—Mini Review—

Freeze-dried Spermatozoa and Freeze-dried Sperm Injection into Oocytes

Hiroshi Suzuki[1, 2]*

[1]Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan
[2]Department of Developmental and Medical Technology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

Abstract: *Attempts to freeze-dry spermatozoa are not new. Successful conception and full-term development using freeze-dried spermatozoa were reported in the cow and rabbit prior to 1960. However, these preliminary results have not been confirmed. Spermatozoa become defective in motility after freeze-drying and are unable to fertilize eggs both in vivo and in vitro. However, in 1998, freeze-drying of mammalian spermatozoa was demonstrated without loss of genetic or reproductive potential. Freeze-dried spermatozoa support normal development when injected directly into oocytes by means of an intracytoplasmic sperm injection (ICSI) technique. Recent investigations of freeze-dried sperm have focused on the factors affecting freeze-drying in spermatozoa in an effort to make it possible to store the male gamete at ambient temperature. The pressure level at primary dryied of freeze-drying spermatozoa, in addition to the components of the suspending solution, appears to be an important factor for long-term preservation of freeze-dried spermatozoa.*

Key words: *Spermatozoa, Freeze-drying, ICSI, Preservation*

History of Freeze-drying Spermatozoa

The technique of freeze-drying or drying by sublimation is frequently used to preserve certain bacteria, yeasts, viruses, and a great number of other biological products. Attempts to freeze-dry spermatozoa are not new. Application of a freeze-drying technique to sperm was first attempted to preserve fowl spermatozoa by Polge *et al.* in 1949, who reported that 50% of the freeze-dried spermatozoa regained their motility [1]. However, the fertility of those motile spermatozoa was not examined [1]. Since no live cells were observed after two hours of storage at room temperature [1], drying might have been incomplete. In the 1950s and 1960s, several reports were published on human [2], rabbit [3], bovine [4–10], and buffalo [10] freeze-dried spermatozoa. Successful full-term development derived from rabbit freeze-dried spermatozoa was reported by Yushchenko in 1957, who showed 15–20% motile spermatozoa after rehydration that yielded 12 offspring [3]. In bovine spermatozoa, Leidl [4], Bialy and Smith [5], Albright *et al.* [9], and Singh and Roy [10] reported that hydrated spermatozoa regained their motility slightly. Leidl recovered live bull spermatozoa after freeze-drying and reconstitution when the final water content of the residual spermatozoa did not fall to a level lower than 5% and the glycerol content did not exceed 50% [4]. In 1959 and 1960, Meryman and a colleague [7, 8] showed that 40–50% of freeze-dried bovine spermatozoa exhibited motility after rehydration and artificial insemination such that use of these spermatozoa resulted in successful conception. In addition, Larson and Graham [11] reported pregnancy resulting from insemination of freeze-dried bovine spermatozoa that had been stored at 25°C for one month. Unfortunately, the studies described above have not been demonstrated to be reproducible in the period since their publication [12, 13]. It has been indicated that the motility of spermatozoa declines with dehydration in freeze-drying [5, 13–15]. Bovine semen freeze-dried to 2% residual moisture lost fertility [15] and displayed an altered tertiary structure in seminal proteins [16]. Since electron microscopic observation has shown that freeze-dried mouse spermatozoa lost or had extensively damaged acrosomes and plasma membranes [17, 18],

Received: May 1, 2006
Accepted: May 16, 2006
*To whom correspondence should be addressed.
e-mail: hisuzuki@obihiro.ac.jp

it is thought that spermatozoa become defective in motility after freeze-drying and thus are unable to fertilize eggs both *in vivo* and *in vitro*.

Successful Full-term Development from Freeze-dried Spermatozoa with Intracytoplasmic Sperm Injection (ICSI)

It is, however, possible to overcome these difficulties by means of ICSI. In 1995, Kimura and Yanagimachi [19] developed an efficient ICSI method that uses a piezo-micromanipulator in which 80% of oocytes survived after ICSI and 30% of transferred fertilized eggs developed to term. In 1998, Wakayama and Yanagimachi [17] reported that 30% of transferred eggs fertilized with freeze-dried sperm by ICSI successfully developed into young in mice. In a conventional sense, freeze-dried spermatozoa are all considered dead cells because their heads and tails are often separated and their plasma membranes are broken. However, they are able to produce normal offspring using ICSI. In this sense, freeze-dried spermatozoa must still be alive. Thus, defining spermatozoa as alive or dead seems rather arbitrary. Since the report by Wakayama and Yanagimachi in 1998 [17], possible application to the freeze-dried spermatozoa of other animals in combination with ICSI and the optimum conditions for freeze-drying of spermatozoa have been investigated. In addition to the mouse, it has been shown in the rabbit [18] and rat [20] that freeze-dried spermatozoa are able to produce normal offspring using ICSI. Development to morphologically normal blastocysts derived from oocytes injected with freeze-dried spermatozoa has been observed in the cow [21, 22] and pig [23]. In addition, pronuclear formation in oocytes by ICSI with freeze-dried spermatozoa has been reported in the hamster [24].

Freeze-dried spermatozoa are widely regarded as a suitable method for transportation of genetic materials. Wakayama and Yanagimachi reported that 16% of transferred embryos derived from ICSI with freeze-dried spermatozoa developed to term after a 3-week trip and additional storage for one week [17]. The temperature varied between 5°C and 30°C during the trip.

Factors Affecting Freeze-drying of Spermatozoa

Factors affecting fertilization and subsequent development after ICSI with freeze-dried spermatozoa have been examined. Normal fertilization in mammals is associated with a series of periodic Ca^{2+} increases in the oocyte cytoplasm. These Ca^{2+} oscillations play a key role in the development of fertilized oocytes. Recently, it has been shown that mouse and bovine freeze-dried spermatozoa maintain their full calcium oscillation-inducing capability in mouse oocytes [25]. Since the sperm-induced calcium oscillations are induced by a sperm protein factor that diffuses into oocyte cytoplasm upon sperm-oocyte membrane fusion, sperm that have undergone the freeze-drying, rehydration, and ICSI procedures appear to still retain the activity of this sperm protein factor. Chromosome analysis has shown that freeze-dried sperm maintain their genetic integrity [26–30]. Therefore, the freeze-drying method seems to be a viable means of preserving mammalian spermatozoa. It has been suggested that a simple Tris-HCl buffered solution with a high concentration of calcium-chelating agent [50 mM ethylene glycol-bis (-amino ethylether) -N, N, N', N' -tetraacetic acid tetrasodium EGTA] is much more effective for maintenance of chromosomal integrity during freeze-drying than CZB medium [26]. Furthermore, it has been shown that use of a slightly alkaline solution (pH 8.0) for freeze-drying is much better than neutral or acidic (pH 7.4–6.0) solutions for maintenance of both chromosomal integrity and the developmental capacity of mouse spermatozoa [27]. Although the frequency of normal metaphase plates (chromosomal integrity) is lower in freeze-dried mouse spermatozoa (61–83%) than in fresh spermatozoa (92–100%) [26–28, 30], freeze-dried mouse spermatozoa are more resistant to ^{137}Cs γ-ray irradiation than fresh spermatozoa [28]. The chromosomal integrity of freeze-dried spermatozoa was not found to be affected by ^{137}Cs γ-ray irradiation of up to 8 Gy [28]. Immature spermatozoa recovered from the testis were damaged and lost their genetic integrity as a result of freeze-drying but became resistant to this damage when their free thiols were oxidized to disulfides by diamide [31]. In contrast, epididymal spermatozoa are susceptible to damage by freeze-drying when treated with the disulfide-reducing agent dithiolthreitol, which reduces protein -SS- to -SH- [31]. The potential for freeze-drying damage seems to be related to the -SS- status of sperm proteins, in particular its protamines. Since the protamines of non-eutherian vertebrates (from most fish to marsupials) have little or no cysteine, it seems likely the spermatozoa of these species are susceptible to freeze-drying damage [31]. When Nile tilapia spermatozoa were stained with Hoechst 3342 after freeze-drying, the stained sperm heads seemed to be the same size as those of fresh sperm, but the nuclei

Table 1. Estimated rates of development to the blastocyst stage by extrapolation of the Arrhenius plot

Storage temp. (°C)	Storage term						
	0 mo	1 mo	3 mo	6 mo	1 yr	10 yr	100 yr
25	59.00	1.66	0.00	0.00	0.00	0.00	0.00
4	59.00	42.21	21.60	7.91	1.00	0.00	0.00
–20	59.00	58.19	56.00	54.30	49.86	10.96	0.00
–80	59.00	59.00	59.00	59.00	59.00	59.00	58.99

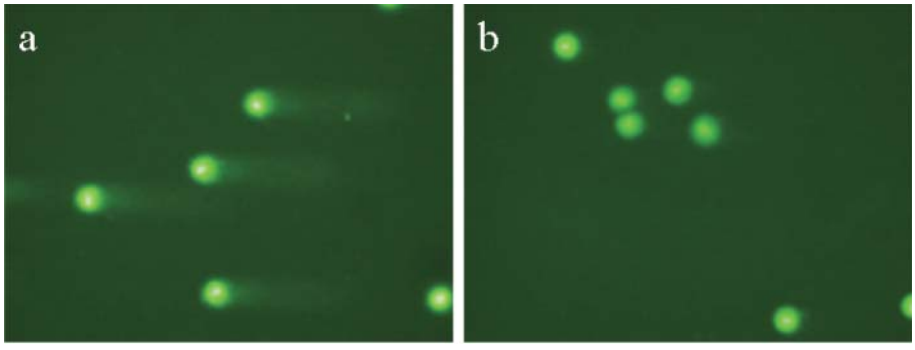

Fig. 1. Comet assay of freeze-dried mouse spermatozoa. Spermatozoa were stored at (a) 4°C, and (b) –80°C for 6 months each. The presence of comet tails in (a) indicates that fragmented DNA is present in these spermatozoa.

appeared more diffuse [32]. Injections of freeze-dried Nile tilapia spermatozoa could not be performed as a result of a technical problem related to manipulation of the rehydrated sperm cells [32].

Possible Long-term Preservation of Freeze-dried Spermatozoa at Ambient Temperature

When freeze-dried spermatozoa are used as a method for storage of genetic materials, it is particularly important to allow long-term preservation for several decades or even centuries. Although Word *et al.* [30] reported that live fertile offspring were obtained after 18 months sperm storage at 4°C, little is known regarding the possibility of much longer terms of preservation of freeze-dried spermatozoa. At present, no reports have been published concerning successful full-term development derived from freeze-dried spermatozoa preserved for a long term at room temperature. Thus, there is a great need for studies of the conditions required for freeze-drying to produce successful, effectively "permanent" preservation of freeze-dried spermatozoa at ambient temperature. Recently, Kawase *et al.* [33] applied determination of accelerated degradation kinetics to the preservation of freeze-dried spermatozoa. Accelerated degradation kinetics were calculated by extrapolation of arrhenius plots typically applied to the long-term stability of drugs. The results of their study indicate that successful preservation of freeze-dried spermatozoa for 100 years or more requires storage at temperatures of less than –80°C (Table 1) [33]. Since the estimated development rates to the blastocyst stage and the actual rates of oocytes derived from ICSI during preimplantation are similar, it appears to be possible to apply determination of accelerated degradation kinetics to preservation of freeze-dried mouse spermatozoa. Arrhenius plots also indicate that prolonged preservation of freeze-dried spermatozoa at 4°C, but not at –80°C, might be harmful to the preimplantation development of fertilized eggs by ICSI. Comet assay clarified that the higher temperature (4°C) induced DNA damage in stored freeze-dried spermatozoa (Fig. 1). Thus, further modification to the freeze-drying procedure and/or solution is required for successful permanent preservation of mouse spermatozoa at much higher temperatures. More recently, the relationship between the pressure at primary drying and the preservation potential of freeze-dried mouse spermatozoa has been examined. In previous studies, a pressure of 0.03–0.04 mbar (1 bar =

Table 2. Effect of vacuum pressure at primary drying on *in vitro* development of fertilized eggs derived from ICSI with freeze-dried mouse spermatozoa stored for 6 months at 4°C

Vacuum pressure (mbar)	No. of surviving oocytes/no. of oocytes injected (%)	No. (%) of oocytes fertilized*	No. (%) of embryos developed to the 2-cell stage**	No. (%) of embryos developed to the blastocyst stage**
0.04***	404/522 (77)	367 (91)	346 (94)	48 (13)
0.37	156/213 (73)	145 (93)	142 (98)	73 (50)
1.03	187/267 (70)	182 (97)	179 (98)	66 (36)

*: Calculated from the number of oocytes that survived. **: Calculated from the number of fertilized eggs. ***: Data from Kawase *et al.* (2005).

100 kPa) has commonly been used at primary drying [18, 23, 26–31, 33]. Primary drying is characterized by a visibly receding boundary from the top of the frozen layer. Once the ice has sublimed, heat for sublimation is no longer needed, and the temperature of the product usually increases sharply toward the shelf temperature. Since the driving force for freeze-drying is the vapor pressure of ice, it is important from the standpoint of process efficiency to keep the product temperature as high as is practical during primary drying. When mouse spermatozoa were freeze-dried at a pressure of 0.04 mbar, 0.37 mbar or 1.03 mbar for primary drying and 0.001 mbar for secondary drying, the developmental rates to the blastocyst stage of the fertilized eggs from ICSI with the freeze-dried spermatozoa were 59%, 71%, and 33% for primary drying, respectively (our unpublished data). The proportion of development to the blastocyst stage at a pressure of 0.37 mbar was significantly higher than for the other experimental groups. In addition, when these spermatozoa freeze-dried at a pressure of 0.04 mbar, 0.37 mbar, or 1.03 mbar for primary drying were stored for 6 months at 4°C and subjected to ICSI, the developmental rates to the blastocyst stage were 13%, 50%, and 36%, respectively (Table 2, unpublished). Similar to the case without storage, the proportion of development to the blastocyst stage at a pressure of 0.37 mbar was significantly higher than for the other experimental groups. The overall success rates (the proportion of live born mice developed from oocytes fertilized) were 1.1%, 10.3%, and 8.8% for freeze-dried spermatozoa stored for 6 months at 4°C at a pressure of 0.04 mbar, 0.37 mbar, and 1.03 mbar for primary drying. Since the change in freeze-drying pressure at primary drying from 0.04 mbar to 0.37 mbar or 1.03 mbar significantly improved the developmental potential of mouse spermatozoa with and without storage at refrigerator temperature after freeze-drying, the pressure at primary drying appears to be an important factor affecting time and temperature in the preservation of freeze-dried spermatozoa. Further modification to freeze-drying conditions, including the pressure at primary drying, will contribute to the effort to achieve permanent preservation of mammalian spermatozoa stores at much higher temperatures than currently feasible. The medical safety of future generations is the prime concern behind ICSI with freeze-dried spermatozoa and other assisted reproductive techniques. Further studies are required to clarify whether freeze-drying of spermatozoa increases the frequency of certain extraordinary events, such as epigenetic modification in the course of development.

Acknowledgements

This study was supported, in part, by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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