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Sex-sorted canine sperm cryopreservation: Limits and procedural considerations

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ABSTRACT

The aim of this study was to define a protocol to store dog sperm before and after sorting to obtain an insemination dose sufficient to allow the conception by artificial insemination. Experiment 1 and 2 were performed to evaluate the more appropriate extender for preserving at room temperature dog sperm before and after sorting. Four extenders were tested: (1) Tris-fructose-citrate (TFC), (2) Tris-glucose-citrate (TGC), (3) modified Tyrode's albumin lactate pyruvate medium (mTALP), and (4) third fraction of the ejaculate (after centrifugation at $5000\times g$ for 10 minutes; III FRAC). Experiment 3 and 4 were performed to evaluate the ability of dog semen to withstand sex sorting and freezing/thawing. Modified Tyrode's albumin lactate pyruvate medium was the best extender for canine sperm storage at room temperature (20 °C–25 °C) before (total motility: TFC, 8.3 ± 1.7; TGC, 50.0 ± 11.5; mTALP, 70.0 ± 0.1; III FRAC, 25.0 ± 1 0.4; $P < 0.05$) and after sorting (total motility: TFC, 7.3 ± 1.5; TGC, 10.3 ± 1.5; mTALP, 33.3 ± 6.7; III FRAC, 8.7 ± 5.8; $P < 0.05$), even if at 24-hour sorted sperm quality was impaired in all extenders tested herein. Sperm quality decreased after sorting (total motility: control, 92.5 ± 0.9; sorted, 52.9 ± 6.0; $P < 0.05$) and, especially, after freezing/thawing (total motility: frozen control, 25.7 ± 4.1; frozen sorted, 2.4 ± 1.2; $P < 0.05$). In conclusion, mTALP is an appropriate medium for canine sperm storage before and soon after sorting (hours), but a long storage period of sexed sperm at room temperature is not adequate. Cryopreservation greatly impaired sperm quality, and further studies are needed to optimize the freezing protocol for sexed dog sperm.

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1. Introduction

Flow cytometric sperm sorting based on X and Y sperm DNA difference has been established as the only effective method to predeterminate the sex of mammalian offspring before fertilization.

Although sexing technique has already reached a commercial level in the bovine species, sorting efficacy has been reported suggesting future applications in a variety of mammals (pig, horse, sheep, goat, cat, and endangered species) [1].

The production of animals of a predetermined sex by sperm sexing is an interesting target also in dog reproduction as, according to the different fields of breeding and use of animals (purebred pet dog, working dogs such as guide dogs, rescue dogs, and farm dogs), male or female is preferably sought by dog breeders and owners [2–5].

The possible association of reproductive technology, such as cryopreservation and artificial insemination (AI), with sex sorting of sperm could optimize the profitability of the breeding and should have a great potential application also for the preservation of endangered canids maintaining the best male–female ratio for the animal repopulation [6].

To the best of our knowledge, only a couple of studies have been performed until now on dog sperm sexing [3,5].

One of the major limitations for the use of sperm sexing in the dog and in other species is the long sorting time necessary to obtain the number of sexed spermatozoa

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necessary for AI (approximately 150–200 million motile spermatozoa) [7]. Rodenas et al. [5] reported that it is possible to collect 14 to 16 $\times$ 10^6 dog sexed spermatozoa per hour in the best samples; therefore, conventional AI is not feasible in this species. Moreover, it has been reported that spermatozoa can be damaged during the sorting process [1]. The possibility to overcome this limitation by reducing both the number of spermatozoa and the distance from the site of fertilization has been reported by Meyers et al. [3] producing offspring of the desired sex in one bitch by intrauterine low-dose insemination using sexed spermatozoa.

Furthermore, the number of spermatozoa requested in dog for a intrauterine low-dose insemination (at least 50 million) [7] is very high considering the sorting time and, therefore, sperm cells have to be stored properly at room temperature waiting to be sexed.

Another problem to solve is the storage of sexed semen, as the bitch can be far from the sorting facilities or more than one insemination could be required. Until now, no data about storage of canine sexed semen are available in the literature.

Cryopreservation permits long-term sperm storage, but it has to be taken in mind that sexed spermatozoa are cells that are already stressed by the sorting procedure and usually present a greater susceptibility to cryopreservation procedures compared with unsexed semen.

The aim of this study was to (1) define an appropriate extender to liquid store dog sperm before and after sorting and (2) evaluate if sexed canine spermatozoa are able to withstand freezing/thawing procedure. For this purpose, motility, viability, and acrosome integrity were assessed in unsorted and sorted sperm either maintained at room temperature or cryopreserved.

2. Materials and methods

2.1. Experimental design

This study was divided into four experiments.

As the sorting process is slow, the aim of the experiment 1 was to evaluate which of four extenders is the more appropriate for storing at room temperature the dog spermatozoa waiting to be sexed. Total motile spermatozoa, movement, viability, and acrosome integrity were evaluated at 0, 2, 4, 6, 8, and 24 hours.

In the experiment 2, the best extender from experiment 1 (modified Tyrode's albumin lactate pyruvate medium [mTALP], see the following) was used for diluting presorted spermatozoa, whereas the four extenders evaluated in the experiment 1 were tested for sperm storage at room temperature (20 °C–25 °C) after sorting. The above mentioned parameters were evaluated at 0, 2, 4, and 24 hours after sorting.

The aim of the experiment 3 was to evaluate if dog spermatozoa are able to withstand sexing procedure followed by freezing. For that purpose, ejaculates from purebred dogs (Australian Shepherd) were analyzed: immediately after collection (CTR), after the sorting (SORT), and after freezing and thawing of sperm for all groups (CTR-FR and SORT-FR).

In the experiment 4, the same protocol of the experiment 3 was performed on semen from mixed-breed dogs.

2.2. Extenders

All reagents were purchased from Sigma–Aldrich (Milan, Italy) unless otherwise stated.

Extenders tested in experiment 1 were Tris-fructose-citrate (TFC; 3% wt/vol Tris, 1.7% wt/vol citric acid, 1.25% wt/vol fructose, 0.06% wt/vol Na benzylpenicillin, 0.1% wt/vol streptomycin sulfate in distilled water), Tris-glucose-citrate (TGC) (2.4% wt/vol Tris, 1.4% wt/vol citric acid, 0.8% wt/vol glucose, 0.06% wt/vol sodium benzylpenicillin, 0.1% wt/vol streptomycin sulfate in distilled water), mTALP (3.3-mM $MgCl_2$, 99-mM NaCl, 3.1-mM KCl, 25-mM $NaHCO_3$, 0.35-Mm NaH_2PO_4, 1-Mm sodium pyruvate, 21.6-mM DL lactic acid, 0.125-mM Kanamycin in distilled water), and third fraction (III FRAC) of the ejaculated after centrifugation at 5000$\times$ g for 10 minutes.

Freezing extender for the first step was TGC supplemented with 20% (v:v) egg yolk, 3% (v:v) glycerol (EYTGC1), whereas for the second step TGC was supplemented with 20% (v:v) egg yolk, 7% (v:v) glycerol, and 1% (v:v) Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA; EYTGC2).

2.3. Semen collection and evaluation

A total of 14 adult mixed-breed and purebred male dogs aged between 1 and 7 years were enrolled in these experiments, between January 2008 and March 2013. The three fractions of the ejaculates were collected in calibrated plastic vials by digital manipulation. The experiment was approved by the Ethic-scientific Committee of Alma Mater Studiorum, University of Bologna.

The sperm-rich fraction was evaluated for volume, concentration, total sperm motility, movement, sperm morphology, and membrane integrity to estimate sperm viability.

The volume was measured by a calibrated micropipette, and sperm concentration was determined with a Bürker chamber, after dilution of the sperm suspension 1:40 with buffered formol saline to immobilize spermatozoa.

The percentage of total motile spermatozoa and movement score, based on the type of the forward movement of sperm (scale of 0–5; 0 = no forward movement, 5 = steady, rapid forward progression), [8] were subjectively estimated at a phase contrast microscope ($\times$400; Axiolab; Zeiss, Italy) equipped with a warming plate (37 °C; Thermo Plate; Tokai Hit, Japan). Percentages of morphologically normal spermatozoa were determined at the same microscope ($\times$1000) after dilution of semen 1:1 with buffered formol saline, and at least 200 spermatozoa per sample were examined.

To evaluate plasma membrane integrity, 25 μL of semen was incubated with 2 μL of a 300-μM propidium iodide (PI) stock solution and 2 μL of a 10-μM SYBR-14 (green) stock solution, both obtained from the live/dead sperm viability kit (Life Technologies, Molecular Probes, Monza, Italy) for 5 minutes at 37 °C in the darkness. Aliquots of the stained suspensions were placed on clean microscope slides, overlaid carefully with coverslips, and at least 200 spermatozoa per sample were scored with the Nikon Eclipse E600 epifluorescence microscope (Nikon Europe BV, Badhoevedorp, the Netherlands). Spermatozoa stained with

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