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Research Article/Artículo de Investigación

EFFECTS OF THE LOW INTENSITY CENTRIFUGATION AND THE BREED ON THE QUALITY OF FRESH CANINE SEMEN

EFECTO DE LA CENTRIFUGACIÓN A BAJA INTENSIDAD Y DE LA RAZA SOBRE LA CALIDAD DEL SEMEN FRESCO CANINO

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ABSTRACT

The effect of the low intensity centrifugation (300xg), as well as the breed variation were evaluated. To this, semen of 12 dogs of the Beagle (n = 3), Schnauzer (n = 3), Doberman (n = 3) and Boxer (n = 3) breeds, 3 ejaculates collected per individual, totaling 36 ejaculates were used. The direct microscopic evaluation of the motility parameters and the hypoosmotic and supravital staining tests were used. The individual means of sperm vigor and motility varied from 3.5 to 5.0 and from 63 to 90% respectively, while the breed means ranged from 4.4 to 4.9 and from 80 to 90%, respectively. No significant difference was observed in any of the parameters evaluated in fresh or centrifuged semen of dogs among the different breeds studied. Centrifugation of semen resulted in a reduction of about 13% in sperm vigor and 14% in motility and sperm index. The analysis of the parameters of viability and sperm membrane integrity shows a 38% and 14% reduction in the percentage of reactive cells to the hypoosmotic test and supravital staining respectively. These results allow us to conclude that the centrifugation protocol, even at low intensity, significantly reduces sperm motility, the membrane integrity and sperm viability, this effect being more clearly perceived through the hypoosmotic test.

Keywords: fresh semen, canine breeds, low intensity centrifugation.
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RESUMEN

EL efecto de la centrifugación a baja intensidad (300xg), así como la variación racial fueron evaluados. Para esto, semen de 12 perros de las razas Beagle (n=3), Schnauzer (n =3), Doberman (n = 3) y Boxer (n = 3), 3 eyaculados colectados por individuo, totalizando 36 eyaculados fueron usados. La medición por microscopia directa de los parámetros de motilidad, así como el test hipoosmótico y la tinción supravital fueron usadas. La media individual del vigor espermático y la motilidad variaron desde 3,5 a 5,0 y desde 63 a 90% respectivamente, mientras que las medias de las razas variaron desde 4,4 a 4,9 y desde 80 a 90%, respectivamente. No se observaron diferencias significativas en los parámetros evaluados el semen fresco y centrifugado entre las razas estudiadas. El análisis de los parámetros de vitalidad y la integridad de la membrana espermática muestra una reducción de 38% y 14% en el porcentaje de células reactivas al test hipoosmótico y a la tinción supravital respectivamente. Estos resultados llevan a la conclusión de que el protocolo de centrifugación, aun a baja intensidad, significativamente reduce la motilidad espermática y la integridad de la membrana y vitalidad espermática, siendo este efecto percibido más claramente a través del test hipoosmótico.

Palabras clave: semen fresco, razas caninas, centrifugación a baja intensidad.
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INTRODUCTION

In recent decades, the growing interest in pet breeding has been particularly reflected in specialized cynophilia, where there has been an increase in the investment in zootechnical improvement of animals through genetic improvement programs and mainly the importation of breeding animals and this increase the demand of assisted reproduction biotechnologies that potentiate the use of higher quality breeding animals (Peña et al., 2006). Especially in the last 20 years, techniques of artificial insemination and cryopreservation of semen have advanced significantly in the canine specie making possible the mating of animals that present anatomical obstacles to the copula, reducing the need of transportation of animals and, mainly, making possible the storage of material for indefinite periods of time (Silva et al., 1996; Linde-Forsberg et al., 1999; Verstegen et al., 2005; Batista et al., 2006; Prapaiwan et al., 2016). Artificial insemination with fresh semen presents conception rates similar to those observed in natural breeding systems (Linde-Forsberg and Forsberg, 1989; Silva et al., 1996). The use of cryopreserved semen, however, still results in lower conception rates, especially when associated with intravaginal insemination (Fontbonne and Badinand, 1993; Silva et al., 1996, Linde-Forsberg et al., 1999; Verstegen et al., 2005; Rota et al., 2010).

During artificial insemination procedures, the fertility estimation of semen samples is routinely performed by the evaluation of parameters such as concentration, sperm motility and sperm morphology. However, most of dog breeds use natural breeding systems where breeding animals are poorly tested, since the male / female ratio is low and allows several mating at each estrus cycle (Rijsselaere et al., 2005). As a result, little attention has been paid to determining patterns of subfertility and infertility in the canine species. Although the dog is the domestic species with the highest number of recognized breeds, there are few reports on reproductive performance in different breeds (Schelling et al., 2005; Batista et al., 2006; Schrack et al., 2017).

Canine semen is ejaculated in three fractions. Most spermatozoa are ejaculated in the intermediate fraction, the first and last fraction of the ejaculate are mostly of prostatic origin (Rota et al., 2007). 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 (Rota et al., 2007). Thus, in the procedures of artificial insemination with fresh semen, the second fraction is usually collected separately, however, when it is not possible to separate the ejaculate fractions, or even in routine cryopreservation protocols, the centrifugation is an alternative widely used to concentrate the spermatozoa and eliminate the supernatant (Rijsselaere et al., 2002; Verstegen et al., 2005, Rota et al., 2010). In certain situations, species specific or in specific procedures, such as collection directly from the epididymis isolated, centrifugation is essential in the processing of spermatozoa for different techniques in assisted reproduction (Zhang, et al., 2012, Prapaiwan et al., 2016).

Although the studies by Cunha and Lopes (2009) point to the non-negative influence, and even to the improvement of canine semen quality, in certain situations after centrifugation, our preliminary studies and more comprehensive studies (Rijsselaere et al. 2002) indicate a decrease in the final quality of canine semen with the use of medium and high intensity centrifugations. There have been reports in dogs and horses that the centrifugation and the resuspension of the sperm pellet resulting therefrom, can induce sperm membrane damage, generation of reactive oxygen species (ROS), damage to DNA integrity, etc, that, in short, are reflected in the loss of semen motility and fertility (Rijsselaere et al., 2002, Aurich, 2005, Rappa et al., 2016 and Ellerbrock et al., 2017). In this sense, the cost-benefit associated with centrifugation should be observed (Ellerbrock et al., 2017).

According to Rijsselaere et al. (2002), intermediate centrifugation intensities of 180 to 1620 xg, are less harmful during canine semen processing, since they avoid the loss of spermatozoa at low rotations and minimize the presence of dead or moribund spermatozoa related to the use of high rotations. Despite the large amplitude described, these authors used only the speed of 720 xg as the intermediate rotation, and the vast majority of subsequent studies are also restricted to the use of rotations between 660 and 700 xg in canine semen processing protocols (Hermansson et al., 2006, Koderle et al., 2009, Rota et al., 2010, Prapaiwan et al., 2016), In this sense, the present work aims at the evaluation of the effect of rotation with less intensity (300 xg) on the quality of fresh dog semen and also the influence of the breed on these parameters.

MATERIALS AND METHODS

Twelve healthy male adult dogs of the Beagle (n = 3), Schnauzer (n = 3), Boxer (n = 3) and Doberman (n = 3) breeds were used. The animals used belonged to outstanding breeders, specialized in the commercialization and competitive participation in dog shows of national and international scope. The animals selected for the experimentation were certified breeders and considered of high breed standard. All procedures used followed the protocol of ethical conduct established by the Ethics Committee for Animal Use of the Federal University of Viçosa (CEUA-UFV). Before the beginning of the work the dogs underwent a complete clinical and andrological examination, being selected for the

experiment those that presented the physical and morphological seminal parameters judged within the standard considered normal for the species (CBRA, 1998).

The semen was collected in a single aliquot containing the sperm fraction by the digital manipulation method in graduated centrifuge tubes coupled to a plastic funnel, the set being heated before collection and the centrifuge tube kept inside a container containing water at 38°C. Three ejaculates by dog were collected, with 48-hours interval between collections.

Immediately after collecting the semen was kept in a water bath at 38°C, while a 20μL aliquot was placed on a previously heated slide and covered with cover slip for evaluation of vigor (intensity of movement classified from 0 to 5) and sperm motility (expressed like percentage of motile sperm) in optical microscopy (100 and 400x). These values were then used to make the spermatic index (SI) using the formula: IE = [M + (V x 20)] / 2, where M = sperm motility and V = sperm vigor (Morais et al., 2002). The integrity of the sperm membrane was evaluated by hypoosmotic test and supravital staining. To perform the hypoosmotic test, a 20 μL sample of semen was incubated at 38 ° C for half hour in 0.5 mL of 60 mosmol fructose solution and sodium citrate. Then 100 cells were observed under optical microscopy for counting the crude percentage of spermatozoa with coiled tail or reactive to hypoosmotic test. Within this gross total, a portion of the spermatozoa already had coiled tail even before the hypoosmotic test, thus, the gross value of spermatozoa with rolled tail was corrected excluding from the total population the plot with rolled tails previously accounted for. For this purpose, a 20 μL aliquot of the semen was added to 0.5 mL of buffered saline formol and evaluated under the light microscope for the specific proportion of coiled tails.

For the supravital staining test, 20 μL of semen were added to 40 μL of eosin-nigrosine dye previously heated for 40 seconds. Then, a smear was made on a slide and immediately air dried and observed in an increase of 400x for the counting of the stained spermatozoa, which were counted as injured.

The sperm concentration was measured in a hematimetric chamber by diluting 20 μL of semen in 1 mL of buffered saline formaldehyde and counting the cells present within the chamber, the concentration was determined based on the dilution factor. The total number of spermatozoa per ejaculate was obtained by multiplying the sperm concentration by the total volume collected. The total number of mobile spermatozoa in the ejaculate was calculated by multiplying the total number of sperm ejaculated by the percentage of motile spermatozoa. Then the semen was centrifuged at 300 x g for ten minutes.

After centrifugation the supernatant was discarded, and the pellet gently resuspended in Tris-citrate base diluent medium to give a final concentration of 100 x 10^6 sperm / ml. Then the parameters of sperm motility and sperm membrane integrity were reevaluated as described for fresh semen.

The data evaluated were described for the mean, respective standard deviation and coefficient of variation. For the comparison of means, the mean confidence interval with 5% margin of error was calculated through the statistical function of the Excel program Windows XP.

RESULTS AND DISCUSSION

Many studies have been conducted on the canine specie in order to evaluate sperm motility either in fresh semen or after cryopreservation (Silva et al., 1996; Nöthling et al., 1997; Rota et al., 1999). However, most of these studies used semen from animals of different breeds or even from mongrel dogs grouped in pools (Rota et al., 1995; Peña et al., 1998; Peña e Linde-Foresberg, 2000; Santos et al., 2003; Martins-Bessa et al., 2006). In the present study, the individual means of sperm vigor and motility varied from 3.5 to 5.0 and from 63 to 90%, respectively, while the breed means ranged from 4.4 to 4.9 and from 80 to 90%, respectively. (Table 1). These motility values are similar to those described in Beagle dogs (Silva et al., 1996, 90%, Verstegen et al., 2005, 85-95%), Mastiff (Batista et al., 2006, 90%), Labrador retriever (Bueno et al., 2002, 93%) and Poodle (Yamashiro et al., 2007, 70%). The evaluation of sperm motility parameters by semen visualization under a microscope is a subjective evaluation method and, therefore, may be subject to variations between different laboratories and examiners (Rijsselaere et al., 2005). However, the use of computerized methods, such as the CASA system, although it presents a greater accuracy and reproducibility in obtaining data, the evaluation of the results and the quality of the results are, however, highly problematic, in this sense, computerized systems do not seem to be superior to visual assessment of motility regarding the fertilizing capacity of spermatozoa (Krause and Viethen, 1999). However, results obtained in both evaluation systems are highly and significantly correlated (Peña et al., 2003; Vyt et al., 2004), and are routinely used (Aquino-Cortez et al., 2017). In the present study, the sperm index, which consist in a combination of sperm motility and vigor (Morais et al., 2002), varied from 86 to 94% (Table 1).

The number of sperm produced daily is a function of testicular mass (Paula and Cardoso, 1995). In general, large dogs produce more sperm than small dogs since the relationship between body mass and testicular mass is constant in this species (Mascarenhas et al., 2006). However, in addition to testicular mass, factors such as frequency of collection, behavior, reproductive experience and presence of female in estrus influence the total of ejaculated spermatozoa. As expected, in the present study a large variation in total sperm concentration per ejaculate was observed between individuals and between breeds (Table 1). Doberman dogs presented total sperm concentration per ejaculate similar to that observed in small dogs (Table 1). Semen centrifugation is a commonly used methodology for artificial insemination and cryopreservation of semen in order to concentrate spermatozoa and remove seminal plasma, but routinely neglects its negative influence on semen quality (Rijsselaere et al., 2002; Verstegen et al., 2005). According to these same authors, the need to use canine semen centrifugation should be carefully evaluated, especially to the deleterious effects associated with the oxidation of unsaturated fatty acids and sperm membrane integrity, but at the same time, regarding the recovery of spermatozoa after centrifugation. In other words, although high spin speeds are detrimental to seminal quality, slow centrifugations are associated with low sperm sedimentation rates, especially in high viscosity media (Rijsselaere et al., 2002). Moreover, the resuspension of strongly compacted spermatozoa in the pellet formed during centrifugation exposes the cells to severe mechanical injuries (Matás et al., 2007).

Table 1. Vigor, motility and spermatic index and percentage of cells reactive to the supravital staining test and hypoosmotic test in fresh semen of Beagle, Schnauzer, Doberman and Boxer dogs. Total ejaculated sperm and total motile sperm ejaculated in different breeds. Data presented in Mean ± Standard Deviation (Coefficient of Variation).

Ejaculate	Vigor (0-5)	Motility (%)	Sperm index (%)	Supravital staining test (dead sperm) (%)	Hypoosmotic Test (%)	Total ejaculated sperm (10^6)	Total motile sperm ejaculated (10^6)
Beagle							
1	5,0	86,7	93,3	10,5	88,2	453,3	394,0
2	4,3	83,3	85,0	4,3	77,2	200,0	166,7
3	5,0	90,0	95,0	5,5	94,7	380,0	342,0
Mean	4,8 ± 0,4 (8,0)	86,3 ± 4,4 (5,1)	90,6 ± 5,5 (6,0)	6,4 ± 4,1 (64,0)	85,7 ± 10,0 (11,6)	340,0 ± 126,0 (37,1)	295,8 ± 116,3 (39,3)
Schnauzer							
1	5,0	90,0	95,0	4,3	97,9	466,7	420,0
2	4,2	85,0	86,7	8,7	78,5	310,0	262,7
3	3,5	80,0	80,0	17,0	52,1	150,0	120,0
Mean	4,4 ± 0,6 (13,7)	86,4 ± 4,8 (5,5)	89,3 ± 6,1 (6,8)	8,0 ± 5,9 (73,6)	83,1 ± 16,9 (20,4)	354,3 ± 138,3 (39,0)	309,7 ± 130,3 (42,1)
Doberman							
1	5,0	90,0	95,0	4,3	92,9	516,7	465,0
2	4,7	86,7	90,0	8,5	95,2	446,7	387,7
3	4,2	63,3	73,3	24,3	81,6	370,0	239,0
Mean	4,6 ± 0,6 (13,0)	80,0 ± 15,0 (18,8)	86,1 ± 11,4 (13,2)	12,9 ± 11,4 (88,5)	89,9 ± 9,9 (11,0)	444,4 ± 98,2 (22,1)	363,9 ± 123,3 (33,9)
Boxer							
1	5,0	90,0	95,0	4,7	93,1	373,3	336,0
2	4,7	90,0	91,7	21,0	95,6	683,3	615,0
2	5,0	90,0	95,0	6,7	94,4	733,3	660,0
Mean	4,9 ± 0,3 (6,8)	90,0 ± 0,0 (0,0)	93,9 ± 3,3 (3,6)	7,9 ± 6,9 (87,4)	94,4 ± 3,8 (4,1)	596,7 ± 291,7 (48,9)	537,0 ± 262,5 (48,9)

Rijsselaere et al. (2002) evaluated the effect of centrifugation intensities of 180; 720; 1620 and 2880 xg, on sperm motility, sperm membrane integrity and viability, and cell loss in the supernatant elimination, and concluded that centrifugation at 720 xg resulted in no significant loss of spermatozoa by elimination of the supernatant, however, from 1620 xg there are large percentages of spermatic membrane integrity loss. In the present study, no significant change was observed in any of the analyzed parameters regarding the effect of the centrifugation between the different canine breeds studied. Thus, in all the animals studied, semen centrifugation at 300g for 10 minutes did not present significant loss of sperm recovery, but resulted in significant falls of about 13% in sperm vigor and 14% in sperm motility which is reflected in a decrease of about 14% in the sperm index after this centrifugation protocol (Table 2). These results reinforce that in the dog and other domestic species,

the centrifugation have deleterious effects on motility and consequently the quality of the semen (Aurich, 2005; Sieme et al., 2006; Matás et al., 2007; Rappa et al., 2016; Ellerbrock et al., 2017; Davis et al.,2007).

Table 2. Vigor, motility, sperm index and percentage of cells reactive to the hypoosmotic test and supravital staining in fresh and centrifuged canine semen.

	Vigor (0-5)	Motility (%)	Sperm Index (%)	Supravital staining (dead sperm) (%)	Hypoosmotic test (%)
Fresh semen	4,7±0,4[a] (9,5)	85,6±9,8[a] (11,5)	90,2±8,1[a] (8,9)	8,8±8,5[a] (96,5)	92,9±7,7[a] (8,3)
Centrifuged semen	4,1±0,3[b] (8,4)	73,8±13,5[b] (18,3)	77,9±9,0[b] (11,5)	7,6±3,0[b] (56,3)	57,9±43,0[b] (5,3)

Data presented in Mean + Standard Deviation (Coefficient of variation).Different letters in the same column represent significantly different means ($p < 0.05$).

The analysis of the parameters of viability and sperm membrane integrity in the studied animals also shows a significant decrease in the percentage of reactive cells to the hypoosmotic and supravital staining test (Table 2), showing a decrease in the integrity and viability of the sperm membrane of 38% and of 14% respectively. Although Rijsselaere et al. (2002), implies plasma membrane integrity as the functional parameter most directly influenced by centrifugation, Davis et al. (2007) measured the concentration of reactive oxygen before and after centrifugation of the semen of dogs at the intensity of 700 x g for 10 minutes and verified a 200% increase in their concentration after centrifugation. These deleterious effects are probably associated with damage to DNA integrity (Rappa et al., 2016). In this sense, there is apparently no centrifugation intensity totally inert to the quality of canine semen, that is, even at intensities below the usual range, the decrease in sperm quality is observed

CONCLUSIONS

In the present work, no significant difference was observed between sperm motility, integrity and sperm membrane viability in fresh or centrifuged semen among the Beagle, Schnauzer, Doberman and Boxer breeds. The sperm centrifugation protocol with intensity of 300 x g for 10 minutes significantly reduced the sperm motility and the integrity and viability of the sperm membrane, being this difference more clearly perceived through the hypoosmotic test.

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