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Further Optimization of Mouse Spermatozoa Evaporative Drying Techniques

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Abstract

It has been shown in the past that mouse spermatozoa could be dried under a stream of nitrogen gas at ambient temperature and stored at 4°C or 22 °C for up to 3 months and was capable of generating live-born offspring. In previous desiccation work, dried sperm were stored in a vacuum-sealed plastic bag placed in a vacuum-packed Mylar bag. However, dried specimens stored in this way often lost moisture, particularly in samples stored at higher temperatures (22°C) compared to lower temperatures (4°C). The present report describes a method which minimizes this water loss from the dried sperm samples. Its use is described in a preliminary study on the effect of supplementing the trehalose with glycerol. The results have demonstrated that mouse sperm can be stored at 4°C over saturated NaBr without the uptake of water which occurs when they are stored in Mylar packages. In addition, we were able to get some survival of sperm (9–15%) at room temperature storage after three months. The addition of glycerol to trehalose had little effect on the survival of dried mouse sperm stored over NaBr for 1 and 3 months.

Keywords

Evaporative drying; desiccation; mouse sperm; glycerol; trehalose; intracytoplasmic injection; mammalian cells; preservation; temperature; long-term storage

The rapid advances in molecular genetics and reproductive technology during the past decade have led to the production of a large number of artificially produced mouse genotypes. The traditional way of storing mouse genotypes is by maintaining breeding colonies which has become prohibitively expensive. It is now being widely recognized that sperm preservation is a substantially more efficient and cost-effective approach. Since the advent of mouse intracytoplasmic sperm injection (ICSI) techniques, alternative approaches to simplify the methodology of mouse sperm preservation have been explored beyond the traditional method

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of cryopreservation. Mouse spermatozoa can be dried under a stream of nitrogen gas at ambient temperature and stored overnight at 4°C without losing their capability, after rehydration and ICSI, to support 1- fertilization, 2- embryo development in vitro to the blastocyst stage in vitro and 3- development in vivo to Day 15 after embryo transfer into foster mothers [1]. Mouse spermatozoa permeabilized with α -hemolysin to allow entry of trehalose before evaporative drying then stored at 4°C for 3 months, were capable of generating live-born offspring [7]. More recently, it was demonstrated that evaporatively-dried mouse spermatozoa can be stored at -20 and -80°C without deterioration for up to five months. Blastocysts derived from these spermatozoa were transferred to pseudopregnant recipient females and developed into healthy live-born offspring [4,5].

In all of this work the dried sperm were stored in a vacuum-sealed plastic bag placed in a vacuum-packed Mylar bag (Impak Corp, CA). However, dried specimens stored in this way often lost moisture, particularly in samples stored at higher temperatures (22°C) compared to lower temperatures (4°C) [7]. The present report describes a method which minimizes this water loss from the dried sperm samples. Its use is described in a preliminary study on the effect of supplementing the trehalose with glycerol.

Complete details of all of the techniques used in this study are described elsewhere [1,4,5,7]. Briefly, the sperm preparations were evaporatively-dried in a chamber, after poration with α -hemolysin and exposure to trehalose, by blowing compressed ultra-pure-grade nitrogen gas for 6.5 min at 10 L min⁻¹. The water content of samples after drying and storage was determined gravimetrically. At the end samples were baked for 5–7 days at 85°C to determine their dry weight (DW). These measurements were then used to calculate the moisture content, expressed as g H₂O/g DW, of the sperm samples before and after storage. Other samples, done in parallel, were vacuum-packed in Mylar bags and shipped overnight to UC Davis to test the viability of the spermatozoa after hydration by fertilizing mouse ova using ICSI and pre-implantation embryo culture to the blastocyst stage. Relative humidities were measured using a hydrometer (Omega RH 201, Stamford, CT).

Sorption isotherms

Saturated solutions of LiCl, K acetate, MgCl₂, K₂CO₃, Mg(NO₃)₂, NaBr, SrCl₂, NaCl, KCl, and BaCl₂, each with different water activities (a_w), were prepared in sealed glass jars. Two hundred grams of 10 different salts were added to various quantities of water according to Table 10.6 (Spiess and Wolf, 1987) [9]. Each solution was placed in specially designed wide mouth screw-top glass jars (16 oz capacity, neck ID 89 mm) (Cole Palmer Instruments Co.) and allowed to equilibrate at 4°C and 22°C for a minimum of two weeks prior to use. Sperm samples, porated with α -hemolysin followed by exposure to trehalose and desiccated, were then placed in the glass jars containing the 10 equilibrated salt solutions at 4°C and 22°C. After 2–3 weeks, the sperm samples were weighed. Each sample was weighed at least three times. After each weighing, sperm samples were returned to the isotherm jars and re-equilibrated a minimum of 48 h before samples weights were measured again. Following the final weighing, sperm samples were baked and weighed again to determine moisture levels using the methods described elsewhere [1]. Triplicate experiments were done. The results were plotted as two sorption isotherms obtained at 4°C and 22°C (Figure 1). The sperm samples were always wetter when stored at 4°C compared with those stored at 22°C, particularly in the higher ranges of a_w . It was shown elsewhere that ova fertilized by sperm containing less than ~0.3 g H₂O/g DW developed at a low rate into blastocysts [7]. This water content is similar in value to that found to be tolerated by other mammalian cells [6]. There are two candidate molecules which could be used to create conditions giving dry weights near this value when stored at 4°C. These are Mg(NO₃)₂ and NaBr. A preliminary experiment examined the viability of sperm stored at 4°C for four weeks in an atmosphere over saturated Mg(NO₃)₂ and NaBr solutions. Sixty eight

percent (122/179) and 72% (134/186) of ova fertilized by sperm stored over $\text{Mg}(\text{NO}_3)_2$ and NaBr, respectively, developed into blastocysts. We selected NaBr for use in the rest of our experiments in which the sperm were stored at 4°C. The results shown in Figure 1 [3] also indicated that for future experiments, when storage of sperm is at 22°C, storage over SrCl_2 or NaCl may be a more appropriate sorption isotherm in order to obtain a dry weight $\sim 0.3 \text{ g H}_2\text{O/g DW}$, since the sperm would become too dry when stored over NaBr and $\text{Mg}_2(\text{NO}_3)_2$.

The data shown in Table 1 compares the changes in moisture and the viability of sperm after hydration stored for 1 and 3 months and 4°C and 22°C over saturated NaBr in glass jars, compared with previously published data on the effects of storage in foil packets [7]. The results show that the sperm samples lose water slightly during storage over saturated NaBr. This result contrasts significantly from the comparatively large increase in moisture content in the samples stored in foil packets. The rate of fertilization, measured by the development of 2-cell embryos, is high when sperm is stored for 1 month using both methods of storage at 4°C and 22°C. The rate of blastocysts development is higher using sperm storage for 1 month at both 4°C and 22°C when the sperm are stored over saturated NaBr compared to storage in foil packets. The combined evidence shows that storage of the sperm samples over saturated NaBr provides a more stable environment than that which occurs in foil packets.

Glycerol supplementation

Glycerol is a sugar alcohol commonly used for cryopreservation. However, because of its low glass transition temperature ($T_g = -93^\circ\text{C}$), it is not a desirable molecule to be used for desiccation. Although glycerol is a stronger glass former than trehalose, which is known to be a fragile glass, the addition of small amounts of glycerol ($\sim 5\%$) to trehalose decreases its fragility significantly [2]. Further it was shown previously that glycerol, in low concentrations, acts additively with trehalose to promote desiccation tolerance [8]. Therefore, in the second part of this study, we investigated whether adding small concentrations of glycerol (either 3% or 5%) to trehalose offers better protection against desiccation for the mouse sperm.

The fertilization rates (% 2 cell) of dried sperm stored for 1 month at both 4°C and 22°C showed no change with the addition of glycerol (Table 2). Although it seemed that an increase in glycerol concentration lead to more blastocysts after storage at 22°C for 1 month, it did not prove to be statistically significant. After storage for 3 months, the fertilization rates (% 2 cell) at 22°C was much reduced when both 3% and 5% glycerol was added (Table 2). Blastocyst formation rates from sperm dried with 3% or 5% glycerol was lower than the controls stored at 4°C, while at 22°C, the added glycerol concentration seemed to help blastocyst formation. However, this did not prove to be statistically significant.

The results have demonstrated that mouse sperm can be stored at 4°C over saturated NaBr without the uptake of water which occurs when they are stored in Mylar packages. In addition, we were able to get some survival of sperm (9–15%) at room temperature storage after three months. The addition of glycerol to trehalose had little effect on the survival of dried mouse sperm stored over NaBr for 1 and 3 months.

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Figure 1.

Sorption isotherms relating the water activity of LiCl, K acetate, MgCl₂, K₂CO₃, Mg(NO₃)₂, NaBr, SrCl₂, NaCl, KCl, and BaCl to the moisture content of sperm samples dried at 4°C and 22°C over saturated solutions of these compounds. The water activities were approximated by those given for 5°C and 20°C from Greenspan [3].

Table 1
Trehalose storage moisture changes (g water/g dry weight) and blastocyst formation

Storage Method	Temperature (°C)	1 month Pre- Storage Moisture	1 month Post- Storage Moisture	1 month Change in Moisture	1 Month % 2 cell	1 month % Blastocyst
Foil Packages*	4 (n=4)	1.063	0.374	0.689	80 (49/61)	63 (31/49)
Glass Jar (NaBr)	4 (n=5)	0.32	0.52	-0.20	100 (56/56)	82 (46/56)
Foil Packages*	22 (n=4)	1.063	0.051	1.012	82 (51/62)	6 (3/51)
Glass Jar (NaBr)	22 (n=5)	0.32	0.40	-0.08	93 (55/59)	20 (11/55)

* Data from McGinnis et al., 2005.

Table 2
The effect after 1 and 3 months dried sperm storage at 4°C and 22°C, of adding glycerol to trehalose on the fertilization rate (% 2-cells) and the rate of blastocyst development

Media	4°C 1 Month		22°C 1 Month		4°C 3 Month		22°C 3 Month	
	% 2 Cell	% Blastocysts	% 2 Cell	% Blastocysts	% 2 Cell	% Blastocysts	% 2 Cell	% Blastocysts
Trehalose	100	82	93	20	97	32	92	9
Trehalose + 3% Glycerol	92	59	98	24	92	19	46	15
Trehalose + 5% Glycerol	100	74	100	35	100	10	69	12

* Exact Kruskal-Wallis Test