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CRYOPRESERVATION OF HORSE SEMEN UNDER LABORATORY AND FIELD CONDITIONS USING A STIRLING CYCLE FREEZER

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Abstract

A Stirling Cycle freezer has been developed as an alternative to conventional liquid nitrogen controlled rate freezers. Horse semen samples were cooled in 0.25 ml straws and 15 ml bags in the Stirling Cycle freezer under laboratory conditions and as a portable device, powered from a car battery. For comparison, straws were frozen in a conventional liquid nitrogen controlled rate freezer. Upon thawing, motility and viability of samples frozen in the Stirling Cycle freezer were not significantly different when compared to samples frozen in the liquid nitrogen freezer. Unlike liquid nitrogen systems, the Stirling Cycle freezer does not pose a contamination risk, can be used in sterile conditions and has no need for a constant supply of cryogen. The freezer has potential for use in veterinary and genetic conservation applications.

Keywords: Cryopreservation, liquid nitrogen-free, spermatozoa, Stirling Cycle freezer, portability, bag freezing

INTRODUCTION

Liquid nitrogen-free programmable freezers, using a Stirling Cycle Cryocooler as a cooling source, have already been demonstrated as a viable alternative to liquid nitrogen controlled-rate freezers for the cryopreservation of reproductive cells in human *in vitro* studies (6). Such equipment does not require a constant source of gaseous cryogen, reduces handling risks and logistical problems and lowers the risk of pathogen transfer from non-sterile cryogens. Consequently, a Stirling Cycle freezer can also readily be used in cleanrooms.

The need for large volumes of liquid nitrogen is a significant constraint on semen collections for veterinary and/or genetic conservation purposes in wild and domesticated populations of mammals, birds and aquatic organisms (2,3,5,7,8,9,10). This constraint could be eliminated if the efficacy of a Stirling Cycle freezer under field conditions can be established.

Semen cryopreservation studies are also limited in the volumes that can be frozen. Typically, low volume plastic straws (0.25 ml and 0.5 ml) are used, which dramatically increase handling time and risk, and the available commercial equipment is configured to use

these containers with relatively little flexibility. A Stirling Cycle freezer can be configured readily to freeze foil sachets and plastic cryopreservation bags containing volumes of between 5 ml and 15 ml, as well as straws.

This study evaluates the performance of the Stirling Cycle freezer for the cryopreservation of horse semen in straws and bags under laboratory conditions compared to a conventional liquid nitrogen controlled rate freezer. Straws were also frozen in the Stirling cycle freezer under field conditions: the freezer was installed in the back of the pick-up truck and powered from the vehicle's battery.

MATERIALS AND METHODS

Semen was collected from a 4 year old Fell Pony stallion and processed in the conventional way to provide a gel-free sperm pellet, centrifuged from Kenny extender (Minitub, Abfull-und Labortechnik GmbH & Co., Tiefenbach, Germany) at 2:1 extender:sperm. The recovered pellet was re-extended with Gent freezing medium (Minitub, Abfull-und Labortechnik GmbH & Co, Germany) to a concentration of 1.6×10^8 sperm ml^{-1} and loaded into straws (0.25 ml) or 15ml plastic-lined, foil bags as required. The loaded semen containers were placed horizontally at 4°C for 3h in a standard laboratory refrigerator, or on the cooling plate of the Stirling Cycle freezer in the field study to achieve the same conditions.

Freezers

The freezer used was a programmable freezer employing a Stirling Cycle Cryocooler to generate temperatures down to -100°C (EF600 Asymptote Ltd., Cambridge, UK). The freezer was connected to a conventional 240V AC supply in the laboratory and operated as a portable unit connected to a car battery via a regulated inverter (12v DC to 240v AC at 150W) in the field study (Figure 1). The sample plate was machined to accommodate 0.25 ml straws or exchanged for a flat plate when freezing bags. External data logging monitored the control temperature, the actual temperature within the sample plate and the operating voltage to the Stirling cycle cooler.

In the laboratory study a programmable freezer using liquid nitrogen (Kryo 10-16, Planer Products Ltd, Sunbury-on-Thames, UK) was used for comparison.

Freezing containers and temperature measurements

The semen was held in 0.25 ml straws (IMV, L'Aigle, France) plugged using polyvinylalcohol powder. Cryopreservation bags (Product 4R9951, Baxter International, Deerfield, Illinois, USA) were used for larger semen volumes (10 and 15 ml). Temperatures were measured and logged in the samples using a type T 28 SWG thermocouples connected to a 1200 series Grant data logger (Grant Instruments, Cambridge, UK) or a Pico TC-08 datalogger (Pico Technology Ltd, St Neots, Cambs, UK).

Controlled ice nucleation

A small cryosurgical device (Asymptote Ltd., Cambridge, UK), which emits sterile nitrous oxide, was used to nucleate individual straws and bags *in situ* on the cooling plate of the Stirling Cycle freezer. The straws frozen in the Planer programmable freezer were not individually nucleated, as is common practice with multiple samples under such conditions.

Equipment vibration and particulate ice contacting the straw caused nucleation and was observed to occur in straws at temperatures between -6.8°C and -10.4°C .

Cryopreservation of samples

Following a minimum of 3h equilibration at 4°C , straws were cooled by the following protocol: linear cooling at $2^{\circ}\text{C min}^{-1}$ to a holding temperature of -7°C ; manual nucleation at -7°C ; hold at -7°C for 10 minutes; linear cooling at $4^{\circ}\text{C min}^{-1}$ to -80°C ; transfer to liquid nitrogen storage vessel. The same protocol was applied to bags except that a 20 minute hold at -7°C was required to ensure equilibrium ice growth after nucleation.

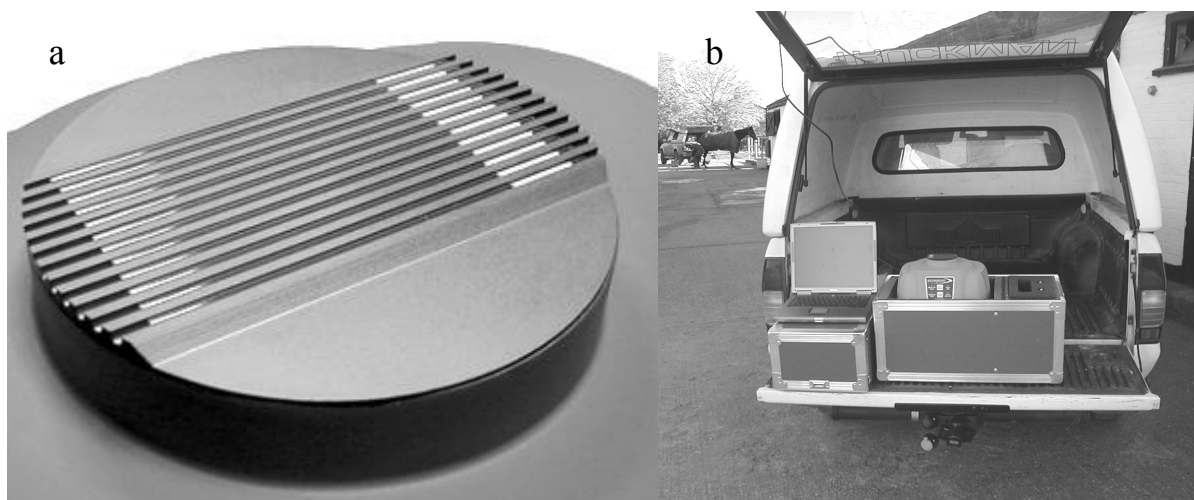


Figure 1. a) Sample plate of the Stirling Cycle freezer machined to accept straws b) Stirling Cycle freezer as a portable device connected to a car battery.

Assessment of post-thaw performance

Straws were thawed in a water bath at 37°C for 30 seconds, bags for 3 minutes. Sperm motility and viability were assessed using a SpermVision™ Computer Assisted Sperm Analysis (MTG-Medical Technology Vertriebs-GmbH, Altdorf, Germany) based on a Zeiss Axiosko 40FL fluorescence microscope. Percentage motility was recorded as all moving sperm in the semen sample. Percentage viability was assessed, using the CASA system, following dual staining with SYBR14 and propidium iodide using a proprietary sperm viability kit (L-7011 Invitrogen Ltd, Paisley, UK).

RESULTS

Cryopreservation in straws

In the laboratory there was no significant difference ($p>0.05$, $n=10$) between the performance of the Stirling Cycle freezer and the liquid nitrogen freezer for semen in 0.25 ml straws (Figure 2). The field studies with the Stirling Cycle freezer produced semen motility and viability comparable to laboratory frozen material using either type of freezer.

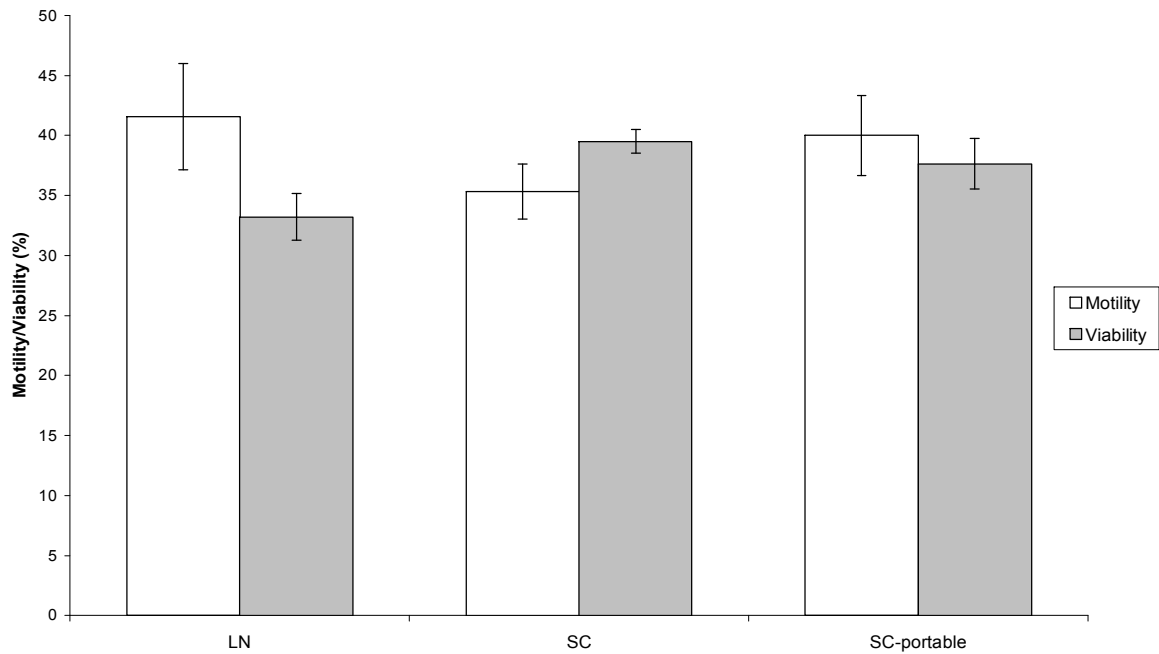


Figure 2. Motility and viability of horse sperm (+/- s.e.) following freezing using the same protocol in either a liquid nitrogen controlled rate freezer (LN), or a Stirling cycle controlled rate freezer operated in the laboratory (SC) or as a portable device (SC-portable).

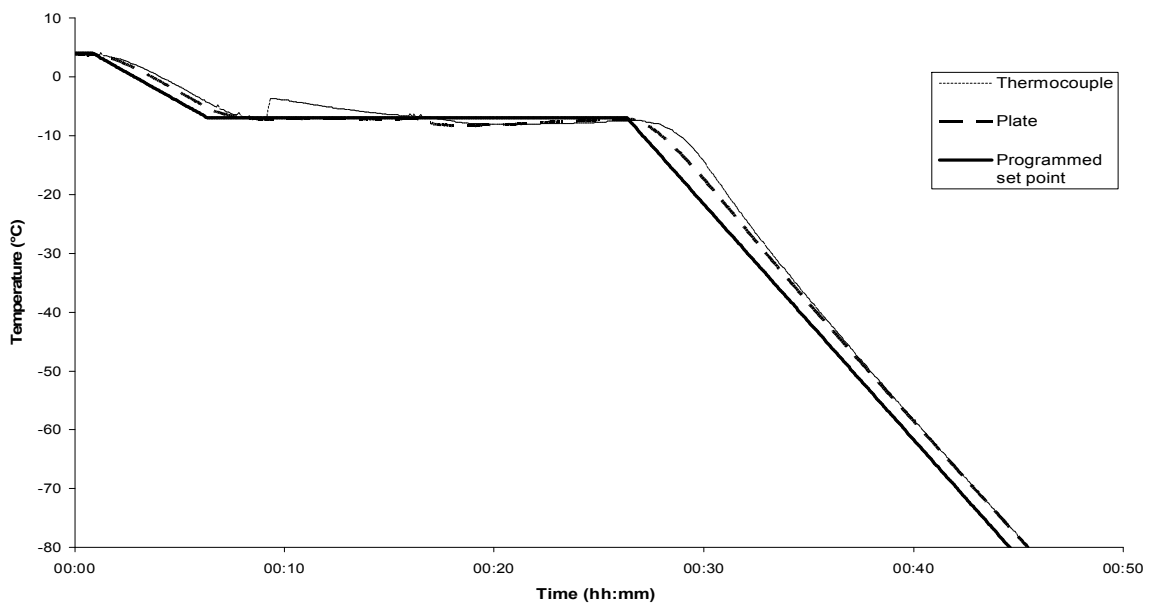


Figure 3. Measured plate and thermocouple temperature using a 10 ml plastic bag and cooling at $4^{\circ}\text{C min}^{-1}$.

Large volume freezing

The temperature measured in the bags was in good agreement with the programmed temperature in the Stirling Cycle freezer down to a temperature of -80°C (Figure 3), at which point bags were transferred to liquid nitrogen.

Upon thawing, the viability and motility levels of spermatozoa frozen in bags were similar to the values attained in straws (Figure 4). Little variation was observed in bag to bag viability or motility.

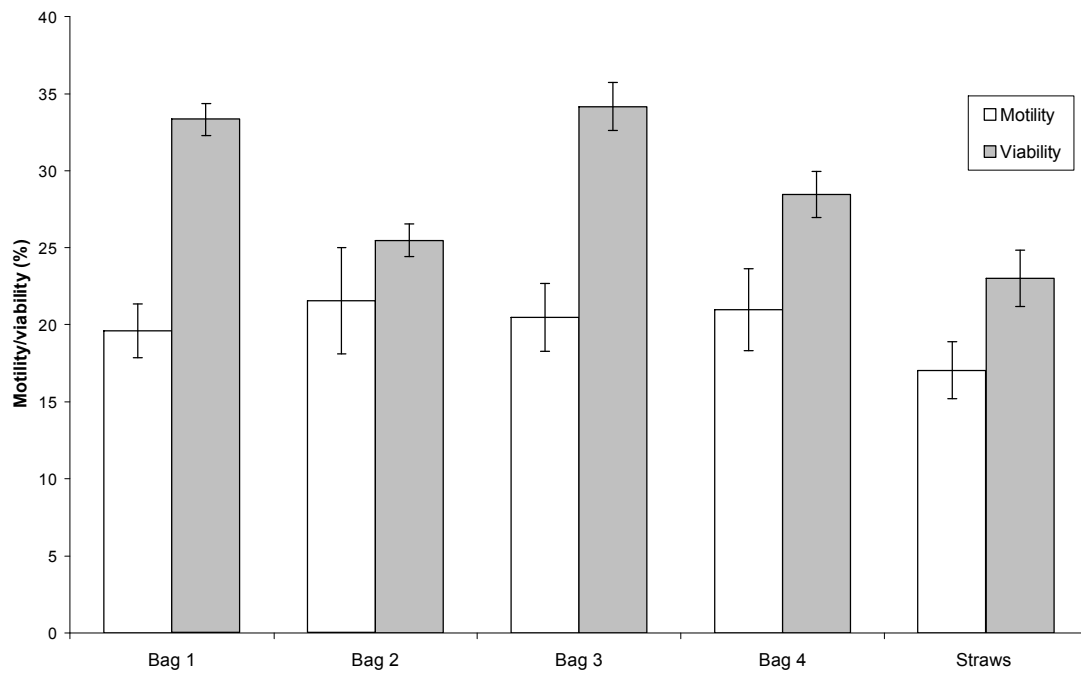


Figure 4. Motility and viability of horse sperm (+/- s.e.) frozen in 4 x 15 ml bags or in 0.25 ml straws in the Stirling Cycle freezer, following the same protocol under laboratory conditions.

DISCUSSION

This study has demonstrated that it is possible to achieve a good recovery rate of horse spermatozoa, following freezing and thawing, without using liquid nitrogen during the controlled rate cooling step of the cryopreservation process. The viability observed was similar to that reported in other studies of horse sperm cryopreservation where a range of cooling rates were examined (1). The equipment may be operated in the laboratory or as a portable device, showing its potential for veterinary and conservation applications. The use of a cryosurgical nucleating device allowed controlled sample nucleation in situations in which liquid nitrogen is not available. More usual methods of ice nucleation, for example using forceps cooled in liquid nitrogen, are possible if liquid nitrogen is available.

Larger volumes, up to 15 ml, of horse semen may be processed and achieve similar viability to samples frozen in 0.25 ml straws. This observation is interesting because the bags were cooled from one side, which would have resulted in a vertical temperature gradient within the sample. However this was not detrimental to recovery upon thawing. The larger samples took longer to thaw than straws, but again this did not reduce cell viability. This is in agreement with previous studies that demonstrated warming rate had little effect on the viability of human spermatozoa samples when cooled at rates close to the optimum (4).

This study has further demonstrated the potential for cooling sources other than liquid nitrogen to be used for the cryopreservation of reproductive cells. The applications of such equipment could expand its use into sterile environments and as a portable device.

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