High post-thaw survival of ram sperm after partial freeze-drying

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Received: 23 October 2017 / Accepted: 19 February 2018 / Published online: 14 March 2018
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Abstract

Background Recrystallization damages occur when a frozen sample is held at high subzero temperatures and when the warming process is too slow.

Methods In this work, ram semen diluted in two different concentrations of sugar solutions (Lyo A consisted of 0.4 M sorbitol and 0.25 M trehalose, and the second, Lyo B composed of 0.26 M sorbitol and 0.165 M trehalose) in egg yolk and Tris medium were compared after freezing 10 µL samples to: (1) –10, –25, and –35 °C and thawing. (2) Freezing to –10 and –25 °C, holding for 1 h and then thawing, and (3) freezing to –10 and –25 °C and drying for 1 h at these temperatures at a vacuum of 80 mTorr, prior thawing. For drying, we used a new freeze-drying apparatus (Darya, FertileSafe, Israel) having a condensation temperature below –110 °C and a vacuum pressure of 10–100 mTorr that is reached in less than 10s.

Results Results showed that samples in Lyo B solution frozen at –25 °C had significantly higher sperm motility in partially freeze-dried samples than frozen samples ($46.6 \pm 2.8\%$ vs $1.2 \pm 2.5\%$, $P < 0.001$). Moreover, partially dried samples in Lyo B showed higher motility than Lyo A at –25 °C ($46.6 \pm 2.8\%$ vs $35 \pm 4\%$). Cryomicroscopy and low-temperature/low-pressure environmental scanning electronic microscope demonstrated that the amount of the ice crystals present in partially dried samples was lower than in the frozen samples.

Conclusion Holding the sperm at high subzero temperatures is necessary for the primary drying of cells during the freeze-drying process. Rapid freeze-drying can be achieved using this new device, which enables to reduce recrystallization damages.

Keywords Freeze-drying · Sperm · Motility · Preservation · Freezing

Introduction

The currently most used technique for the storage of semen is cryopreservation in liquid nitrogen. Cryostorage has high costs and requires dedicated specialized facilities, trained staff, and continuous liquid nitrogen (LN₂) supply [1]. Moreover, cryostorage is energy-dependent, causes safety concerns, and presents a risk of pathogen transmission between samples: all serious issues in clinical practice [2]. For all these reasons, seeking an alternative system of sample storage that may help overcome these limitations is highly desirable. Freeze-drying is a valuable method and could be an alternative system in overcoming the problems and risks described above [3, 4]. Polge et al. reported in 1949 the successful freeze-

drying of poultry spermatozoa [5]. Although the glycerol received the greater attention from this article [5], the studying of freeze-drying of sperm has continued during the 1950s and 1960s of the previous century especially on bovine and human sperm [6–11]. After that, the freeze-drying of sperm was also studied on cats, dogs, bulls, pigs, and human sperm cells [12–20]. Furthermore, the production of live offspring following the use of freeze-dried spermatozoa was first demonstrated in 1998 by Wakayama and Yanagimachi in mice [21], then on rabbits [22], and on rats [23, 24]. Some work was also done on different cells, such as red blood cells [25, 26], cord blood-derived mononuclear cells [27], fibroblasts [28, 29], and other cell types [30, 31]. Ice crystallization and recrystallization during freezing and thawing techniques are significant contributors to cellular damage and death. For this reason, avoiding or minimizing crystallization and recrystallization injury could facilitate the maintenance of physiological properties in mammalian cells after freeze-thawing and freeze-drying. These induced cell damages occur when large sample volumes are frozen [32] or when thawing or warming are not performed quickly enough [33] and at high subzero storage temperatures. During the first phase of freeze-drying, referred

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to as primary drying, the frozen sperm has to be exposed to high subzero temperatures for several hours, during which all of the ice crystals sublimate. We developed a small volume (1 Liter) freeze-drying device. This device, named Darya (FertileSafe Ltd., Nes-Ziona, Israel), has a temperature-controlling unit which can maintain the samples at high subzero temperatures. The device can reach very low pressures, down to 10 mTorr, at a very fast rate. Therefore, it may reduce recrystallization damages occurring at high subzero temperatures since the sublimation process outruns the recrystallization rate and, in a short time, it reaches a point where the amount of the unfrozen fraction, where the cells are embedded, is larger. Mazur and Cole (1989) showed that the size of the unfrozen fraction is inversely correlated to cell damages [34].

Lyophilization processes, similarly to freezing, require cryoprotectant solutions (CPs) that may contain mainly carbohydrates, polymers, and proteins as protectants for the cells [35, 36].

In a paper from 2010, the authors demonstrated that using bovine sperm partially desiccated using a combination of trehalose and sorbitol-based solution yielded high post-thaw survival and motility rates [37]. In this paper, the authors suggest that it may be that sorbitol penetrates the cells, thus conveying better protection during the freeze-drying process. In this study, we sought to further investigate the role of sorbitol and trehalose during the partial desiccation of ram sperm. Ram sperm is much more sensitive to osmotic stress than human sperm [38]; therefore, we assume that if sorbitol enters the cells, it will also reduce the osmotic damages combined with the known beneficial effects of trehalose on freeze-drying [36]. The goal of this study was to achieve partial freeze-drying of ram semen at a high subzero temperature yielding high post-thaw sperm motility.

Materials and methods

The experiments performed and the parameters evaluated are described in Fig. 1 as a schematic workflow.

Sperm collection

Sperm samples were collected from $n = 3$ rams of Sarda breed and pooled together to be analyzed as a single sample. Concentration and motility were evaluated using CASA (Ivos, Hamilton Thorne, Biosciences). The motility of each semen ejaculate was at least 85% or higher. The sperm samples were diluted to a concentration of 50 million cells/mL in one of the following two solutions: (1) Lyo A—0.25 M trehalose and 0.4 M sorbitol in Tris medium and 20% egg yolk; (2) Lyo B—0.16 M trehalose and 0.26 M sorbitol in Tris medium and 20% egg yolk. Then sperm was cooled to 4 °C at a rate of 1 °C/min followed by a re-evaluation for motility using CASA.

Freeze-thawing experiments

The first experiment was aimed to evaluate post-thaw semen motility that was frozen at different temperatures. Freezing was done by pipetting 10 µL drops of sperm either in Lyo A or in Lyo B solutions on a coverslip which was precooled to various temperatures (−10, −25, or −35 °C) and left for 10 min. Then, the coverslip was removed and thawed by placing it on a warm plate (38 °C). On each coverslip, there were between 5 and 10 drops. The experiment was repeated three times in different days.

Freeze-thawing and partial freeze-drying experiments

The second experiment aimed to compare freeze-thawing with partial freeze-drying at various temperatures (−10 and −25 °C) for 1 h.

Freezing was done as described in experiment 1 above, by pipetting 10 µL drops of sperm on a coverslip which was precooled to the prefixed temperatures and left for 1 h at these temperatures. Then, the coverslip was removed and thawed by placing it on a warm plate (38 °C). Freeze-drying was done using a novel device (Darya, FertileSafe, Nes-Ziona, Israel), which allows a very rapid decrease in vacuum pressure down to 10 mTorr. It comprises a small metal cylinder with a volume of 1 L which has two compartments (Fig. 2): a condenser which is placed in LN₂ and the samples compartment which is controlled by a heater and connected to a vacuum pump. We set the vacuum to 80 mTorr and the sample heater to various temperatures according to the experimental group (−10 or −25 °C). The frozen samples were placed in the lyophilizer after 5 min of freezing (at each temperature) for a duration of 1 h. This was done very fast (1–2 s) since the freezing was done in a very close proximity to the Darya lyophilizer which was open and ready to receive the samples. In every drying cycle between 3 to 5 slides were placed in the Darya device. After 1 h, the samples were thawed by placing the coverslip on a warm plate (38 °C) for a few seconds since thawing was very fast. The experiments were repeated three times in different days.

Cryomicroscope and environmental scanning electron microscope evaluations

The third experiment aimed to evaluate the physical aspect of ice formation and the amount of unfrozen fraction via a cryomicroscope and an environmental scanning electron microscope (ESEM). Cryomicroscopy analyses were performed through an optical microscope equipped with the cryo-stage BCS196 (Linkam, Waterfeeld, UK). Five microliters of sperm were cooled in Lyo A and Lyo B solutions down to −10 °C and held at this temperature for 10 min. Likewise, samples were freeze-dried in Darya for 10 min at the same temperature

Fig. 1 A schematic workflow of the freeze-thawing and freeze-drying experiments performed

of -10°C but applying a vacuum of 80 mTorr. Both frozen and partially dried samples were then placed in the cryomicroscope. Randomly, fields of the sample were selected to acquire images and video recordings. Within this field, the area of the ice crystals was calculated by the measurements realized with the ruler tool of the cryomicroscopy software Linksys 32. The ratio of unfrozen/entire area was calculated according to the following equation: $\%U = (A_t - A_c)/A_t \times 100$ where U is the unfrozen fraction, A_t is the total surface area, and A_c is the crystals surface area.

Low-temperature analyses was performed using ESEM (SEM, Zeiss, Oberkochen, Germany) equipped with a vacuum chamber, which avoided condensation and carefully isolated the sample drop from the environment. Five microliters

of sperm in Lyo B were placed in the device chamber at -10°C and held at this temperature and atmospheric pressure for 20 min. Likewise, identical conditions of temperature and time were applied in the second experiment, which differed only in pressure settings (10 Pa = 80 mTorr).

Volume and weight measurements

Before and after the drying of samples held at temperatures of -10 and -25°C (1 h), the amount of volume and the weight of the drops were measured by using a calibrated pipette and a high-precision analytical scales (Sartorius ED224S, Göttingen, Germany).

Fig. 2 Mini freeze-drying apparatus (Darya, FertileSafe Ltd., Nes-Ziona, Israel). This figure depicts a schematic sketch of the Darya freeze-drying device

Statistical analysis

The difference in sperm motility between groups was analyzed using a Student's *t* test. Significance was set at $P < 0.05$. Data is expressed as mean $\pm$ standard deviation.

Results

Post-thaw motility after freeze-thawing and partial freeze-drying

After sperm collection and CASA analysis, we found that the motility of spermatozoa was reduced when they were exposed to Lyo A solution. Motility decreased from 86 to 30%, but only temporarily. In fact, after 2 min, it went up to 60%. Motility remained unchanged after sperm was chilled to 4 °C (61%). The sperm exposed to Lyo B solution did not show any major changes and displayed a post-chilling motility of 67% (Table 1).

After freezing to different high subzero temperatures, -10 , -25 , and -35 °C, the post-thaw motility (PTM) of the semen exposed to Lyo A was 35, 36, and 38%, respectively (Table 1).

The semen exposed to Lyo B solution was better than that exposed to Lyo A after freezing and thawing to -10 and -25 °C (64.5%, 64%), but showed a decrease at -35 °C (31%), as shown in Table 1.

We recorded the changes occurred in semen when exposed and maintained for 1 h at high subzero temperatures.

PTM was very low ($<10\%$) when the frozen sperm was maintained at subzero temperatures of -10 or -25 °C for 1 h in both solutions. At -35 °C, no motility was recorded. (Table 2).

Freeze-drying for 1 h at a temperature of -10 °C was not beneficial for semen, in fact PTM was, in both solutions, under 10% (Table 2).

However, when freeze-drying was done at -25 °C at 80 mTorr in Lyo A solution, PTM was 35%, while in Lyo B

Table 1 Post-thaw sperm motility after freezing to different temperatures using two different solutions (Lyo A and Lyo B)

Temperature	Post-thaw sperm motility	
	Lyo A	Lyo B
Room temperature	86% $\pm$ 2% a	86% $\pm$ 2% a
Fresh 4 °C	61% $\pm$ 3.21% b	67% $\pm$ 2% b
-10 °C	35% $\pm$ 7.07% c	64.5% $\pm$ 5.25% b
-25 °C	36% $\pm$ 4.78% c	64% $\pm$ 4.89% b
-35 °C	38% $\pm$ 2.44% c	31% $\pm$ 2.5% c

Results are shown as means $\pm$ SD. Different letters (a, b, c) indicate significant differences in sperm motility within solutions and within temperatures ($P < 0.05$)

solution, it was slightly better with 46.6% (Table 2). Partial freeze-drying (PFD) was not performed at -35 °C.

Volume and weight before and after partial freeze-drying

Volume and weight reduction after freeze-drying at -10 °C with Lyo A was as follows: An initial volume of 80 μ L was reduced to 76 μ L (5% volume reduction), and the weight, which was initially 92 mg, was reduced to 86 mg (6.5% weight reduction).

Volume and weight reduction after freeze-drying at -25 °C with Lyo A was as follows: An initial volume of 80 μ L was reduced to 70 μ L (12.5% volume reduction), and the weight, which was initially 92 mg, was reduced to 80 mg (13% weight reduction).

Volume and weight reduction after freeze-drying at -10 °C with Lyo B was as follows: An initial volume of 80 μ L was reduced to 75 μ L (6.25% volume reduction), and the weight, which was initially 92 mg, was reduced to 84 mg (8.7% weight reduction).

Volume and weight reduction after freeze-drying at -25 °C with Lyo B was as follows: An initial volume of 80 μ L was reduced to 65 μ L, and the weight (18.75% volume reduced), which was initially 92 mg, was reduced to 71 mg (22.8% weight reduction).

Cryomicroscopy

There were clear differences in the amount of the unfrozen fraction (*U*) and in ice-crystal size shown in cryomicroscopy data collected from ram semen samples held for 10 min at -10 °C, with and without previous freeze-drying. The *U* rate in Lyo A after freeze-drying ($U = 28\%$, Fig. 3d) was very large compared to the frozen sample (19%, Fig. 3b).

A more evident difference was shown in Lyo B samples, where the freeze-dried sample exhibited a very large *U* (30%, Fig. 3c) compared to the frozen sample ($U = 13\%$, Fig. 3a). In addition, much smaller ice crystals were found in freeze-dried samples (Fig. 3c, d) than in the frozen samples (Fig. 3a, b).

Environmental low-temperature scanning electron microscopy (ESEM)

ESEM analysis of Lyo B samples exposed to sublimation process under vacuum pressure (80 mTorr) and held at -10 °C for 20 min showed smaller ice crystals (Fig. 4b) compared to the large crystals found in samples cooled at atmospheric pressure (Fig. 4a).

Table 2 Post-thaw sperm motility: (A) after freezing and holding for 1 h at two different temperatures using two different solutions (Lyo A and Lyo B) and (B) after partial freeze-drying (PFD) at two different temperatures using two different solutions (Lyo A and Lyo B)

Temperature	A. Post-thaw sperm motility after holding 1 h		B. Sperm motility after PFD	
	Lyo A	Lyo B	Lyo A	Lyo B
– 10 °C	3% ± 1.4% a	3% ± 2% a	6.5% ± 2% a	8% ± 1.2% a
– 25 °C	3.3% ± 2.8% a	1.2% ± 2.5% a	35% ± 4% b	46.6% ± 2.8% b

Results are shown as means ± SD. Different letters (a, b) indicate significant difference in sperm motility between rows and between columns ($P < 0.001$)

Discussion

A good freeze-drying procedure requires an optimization of three major procedures: freezing, drying (includes primary drying and secondary drying), and rehydration. In the first part of the experiment, we focused our attention on the effect of subzero temperatures on ram semen diluted with two solutions composed with different concentrations of trehalose and sorbitol, mixed with egg yolk and Tris solution, named Lyo A and Lyo B. Semen samples frozen with Lyo A solution showed a reduction of PTM which can be explained by an osmotic stress due to the higher concentration of CPs. Moreover, we observed that the higher CPs concentrations in Lyo A dramatically reduced sperm motility just after semen dilution. However, this decrease in motility, detected after the dilution, was not observed when the semen was chilled to 4 °C.

Subzero temperatures also had a significant influence on PTM in thawed semen samples. In fact, when the semen samples diluted in Lyo A solution were exposed to – 10 and –

25 °C, we observed significantly lower values of PTM compared to those diluted in Lyo B (Table 1). When exposed to – 35 °C, the results were similar between the Lyo A and Lyo B groups, but for the Lyo B group, they were significantly lower than those received when frozen to higher temperatures (Table 1).

Therefore, we decided to continue with the two higher temperatures for the freezing-holding and freeze-drying experiments. When semen samples were exposed to subzero temperatures (– 10 and – 25 °C) for 1 h, both Lyo B and in Lyo A showed a severe decrease in PTM (Table 2). This is probably due to recrystallization damages caused at these high subzero temperatures. However, when semen samples were dried at 80 mTorr for 1 h, the results showed higher PTM after freezing to – 25 °C, which was higher in the Lyo B group (PTM = 46.6%) than in the Lyo A (PTM = 35%). Both Lyo A and Lyo B samples showed significantly lower PTM after partial drying at – 10 °C which was a bit higher than the results received after freezing and holding for 1 h (Table 2).

Fig. 3 Cryomicroscopy images of samples frozen (a, b) and freeze-dried (c, d) showing differences in unfrozen fraction amounts. Samples were cooled to – 10 °C for 10 min in Lyo A (Fig. 2b, d) and Lyo B (Fig. 2a, c). Magnification of ×10. The black and white arrows point to the ice crystals and the unfrozen fraction

Fig. 4 Low-temperature scanning electron microscope images. Sample frozen in Lyo A solution, magnification of $\times 500$ (a). Sample frozen with Lyo A solution and dried for 20 min, magnification of $\times 100K$ (b)

A successful sublimation process is affected by a combination of the vacuum and the temperature at which the sample is maintained. Since the same vacuum pressure was applied in these experiments and only the shelf temperature was different, i.e., -10 or -25 °C, we assume that the damage seen at -10 °C was due to a higher sublimation rate which is known to cause damages [39]. Sublimation is also influenced by the sample's volume, and in our experiment, sperm samples were frozen as droplets of 10 μ L. This volume has been considered to ensure a fast freezing process as well as a fast sublimation and thawing processes, thus reducing the damages caused by recrystallization.

Volume and weight measurements revealed that the reduction in these parameters was more profound when drying was done at -25 °C than at -10 °C for 1 h, and in Lyo B solution, the volume and weight reduction were higher than in Lyo A. Although these experiments were done with samples having a volume of 80 μ L (in order to maintain accuracy of the measurements) and not 10 μ L, we assume that the pattern is the same. Therefore, in the samples that had highest volume/weight reduction (Lyo B at -25 °C), we had the highest PTM. Thus, strengthening the assumption that maintaining a relatively high sublimation process at a high subzero temperature will result with reduced recrystallization damages resulting with higher PTM.

The rapid reduction in drop volume exposed sperm cells to excessive osmotic stress. When samples were freeze-dried for 10 min, about 15% of water content was removed. This period was sufficient to avoid recrystallization and to keep the unfrozen fraction at one third of the frozen area, as seen by low-temperature cryomicroscopy (Fig. 3).

Severe dehydration causes solution-effect injury as well as macromolecule denaturation and extreme cell shrinkage, which can lead to irreversible membrane collapse [40]. A further damaging effect is the mechanical stress caused by ice formation around the cells, which force the cells into an extremely limited space: that of the unfrozen solution [41].

Evaluation with cryomicroscopy after holding the sperm at -10 °C confirms that the damage that occurs at this temperature can be attributed to the fusion of large ice crystals and the decrease in the unfrozen fraction to less than 20%. The effect of the small unfrozen fraction could be explained by mechanical damage, as was shown in Saragusty et al. [42], or due to the increased concentration in solutes which results in osmotic stress injury. The formation of large ice crystals when holding samples at high subzero temperatures is confirmed by low-temperature ESEM; in addition, using low-pressure (80 mTorr) and low-temperature ESEM confirmed the partial drying of the drop (Fig. 4).

This is a first step toward freeze-drying sperm with high motility after rehydration. The new device, which allows rapid sublimation at high subzero temperatures, combined with sugar solutions that have low osmotic effects and facilitate the dehydration of the sample, might also be beneficial to other cell types.

Acknowledgements We thank Dr. Monica Strina for the proofreading and editing of the manuscript. This work was supported by Progetto RAS MIGLIOVIGENSAR 2016/2018 and by Programma visiting professor RAS 2016. This work was also supported by FertileSafe Ltd.

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