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Single-layer centrifugation through PureSperm® 80 selects improved quality spermatozoa from frozen-thawed dog semen

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ABSTRACT

The aim of this study was to investigate whether single-layer centrifugation (SLC) with PureSperm® 80 could select good quality spermatozoa, including those with specific motility patterns, from doses of frozen dog semen. Semen from 5 dogs was collected and cryopreserved following a standard protocol. After thawing, semen samples were divided into two aliquots: one of them was used as control and the other one processed by SLC. Assessment of sperm motility (assessed by computer-assisted semen analysis), morphology (Diff-Quick staining) and viability (triple fluorescent stain of propidium iodine/isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin/Rhodamine 123), were performed on aliquots of fresh semen, frozen-thawed control and frozen-thawed SLC treated samples. A multivariate clustering procedure separated 26,051 motile spermatozoa into three subpopulations (sP): sP1 consisting of highly active but non-progressive spermatozoa (40.3%), sP2 consisting of spermatozoa with high velocity and progressive motility (30.0%), and sP3 consisting of poorly active and non-progressive spermatozoa (29.7%). SLC with PureSperm® 80 yielded sperm suspensions with improved motility, morphology, viability and acrosome integrity ($P < 0.001$). The frozen-thawed SLC treated samples were enriched in sP2, reaching a proportion of 44.1% of the present spermatozoa. From these results, we concluded that SLC with PureSperm® 80 may be an alternative and successful method for improving the quality of frozen-thawed dog spermatozoa. Moreover, sP2 (high-speed and progressive spermatozoa) was more frequently observed after SLC. Finally, this study also demonstrated that the general motile sperm structure present in dogs remained constant despite the effect caused by either cryopreservation or separation by SLC through PureSperm® 80.

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1. Introduction

Sperm cryopreservation is an integral part of assisted conception programs and has its application in many circumstances, such as situations involving impaired male fertility, assisted reproduction techniques (ART), and donor semen banking (Kim et al., 2010). However, cryopreservation induces a series of osmotic, chemical, and mechanical stresses to sperm, causing death of some sperm and severe post-thaw damage in surviving cells, reducing fertilizing

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ability (Watson, 1995, 2000). Moreover, damage caused by semen processing procedures is accumulative, and small injuries may result in important deleterious changes at the end of the process (Nicolas et al., 2012). Therefore, current pregnancy and live birth success rates of ART are not completely satisfactory with frozen-thawed dog semen (Kim et al., 2010). For all these reasons, the selection of suitable spermatozoa from processed semen (frozen-thawed) where the majority has been damaged or is dead should be one of the prerequisites for achieving optimal conception rates after artificial insemination (AI) or other ART with frozen-thawed dog semen.

There are several methods (*i.e.* migration, filtration, and colloid centrifugation) available for selecting functional spermatozoa on the basis of certain sperm attributes (Morrell and Rodriguez-Martinez, 2011). Density gradient centrifugation (DGC) has been suggested as a means of selecting spermatozoa for artificial breeding (Morrell et al., 2009). This technique has been satisfactorily used to separate motile, chromatin-intact and morphologically normal spermatozoa from the extended semen (Morrell and Rodriguez-Martinez, 2009). Specially, the PureSperm® (Nidacon International AB, Gothenburg, Sweden) density-gradient centrifugation technique has been designed to select viable and morphologically intact human spermatozoa and to purify them for ART (Morrell et al., 2004; Mousset-Siméon et al., 2004). Moreover, PureSperm® gradients have also been satisfactorily applied to ejaculates and processed semen from many species, such as primates (O'Brien et al., 2003), marmosets (Hernández-López et al., 2005), bulls (Underwood et al., 2009), rams (Hollinshead et al., 2004), bears (Nicolas et al., 2012) and dogs (Dorado et al., 2011a, 2011b; Phillips et al., 2012), in order to clean the spermatozoa from seminal plasma or extenders and also to enrich the amount of cells with normal morphology and/or motility to be then used in ART.

A new method, single-layer centrifugation (SLC) through a colloid, has been recently developed by Morrell et al. (2008a), which uses only one layer of colloid. This technique is simpler to use than DGC, while apparently being equally effective (Morrell et al., 2009). Therefore, using different colloids, SLC has successfully been used to improve the quality of sperm samples in humans (Zhou et al., 2010), stallions (Gutiérrez-Cepeda et al., 2011; Morrell et al., 2011), boars (Martinez-Alborcia et al., 2012), bulls (Thys et al., 2009), dogs (Morrell et al., 2008b) and cats (Chatdarong et al., 2010). A modification of the PureSperm® density gradient technique, centrifugation through a single-layer of colloid, has also been reported for human spermatozoa (Zhang et al., 2004). However, to our knowledge, studies on the effect of sperm selection by means of SLC through PureSperm® on sperm quality after cryopreservation of dog semen have not been reported. Likewise, there are no available references relative to the influence of the centrifugation of frozen-thawed semen through a single-layer of PureSperm® on the structure of dog motile sperm subpopulation.

The aim of this study was therefore to evaluate whether SLC through PureSperm® 80 improves the post-thaw sperm quality of dog spermatozoa, concerning its ability to separate the subpopulation of spermatozoa with good motility, morphology, viability and acrosome integrity.

2. Materials and methods

2.1. Animals

Semen was collected from 5 clinically healthy experimental dogs (4 Beagles and 1 German Pointer) ranging between 2 and 8 years and of unknown fertility. The study was carried out according to the Spanish laws for animal welfare and experimentation.

2.2. Semen collection and processing

A total of 10 ejaculates (2 ejaculates per dog) were obtained by digital manipulation on different and non consecutive experimental days, once or twice per week, and the sperm-rich second fraction of the ejaculates was collected (Linde-Forsberg, 1991). Semen volume was determined in a calibrated tube. Sperm concentration was determined with a photometer (SpermaCue, Minitüb, Tiefenbach, Germany), as described by Peña et al. (2003). Only ejaculates with volume $\geq$ 0.8 mL, sperm concentration $\geq 200 \times 10^6$ spermatozoa/mL, motility $\geq$ 70% and normal morphology $\geq$ 70% were included in the study.

Semen was then frozen using the Uppsala method (Peña and Linde-Forsberg, 2000) modified by Dorado et al. (2011a) as follows: after collection, semen was diluted 1:1 (v/v) with Tris-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) and centrifuged at $700 \times g$ for 10 min at 22 °C. The resulting sperm pellet was suspended to a final sperm concentration of $300–400 \times 10^6$ spermatozoa/mL with CaniPRO™ Freeze A plus 20% centrifuged egg yolk at 22 °C. Extended semen was slowly cooled to 5 °C within an hour and then diluted to a final sperm concentration of $150–200 \times 10^6$ spermatozoa/mL in CaniPRO™ Freeze B plus 20% centrifuged egg yolk at 5 °C. Finally, the spermatozoa were loaded in 0.5 mL plastic straws and frozen horizontally in ranks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 min, after which they were directly placed in LN_2. After 24–48 h of storage, straws were thawed in a water bath at 37 °C for 30 s, for analyses.

2.3. Sperm separation procedure

The technique used for SLC was a modification of the procedure described by Morrell et al. (2012). In brief, 2 mL of PureSperm® 80, equilibrated to 22 °C, were poured into a 15 mL Falcon centrifuge tube (BDFalcon™ Tubes, BD Biosciences, Erembodegem, Belgium). Thereafter, an aliquot of the frozen-thawed semen (straws containing $80–100 \times 10^6$ spermatozoa/mL) was layered (1 mL) on top of the colloid. The tubes were centrifuged for 20 min at $300 \times g$ in a bench centrifuge (Eppendorf Centrifuge 5702RH, Eppendorf Ibérica SLU, Madrid, Spain) with a swing-out rotor. The sperm pellet was re-suspended in a new conical tube to obtain a final sperm concentration of 30×10^6 spermatozoa/mL with

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