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SRD Young Investigator Award

A Study on Freeze-Drying as a Method of Preserving Mouse Sperm

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Abstract. This review describes the study of freeze-dried mouse sperm for practical application in preserving and transporting genetic resources. Freeze-dried sperm can be used to preserve and transport genetic resources; however, there still remain many areas which need to be studied. In particular, it is essential to assure long-term preservation over several decades or centuries. Recently, the theory of accelerated degradation kinetics to freeze-dried mouse sperm has been applied, and found that long-term preservation by conventional methods requires temperatures lower than –80 C. When the relationship between the pressure at primary drying and the preservation potential of freeze-dried mouse sperm was examined, a pressure of 0.37 mbar at primary drying significantly improved the developmental rate to the blastocyst stage. In addition, it has been shown that freeze-dried sperm stored at –80 C with and without transportation can retain their ability to generate viable offspring after storage for up to 2 years. Sperm chromatin structure assay (SCSA) was applied to mouse sperm freeze-dried under several conditions and compared the results with the embryonic developmental rates of freeze-dried sperm after intracytoplasmic sperm injection (ICSI) and with comet assay results. Furthermore, SCSA might be useful for estimation of developmental potential of fertilized eggs derived from ICSI using freeze-dried sperm in mice.
Key words: Comet assay, Freeze-dried sperm, ICSI, SCSA

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The number of transgenic and knockout mice being created and studied is increasing explosively with saturation mutagenesis projects and the International Knockout Mouse Consortium, which are producing mutants for all protein-coding genes. Consequently, an efficient method of preserving gene resources is necessary. Cryopreservation of mouse sperm has been widely applied to the maintenance of transgenic and knockout lines but, although cryopreservation of sperm is simpler and less time-consuming than that of embryos in maintaining those genetically modified mouse strains, keeping cryopreserved sperm has high running costs because of the need for a constant supply of liquid nitrogen (LN_2). In certain areas of the world, it also may be difficult or very expensive to store sperm in liquid nitrogen. Because it has been reported that freeze-dried mouse sperm are capable of producing normal embryonic development after injection into oocytes [1–5], they have attracted a great deal of attention as storable gene resources. Freeze-dried sperm could provide enormous reductions in maintenance and shipping costs but little is known regarding the possibility of long-term preservation. In particular, it is essential to assure long-term preservation for several decades or centuries. This review describes the following four points: 1) the accelerated degradation kinetics of preserved freeze-dried sperm, 2) improvement of freeze-drying conditions, 3) the influence of transportation and preservation conditions on freeze-dried sperm, 4) DNA fragmentation of freeze-dried mouse sperm with sperm chromatin structure assay (SCSA).

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Possibility of Long-term Preservation of Freeze-dried Mouse Sperm

When freeze-dried sperm are used as a method for storing gene resources, it is particularly essential to allow for long-term preservation over several decades or centuries. Thus, the accelerated degradation kinetics of preserved freeze-dried sperm by calculating an extrapolation of Arrhenius plots, a theory which is also being applied to the long-term stability of drugs, were measured. The percentage rates of freeze-dried sperm preserved for 100 years at 4 or –80 C developing to the 2-cell and blastocyst stage were estimated and then validated the estimated values by intracytoplasmic sperm injection (ICSI) using freeze-dried sperm stored for 3 or 6 months at 4 or –80 C [6].

The estimated percentage rates of development to the blastocyst stage by Arrhenius plot were 21.6, 7.9, 1 and 0% at 3 and 6 months and 1 and 10 years, respectively, after sperm storage at 4 C (Table 1). However, when stored at –80 C, the projected rates of development to the blastocyst stage that would be expected after 100 years of storage did not decline significantly from normal rates (Table 1). To evaluate the degradation kinetics described above, freeze-dried sperm stored at 4 or –80 C for 3 or 6 months were injected into oocytes, and the fertilized oocytes were cultured for 96 h after ICSI. The population of oocytes developing to the blastocyst stage following ICSI with sperm stored for 3 and 6 months at 4 C was predicted to be 21.6 and 7.9 % from the Arrhenius plots (Table 1).

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

Term	Months				Years		
C	0	1	3	6	1	10	100
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	56.60	54.30	49.86	10.96	0.00
–80	59.00	59.00	59.00	59.00	59.00	59.00	58.99

Table 2. Fertilization and development of oocytes by ICSI using freeze-dried sperm with a primary pressure of 0.04 mbar

Sperm storage term (Months)	Storage temp. (C)	No. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes fertilized[1)]	No. (%) of embryos developed to 2-cell[2)]	No. (%) of embryos developed to blastocyst[2)]
0	–	864	608 (70) [a]	576 (95) [a]	557 (97) [a,b]	337 (59) [a]
3	4	493	366 (74) [a,b]	345 (94) [a]	319 (92) [c]	73 (21) [b]
6	4	522	404 (77) [b]	367 (91) [a]	346 (94) [b,c]	48 (13) [b]
3	–80	485	373 (77) [a,b]	353 (95) [a]	348 (99) [a]	219 (62) [a]
6	–80	495	378 (76) [a,b]	351 (93) [a]	348 (99) [a]	208 (59) [a]

[1)] Percentage of oocytes survived. [2)] Percentage of oocytes fertilized. Different superscript letters within a column indicate significantly different values ($P<0.05$).

In the validation experiments, 21% of the fertilized oocytes derived from ICSI stored for 3 months and 13% of those stored for 6 months, with both groups stored at 4 C, developed to the blastocyst stage in culture (Table 2). The estimated development rates and the actual rates of the ICSI oocytes during preimplantation were similar in this study (Tables 1and 2) and in previous reports [2, 3, 5–11]. As shown in Table 2, development rates to the 2-cell and blastocyst stages after ICSI were significantly higher in freeze-dried sperm stored at –80 C than in those stored at 4 C. Furthermore, no significant differences were found between freeze-dried sperm stored at –80 C and the controls with regard to development to the 2-cell or blastocyst stage (Table 2).

The comet assay was used to assess the DNA integrity of individual freeze-dried sperm. Evaluation of the DNA "comet" tail shape and migration pattern allows assessment of DNA damage. The assay was performed using alkaline electrophoresis to measure single-stranded DNA breaks and alkali-labile sites of the DNA. Most freeze-dried sperm stored at 4 C for 3 or 6 months had comet tails, but fresh as well as freeze-dried sperm stored at –80 C for 3 and 6 months did not (Fig. 1).

In this study, the determination of accelerated degradation kinetics to freeze-dried mouse sperm was applied. The results indicate that successful preservation of freeze-dried sperm for 100 years or more requires storage at temperatures less than –80 C (Table 1). To evaluate the estimation of development rates to the 2-cell and blastocyst stages, actual development rates derived from ICSI by using freeze-dried sperm stored for 3 and 6 months at 4 and –80 C were examined. These results indicate that it appears to be possible to apply the determination of accelerated degradation kinetics to the preservation of freeze-dried mouse sperm. Arrhenius plots also indicate that prolonged preservation of freeze-dried sperm at 4 C, but not at –80 C, might be harmful to the preimplantation development of fertilized eggs by ICSI. The comet assay clarified that the higher temperature (4 C) induced DNA damages in stored, freeze-dried sperm (Fig. 1). It appears that damage to the DNA of freeze-dried sperm stored at temperatures greater than –80 C resulted in reduced rates of preimplantation development after ICSI. Thus, freeze-dried mouse sperm are most efficiently stored for extended periods, several tens of decades, at temperatures lower than –80 C. Further modifications in the freeze-drying method and/or solution are required for successful permanent preservation of mouse sperm at much higher temperatures.

Effect on Reproduction Ability and Preservation Potential of Pressure at Primary Drying Stage of Freeze-drying Mouse Sperm

The freeze-drying process is of importance, especially the primary drying process but no study of pressure at primary drying of sperm has been reported. 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 [12]. In previous studies, a pressure of about 0.04 mbar has commonly been used at primary drying. Thus, the pressure at primary drying was focused on and found 0.37 mbar to be the optimum pressure for preservation of freeze-dried mouse sperm at much higher temperatures [9].

The developmental rates to the blastocyst stage of embryos from ICSI by freeze-dried sperm without storage, and with storage at 4 C for 6 months showed the highest rate of embryonic development when primary drying was performed at 0.37 mbar (Tables 3 and 4). In addition, the number of live-term fetuses produced was higher at

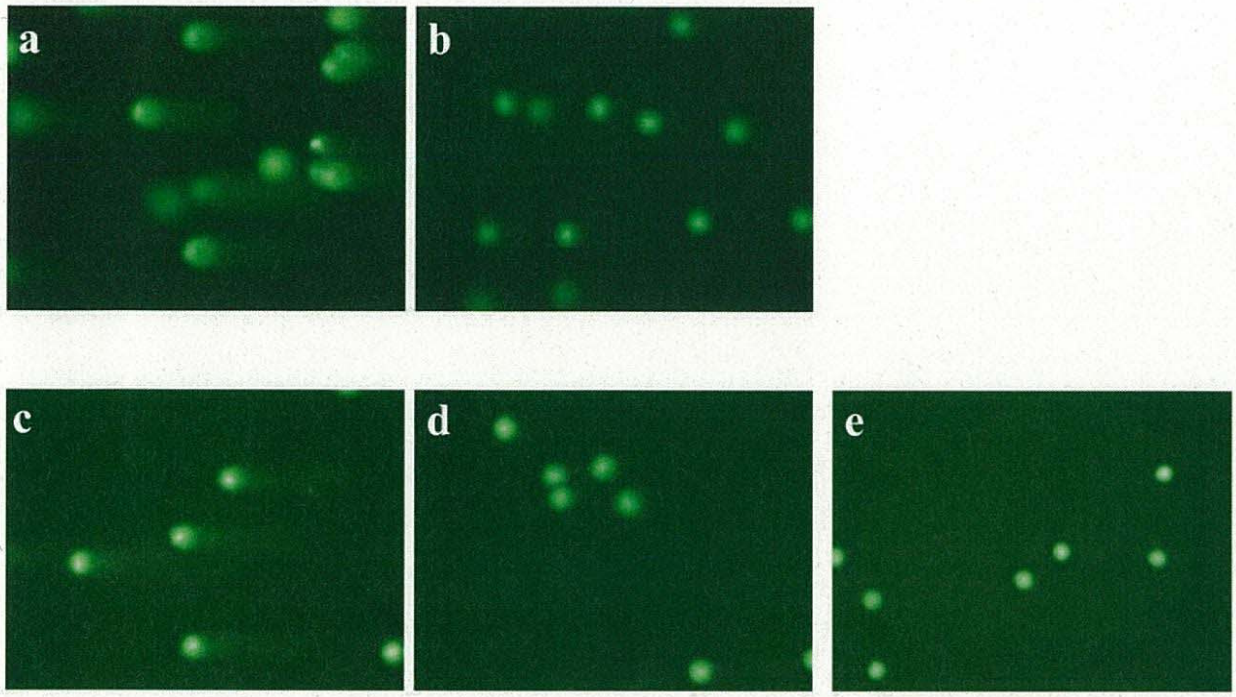

Fig. 1. Comet assay of freeze-dried sperm [6]. Sperm were stored at (a) 4 C for 3 months, (b) at –80 C for 3 months, (c) at 4 C for 6 months, and (d) at –80 C for 6 months. Fresh sperm were used as a control (e). The presence of comet tails in (a) and (c) indicates fragmented DNA is present in these sperm. Original magnification: ×200.

0.37 mbar than at other pressures for groups without storage and with storage at 4 C for 6 months although the difference was not significant (Table 5). All fetuses were morphologically normal—some of the mice have since been mated and have delivered normal offspring. A numerical indicator for overall efficiency of mouse production after ICSI using freeze-dried mouse sperm was determined by dividing the ratio of the number of embryos that developed blastocysts to the number of fertilized oocytes by the ratio of the number of live-term fetuses to the number of blastocysts transferred (Fig. 2). With storage at 4 C for 6 months, the overall efficiency from a pressure of 0.37 mbar (10.3) or 1.03 mbar (10.0) was significantly higher than 0.04 mbar (1.1). Moreover, with a pressure of 0.37 mbar (25.7) without storage, the overall efficiency after ICSI was significantly higher than for 0.04 mbar (17.5) or 1.03 mbar (6.62). The results showed that blastocyst formation was significantly higher when primary drying was performed at 0.37 mbar than at 0.04 mbar and 1.03 mbar irrespective of the storage conditions (without storage, or 4 C for 6 months) (Tables 3, 4, and Fig. 2). Although the precise mechanisms affecting storage potential of pressure at primary drying of freeze-drying mouse sperm was not clarified in this study, differences of an alteration of tertiary structure in seminal proteins by freeze-drying might influence their potential [13]. Sperm freeze-dried at 1.03 mbar yielded the same proportion of development to blastocysts and live born after transfer when injected immediately after preservation (without storage) and after 6 months storage (Tables 3–5). However, overall efficiency at 1.03 mbar was not significantly different between the experimental groups.

The overall efficiency determined from the data in this study clearly indicates that the pressure at primary drying is an important factor affecting the outcome after storage. Modifications to the freeze-drying method, with attention to the pressure at primary drying, will contribute to the success of permanent preservation of mammalian sperm stored at higher temperatures. However, the relationship between the primary drying mechanism and embryonic developmental rate was not clarified [9]. Further studies are needed for a better understanding of this relationship.

Transportation of Freeze-dried Mouse Sperm

Freeze-dried sperm is a useful method not only for preserving genetic resources but also for transporting genetic resources from animals. Freeze-dried sperm are easily transported around the world, because their preservation does not need LN_2 or dry ice. In fact freeze-dried sperm can be carried in an envelope or it someone's pocket. For practical use of transportation of genetic resources, this study was designed to determine the influence of storage temperature, transportation time and primary drying pressure on the development of embryos derived from freeze-dried mouse sperm. Freeze-dried sperm stored for 2 or 2.5 years at 4 or –80 C were transported round trip overland between Shizuoka and Hokkaido prefectures in Japan or by air between Japan and Bel-

Table 3. Effect of vacuum pressure at primary drying on the *in vitro* development of embryos generated by ICSI from freeze-dried, non-stored, sperm

Vacuum pressure (mbar)	No. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes fertilized[1)]	No. (%) of embryos developed to 2-cell[2)]	No. (%) of embryos developed to blastocyst[2)]
0.04	864	608 (70)[a]	576 (95)[a]	557 (97)[a]	337 (59)[a]
0.37	253	194 (77)[ab]	187 (96)[a]	181 (97)[a]	132 (71)[b]
1.03	404	317 (78)[b]	302 (95)[a]	288 (95)[a]	99 (33)[c]

[1)] Percentage of oocytes survived. [2)] Percentage of oocytes fertilized. Different superscript letters within a column indicate significantly different values ($P<0.05$).

Table 4. Effect of vacuum pressure at primary drying on the *in vitro* development of embryos generated by ICSI from freeze-dried sperm stored at 4 C for 6 months

Vacuum pressure (mbar)	No. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes fertilized[1)]	No. (%) of embryos developed to 2-cell[2)]	No. (%) of embryos developed to blastocyst[2)]
0.04	522	404 (77)[a]	367 (91)[a]	346 (94)[a]	48 (13)[a]
0.37	213	156 (73)[a]	145 (93)[ab]	142 (98)[a]	73 (50)[b]
1.03	267	187 (70)[a]	182 (97)[b]	179 (98)[a]	66 (36)[c]

[1)] Percentage of oocytes survived. [2)] Percentage of oocytes fertilized. Different superscript letters within a column indicate significantly different values ($P<0.05$).

Table 5. Effect of vacuum pressure at drying on the *in vivo* development of embryos generated by ICSI using freeze-dried sperm

Vacuum pressure (mbar)	Storage temperature (C)	Sperm storage time (months)	No. of blastocysts transferred	No. (%) of implantation sites	No. (%) of live term fetuses
0.04	RT	non-stored	194	137 (71)[a]	58 (30)[a]
0.37	RT	non-stored	132	93 (70)[a]	48 (36)[a]
1.03	RT	non-stored	99	70 (71)[a]	20 (20)[a]
0.04	4	6	48	28 (58)[a]	4 (8)[a]
0.37	4	6	73	39 (53)[a]	15 (21)[a]
1.03	4	6	66	56 (85)[b]	16 (24)[a]

Different superscript letters within a column indicate significantly different values ($P<0.05$).

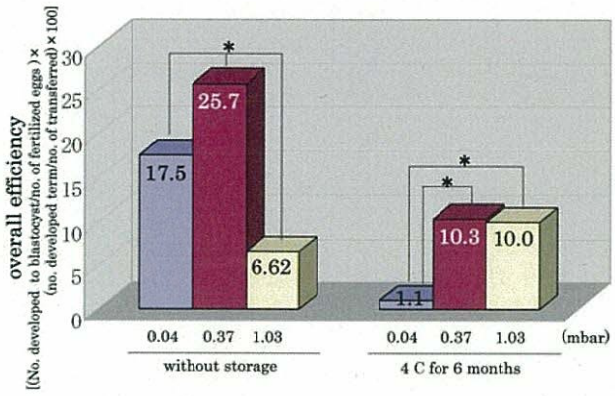

Fig. 2. Overall efficiency of mouse production from ICSI using freeze-dried sperm [9]. Sperm were dried at 0.04 mbar (blue bar), 0.37 mbar (purple bar) or, 1.03 mbar (yellow bar) and then used for ICSI without storage (left) or after storage at 4 C for 6 months (right). $P<0.05$.

gium [10].

With primary drying of freeze-dried sperm at 0.04 mbar, the embryonic developmental rates to the 2-cell stage from ICSI after storage at 4 C with and without transport were significantly lower than at –80 C ($P<0.05$, Table 6). For storage at –80 C for up to 2 years, the developmental rates to the 2-cell stage were similar for freeze-dried sperm without transport and with either land or air transport ($P>0.05$, Table 6). However, the rate of full-term fetuses derived from the same conditions (–80 C, storage up to 2 years) but without transportation was 28% and was only 8% and 1% with land and air transport, respectively. No fetuses developed from freeze-dried sperm stored at 4 C (Table 6).

To investigate the effect of air transport and primary drying pressure, the experiment performed ICSI using freeze-dried sperm that had been transported roundtrip between Japan and Belgium after primary drying at pressures of 0.04, 0.37 and 1.03 mbar. The developmental rates to the 2-cell stage were 96, 95 and 95%, respectively. The developmental rates to the 2-cell stage were not significantly different ($P>0.05$, Table 7); however, development to

Table 6. Fertilization and development of oocytes by ICSI using freeze-dried spermatozoa with a primary pressure of 0.04 mbar

Sperm storage (years)	Storage temperature (C)	Mode of transport	No. of oocytes injected	No. (%) of surviving oocytes	No. (%) of oocytes fertilized[1)]	No. (%) of embryos developed to the 2-cell stage[2)]	No. of 2-cell embryos transferred	No. (%) of implantation sites	No. (%) of live term fetuses
–	–	–	151	109 (72)[a]	106 (97)[a]	103 (97)[a]	103	68 (66)[a]	21 (20)[a]
2	4	–	145	102 (70)[a]	101 (99)[a]	84 (83)[b]	84	3 (4)[b]	0 (0)[b]
2	4	Land	157	101 (64)[a]	98 (97)[a]	81 (83)[b]	81	0 (0)[b]	0 (0)[b]
2	–80	–	155	99 (64)[a]	99 (100)[a]	97 (98)[a]	97	56 (58)[a]	27 (28)[a]
2	–80	Land	150	108 (72)[a]	107 (99)[a]	104 (97)[a]	104	37 (36)[c]	8 (8)[b]
2.5	–80	Air	180	125 (69)[a]	120 (96)[a]	115 (96)[a]	115	7 (6)[b]	1 (1)[b]

[1)] Percentage of surviving oocytes. [2)] Percentage of fertilized oocytes. Different superscript letters within a column indicate significantly different values ($P<0.05$).

Table 7. Fertilization and development of oocytes by ICSI using air-transported (Japan↔Belgium) freeze-dried sperm

Vacuum pressure (mbar)	No. of oocytes injected	No. (%) of surviving oocytes	No. (%) of oocytes fertilized[1)]	No. (%) of embryos developed to the 2-cell stage[2)]	No. of 2-cell transferred	No. (%) of implantation sites	No. (%) of live term fetuses
0.04[3)]	180	125 (69)[a]	120 (96)[a]	115 (96)[a]	115	7 (6)[a]	1 (1)[a]
0.37	198	145 (73)[a]	141 (97)[a]	134 (95)[a]	134	43 (32)[b]	22 (16)[b]
1.03	180	119 (66)[a]	114 (96)[a]	108 (95)[a]	108	16 (15)[a]	5 (5)[a]

Freeze-dried spermatozoa were stored at –80 C until use. [1)] Percentage of surviving oocytes. [2)] Percentage of fertilized oocytes. 3) Data from Table 6. Different superscript letters within a column indicate significantly different values ($P<0.05$).

full-term fetuses at 0.37 mbar was higher than at 0.04 and 1.03 mbar ($P<0.05$, Table 7).

The rate of full-term fetuses using freeze-dried sperm stored at –80 C was significantly lower when the samples were transported compared to those which were not transported. However, the results showed that freeze-dried sperm stored at –80 C with and without transport can retain their ability to generate viable offspring after storage for up to 2 years (Table 6). In this study, fertilization and full-term development were successful using sperm stored for 2.5 years. This storage duration is longer than that reported previously, where live and fertile offspring were obtained from sperm stored for 1.5 years at 4 C [5].

For freeze-dried sperm with primary drying pressure at 0.04 mbar, the embryonic development rates from ICSI to the 2-cell stage with storage at –80 C for 2 and 2.5 years were almost the same as that from sperm which was not stored ($P>0.05$, Table 6). The results correspond to the accelerated degradation kinetics determined by the Arrhenius equation [14]; the embryonic development rate from ICSI to the 2-cell stage that would be expected after 100 years of storage at –80 C does not decrease significantly [6]. Consequently, for long-term preservation and transportation after preservation, freeze-dried sperm requires storage at –80 C. The embryonic developmental rate (Tables 6 and 7) was influenced mainly by temperature during transportation and to a lesser extent by storage duration. The data show that freeze-dried sperm stored at temperatures lower than –80 C are viable for ICSI; namely, lower temperatures during storage and transportation will result in higher developmental rates.

Primary drying at a pressure of 0.37 mbar had better results than 0.04 mbar or 1.03 mbar after air transport. A pressure of 0.37 mbar significantly improved the rate of development to the blastocyst stage [9] and the rate of development of fetuses to full term using freeze-dried sperm with longer term preservation (Table 7).

Establishing a Method for Evaluating DNA Fragmentation of Freeze-dried Mouse Sperm Using a Modified SCSA

Freeze-drying solutions, primary drying pressure, and all other conditions of the technique need further exploration but, in addition and largely unexplored, is chromosome analysis after ICSI under various conditions. It is essential to determine the level of DNA fragmentation of freeze-dried sperm before use. Sperm chromatin assessment is an independent measure of sperm quality that provides better diagnostic and prognostic capabilities for potential fertility than standard sperm parameters [15]. Janny and Menezo reported that development to the blastocyst stage is linked to sperm quality [16] and it has been reported that increased levels of DNA fragmentation significantly decrease embryonic development to the blastocyst stage in mice [17]. The flow cytometric SCSA, can detect DNA fragmentation of several thousand sperm [18], unlike conventional microscopy methods, and is currently the most accurate for predicting the outcome of various assisted reproduction technologies such as fertilization and implantation [19]. The SCSA has potential as a powerful tool for evaluating freeze-dried sperm and as an aid in determining the best conditions for long-term preservation at ambient temperature because it can indicate sperm DNA fragmentation of many thousands of sperm samples, thus saving

Table 8. Analysis of *in vitro* development of embryos from spermatozoa freeze-dried under various conditions and stored for 18 months

Vacuum pressure (mbar)	Storage temperature (C)	ICSI		Comet Assay		SCSA	
		No. developed to two-cell / Total fertilized (%)	No. developed to blastocyst / Total fertilized (%)	No. damaged sperm / Total no. of sperm (%)	Average tail length (μm)	Definite DFI (%)	DFI (%)
0.04	4	67/86 (78) [a]	2/86 (2) [a]	65/65 (100) [a]	22.1 ± 10.5 [a]	38.93 [a]	81.57 [a]
	−80	95/98 (97) [b]	54/98 (55) [bc]	0/57 (0) [b]	0 [b]	2.89 [bc]	3.76 [b]
0.37	4	110/115 (96) [b]	54/115 (47) [c]	3/51 (6) [b]	0.5 ± 2.1 [b]	4.27 [c]	46.72 [c]
	−80	135/137 (99) [b]	95/137 (69) [b]	0/55 (0) [b]	0 [b]	1.79 [b]	2.52 [b]

DFI: Percentage of cells outside the main population. Different superscript letters within a column indicate significantly different values ($P<0.001$).

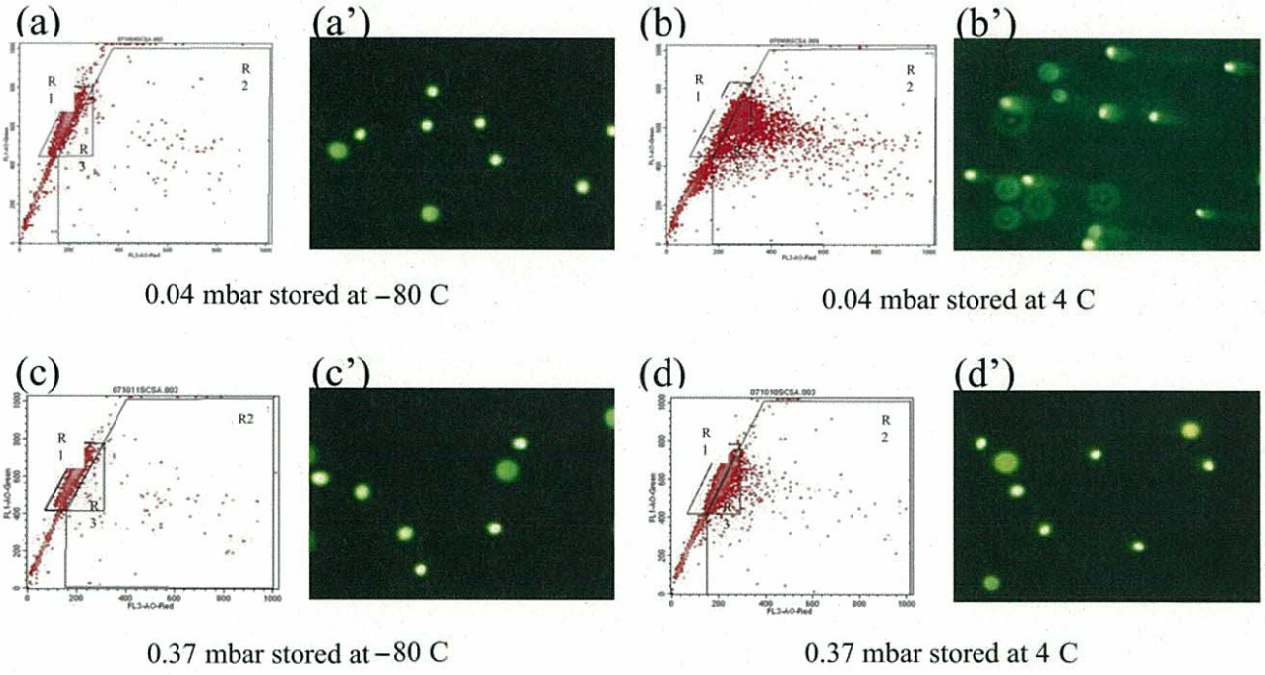

Fig. 3. SCSA scattergrams (a–d) and comet assays (a'–d') of freeze-dried sperm under various conditions of pressure and temperature stored for 1.5 years [11]. The SCSA scattergrams show the intensity of fragmented DNA (red) *vs.* double-strand DNA (green) fluorescence of the freeze-dried sperm. Each dot represents a single sperm and the areas indicate sperm that are intact (R1), highly fragmented (R2), and not highly fragmented (R3). The 'halo effect' in the comet assay (a') indicates the presence of fragmented DNA. Original magnification: ×200.

both cost and time [11].

It has been shown that a modified SCSA of freeze-dried sperm is a useful method for DNA fragmentation analysis. Namely, applying SCSA to freeze-dried mouse sperm is a viable method for examining the developmental rates derived from ICSI. Modifications to the SCSA included an increase in the acid-detergent solution and a shorter reaction time in order to better detect the DNA fragmentation of freeze-dried sperm. The modified SCSA was not detected self fluorescence and could be separated single-strand DNA from double-strand DNA. As a result, a correspondence between the definite DNA fragmentation index (DFI) of the rate of development to the blastocyst stage and the SCSA results was indicated. There was also correlation between the DFI and ICSI results from different primary pressures and storage temperatures (Table 8). The definite DFI clearly indicated the differences in sperm DNA fragmentation between the two primary drying pressures with storage at 4 C. On the other hand, the comet assay detected only obvious DNA fragmentation and did not detect the subtle fragmentation found by SCSA, exemplified by the difference in the results for sperm freeze-dried at a pressure of 0.04 mbar with storage at 4 C for 18 months (Fig. 3, Table 8). The SCSA better predicted the various outcomes of ICSI using freeze-dried sperm. The validity of SCSA was confirmed by the high level of consistency of the developmental rates found with previous DNA fragmentation analysis of mouse sperm. Of the four conditions tested, storage at 4 C with primary drying at 0.04 mbar resulted in a

significantly lower rate of embryo development to the blastocyst stage and a significantly higher definite DFI (Table 8). It is concluded that the developmental rates and definite DFI found from SCSA clearly correlated with embryo development to the blastocyst stage. An abnormal paternal genome can have an adverse effect on embryonic development to the blastocyst stage and thus early detection in freeze-dried sperm is important.

In order to achieve permanent preservation of mouse sperm at ambient temperature further improvements in the freeze-drying process are required. In addition to the actual process of freeze-drying, storage times and modes of transportation also require further analysis. Application of a modified SCSA to mouse freeze-dried sperm might be useful to explore the conditions of freeze-drying mouse sperm. It is concluded that SCSA has the potential to be a powerful tool for determining conditions to improve the fertility rate of freeze-dried sperm.

Conclusion

Over the past ten years, numerous reports have shown that freeze-dried mouse sperm are capable of producing normal embryonic development after injection into oocytes [1–5]. Moreover, a lot of researchers have made various improvements to the practical aspects of the freeze-drying process, with beneficial effects on the embryo developmental rate. Improving the solution [2, 3, 8, 20], the primary drying pressure [9], and adding a cryoprotectant for rhesus macaque [21], etc. are examples of these studies. This review described the results concerning the following four points, 1) the storage of freeze-dried mouse sperm at the commonly-used primary drying pressure (0.04 mbar) required temperatures lower than –80 C to withstand long-term preservation, 2) since changing the pressure at primary drying from 0.04 mbar to 0.37 mbar significantly improved the developmental potential of mouse sperm 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 sperm, 3) freeze-dried sperm stored at –80 C with and without transportation can retain their ability to generate viable offspring after storage for up to 2 years, 4) the SCSA method applied to freeze-dried mouse sperm after storage will not only clarify the developmental rate of embryos derived from ICSI using freeze-dried sperm but also improve the conditions of the freeze-drying and storage process. In these findings, it was suggested that freeze-dried mouse sperm are coming of age as a practical method of preserving gene resources. Especially, freeze-dried mouse sperm may largely contribute to diversify the risks of preserving gene resources with liquid nitrogen. It is suggested that freeze-drying sperm become more widespread as an effective method of maintaining gene resources and feel that the freeze-drying of sperm should be used as an additional method of preservation for the storage of gene resources in the immediate future.

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