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Technical Note

Single-layer centrifugation separates spermatozoa from diploid cells in epididymal samples from gray wolves, *Canis lupus* (L.)

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ABSTRACT

Sperm samples may be used for assisted reproductive technologies (e.g., farmed or endangered species) or as a source of haploid DNA or sperm-specific RNA. When ejaculated spermatozoa are not available or are very difficult to obtain, as is the case for most wild endangered species, the epididymides of dead animals (e.g., animals that have been found dead, shot by hunters or poachers, or that that require euthanasia in zoological collections) can be used as a source of sperm. Such epididymal sperm samples are usually contaminated with cellular debris, erythrocytes, leukocytes, and sometimes also bacteria. These contaminants may be sources of reactive oxygen species that damage spermatozoa during freezing or contribute undesired genetic material from diploid cells. We used single-layer centrifugation through a colloid formulation, Androcoll-C, to successfully separate wolf epididymal spermatozoa from contaminating cells and cellular debris in epididymal samples harvested from carcasses. Such a procedure may potentially be applied to epididymal sperm samples from other species.

1. Introduction

Sperm samples can be used in assisted reproduction in animal breeding and are also a source of haploid DNA or sperm-specific RNA. When fresh ejaculates are difficult to obtain, such as when working with wild animals, the epididymides of animals that have died unexpectedly or that require euthanasia become an alternative source of spermatozoa. Samples obtained this way, however, are compromised because they carry cellular debris, erythrocytes, and leukocytes in a proportion that is much higher than in ejaculates, and which are generally undesirable in downstream applications. For example, in sperm typing studies that aim at measuring the recombination rate, the presence of diploid cells, such as epithelial cells, is of particular concern, as they may result in false recombinants [1]. In the context of assisted reproductive technologies, these contaminants may act as sources of reactive oxygen species that damage spermatozoa during freezing. For instance, separating stallion epididymal spermatozoa from cell debris aids cryosurvival [2]. For assuring the isolation of sperm-specific RNA, separation of spermatozoa from somatic cells is critical [3]. In cases of rape, separating sperm cells of the attacker from vaginal cells is critical to obtain enough material for DNA identification [4]. Therefore, it is important to assess the presence of undesirable contaminants in sperm samples and design appropriate methods to remove them.

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The technique of single-layer centrifugation through species-specific colloids has been used to improve sperm quality in ejaculated sperm samples from many species [5] and in epididymal sperm samples from cats [6] and red deer [7]. However, because the density of the sperm cell changes during maturation, and because there is species-specific variation, available colloid centrifugation protocols aimed at selecting mature sperm in other species may not be optimal for gray wolf epididymal sperm samples. In addition, a large proportion of the spermatozoa are lost during the process. Here we intended to maximize the amount of sperm cells remaining after the cleaning procedure, independent of their maturation stage, while successfully removing cells and cell debris. We developed a protocol based on Androcoll-C, a silane-coated silica colloid formulation, for separating wolf epididymal spermatozoa from contaminating cells and cellular debris in epididymal samples harvested from carcasses. This method was successful at separating both motile and nonmotile spermatozoa from other cells and tissue.

2. Materials and methods

We obtained wolf testes opportunistically from animals killed or found dead in the field or euthanized in zoos for reasons other than this study (Table 1). Testes were sampled and refrigerated as soon as possible (often hours after the death of the individual) and were taken to the laboratory. In these conditions, canid spermatozoa may be alive, and therefore motile, for about a week [8]. In some cases, testes arrived at the laboratory frozen due to environmental conditions (animals killed or found dead during winter) or logistics (samples could not be shipped or released immediately by the collector and were preserved frozen at −20 °C).

Epididymides were cut and flushed with a Tris extender [9] or the spermatozoa aspirated with a pipette and released into 4 mL of the same extender. Some of these samples were red in color because of the presence of erythrocytes. Under the microscope, diploid cells and undetermined particles were observed. In order to clean the samples, we tested 80%, 40%, 30%, and 20% Androcoll-C (Swedish University of Agricultural Sciences–SLU, Uppsala, Sweden) concentrations semiquantitatively. Androcoll-C is a glycidoxypropyltrimethoxysilane-coated silica optimized for dog spermatozoa, which requires only equilibration to room temperature (22 °C) before use [10–12]. We diluted Androcoll-C with buffer E, and determined that 30% Androcoll-C provided a good balance between removal of diploid cells and loss of spermatozoa. We dispensed 4 mL of the sperm sample on top of 4 mL of the diluted colloid, with particular care to avoid mixing them, as this would destroy the integrity of the interface between the two layers and reduce the efficiency of the sperm selection process. The samples were then centrifuged for 20 minutes at 300× *g*. We aspirated the supernatant, the interface, the colloid, and the pellet separately; the sperm pellet was subsequently resuspended in PBS. To determine the concentration of spermatozoa and cells in the pellet suspension, these samples were examined under a phase-contrast microscope using a Bürker chamber.

In order to compare the relative proportion of spermatozoa to other diploid cells in the pellet suspension with that obtained from fresh ejaculates, we estimated spermatozoa and cell concentration in fresh semen obtained

Table 1
Concentration of spermatozoa and cells in epididymal sperm samples of gray wolves, *Canis lupus*, after single-layer centrifugation with 30% Androcoll-C (treatment SLC below, see text for details), estimated using a Bürker chamber under a phase-contrast microscope. Concentration of the dog ejaculates, *C. familiaris*, are provided for comparison.

Sample	Species	Origin	Source	Treatment	Spz/mL	Cells/mL
CaLu1	Wolf	Sweden	Epididymus	SLC	Spz aggregates	?
CaLu2	Wolf	Spain	Epididymus	SLC	Spz aggregates	?
CaLu3	Wolf	Spain	Epididymus	SLC	Spz aggregates	?
CaLu4	Wolf	Sweden	Epididymus	SLC	4×10^6	0
CaLu5	Wolf	Sweden	Epididymus	SLC	43.5×10^6	0
CaLu6	Wolf	Sweden	Epididymus	SLC	9.0×10^6	0
CaLu7	Wolf	Sweden	Epididymus	SLC	16.7×10^6	1×10^6
CaLu8	Wolf	Spain	Epididymus	SLC	5.3×10^6	0
CaLu9	Wolf	Spain	Epididymus	SLC	51.1×10^6	0
CaLu10	Wolf	Spain	Epididymus	SLC	Spz aggregates	?
CaLuCap1	Wolf	Captive	Epididymus	SLC	11.5×10^6	0
CaLuCap2	Wolf	Captive	Epididymus	SLC	7.4×10^6	0
CaLuCap3	Wolf	Captive	Epididymus	SLC	7.4×10^6	2×10^6
CaFa1	Dog	Pet	Ejaculate	None	21×10^6	0
CaFa2	Dog	Pet	Ejaculate	None	12×10^6	0
CaFa3	Dog	Pet	Ejaculate	None	6.6×10^6	?[a]
CaFa4	Dog	Pet	Ejaculate	None	71.5×10^6	0
CaFa5	Dog	Pet	Ejaculate	None	37×10^6	0
CaFa6	Dog	Pet	Ejaculate	None	Too diluted	?
CaFa7	Dog	Pet	Ejaculate	None	Spz aggregates	?
CaFa8	Dog	Pet	Ejaculate	None	Spz aggregates	?
CaFa9	Dog	Pet	Ejaculate	None	Spz aggregates	?
CaFa10	Dog	Pet	Ejaculate	None	41×10^6	1×10^6

Abbreviations: SLC, single-layer centrifugation; Spz, spermatozoa.
[a] Undefined particles present. Number of cells cannot be estimated and are difficult to count.

from 10 privately-owned dogs sampled for reasons other than this study. Semen had been obtained as in [13], and samples were similarly examined under a phase-contrast microscope using a Bürker chamber.

3. Results

Visual inspection under the microscope revealed the presence of cell debris and spermatozoa in the supernatant and interface, whereas the pellet, resuspended in PBS, contained spermatozoa basically free of other cells. This contrasts to the precentrifugation epididymal samples, in which spermatozoa, cells and undetermined particles were observed. For the 13 "clean" wolf epididymal sperm samples, we found spermatozoa aggregates in four (Table 1). For the 10 fresh ejaculates from dogs used as reference, spermatozoa aggregates were found in three, undefined particles were observed in an additional one, and another one was too diluted for accurate quantification. The reamining samples could be quantified, and the concentration estimates of spermatozoa and cells as well as the proportion of samples with cells were similar in the wolf resuspended pellets and dog ejaculates (Table 1). Spermatozoa ranged between 4×10^6 to 51.1×10^6 spermatozoa/mL in the pellet suspension, compared with 6.6×10^6 to 71.5×10^6 spermatozoa/mL in the fresh ejaculates, while cells were observed in two out of the nine pellet suspensions, and in one out of five dog ejaculates, in a concentration of 1×10^6 to 2×10^6 cells/mL and 1×10^6 cells/mL, respectively.

4. Discussion

Density gradients have been used successfully for selecting good quality spermatozoa (reviewed in [5,14]). However, the requirement of layering at least two densities of colloid in the tube and the small sample volumes that can be processed per tube make the process impractical. On the other hand, single-layer centrifugation using species-specific colloid formulations based on silane-coated silica is more practical and easier to use because only one colloid layer is needed [5]. Moreover, single-layer centrifugation can be scaled-up to process larger volumes, which is more difficult and time-consuming to do with density gradients. Traditionally, density gradient centrifugation was performed with Percoll, which contains polyvinylpyrrolidone-coated silica particles and requires the addition of salts, sugars, and buffers for use with spermatozoa [5]. Although Percoll-based protocols have been used successfully to select robust spermatozoa from the rest of the ejaculate in poor quality samples, they do not appear to work well with normal stallion ejaculates [15], but the colloid formulation used may not have been optimal for this species. In contrast, a species-specific colloid such as Androcoll-E selects good quality spermatozoa from the rest of the ejaculate even when normal ejaculates are processed [16,17], suggesting that the physical characteristics of the colloid formulation are important for achieving good selection. Comparisons between Androcoll and Percoll (e.g., [18]) and Androcoll and other silane-coated silica colloids [17,19] have been made previously, with Androcoll performing better in all cases. In the study by Colleoni et al. [19], more embryos developed to the blastocyst stage when Androcoll-E–selected spermatozoa were injected into oocytes (intracytoplasmic sperm injection) than when another formulation was used as a density gradient. Sperm also retained their motility for longer in samples selected by Androcoll-E than by another silane-coated silica product used as a density gradient [17]. Single-layer centrifugation has been successfully applied for animal breeding purposes to select good quality spermatozoa and to remove cellular debris and pathogens that may be present in the seminal plasma of ejaculates, [16] to prepare epididymal sperm because the spermatozoa survive freezing better if the debris is removed, [6] or to remove proteins coating the surface of the spermatozoa [20].

The colloid formulation used in this study—Androcoll-C, where C denotes canine—has been used to separate robust dog spermatozoa from the rest of the ejaculate [10–12]. Similar results, in terms of selection for good motility, normal morphology, intact sperm membranes, and good chromatin integrity, have been described for other species where species-specific colloid formulations have been used to prepare semen [14]. However, in the present study, it was not possible to test a variety of colloid formulations because of the paucity of material available. Wolf epididymal sperm samples are in short supply and all the sperm were needed for the extraction of DNA. Hence, it is not known whether the colloid formulation was optimal for wolf epididymal sperm compared with alternative approaches; here we report that this method worked to provide us with "clean" sperm samples free of somatic cells, which may be of interest to other researchers and technicians working with DNA or RNA in sperm samples.

In this study, we found that using Androcoll-C at 30% allowed us to achieve a good balance between removal of diploid cells and loss of spermatozoa in wolf epididymal sperm samples. Quantitative determination of spermatozoa and diploid cells in the pellet suspension obtained after single-layer centrifugation resulted in similar ratios to those observed in fresh dog ejaculates. Ejaculates are considered adequate, for example, in sperm typing studies aimed at measuring recombination rate (e.g., [1]), in which the sample needs to be free of somatic cells. The method recovered both motile spermatozoa from fresh testes and nonmotile spermatozoa from frozen testes.

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The authors declare no conflict of interest. JMM has applied for a patent for Androcoll-C and Buffer E; both are available from the Swedish University of Agricultural Sciences–SLU, Uppsala, Sweden.

Author contributions: VMF, CLF, and JMM designed research; VMF, CLF, and CV collected samples; VMF, CLF, and JMM performed experiments; VMF wrote the first draft of the article. All authors revised the article and contributed to the text.

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