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Freezing dog semen using −80 °C ultra-freezer: Sperm function and *in vivo* fertility

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ABSTRACT

Long term storage of canine frozen semen is conventionally performed in liquid nitrogen (LN_2). However, previous works in freezing canine semen using a −80 °C ultra-freezer (−80°C-UF) showed no differences on sperm quality after thawing. The main objective of this study was to compare the effects of the freezing techniques using LN_2 or -80°C-UF on sperm function and *in vivo* fertility of frozen−thawed dog semen. The sperm-rich fraction of the ejaculate was collected separately from five Chihuahua breed, and each one divided into two aliquots, and frozen and stored in LN_2 or -80°C-UF. Sperm function was analyzed for motility and viability, acrosome integrity, mitochondrial function and phosphatidylserine translocation by flow cytometry before and after cryopreservation. A total of 10 bitches were intravaginal inseminated (IVAI; LN_2 frozen-thawed semen = 5 and −80°C-UF frozen−thawed semen = 5). Pregnancy status was confirmed 30 d after IVAI by transabdominal ultrasonography and live born puppies at term were recorded. Sperm function parameters were affected for both freezing protocols. Differences ($P < 0.05$) were found between freezing and storage methods in most of the parameters of sperm function analyzed, except in the phosphatidylserine translocation. The percentages of pregnancies were not different between the two freezing and storage protocols used. Semen freezing and storage using −80 °C UF is an effective technique for long-term preservation of canine spermatozoa.

1. Introduction

Long term storage of canine frozen semen is conventionally performed in liquid nitrogen (LN_2), obtaining pregnancy rates near to 60% [1]. Cryopreservation processing, however, exposes cells to stress resulting in cellular damage compromising sperm function, these alterations decrease sperm life span, ability to interact with the female reproductive tract, and fertilizing potential [2–4].

The use of ultra-low temperature freezers for semen cryopreservation have been previously described [5–9]. These equipments allow the storage of a large number of straws, decrease the time between thermic equilibration and freezing, reduce the risk of accidents due to mishandling of LN_2 and the economic cost due to its continuous reposition [5].

In canine, studies have confirmed that the use of ultrafreezers (UF) at −152 °C for freezing and storing canine semen could represent a potential alternative to LN_2 [1,5]. Similarly, in goat semen has been validated the use of UF at −150 °C for freezing and storing semen, this study showed that the *in vitro* seminal quality (2 months after freezing) was not significantly different compared with frozen and stored in LN_2 [9], at present, live birth goats using the same technique is described [6]. In a previous study [8], it was demonstrated the use at −80°C-UF for freezing and storing semen, this study showed that the sperm function (*in vitro* sperm quality) with respect to plasma membrane integrity, acrosome intactness, mitochondrial function and phosphatidylserine translocation, were not significantly different for semen frozen and stored in LN_2 when compared with those frozen and stored in the ultra-freezer at −80 °C.

To our knowledge, no published data exist regarding *in vivo* fertilizing capacity of sperm frozen and stored by ultra-freezer at −80 °C. Therefore, our aim was to compare the effects of the

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freezing and storage of semen using LN_2 and -80°C-UF on sperm function and *in vivo* fertility of frozen–thawed dog semen following intravaginal insemination.

2. Materials and methods

All experimental protocols received institutional review board approval by the Scientific Ethics Committee from University of La Frontera and were conducted according to Chilean Law No. 20.380 for Animal Protection. The entire study was developed at the Center of Biotechnology on Reproduction (CEBIOR), Faculty of Medicine, University of La Frontera, Temuco, Chile.

All chemicals and reagents used in this study were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Experimental design

The study consisted of two experiments: the aim of experiment 1 was to test the effects of the storage of frozen semen in LN_2 (Control) or −80 °C UF on canine frozen-thawed semen quality with a special focus on sperm function parameters such as motility, viability, acrosome integrity, mitochondrial function and phosphatidylserine translocation. The aim of experiment 2 was to test the effects of the storage of frozen semen in LN_2 (Control) or −80°C-UF on *in vivo* fertilizing capacity of frozen-thawed spermatozoa. Ten bitches were inseminated IVAI (LN_2 frozen–thawed semen = 5; −80 °C UF frozen–thawed semen = 5).

2.2. Animals

Five healthy adult male dogs (weight: 2.5 ± 0.5 kg, 1–4 years old) of the Chihuahua breed, of proven fertility, were used as semen donors. Also, ten healthy adult bitches, weight: 3 ± 0.5 kg, 2–6 years old (Chihuahua breed) of proven fertility (with regular estrous cycles), were used in this study. All animals belong to a private dog kennel and were fed with dry commercial dog food (Purina Pro Plan puppy, San Luis, Missouri, USA), water *ad libitum* and exercised daily throughout the experiments. The animal health status was periodically checked by a veterinarian.

2.3. Semen collection and evaluation

The sperm rich fraction of the ejaculate (n = 6) from each dog was collected by manual manipulation after sexual/mating abstinence for at least a week, as previously described [2], into a pre-warmed sterile glass [10]. The semen quality of each ejaculate was assessed immediately after collection and the following parameters were determined: sperm concentration and total motility [10] and sperm function parameters were assessed by flow cytometry (see 2.6. Sperm function evaluation) [3,8]. Only semen samples with progressive sperm motility >70%, were included in this study.

2.4. Semen freezing

Canine semen freezing was performed as previously described [3], with some modifications. The collected ejaculates were centrifuged at 300 × g for 5 min and the seminal plasma was removed. Sperm pellets were diluted in Extender 1 [TRIS supplemented with 20% [v/v] egg yolk and 3% [v/v] glycerol] to obtain a sperm concentration of 200 × 10^6/ml at room temperature. Semen samples were cooled at 4 °C for 1 h. After equilibration, they were rediluted (1:1 v/v) in Extender 2 [TRIS supplemented with 20% [v/v] egg yolk, 7% [v/v] glycerol and 1% [v/v] Equex STM paste (Nova Chemical Sales, Scituate, MA, USA)] to reach a final concentration of 100 × 10^6/ml. After 10 min at 4 °C, 0.5 ml straws (Minitüb, Tiefenbach, Germany) were filled with the extended semen and sealed with heat pins.

Six semen freezes were performed during the study. In each experimental trial, two different protocols of freezing were tested. 1) LN_2 (Conventional technique): The straws (n = 20) were placed on a freezing rack (Minitüb, Tiefenbach, Germany) at 4 cm above the surface of the LN_2 in a polystyrene box and were frozen over the LN_2 vapour for 15 min; finally, straws were packaged into rack and plunged directly into LN_2 tank for storage until thawing, 2) −80°C-UF: the straws (n = 20) were packaged into rack and plunged directly into ultra-freezer at −80 °C (model Revco ULT1386-5V; Thermo®, Waltham, Massachusetts, USA) and stored until thawing [8]. The time elapsed to move the straws from the cooler to the ultra-freezer was approximately 10–15 s [5]. Before initiating the experiments, the cooling velocity of the ultra-low temperature was defined in the −80°C-UF technique, the freezing rate into straws was obtained using a Digi-Sense® Type-K thermocouple with a thermosensitive sonde. The sonde was placed inside the straws filled with sperm suspension and showed at −80°C-UF a fast freezing rate of −10 °C/min from 4 °C to −70 °C, and a slow freezing rate of −0.26°C/min from −70 °C to −80 °C, the data obtained for the freezing rates were expressed as the mean ± standard deviation (Fig. 1). In both freezing protocols, straws were stored at least for 45 days until sperm function evaluation or IVAI. Frozen semen was thawed in a water bath at 37 °C for 45 s.

2.5. Sperm function evaluation

For fresh and thawed semen samples, spermatozoa were

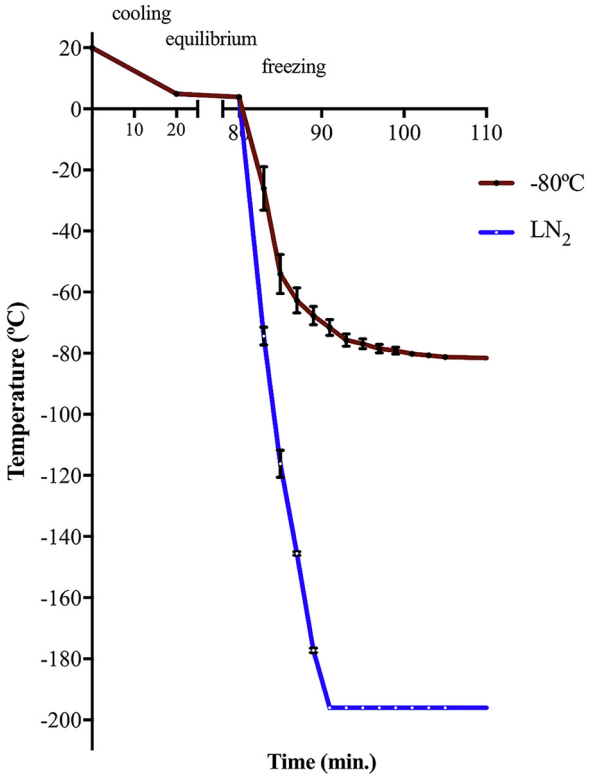

Fig. 1. Freezing curves of 0.5-ml straws obtained in different uncontrolled freezing devices a) Ultra-freezer (−80 °C), non-programmable model Revco ULT1386-5V (Thermo®, Waltham, Massachusetts, USA), b) Liquid nitrogen (LN_2). The data of five measurements obtained for the freezing rates are presented as mean ± standard deviation (SD).