Animal Reproduction Science 127 (2011) 106–113

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Identification of sperm subpopulations in canine ejaculates: Effects of cold storage and egg yolk concentration

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ARTICLE INFO

Article history:
Received 28 November 2010
Received in revised form 6 June 2011
Accepted 13 July 2011
Available online 23 July 2011

Key words:
Dog spermatozoa
Motility pattern
CASA
Sperm subpopulations
Chilled semen

ABSTRACT

The aim of this study was to evaluate the effects of cold storage and egg yolk concentration on the distribution of spermatozoa within the different subpopulations. Twenty ejaculates from 4 dogs were collected, diluted in either TRIS buffer containing 20% (TEY20) or 10% centrifuged egg yolk (TEY10) and cooled following a conventional protocol. The kinematic parameters of individual spermatozoa were evaluated in fresh ejaculates and after 24 and 72 h of preservation at 5 °C. A multivariate clustering procedure separated 54,261 motile spermatozoa into four subpopulations: Subpopulation 1 consisting of poorly active and non-progressive spermatozoa (19.80%), Subpopulation 2 consisting of slow and low-linear spermatozoa (25.21%), Subpopulation 3 consisting of high speed and progressive spermatozoa (23.88%), and Subpopulation 4 consisting of highly active but non-progressive spermatozoa (31.11%). Although, cold storage had a significant ($P < 0.05$) effect on both the frequency distribution of spermatozoa within subpopulations and the motion characteristics of each subpopulation, the sperm subpopulation structure was perfectly maintained after cold storage. Subpopulations 1 and 2 significantly ($P < 0.001$) decreased during cold storage (Subpopulation 1: 26.6, 16.9 and 18.4%; and Subpopulation 2: 33.6, 21.3 and 24.0%, respectively, for fresh, 24 and 72 h post-cooled), whereas Subpopulations 3 and 4 significantly ($P < 0.05$) increased (Subpopulation 3: 16.7, 27.6 and 24.3%, and Subpopulation 4: 23.1, 34.1 and 33.4%, respectively, for fresh, 24 and 72 h post-cooled). Regarding the relative percentage of spermatozoa within each extender, Subpopulation 3 was more frequently observed in TEY20 after both 24 and 72 h of cold storage. Significant correlations ($P < 0.05$) were found between the proportions of spermatozoa assigned to Subpopulation 3 in the fresh ejaculates and those in stored samples after 24 h ($r = 0.48498$). In conclusion, cold storage significantly modified both the specific parameters and the distribution of spermatozoa within subpopulations. These changes did not affect the general motile sperm structure present in dog, which is conserved during cold storage. The analysis of the changes observed in structures of subpopulations also suggests that the TEY20 provide more effective preservation of dog semen during cold storage.

1. Introduction

The cooling, storing, and transport of semen for use in subsequent insemination is very important in the reproductive management of a number of species. Preserving canine semen by adding an appropriate extender and chilling it ensures the semen can be available for artificial

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doi:10.1016/j.anireprosci.2011.07.005

insemination (AI) of the bitch at the optimum time during estrus even if the male is not present, or lives far from where the bitch lives. Handling and shipping of chilled semen is both easier and cheaper than using frozen semen. Furthermore, it has been demonstrated that dog semen can be successfully frozen after cold storage for 1–2 days (Hermansson and Linde-Forsberg, 2006). Moreover, the success rates of AI are higher for chilled semen than frozen semen when equally good methods for timing of the oestrus cycle, and for AI, are used (Linde-Forsberg, 1995). The main limitation for the use of chilled semen is the survival time of the preserved spermatozoa, as the extended sample should be used within approximately 4.9 days after collection (England and Ponzio, 1996).

The objective evaluation of motility parameters of dog semen, as an important indicator of the viability of spermatozoa, has been previously used in selecting extenders and sperm processing techniques (Gunzel-Apel et al., 1993; Iguer-Ouada and Verstegen, 2001; Smith and England, 2001; Rijsselaere et al., 2003; Verstegen et al., 2005; Schäfer-Somi and Aurich, 2007). The classical approach, considering the whole ejaculate as a homogeneous population with a normal statistical distribution, and the use of mean values to classify the ejaculates, or to asses the effect of a treatment or a biotechnological procedure is, nowadays, considered erroneous (Mortimer, 1997).

In recent years, it has been reported that ejaculates from a large number of mammalian species are composed of well-defined subpopulations (Holt, 1996; Abaigar et al., 1999; Rigau et al., 2001; Quintero-Moreno et al., 2003, 2007; Miró et al., 2005; Dorado et al., 2010), which are characterized by precise values of the motion parameters obtained after a computer-assisted sperm analysis (CASA). Although, there is no consensus about the physiological role of these motile sperm subpopulations in the ejaculate, the presence of defined motile sperm subpopulations has been related to resistance to cryopreservation (Martinez-Pastor et al., 2005; Núñez-Martínez et al., 2006a,b; Flores et al., 2009), presence of stimulants (Abaigar et al., 1999), storage and fertility (Quintero-Moreno et al., 2003, 2004). However, to our knowledge, there are no available references relative to the influence of cold storage on the structure of dog motile sperm subpopulation.

The aim of this study was therefore to evaluate the effects of cold storage and egg yolk concentration on the distribution of spermatozoa within the different subpopulations.

2. Materials and methods

2.1. Animals

Four clinically healthy experimental dogs of unknown fertility were used in the study: two Spanish Greyhounds, one German Pointer and one Crossbreed. Their weight was 10–26 kg, with ages ranging from 4 to 5 years. All dogs were obtained from the kennel of the Clinical Veterinary Hospital of the University of Cordoba, Spain. The study was carried out according to the Spanish laws for animal welfare and experimentation.

2.2. Experimental design

Semen (five ejaculates per dog) was obtained from 4 dogs on different and non consecutive experimental days, once or twice per week. After collection, one aliquot was removed for computer-assisted evaluation of sperm motility, and the rest of the ejaculate was cooled. Motility parameters of individual spermatozoa were determined by using a CASA system (Sperm Class Analyzer, Microptic SL, Barcelona, Spain). Chilled semen samples were warmed and sperm motility was evaluated again after 24 and 72 h of preservation at 5 °C, to evaluate the effects of cold storage and egg yolk concentration on the distribution of spermatozoa into different subpopulations.

2.3. Semen collection and processing

A total of 20 ejaculates were collected by masturbation (5 ejaculates per dog) in a pre-warmed (+38 °C) plastic tubes (BDFalcon^{TM}Tubes, BD Biosciences, Erembodegem, Belgium), as described by Linde-Forsberg (1995). After collection, the undiluted sperm-rich fraction of each ejaculate was evaluated for volume and for sperm concentration with a photometer (Spermacue, Minitüb, Tiefenbach, Germany), as described by Peña et al. (2003). An aliquot of semen was immediately removed for the evaluation of CASA motility and then diluted with TRIS-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) (Peña et al., 2003) to reach a sperm concentration of approximately 25×10^6 spermatozoa/mL. The same aliquot of diluted semen was used to assess sperm morphology and acrosome integrity by light microscopy evaluation of smears stained with Spermac staining (Minitüb, Tiefenbach, Germany), as described by Oettlé (1986). Only samples with at least 70% of total motile spermatozoa (assessed by CASA) and 80% of morphologically normal spermatozoa were included in the study.

The rest of the ejaculate was processed as described previously by Beccaglia et al. (2009), with modifications. In brief, semen was divided into two aliquots and diluted 1:1 in Biladyl A at room temperature. They were then centrifuged at 700 g for 8 min and the sperm pellets were suspended in either TRIS buffer containing 20% (TEY20) or 10% centrifuged egg yolk (TEY10) at room temperature to a final sperm concentration of 50×10^6 spermatozoa/mL. Extended semen was then stored at 5 °C for 24 or 72 h before analysis.

2.4. Computer-assisted sperm analysis

Motility was measured in fresh and stored samples, as described Núñez-Martínez et al. (2006a) for dog semen. The CASA system used was based on the analysis of 25 consecutive, digitized images, which were taken in a time lapse of 1 s, which implied a velocity of image-capturing of 1 photograph every 40 ms. A 0.5 ml aliquot of the diluted semen was previously incubated for 5 min at 38 °C. Two consecutive 5 μL drops of each semen sample were then placed in a 38 °C prewarmed Makler chamber and evaluated using a phase contrast microscope (Eclipse 50i,