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# IS SPERM CRYOPRESERVATION AT –150°C A FEASIBLE ALTERNATIVE?

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**Abstract**

A series of experiments was carried out to validate a –150°C ultra-low temperature freezer for its possible use to properly freeze and store semen. In the first part, crude sample handling was simulated to see whether temperature of stored samples was maintained within a safe range; also, the freezing point and latent heat of fusion plateau of a semen extender were monitored. In the second part, buck semen was (i) frozen in liquid nitrogen and stored in the ultra-low freezer, (ii) frozen and stored in the ultra-low freezer, and (iii) frozen and stored in liquid nitrogen, to compare sperm cryosurvival between freezing methods. Both, frequent removal of samples and long opening of the freezer door did not negatively affect stored sample temperature; latent heat of fusion plateau was 5 minutes long. Semen stored either at –150°C or at –196°C cryosurvived similarly after 2 days and after 2 months of cryopreservation.

**Keywords:** semen, goat, freezing, ultra-low temperature freezer.

## INTRODUCTION

Freezing and storage of semen in liquid nitrogen is the best known method to preserve it for long periods of time (8). In some areas, however, liquid nitrogen, nitrogen containers and the related technical support are very expensive and difficult to obtain. Thus, research on new alternatives to store frozen semen is needed to overcome difficulties in obtaining liquid nitrogen and to provide an acceptable substitute to the traditional method.

Storage of semen avoiding freezing, i.e. desiccation, has been proposed as a possible alternative to the traditional method (3). However, another line of research may be the use of ultra-low temperature freezers that can reach –150°C, not too far from liquid nitrogen temperature –196°C.

Previous work in this laboratory showed that temperature of a semen sample, stored in an ultra-low freezer at –150°C, was maintained despite temperature variations in the freezer chamber caused by frequent opening of the freezer door. This could be a limiting problem for the use of this device to store frozen semen. That initial observation stimulated more detailed study of the thermal variables involved in the process of keeping semen at –150°C.

The objective of this work was to see whether an ultra-low –150°C freezer could be used to properly freeze and store spermatozoa.

## MATERIALS AND METHODS

*The ultra-low freezer*

The ultra-low temperature freezer was a Sanyo –152°C (MDF-1155ATN, Sanyo Electric Co. Japan), equipped with a system to monitor the temperature inside the freezer chamber; the chamber capacity is 120 l. Temperature in the vicinity of the semen containers (plastic straws) was monitored by means of a probe (638 Pt, Crison, Spain).

*Source of semen*

Semen was collected from 3 Canarian bucks (*Capra hircus*) by artificial vagina, once a week, during Spring (March to May).

*Semen assessment*

Immediately after collection, the ejaculate volume, sperm concentration and progressive motility were assessed. Progressive motility was estimated from semen diluted in saline solution (0.9% w/v), on a warm microscope stage at 37°C, viewed at x 10 magnification in an optical microscope. Sperm concentration was calculated with the aid of a haemocytometer from semen samples diluted 1:400 (v/v) in 0.3% (v/v) formaldehyde-saline solution (0.9% w/v).

The percentage of plasma membrane-intact spermatozoa was estimated by counting 100 cells per sample from smears stained with the Blom´s eosin-nigrosin stain (1). Acrosome-intact spermatozoa expressed as the percentage of cells showing normal apical ridge was estimated by counting 100 cells per sample fixed in 0.4% v/v glutaraldehyde in PBS (1) under a differential interference-contrast microscope (Olympus) at x 100 magnification.

*Washing of semen*

After the initial assessment of each ejaculate, seminal plasma was removed by twice diluting each ejaculate 1:9 (v/v) in washing solution (250 mM Tris $l^{-1}$, 28 mM glucose $l^{-1}$, 104 mmol citric acid $l^{-1}$, 0.05% streptomycin and 500 UI penicillin $ml^{-1}$) at 37°C and double centrifugation at 700 g for 15 min at room temperature.

*Semen dilution and packaging*

After washing, ejaculates were diluted in a Tris-based freezing medium (washing solution plus 12% egg yolk) and a final concentration of 4% glycerol. Dilution was done in two steps; glycerolated extender was slowly added at room temperature. Diluted semen was packaged in 0.5 ml plastic straws containing 200 x $10^6$ spermatozoa each.

*Experimental design*

Validation of the ultra-low freezer capacity to freeze and store semen:

*1. Effect of frequent opening of the freezer door at regular time intervals on sample temperature.*

The ultra-low freezer was set up at –150°C, one straw was then removed from the freezer leaving the freezer door opened for one minute. This operation was repeated every five minutes during one hour. Temperature from the freezer chamber and the straws container was monitored each minute throughout the experiment.

*2. Effect of a single long opening of the freezer door on sample temperature*

The ultra-low freezer was set up at –150°C, one straw was then removed from the freezer and the straw container was moved from one corner of the chamber to its opposite to simulate crude manipulation of the samples. The freezer door remained then opened for 10 minutes.

Temperature from the freezer chamber and the straw container was monitored each minute throughout the experiment until the initial temperature was recovered.

*3. Latent heat of fusion plateau of a semen extender frozen into the ultra-low freezer*

A Tris-egg yolk based semen extender containing (i) 2%, (ii) 4%, and (iii) 8% glycerol was packaged in 50 ml plastic tubes (30 ml extender each), cooled to +5°C into a standard refrigerator, and directly introduced inside the ultra-low freezer at –150°C. Temperature from the freezer chamber and the semen extender was constantly monitored, both freezing point and latent heat of fusion plateau were registered in each case.

*Freezing of semen*

Seven semen samples each pooled from several males (replicates) were washed, diluted, packaged in plastic straws (3 per treatment), slowly cooled over a two hours period, held two hours at that temperature, and then allocated to the following treatments: (T1) straws were frozen over nitrogen vapour for 15 minutes, plunged in liquid nitrogen and stored into the freezer at –150°C; the straws were moved from the liquid nitrogen to the freezer into goblets filled with nitrogen, (T2) straws into a polystyrene box were directly introduced into the freezer at –150°C, (T3) straws were frozen over nitrogen vapour for 15 minutes, plunged and stored in liquid nitrogen; this treatment was the control.

Thawing was carried out after: (i) two days and (ii) two months of cryopreservation, plunging the frozen straws in a water bath at 37°C for 30 seconds; thawed semen was then poured in plastic tubes, diluted 1:10 (v/v) in either BTS (6) or saline solution (0.9% w/v) at 37°C, and progressive motility, plasma membrane integrity and acrosome membrane integrity were assessed immediately.

*Statistical analysis*

Data expressed as percentages was arcsine-transformed before ANOVA (General Linear Model, SPSS 10).

## RESULTS

*Validation of the ultra-low freezer capacity to freeze and store semen*

1. Freezer and sample temperature increased after each opening of freezer door and straw removal (Figure 1). When the first six straws were removed, temperature in the freezer and the sample container increased only 7°C (from –150°C to –143°C), but at the end of the experiment freezer was about –139°C and samples –137°C.

2. The opening of freezer door for 10 minutes caused a sharp increase in its temperature, reaching about –110°C at the end of that period; initial temperature was recovered 50 minutes later (Figure 2). In contrast, sample temperature increased only to –135°C and the initial temperature was recovered 60 minutes later.

3. Freezing point of the extender containing 2%, 4%, and 8% glycerol was about: –4°C, –5°C and –5°C respectively (Figure 3). Latent heat of fusion plateau was 5 minutes long in the extender containing 2% and 4% glycerol, and 3 minutes long in the extender containing 8% glycerol.

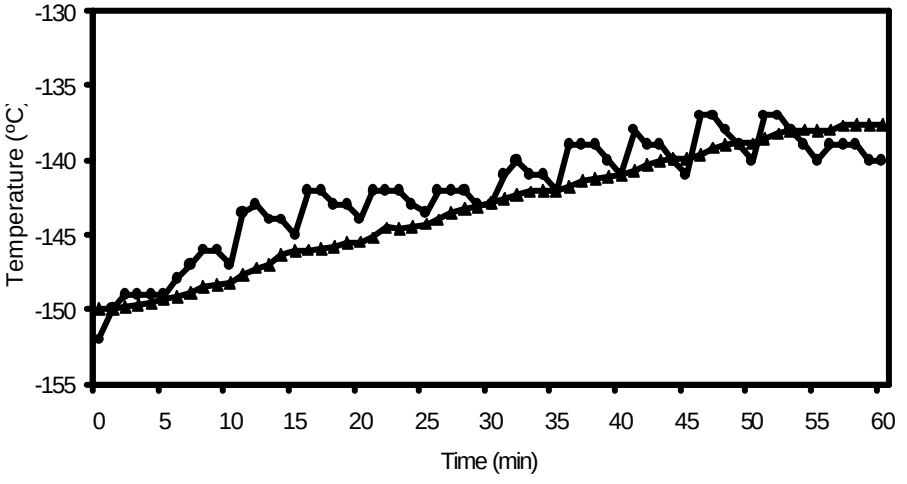


Figure 1. Effect of frequent opening of the ultra-low freezer door on sample (? ) and freezer chamber (?) temperature during one hour. Freezer door was opened each five min, one straw was removed and the door remained one min opened.

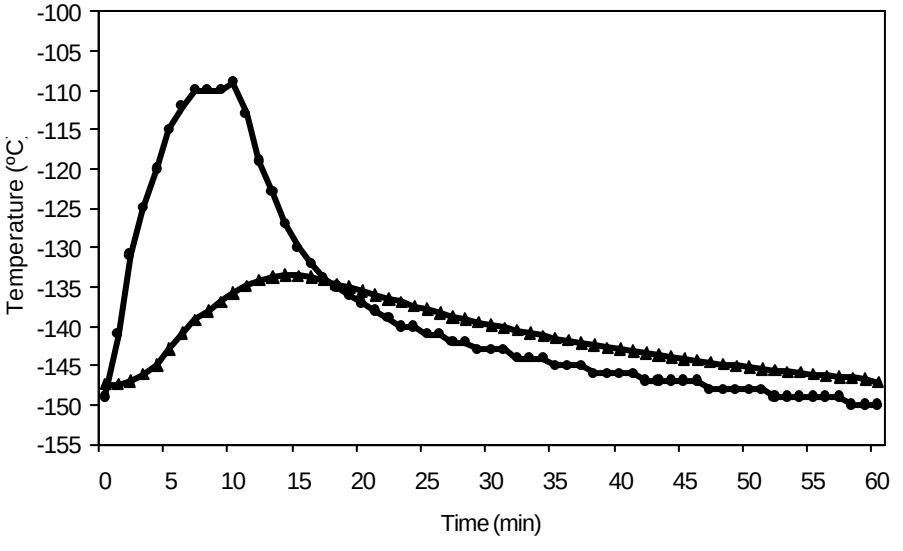


Figure 2. Effect of a single and long opening of the ultra-low freezer door on sample (? ) and freezer chamber (?) temperature. The freezer door remained opened for ten min, it was then closed and temperature was monitored until the initial was recovered.

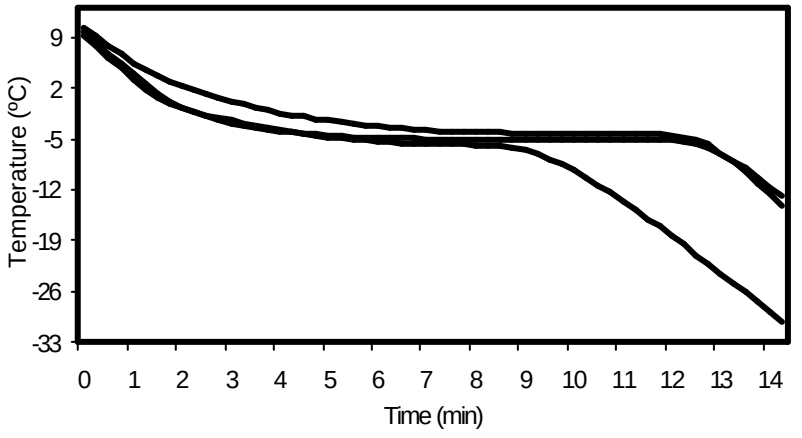


Figure 3. Latent heat of fusion plateau of a semen extender containing three concentrations of glycerol: 2% (top line), 4% (middle line), 8% (bottom line). The cooled extender was directly introduced into the ultra-low freezer at –150°C.

There were no differences between treatments regarding post-thawing motile, plasma membrane-intact, and acrosome membrane-intact spermatozoa, after two days and after two months of cryopreservation (Table 1 & 2).

Table 1. Post-thawing motile, plasma membrane-intact, and acrosome membrane-intact spermatozoa, after two days of cryopreservation in three freezing methods

| Treatment | Motile spermatozoa (%) | Plasma membrane-intact spermatozoa (%) | Acrosome membrane-intact spermatozoa (%) |
|---|---|---|---|
| T1 | 31.6 ± 3.04 | 43.4 ± 3.41 | 52.1 ± 4.84 |
| T2 | 33.4 ± 1.77 | 47.6 ± 4.22 | 54.0 ± 4.43 |
| T3 | 36.8 ± 3.36 | 50.8 ± 4.13 | 56.6 ± 6.37 |

Values are means ± SEM

Table 2. Post-thawing motile, plasma membrane-intact, and acrosome membrane-intact spermatozoa, after two months of cryopreservation in three freezing methods

| Treatment | Motile spermatozoa (%) | Plasma membrane-intact spermatozoa (%) | Acrosome membrane-intact spermatozoa (%) |
|---|---|---|---|
| T1 | 42.5 ± 2.32 | 51.7 ± 1.54 | 49,4 ± 3.18 |
| T2 | 44.8 ± 1.50 | 53.4 ± 1.90 | 55.7 ± 1.96 |
| T3 | 42.2 ± 1.93 | 52.5 ± 2.10 | 47.8 ± 3.19 |

Values are means ± SEM

## DISCUSSION

Semen cryopreservation at –150°C seems to be a feasible alternative to the traditional method, liquid nitrogen. In addition, this new method is easier to carry out. However, it is necessary to study long-term cryopreservation, more than one year, to see whether this sort of freezer can be used as a long-term semen bank or as a short-term storage. It is thought that sperm metabolic activity at –196°C is not important (2) but at –150°C it might be different.

In our knowledge, there are no reports on the use of ultra-low freezers to freeze or store semen at –150°C. Nevertheless, others have frozen and stored boar semen at –80°C using a conventional freezer (7). Sperm survival after 30 days of cryopreservation, although low, was similar to that obtained in standard freezing procedures.

Opening of freezer door for frequent or long periods did not cause a critical increase in temperature inside the freezer chamber nor the straws container. That is, temperature at which recrystallisation occurs, –130°C (4), was not reached. This suggest, that straws stored in this freezer may remain frozen within a safe range of temperature regardless the continuous use of the freezer to introduce or remove any sample.

Semen extender containing 4% glycerol, the used to freeze buck semen, froze 5 minutes after being introduced into the freezer (2°C/minute approximately); this may be considered a slow rate for this stage of the freezing protocol. In addition, latent heat of fusion plateau was too long. It is worth noting that a very short plateau is recommended to improve sperm cryosurvival (5). However, sperm cryosurvival assessed in posterior experiments was not negatively affected.

In addition to the ultra-low freezer usefulness to properly store frozen semen, there is a number of factors to take into account to considering it a viable alternative, for example: the device cost, electricity expenses, risk of electricity interruptions, whether defrosting is required or not and difficulties in sample handling.

At this moment, lacking *in vivo* fertility trials with semen frozen at –150°C, it is not possible to be certain that such spermatozoa remain as fertile as those frozen and stored in liquid nitrogen.

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