Paper

Should single layer centrifugation of dog semen be done before or after the semen is cooled?

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The aim of this study was to assess the effectiveness of sperm selection by single layer centrifugation (SLC) on canine sperm quality when SLC was performed before or after the cooling process, or when double SLC (before and after cooling) was performed. Twenty ejaculates from four dogs were divided into four aliquots as follows: unselected: no SLC was performed; SLC prior to cooling (SLC-PC): sperm selection was carried out before cooling; SLC after cooling (SLC-AC): sperm selection was performed after cooling; and double SLC: sperm selection was carried out before and after cooling. Sperm motility (by computer-assisted semen analysis), morphology (Diff-Quick staining), sperm membrane integrity (Vital-Test kit) and acrosome integrity (double fluorescent stain) were assessed in re-warmed semen samples. Four sperm subpopulations (sP) were detected using a pattern analysis technique (sP1: highly active, non-progressive; sP2: low velocity, highly progressive; sP3: less vigorous, poorly progressive; sP4: highly progressive motility). A higher proportion of sperm were classified as sP4 in SLC-AC samples. Most of the sperm parameters assessed showed higher values in the SLC-AC group. We conclude that SLC-AC is the best protocol to improve sperm quality in chilled canine semen in comparison to the other procedures tested.

Introduction

The use of artificial insemination (AI) with chilled semen in canine breeding has become more popular during the last decades (Farstad 2009). The benefits of this technique are, mainly, that breeding can be carried out without transporting the dogs, and that semen can be easily processed and shipped (Verstegen and others 2005, Shahiduzzaman and Linde-Forsberg 2007). However, their main limitation is that sperm quality decreases during cold storage (Iguer-Ouada and Verstegen 2001, Ponglowhapan and others 2004).

Selection of robust spermatozoa from cooled semen samples could improve the conception rates after AI. In this sense, single layer centrifugation (SLC) has been shown to select good quality spermatozoa (Morrell and others 2009). Several colloids have been successfully used to perform SLC in different species (Morrell and others 2008, Gutiérrez-Cepeda and others 2011, Martinez-Alborcia and others 2012, Dorado and others 2013b), in particular, Androcoll-C (AND), a colloid formulation specifically for dog spermatozoa (Morrell and others 2008, Morrell and others 2013a, Dorado and others 2013c).

The identification of different subpopulations of motile spermatozoa has become an issue of interest (Mortimer 2000), considering mammalian ejaculates as a heterogeneous population. Recent studies have used multistep procedures (non-hierarchical and hierarchical methods) to study the sperm population structure in the dog (Núñez-Martínez and others 2006, Dorado and others 2011c). Besides, the presence of separate motile sperm subpopulations in the dog has been associated with the ability to withstand the adverse effects of cryopreservation (Dorado and others 2011b, Dorado and others 2013c) and storage (Dorado and others 2011c). The characterisation of sperm subpopulations in canine ejaculates has been applied in various studies to compare different cooling extenders (Dorado and others 2011c) or different sperm selection techniques (Dorado and others 2013b, Dorado and others 2013c).

Evaluation of sperm quality in the laboratory is essential to improve efficiency in assisted reproduction. In mammals, sperm motility is of overriding importance for sperm transport (Scott 2000). In addition, the integrity of the plasma membrane (Rijsselaere and others 2002) and normal sperm morphology (Oettle 1993) are essential for spermatozoa to be able to fertilise oocytes. Furthermore, acrosome damage can also render spermatozoa infertile, as acrosomal enzymes are necessary for penetration of oocytes (Kawakami and others 1993). Nevertheless, to the authors' knowledge, no studies have been carried out to assess whether SLC with AND before or after the cooling process might influence sperm quality of chilled dog spermatozoa, including the characteristics of the motility patterns of the different sperm subpopulations.

The objective of the present study was to compare the effect of SLC through AND on canine sperm when SLC was performed before or after the cooling process, or when double SLC (before and after cooling) was performed.

Materials and methods

Animals and semen collection

Animal care and research were performed according to the Spanish Animal Protection Regulation. Semen samples (n=20)

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were obtained from four healthy, mature dogs (five ejaculates per dog). Dogs were three Beagles and one West Highland White Terrier, which were four to eight years old and of unknown fertility. The sperm-rich fraction of each ejaculate was collected by masturbation (Linde-Forsberg 1991), once or twice per week, on non-consecutive days.

Analysis of semen quality parameters

After collection, semen samples were evaluated for volume and sperm concentration by using a photometer (SpermaCue, Minitüb GmbH, Tiefenbach, Germany) (Dorado and others 2013c), and an aliquot of each ejaculate was diluted with a Tris-based extender (Biladyl A, Minitüb GmbH, Tiefenbach, Germany) (Peña and others 2003) to a sperm concentration of 25×10^6 spermatozoa/ml. Aliquots of the diluted semen were taken for the following analyses.

Motility was measured by computer assisted sperm analysis (CASA) as described in Dorado and others (2011b). Each evaluation was carried out using the Sperm Class Analyser motility and concentration module (Microptic SL, Barcelona, Spain). The sperm kinematics measured were total motility (MOT; per cent), progressive motility (PMOT; per cent), curvilinear velocity (VCL; μm/second), straight line velocity (VSL; μm/second), average path velocity (VAP; μm/second), linearity (LIN; per cent), straightness (STR; per cent), wobble (WOB; per cent), amplitude of lateral head displacement (ALH; μm) and beat cross frequency (BCF; Hz). Descriptors of sperm movement were described previously by Dorado and others (2007).

The percentage of spermatozoa with normal morphology (NSM, per cent) was determined by counting 200 spermatozoa per slide under bright-field microscopy at 400× (Dorado and others 2013c).

To assay the sperm membrane integrity we used the Vital-Test kit (Halotech SL, Madrid, Spain) as described by Dorado and others (2013c). The nuclei of spermatozoa with intact plasma membrane stained green with acridine orange (AO) whereas dead sperm nuclei were stained red with propidium iodide (PI). For each sample, 200 spermatozoa were examined by fluorescence microscopy (magnification ×1000) and the percentage of membrane-intact spermatozoa (MIS) (AO+; per cent) was reported in the results.

To assess the sperm acrosomes, we used a fluorescent staining with peanut agglutinin (FITC-PNA) and PI (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Dorado and others 2013c). The sperm acrosome was observed using an epifluorescence microscope. At least 200 spermatozoa were counted on each slide under 1000× magnification and then classified into two groups: acrosome-intact spermatozoa (AIS) (the acrosomal region of the sperm head displayed bright green fluorescence; PI+/FICT−PNA+); and acrosome-reacted spermatozoa (ARS) (green fluorescent at the equatorial segment or no staining; PI +/FICT−PNA−). Spermatozoa showed red fluorescence due to counterstaining with PI. Values were expressed as percentages.

Storage of semen at 5°C

Semen quality was assessed before cooling as detailed above. The cooling protocol was based on the one described by Dorado and others (2011c). In brief, semen was split into two aliquots and processed as follows: one aliquot was diluted 1:1 (v:v) with Tris-based extender and centrifuged at $700\times g$ at 22°C for eight minutes (unselected samples); and the other was diluted in Tris-based extender to 100×10^6 spermatozoa/ml and selected using SLC with AND prior to cooling (SLC-PC samples). The resulting sperm pellets were re-suspended to a final sperm concentration of 50×10^6 spermatozoa/ml with Tris-based extender supplemented with 20 per cent centrifuged egg yolk. Sperm samples were equilibrated at 22°C for 10 minutes before placing in an Equitainer I (Hamilton Research, Inc., Danvers, Massachusetts, USA) for the first 24 hours of chilled preservation. Subsequently the samples were transferred to a 5°C refrigerator (Refrigerated Thermostat–FTC 90E, VELP Scientifica, Usmate, Italy) for preservation for up to 72 hours. For assessment, aliquots of each semen sample were re-warmed to 38°C.

Sperm selection procedure

The colloid used for sperm selection by SLC was a glycidoxypropyltrimethoxysilane-coated silica colloid formulation specially designed for dog spermatozoa (AND; patient applied for). The procedure has been previously described (Dorado and others 2013c). Briefly, 0.8 ml of AND, equilibrated to room temperature (22°C), were poured into a 15 ml Falcon tube (BD Falcon Tubes, BD Biosciences, Erembodegem, Belgium). Thereafter, an aliquot of the semen containing $50–100\times10^6$ spermatozoa/ml was layered (1 ml) on top of the colloid. After centrifugation at $300\times g$ for 20 minutes, the resulting sperm pellet was resuspended in a clean conical tube containing 0.5 ml of Tris-based extender for later evaluation. Sperm concentration was measured before and after SLC to calculate the sperm yield.

Experimental design

As represented in Fig 1, semen samples were divided into four aliquots as follows: unselected: no SLC was performed; SLC-PC: colloidal centrifugation was carried out before cooling; SLC-AC: colloidal centrifugation was performed after cooling; and double SLC: colloidal centrifugation was carried out before and after cooling. Sperm quality parameters were assessed after 72 hours of storage in all samples.

Classification, ordination and identification of sperm subpopulations

With the eight sperm motility descriptors (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF), a multistep clustering procedure was used to identify and classify sperm subpopulations (Martinez-Pastor and others 2005), using data from all 55,138 spermatozoa (17,038 for unselected samples; 14,236 for SLC-PC; 13,204 for SLC-AC; and 10,660 for double SLC) in the study.

Statistical analysis

Results are expressed as mean±SEM. All statistical analyses were carried our using SAS V.9.0 (SAS Institute Inc., Cary, North Carolina, USA).

Sperm parameters were compared by general linear model analysis of variance (ANOVA) (PROC GLM), using the arcsine transformation of the percentage values, in which dogs and ejaculate of the same male were considered a random factor, as well as the interaction between them. Duncan's test was used to determine differences between mean values for significant ($P<0.05$) effects. Finally, to study the distribution of spermatozoa within subpopulations we used the ANOVA and χ^2 (FREQ procedure) tests.

Results

The fresh canine ejaculates (n=20) showed the following features: volume, 0.59±0.04 ml (range 0.5–0.6 ml); sperm concentration, $458.5\pm23.3\times10^6$ spermatozoa/ml (range $409.8–507.2\times10^6$ spermatozoa/ml); total motility, 93.67±1.1 per cent (range 91.4–95.9 per cent); PMOT, 70.8±2.3 per cent (range 65.9–75.7 per cent); normal sperm morphology, 79.4±2.4 per cent (range 74.3–84.4 per cent); MIS, 84.6±1.0 per cent (range 82.5–86.7 per cent); AIS, 74.6±2.7 per cent (range 68.9–80.3 per cent).

Effect of SLC using AND on chilled semen quality

The motility parameters of unselected sperm samples and selected preparations with AND (SLC-PC, SLC-AC and double SLC samples) from chilled dog semen are shown in Table 1. Mean values of VCL, VSL, VAP, LIN, STR and WOB were significantly ($P<0.001$) lower in unselected sperm samples than in the SLC-treatment groups. Mean MOT values were significantly ($P<0.001$) higher in the SLC-PC samples compared with double SLC samples, but no significant differences ($P>0.05$) were observed with unselected or SLC-AC samples. As shown in

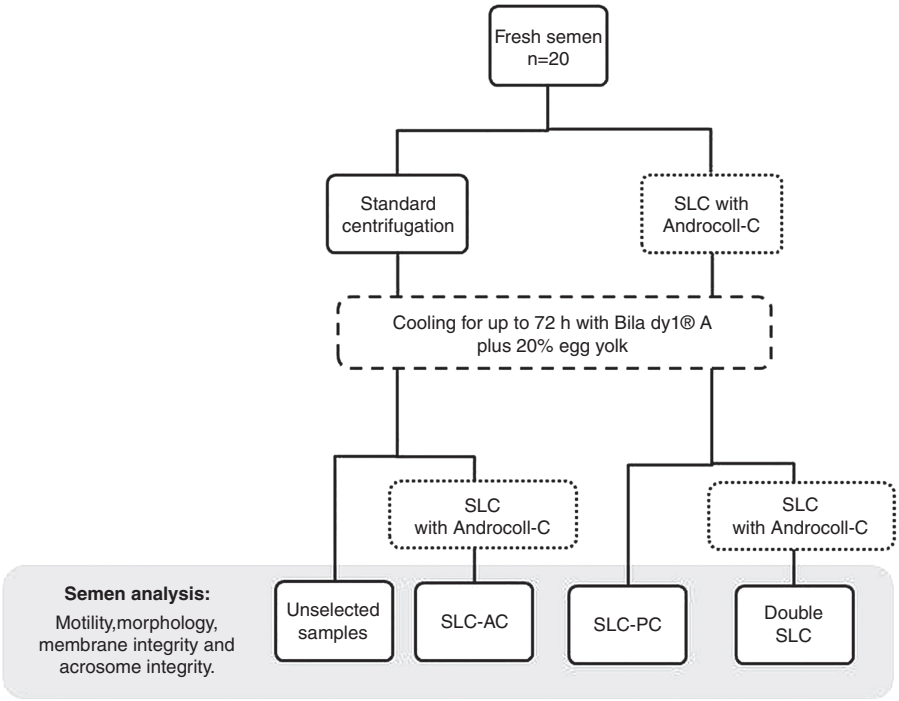

FIG 1: Flow diagram describing the experimental design, showing steps done for different sperm selection methods: SLC-PC (single layer centrifugation prior to cooling), SLC-AC (SLC after cooling) and double SLC. N=20 semen samples for four dogs obtained by digital manipulation

Table 1, SLC-AC samples showed the highest values for the parameters PMOT, VCL, VSL VAP, STR and ALH. However, mean LIN and WOB values were significantly (P<0.001) higher in the double SLC samples.

Sperm morphology, membrane integrity and acrosome integrity parameters are shown in Table 2. Mean MIS values improved significantly (P<0.01) after SLC-PC. Mean NSM values were significantly (P<0.001) higher in the SLC-AC samples compared with SLC-PC samples, but no significant differences (P>0.05) were observed with unselected or double SLC samples. No significant differences (P>0.05) for AIS were found between unselected samples and those selected with AND independently of which protocol was used (SLC-PC, SLC-AC or double SLC).

There were no significant differences (P>0.05) in MOT, PMOT, NSM, MIS and AIS between dogs, in either unselected or selected samples (SLC-PC, SLC-AC and double SLC). However, a significant effect (P<0.01) of replicate was found in all the parameters assessed except for MOT and AIS. Besides, there was an interaction (P<0.01) between dogs and replicate for all sperm parameters.

Changes in the motile sperm subpopulations structure after SLC through AND

Four sperm subpopulations (sP) were identified from the motility data: sP1 (24.5 per cent) included highly active but non-progressive spermatozoa; sP2 (26.9 per cent) included spermatozoa with relatively low velocity but with high progressiveness; sP3 (25.8 per cent) included those spermatozoa whose movement was less vigorous than in all other groups. They were poorly progressive and covered very short distances; sP4 (22.8 per cent) represented the fastest spermatozoa showing the highest degree of PMOT. The spermatozoa exhibited vigorous flagellar action. The motility characteristics of the subpopulations are summarised in Table 3.

Although the percentage of motile sperm included in each subpopulation significantly (P<0.01) changed in each treatment, the sperm subpopulation classification was unchanged for all the protocols carried out in this study (Fig 2). Unselected semen samples showed a significantly higher (P<0.001) percentage of sP3 (34.7 per cent) in comparison to semen samples subjected to SLC. When SLC was performed after cooling (SLC-AC), sP1 and sP4 percentages increased significantly (P<0.001) with

TABLE 1: Mean values (±SEM) of the motility analysis of unselected sperm samples and selected preparations with Androcoll-C (AND) (single layer centrifugation prior to cooling (SLC-PC), SLC after cooling (SLC-AC) and double SLC) from chilled dog semen (20 semen samples from four dogs)

		SLC with AND		
Sperm parameters	Unselected	SLC-PC	SLC-AC	Double SLC
Total motility (MOT, %)	92.66±0.03^{ab}	94.85±0.02^{a}	93.54±0.02^{ab}	89.55±0.02^{b}
Progressive motility (PMOT, %)	53.09±0.04^{b}	57.93±0.03^{b}	69.16±0.03^{a}	60.70±0.04^{b}
Curvilinear velocity (VCL, μm/second)	123.46±0.56^{d}	127.08±0.59^{c}	153.06±0.53^{a}	140.37±0.59^{b}
Straight line velocity (VSL, μm/second)	75.42±0.46^{d}	82.94±0.51^{c}	99.74±0.47^{a}	94.99±0.53^{b}
Average path velocity (VAP, μm/second)	95.17±0.50^{d}	101.41±0.53^{c}	120.02±0.48^{a}	112.67±0.53^{b}
Linearity (LIN, %)	51.43±0.18^{d}	55.91±0.20^{c}	60.18±0.18^{b}	61.15±0.22^{a}
Straightness (STR, %)	69.52±0.18^{d}	72.47±0.20^{c}	78.15±0.18^{a}	77.52±0.23^{b}
Wobble (WOB, %)	71.44±0.11^{d}	74.58±0.11^{c}	75.63±0.11^{b}	77.44±0.12^{a}
Amplitude of lateral head displacement (ALH, μm)	3.86±0.01^{c}	3.78±0.01^{d}	4.54±0.01^{a}	4.14±0.01^{b}
Beat cross frequency (BCF, Hz)	7.84±0.03^{c}	8.46±0.03^{b}	9.40±0.03^{a}	9.48±0.04^{a}

^{a,b,c,d}Different superscripts in the same row indicate significant differences (P<0.05)

TABLE 2: Mean values (±SEM) of the sperm membrane integrity, morphology and acrosome integrity parameters of unselected sperm samples and selected preparations with Androcoll-C (AND) (single layer centrifugation prior to cooling (SLC-PC), SLC after cooling (SLC-AC) and double SLC) from chilled dog semen (20 semen samples from four dogs)

		SLC with AND		
Sperm parameters	Unselected	SLC-PC	SLC-AC	Double SLC
Normal sperm morphology (NSM, %)	67.84±0.03^{ab}	64.69±0.05^{b}	74.46±0.04^{a}	68.28±0.05^{ab}
Membrane-intact spermatozoa (MIS, %)	75.65±0.02^{b}	83.10±0.01^{a}	78.62±0.02^{b}	76.60±0.01^{b}
Membrane-damaged spermatozoa (MDS, %)	24.34±0.02^{a}	16.91±0.01^{b}	21.37±0.02^{a}	23.40±0.01^{a}
Acrosome-intact spermatozoa (AIS, %)	73.45±0.04	78.90±0.03	76.91±0.04	70.80±0.04

^{a,b}Different superscripts in the same row indicate significant differences (P<0.05)

percentages of 31.2 per cent and 28.2 per cent, respectively. However, when semen samples were centrifuged prior to cooling (SLC-PC) most of the spermatozoa were found in sP2 (29.7 per cent) and sP3 (29.9 per cent). sP2 was the most representative sperm subpopulation in semen samples subjected to double colloid centrifugation (32.8 per cent). After 72 hours of cold storage, no significant differences (P>0.05) were found between unselected samples and SLC-PC samples for sP4 (20.3 per cent v 21.0 per cent).

Sperm yield of the SLC procedure

The sperm yield depended on the sperm quality of the original semen sample, the mean recovery rate was 49.6 per cent (range 23–77 per cent) for SLC-PC samples, 51.8 per cent (range 18–90 per cent) for SLC-AC samples and 43.6 per cent (range 18–66.7 per cent) for double SLC samples. Non-significant differences (P>0.05) were found among the protocols SLC-AC, SLC-PC and double SLC for sperm yield.

Discussion

The objective of the present study was to determine the effectiveness of the selection of canine sperm before or after the cooling process, or when double SLC was performed. To this end, sperm quality was evaluated and compared between treatments (unselected, SLC-PC, SLC-AC and double SLC). In our paper, SLC-AC improved all the sperm motility parameters evaluated in comparison to unselected and SLC-PC samples, except for MOT. These results correspond to those obtained in other species (Ortiz and others 2013) where SLC was carried out after the cooling process. Similarly, the three sperm velocities (VCL, VSL and VAP) were significantly higher in SLC-AC samples compared with unselected, SLC-PC and double SLC samples. These variables summarise sperm kinematics and are considered to be reliable indicators of sperm quality (Olds-Clarke 1996). Furthermore, previous studies in canine frozen-thawed semen have demonstrated that sperm velocities (mainly VSL and VAP) are landmarks of fertility in vitro (Silva and others 2006). Our results lead to the conclusion that SLC after cooling improves sperm motility.

In contrast to SLC-AC, SLC-PC did not improve most of the CASA-derived parameters, which could be related to the separation of spermatozoa from seminal plasma before cooling. In fact, other studies also obtained worst results when SLC was performed before freezing in buck (Jiménez-Rabadán and others 2012) and brown bear semen (Nicolas and others 2012). In this regard, it is also plausible that SLC removed proteins that are involved in helping spermatozoa to withstand cold shock (Barrios and others 2005). In a previous study, Barrios and others (2000) demonstrated that the addition of seminal plasma proteins before cold-shock treatment prevents damage to sperm membranes.

Similarly, submitting semen samples to two colloid centrifugations (prior to and after cooling) suppresses the benefits of this technique. It has been demonstrated that centrifugation without a colloid induces sub-lethal damage on the sperm and this adverse effect might be caused by excessive reactive oxygen species (ROS) formation (Mortimer 1991). Morrell and others (2013c) observed that ROS levels were lower in SLC-selected stallion sperm than in uncentrifuged controls. Contrary to our results, better values of PMOT and MOT were obtained in cooled stallion semen subjected to double SLC compared with unselected samples (Morrell and others 2013b). These dissimilar results may be due to the different methods used (consecutive centrifugations, centrifugal force and time, colloid formulation, volumes of sperm and colloid, or size and diameter of centrifuge tubes) and also to the fact that centrifugation has a different effect in different species (Carvajal and others 2004).

In contrast to previous studies in canine fresh semen (Morrell and others 2008, Morrell and others 2013a), no significant differences were observed between unselected and SLC-treated samples for sperm morphology and acrosomal integrity. However, other studies have reported that colloid centrifugation did not significantly improve sperm morphology in frozen-thawed canine semen (Kim and others 2010, Dorado and others 2011a, Phillips and others 2012). These findings suggest that the sperm morphology of the original sample determine how many abnormalities will be removed by colloidal centrifugation. Morrell and others (2009) suggested that the effectiveness of colloid centrifugation is related to the ability of spermatozoa with different morphological characteristics to align themselves in the direction of the centrifugal force.

Of special interest was the observation that the proportion of MIS was greater in SLC-PC samples in comparison to

TABLE 3: Motility parameters for the four sperm subpopulations (sP1, sP2, sP3 and sP4) defined after pattern analysis in unselected and SLC-treated samples from chilled dog semen

		Sperm motility parameters							
Subpopulation (sP)	n (%)	VCL (μm/second)	VSL (μm/second)	VAP (μm/second)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
1	13522 (24.5)	166.28±0.30^{b}	87.44±0.29^{c}	122.92±0.30^{b}	51.43±0.15^{c}	70.70±0.18^{c}	72.78±0.10^{c}	5.33±0.008^{b}	9.38±0.03^{c}
2	14834 (26.9)	130.47±0.30^{c}	96.73±0.31^{b}	108.15±0.29^{c}	72.40±0.11^{b}	88.20±0.10^{b}	81.80±0.07^{b}	3.66±0.007^{c}	10.03±0.03^{b}
3	14217 (25.8)	40.90±0.17^{d}	12.40±0.08^{d}	24.62±0.12^{d}	28.34±0.10^{d}	48.00±0.15^{d}	59.99±0.10^{d}	2.10±0.006^{d}	4.46±0.02^{d}
4	12565 (22.8)	212.26±0.18^{a}	159.48±0.22^{a}	178.00±0.26^{a}	75.40±0.10^{a}	89.82±0.09^{a}	83.82±0.06^{a}	5.38±0.09^{a}	11.17±0.03^{a}

Results are expressed as mean±SEM for 20 semen samples from four dogs. The total number of spermatozoa analysed was 55,138
^{a,b,c,d}Different superscripts in the same column indicate significant differences (P<0.05)
ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; sP1, highly active, non-progressive; sP2, low velocity, highly progressive; sP3, less vigorous, poorly progressive; sP4, highly progressive motility; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble

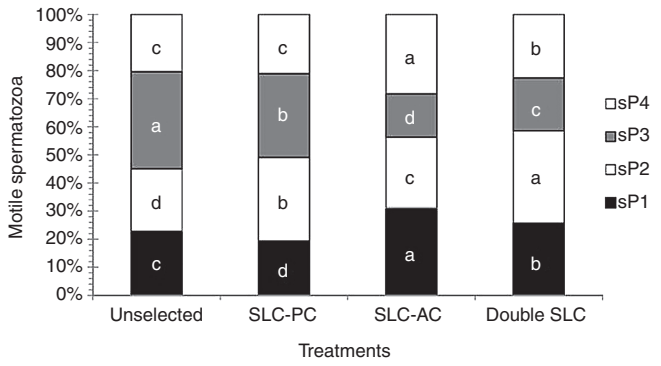

FIG 2: Changes in the sperm subpopulation structure of dog chilled spermatozoa in unselected samples and after different selection methods with Androcoll-C (AND) (single layer centrifugation prior to cooling (SLC-PC), SLC after cooling (SLC-AC) and double SLC samples). Results express the percentages of the sperm populations (sP1, sP2, sP3 and sP4) distributed among the four groups depending upon the treatment (n=20). The total number of spermatozoa analysed was 55,138. The spermatozoa were distributed among the semen samples in the following manner: 17,038 motile spermatozoa were from unselected, 14,236 from SLC-PC, 13,204 from SLC-AC and 10,660 from double SLC samples. sP1, highly active, non-progressive; sP2, low velocity, highly progressive; sP3, less vigorous, poorly progressive; sP4, highly progressive motility. ^{a,b,c,d}Different superscripts inside columns indicate significant differences within subpopulations between treatments ($P<0.05$, χ^2 test)

unselected, SLC-AC or double SLC samples. These results are in agreement with another study (Johannisson and others 2009) where sperm membrane integrity was improved when SLC was carried out in stallion samples before cooling. It is plausible to assume that the lower values found in SLC-AC or double SLC samples for sperm membrane integrity could be due, at least in part, to centrifugal force. In our experimental conditions, it must be considered that SLC-AC and double SLC samples were subjected to double centrifugation, and that, as explained previously, the centrifugation of semen has some drawbacks such as cell damage and the loss of sperm cells in the supernatant (Loomis 2006, Aurich 2008). A previous study in chilled canine semen (Rijsselaere and others 2002) demonstrated that centrifugal force causes a detrimental effect on sperm membrane integrity. In line with this observation, in the present study double centrifugation resulted in higher cell damage and cell death.

The predominance of a particular motile subpopulation has been reported in other studies (Quintero-Moreno and others 2003, Dorado and others 2011c, Mendoza and others 2012). Our study identified four discrete sperm subpopulations in unselected and selected samples from chilled dog semen, which agrees with previous studies in dogs (Dorado and others 2011b, Dorado and others 2011c). In our study, SLC through AND significantly modified the distribution of spermatozoa within subpopulations. On the one hand, the predominant subpopulation in unselected sperm samples was subpopulation 3 (sP3; 34.7 per cent), which consisted of poorly active and non-progressive spermatozoa. On the other hand, sperm selection by SLC through AND enriched the subpopulation depicting higher velocities in the processed dog semen sample (sP4; high-speed and progressive spermatozoa), reaching proportions of 21 per cent, 28.2 per cent and 22.6 per cent for SLC-PC, SLC-AC and double SLC, respectively. Our results are in agreement with previous studies in which frozen-thawed dog semen was submitted to colloid centrifugation with AND (Dorado and others 2013c) and PureSperm (Dorado and others 2011a). Similar results were also obtained when assessing SLC using Androcoll-E in thawed stallion semen (García and others 2009). In this regard, these results demonstrated that the quality of the sperm sample was improved in terms of sperm subpopulation structure and it can be surmised that sperm preparations following SLC with AND contain those spermatozoa with highest potential fertilising ability. It has been demonstrated recently that in vivo fertility in donkeys is related to the identified subpopulations (Dorado and others 2013a). Therefore, further studies should be performed on dog semen to understand the implications of the different motile sperm subpopulations in processed semen on subsequent in vivo or in vitro fertility.

Recent studies have successfully shown an improvement of sperm quality in both raw and chilled dog semen samples by performing SLC using AND, where a yield up to 47 per cent was obtained (Morrell and others 2008). For this reason, SLC could be a suitable option to enhance chilled dog sperm quality before AI. The mean sperm recovery rate in the current study was 49.6 per cent for SLC-PC, 51.8 per cent for SLC-AC and 43.6 per cent for double SLC, which are comparable to those obtained by previous studies for SLC in dogs (Morrell and others 2008) and in stallions (Morrell and others 2011, Morrell and others 2013b). In addition, the sperm recovery yield was greater when SLC was used after cooling than either prior to cooling or double SLC. Our results disagree with those for cat and buck spermatozoa reported by Chatdarong and others (2010) and Jiménez-Rabadán and others (2012), who found greater sperm recovery using SLC before freezing than SLC after thawing. The discordant recovery yields among studies would reflect differences in the initial semen traits of the samples, which could affect the number of functional spermatozoa passing through the colloid (Martinez-Alborcia and others 2012). Moreover, it is difficult to compare assays developed with different sperm selection methodologies, different methods of semen preservation, or species because the physical dynamics may affect sperm recovery differently, as has been suggested previously (Nicolas and others 2012).

Although the yield obtained in the SLC-PC samples was the lowest (43.6 per cent) compared with the remaining treatments, if the whole ejaculate is processed with this protocol, the number of spermatozoa recovered (~117×10^6 sperm) (volume×million sperm/ml×recovery rate/100) is enough to inseminate a bitch with chilled semen according to the insemination protocol described by Pinto and others (1999). This means that sufficient sperm are obtained to inseminate a bitch if intra-vaginal AI is used, regardless of when SLC is performed. In addition, elimination of undesirable spermatozoa from chilled canine semen with SLC after cooling using AND enables sperm doses to be prepared for AI that are enriched in specific sperm characteristics.

Conclusions

With the results of the present study we conclude that SLC-AC is the best protocol to improve sperm quality in chilled canine semen doses in comparison to unselected, SLC-PC or double SLC samples. However, now that SLC through AND after cooling has been shown to be an effective method to enhance sperm quality parameters of cooled dog semen in vitro, further studies are needed to relate sperm quality improvement of dog semen doses to pregnancy rates after AI.

Competing interests None.

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