showed lower total sperm motility percentage (P≤0.01) after dilution (AD) than fresh semen (FS) and single layer centrifugation (SLC); English Setter dogs showed lower total sperm motility percentage (P≤0.01) after SLC than AD and FS; Labrador Retriever dogs showed higher total sperm motility percentage (P≤0.01) AD than FS and SLC. At T24, Dachshund and Labrador Retriever dogs showed higher total sperm motility percentage (P≤0.001) after SLC than FS. At T48, Dachshund, English Setter and Labrador Retriever dogs showed higher total sperm motility percentage (P≤0.001) after SLC than AD, and higher total sperm motility percentage (P ≤ 0.001) after DLC than both AD and SLC.

Table (2): Percentage of sperm motility (mean ± SD) after different treatments in three dog's breeds

Time points	Treatment	Breeds		
		Dachshund	English Setter	Labrador Retriever
T0	Fresh semen (FS)	98.570±0.64^{a}	97.641±0.67^{a}	94.815±1.18^{a}
	After diluition (AD)	97.087±0.59^{b}	98.230±0.60^{a}	96.721±1.15^{b}
	After single layer centrifugation (SLC)	98.197±0.59^{a}	71.01±0.59^{b}	93.011±1.12^{a}
T24	After diluition (AD)	53.896±0.58^{A}	77.601±0.61	45.301±1.17^{A}
	After single layer centrifugation (SLC)	85.846±0.58^{B}	78.042±0.59	55.563±1.13^{B}
T48	After diluition (AD)	30.214±0.59^{A}	33.772±0.77^{A}	31.823±1.18^{A}
	After single layer centrifugation (SLC)	58.269±0.66^{B}	47.232±0.89^{B}	38.271±1.16^{B}
	After double layer centrifugation (DLC)	77.539±1.05^{C}	68.603±0.95^{C}	49.221±1.13^{C}

T0: Baseline time; T24: after 24 h; T48: after 48 h

^{a,b,c}Mean values with different superscript letters in a column and for each Time point indicate significant differences at P≤0.01

^{A,B,C}Mean values with different superscript letters in a column and for each Time point indicate significant differences at P≤0.001

Progressive Sperm Motility Percentage

Progressive sperm motility percentage (mean ± SD) after different treatments in three dog's breeds is shown in Table 3. At T0, Dachshund dogs showed higher progressive sperm motility percentage (P≤0.01) after dilution (AD) than fresh semen (FS) and single layer centrifugation (SLC); English Setter and Labrador Retriever dogs showed lower progressive sperm motility percentage (P≤0.01) after (SLC) than both AD and FS. At T24, Dachshund, English Setter and Labrador Retriever dogs showed higher progressive sperm motility percentage (P≤0.001) after SLC than AD. At T48, Dachshund, English Setter and Labrador Retriever dogs showed higher progressive sperm motility percentage (P≤0.001) after SLC than AD, and higher progressive sperm motility percentage (P≤0.001) after DLC than both SLC and AD.

Table (3): Percentage of progressive sperm motility (mean ± SD) after different treatments in three dog's breeds

Treatment	Breeds		
T_0	**Dachshund**	**English Setter**	**Labrador Retriever**
Fresh semen (FS)	81.332 ± 1.86^{a}	69.850 ± 0.66^{a}	71.490 ± 7.08^{a}
After diluition (AD)	85.450 ± 1.81^{b}	71.481 ± 0.64^{a}	73.521 ± 7.32^{a}
After single layer centrifugation (SLC)	79.943 ± 1.79^{a}	37.836 ± 0.97^{b}	54.011 ± 5.12^{b}
T_{24}			
After diluition (AD)	2.470 ± 0.67^{A}	16.761 ± 1.12^{A}	27.181 ± 5.12^{A}
After single layer centrifugation (SLC)	42.684 ± 1.19^{B}	35.232 ± 0.78^{B}	40.553 ± 5.36^{B}
T_{48}			
After diluition (AD)	0.914 ± 0.29^{A}	3.290 ± 0.78^{A}	11.613 ± 4.52^{A}
After single layer centrifugation (SLC)	1.850 ± 0.45^{B}	6.252 ± 0.76^{B}	13.271 ± 4.55^{B}
After double layer centrifugation (DLC)	6.723 ± 0.43^{C}	12.471 ± 0.99^{C}	16.491 ± 4.33^{C}

T0: Baseline time; T24: after 24 h; T48: after 48 h

^{a,b}Mean values with different superscript letters in a column indicate significant differences (P≤0.01).

^{A,B,C}Mean values with different superscript letters in a column indicate significant differences (P≤0.001).

Discussion

This study represents the particular comprehensive research of improvement of sperm parameters in representative groups of small, medium and large dogs' breed. The obtained data were consisted with the initially hypothesis and objective of the present study. The ultrasound examinations and conventional semen parameters were in line with dogs' physiological range of literature data (Rijsselaere et al., 2007; de la Fuente-Lara et al., 2019; Domoskawska et al., 2019), and with ranges specifically reported for male Labrador Retriever (Kaneko et al., 1998; Thrall et al., 2006; Rijnberk and Kooistra, 2010; Hesser et al., 2017).

The use of the computerized automatic semen analysis system SCA (Sperm Class Analyzer, Microptic Automatic Diagnostic System) and the application of DLC of semen samples showed a significant advantage to improve the semen quality of dog. The percentages of both total sperm motility and progressive sperm motility showed a significant increase both at T24 after SLC and at T48 after SLC, and to a greater extent after DLC.

The most important finding of the present study was that at T24 and T48, the sperm motility and the progressive sperm motility percentages after SLC were higher than in the diluted samples. What is more, at T48 the percentage of sperm motility was

the highest after the DLC. It is possible to presume that the motility reactivation and its maintenance after semen sample centrifugation were probably correlated to the supplementation of glucose of the extender and the removal of samples' product of degradation (Verstegen et al., 2005).

In addition, the execution of the treatment before (T0) and after cooling (T24, T48) sperm samples added an additional and consistent knowledge on the motility percentage of spermatozoa.

At T0 the total sperm motility percentages were more variable among different breeds, with the highest percentage in FS and the lowest in AD in Dachshund dogs, the highest percentage in AD and lowest in SLC in English Setter and Labrador Retriever dogs. On the other hand, the progressive sperm motility percentages showed a homogenous trend, with the highest percentage AD and the lowest percentage after SLC in all the three breeds.

As no expected, semen quality parameters displayed the superimposed trend, with a low intra-individual variation, when considering dogs characterized by heterogeneous small, medium and large breed groups. This result confirms previous data obtained in Beagle, Schnauzer, Doberman and Boxer breeds, which showed no significant difference in any of the parameters evaluated in fresh or centrifuged semen of dogs among the different breeds studied (Mascarenhas and Rego de Paula, 2018).

This study was designed as evaluation and comparison of the effect of different treatment (single- and double-layer centrifugations) at different time points (T0, T24, T48) on the improvement of the dog's semen per se. Given that any effect of semen improvement would be inherently assessed with these treatments, it is appropriate to discuss the differences at different time points and no among different breed.

Hence, in this sense, other authors reported significant different values among breed and among individuals in six dog phenotypes Valverde et al. (2019); moreover, these Authors showed that the intra-male coefficient of variation (CV) was higher than inter-male CV, suggesting that the sperm characteristics cannot be extrapolated from one to another breed. This trend was debated in literature, but it is possible to presume that the technical characteristics of the computer assisted sperm analyzer could affect the results, as previous observed in bull spermatozoa (Contri et al., 2010; Gloria et al., 2016).

Overall, the SLC and in more consistent manner the DLC lead to multiple effects of semen quality, including conventional and kinematic sperm parameters, with a positive effect on the sperm motility and progressive sperm motility percentages, confirming previous results observed in dogs 'sperm quality (Linde-Forsberg et al., 1999; Rota et al., 1999; Rijsselaere et al., 2007).

The conventional sperm sorting methods involve multiple washing and centrifugation steps. The mechanism whereby the centrifugation induces its effect on the semen quality is understood in humans (Rappa et al., 2016) and different animal species (Gloria et al., 2016), with specie-specific effects. The mechanical effect was negative on the spermatozoa of rats (Cardullo and Cone, 1986), mice (Katkov and Ostashko, 1996) and men (Aitken and Clarkson, 1988; Ng et al., 1990; Alvarez et al., 1993), whereas no negative effects of centrifugation were observed for stallion stallion (Contri et al., 2010; Hoogewijs et al., 2010; Stuhtmann et al., 2012) and bull (Katkov and Mazur, 1998; Crockett et al., 2001). In Bovidae, it is reported that, after centrifugation, sperm viability is maintained, without alteration in sperm morphology, and sperm motility may even be greater (Watkins et al., 1996; Lima, 2005). Both SLC and DLC significantly increased the percentage of normal bovine spermatozoa and decreased the percentage of non-sperm cells in poor quality samples, while they were both ineffective in those of normal quality (Gloria et al., 2016).

Selection of robust spermatozoa from cooled semen samples could improve the conception rates after artificial insemination (AI); in this sense, in stallions single layer centrifugation (SLC) has been

shown to select good quality spermatozoa (Morrell et al., 2009).

These results correspond to those obtained in other species (Ortiz et al., 2013) where SLC was carried out after the cooling (AC) process. Similarly, the three sperm velocities (velocity curved line: VCL, velocity straight line: VSL and VAP: velocity average pathway) were significantly higher in SLC-AC samples compared with unselected, SLC prior to coolingand double layer centrifugation (DLC) samples. These variables summarize sperm kinematics and are considered to be reliable indicators of sperm quality (Olds-Clarke, 2003). Furthermore, previous studies in canine frozen-thawed semen have demonstrated that sperm velocities (mainly VSL and VAP) are landmarks of fertility in vitro (Silva et al., 2006).

According to sperm characteristics associated with breed diversity, dogs may represent a pivotal model to evaluate changes in semen parameters, according to different technical approaches. On this basis, because many purebred dogs undergo purposeful breeding, any effect of centrifugation on sperm quality could have a considerable clinical relevance for a good reproductive performance.

Conclusions

These data contribute to the overall information base on the improvement of sperm quality of breeding dogs. Sperm separation techniques by double layer centrifugation (DLC) should be quick, easy, low-cost and have the ability to select motile and normal sperm. However, the DLC effects were recorded soon after centrifugation at T48, but no data on the long-term effects or the impact of this technical treatment on cryopreservation were assessed. Moreover, future approach to significant semen improvement in male dogs could be done, to better understand the effect of DLC also on the kinematic parameters (sperm velocities, percentages of linearity, straightness, oscillation, circular trajectory and hyperactivity).

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