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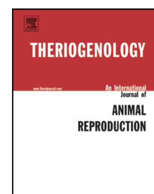

Use of single-layer centrifugation with Androcoll-C to enhance sperm quality in frozen-thawed dog semen

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ABSTRACT

The aim of this study was to investigate whether single-layer centrifugation (SLC) with Androcoll-C could select good quality spermatozoa, including those with specific motility patterns, from doses of frozen dog semen. Semen from five dogs was collected and cryopreserved following a standard protocol. After thawing, the semen samples were divided in two aliquots, one of which was used as a control and the other one processed by SLC. Assessment of sperm motility (assessed by computer-assisted semen analysis), morphology (Diff-Quick staining), viability (dual staining with propidium iodine/acridine orange), and acrosome integrity (dual staining with propidium iodine/isothiocyanate-labeled peanut [*Arachis hypogaea*] agglutinin) were performed on aliquots of fresh semen, frozen-thawed control samples, and frozen-thawed SLC-treated preparations. A multivariate clustering procedure separated 57,577 motile spermatozoa into three subpopulations (sP): sP1 consisted of poorly active and nonprogressive spermatozoa (48.8%), sP2 consisted of moderately slow but progressive spermatozoa (13.3%), and sP3 consisted of highly active and/or progressive spermatozoa (37.8%). SLC with Androcoll-C yielded sperm suspensions with improved motility, viability, and acrosome integrity ($P < 0.01$). The frozen-thawed SLC-treated samples were enriched in sP3, representing 38.5% of the sperm population. Likewise, sP2 was more frequently observed after SLC, but not significantly so. From these results, we concluded that for dog semen samples selected by SLC with Androcoll-C after thawing, the sperm quality parameters, including motility patterns, are better than in frozen-thawed control samples.

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1. Introduction

Sperm cryopreservation is an integral part of assisted conception programs and is used extensively in assisted reproduction techniques (ARTs). In dogs, the use of frozen semen allows breeders to store semen from genetically superior dogs for future use [1]. However, cryopreservation causes major damage to the spermatozoa [2]. In consequence, current pregnancy and live birth success rates of ART are not completely satisfactory with frozen-thawed dog semen [1]. Taking into account all these facts, the selection of suitable spermatozoa from frozen-thawed semen where the majority is dead should be one of the prerequisites for achieving optimal conception rates after artificial insemination (AI) or other ART with cryopreserved 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 [3]. Recently, a simpler colloid centrifugation procedure, so-called single-layer centrifugation (SLC), has been developed to work with only one layer of colloid [4]. In this method, spermatozoa are centrifuged through a column of glycidoxypropyltrimethoxysilane-coated silica in a

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species-specific formulation (Androcoll), resulting in the selection of motile, morphologically normal spermatozoa with intact membranes and good chromatin integrity [5,6]. SLC with Androcoll has been used in several species for harvesting suitable spermatozoa from ejaculates [7–9], epididymal samples [10], or processed semen [11–13], 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 for subsequent use in ART.

The identification of different subpopulations of motile spermatozoa, whose motion parameters are characterized by precise values obtained by computer-assisted sperm analysis (CASA), within the mammalian ejaculate, has become an issue of considerable interest for the evaluation of ejaculates. Moreover, the presence of discrete motile sperm subpopulations has been related to resistance to cryopreservation [14,15], presence of stimulants [16], storage [17], and fertility [18,19]. Recently, Macías García et al. [20] reported that SLC with Androcoll-E is a simple and valuable method of enriching sperm subpopulations with high sperm velocities (particularly VCL) among a suspension of frozen-thawed equine spermatozoa. However, to the best of our knowledge, no studies have so far defined whether the application of SLC with Androcoll influences the sperm quality, including the characteristics of the motility patterns of the different sperm subpopulations, of cryopreserved dog spermatozoa.

With this background, this study was aimed to evaluate whether SLC through Androcoll-C (a colloid for dog spermatozoa) improves the quality of cryopreserved canine spermatozoa, concerning its ability to separate the subpopulation of spermatozoa with good motility, morphology, viability, and acrosome integrity.

2. Materials and methods

2.1. Semen collection

Semen was collected from five clinically healthy experimental dogs (four Beagles and one 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. A total of 20 ejaculates (four ejaculates per dog) were obtained by digital manipulation on different and nonconsecutive experimental days, once or twice per week, and the sperm-rich second fraction of the ejaculates was collected [21]. Immediately after collection, 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. [22].

2.2. Semen freezing and thawing process

Semen quality was assessed before freezing as detailed below. Canine semen freezing was performed as previously described [23]. Briefly, semen was diluted 1:1 (vol:vol) with Tris-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) and centrifuged at $700 \times g$ for 10 minutes at 22 °C. The resulting sperm pellet was resuspended to a final sperm concentration of 200×10^6 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 100×10^6 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 minutes, after which they were directly placed in LN_2. After 24 to 48 hours of storage, straws were thawed in a water bath at 37 °C for 30 seconds for analyses.

2.3. Sperm separation procedure

The colloid used for SLC consisted of glycidoxypropyltrimethoxysilane-coated silica optimized for dog spermatozoa (Androcoll-C; Swedish University of Agricultural Sciences—SLU, Uppsala, Sweden). No preparation is required other than equilibration to room temperature (22 °C) before use. The technique used for SLC was a modification of the procedure described by Morrell et al. [8]. In brief, two straws from frozen-thawed control samples were thawed and their contents were pooled. Androcoll-C (0.8 mL) was poured into a 15-mL Falcon centrifuge tube (BDFalconTubes, BD Biosciences, Erembodegem, Belgium) and an aliquot of pooled semen (1 mL of frozen-thawed semen containing approximately 100×10^6 spermatozoa/mL) was layered on top of the colloid. The tubes were centrifuged for 20 minutes at $300 \times g$ in a bench centrifuge (Eppendorf Centrifuge 5702RH, Eppendorf Ibérica SLU, Madrid, Spain) with a swing-out rotor. The resulting sperm pellet was resuspended in a new conical tube with 0.5 mL of Tris-based extender (Biladyl A, Minitüb) for later evaluations. Sperm concentration was finally measured to calculate the yield of the SLC procedure.

2.4. Sperm quality parameters assessment

Each ejaculate was assessed for sperm quality before freezing (fresh semen), and frozen-thawed sperm samples from each treatment (frozen-thawed control and frozen-thawed SLC-treated samples) were assessed after thawing.

2.4.1. Sperm morphology

Sperm morphology was examined by light microscopy evaluation (Olympus BH-2, Olympus Optical Co., Ltd., Tokyo, Japan) of smears stained with Diff-Quick (Medion Diagnostics AG, Düdingen, Switzerland) staining [1]. At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with abnormal morphology (ASM, %).

2.4.2. Objective sperm motility

Motility was measured using a CASA system (Sperm Class Analyzer, Microptic SL, Barcelona, Spain), as described by Núñez-Martínez et al. [15] for dog semen. The analysis was on the basis of the analysis of 25 consecutive, digitized images obtained from a single field. Images were taken in a time lapse of 1 second, corresponding to a velocity of image capturing of one photograph every 40 ms. Before the analysis, an aliquot of semen was diluted with TRIS-based

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