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—Technical Note—

Long-Term Preservation of Mouse Spermatozoa as Frozen Testicular Sections

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Abstract. We previously demonstrated that testicular spermatozoa can be preserved as frozen testicular sections, allowing us to preserve male gametes in less space than conventional methods. However, it remains unclear whether the testicular spermatozoa can be preserved for a long period using this procedure. In this study, we examined the function of testicular spermatozoa preserved as frozen testicular sections for l year at –30 or –80 C. Testicular spermatozoa were successfully retrieved from frozen testicular sections preserved at either –30 or –80 C, and their function was assessed using intracytoplasmic sperm injection (ICSI). Over 90% of the oocytes injected with long-term preserved testicular spermatozoa formed pronuclei, which was a frequency similar to that obtained with spermatozoa preserved for a short term, indicating that the testicular spermatozoa retained oocyte activation factor(s). Approximately 70% of the fertilized oocytes developed to 2-cell stage embryos, and 9.3 to 12.8% of the embryos developed to term after transfer into pseudopregnant females, regardless of the preservation temperatures examined. These results indicate that the birthrates of progeny did not differ between the preservation temperatures examined. They also indicate that male gametes can be preserved in testicular frozen sections for at least 1 year without loss of function.

Key words: Intracytoplasmic sperm injection (ICSI), Frozen testicular section, Mouse, Testicular spermatozoa, Testis

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The mouse is the primary research animal used in mammalian genetics, and provides valuable models for analyzing human genetic diseases. Transgenic and mutant mice are routinely produced to elucidate gene function *in vivo*. Because it is difficult to maintain all mouse lines as live stock, established mouse lines are cryopreserved as gametes or embryos for future use or distribution. As such, efficient and dependable methods for gamete or embryo cryopreservation are needed to avoid inadvertent loss of these unique materials through disease or other hazards. As spermatozoa are produced in much larger numbers than oocytes and embryos, sperm cryopreservation is less labor intensive than embryo freezing. Thus, a simple, inexpensive and space-effective means to preserve mouse sperm would be an effective way to store the exponentially increasing numbers of mutant and transgenic mouse lines.

Recently, we established a technique in which mouse testicular spermatozoa are preserved as frozen testicular sections at –30 C for at least 3 months [1]. Although the technique enabled us to preserve male gametes in a more space-efficient manner compared with conventional methods [1], it remains unclear whether testicular spermatozoa can be preserved over a longer period. In this study, we assessed the function of testicular spermatozoa prepared from frozen testicular sections preserved for 1 year. In addition, we examined the effects of a relatively low preservation temperature (–80 C) on cryopreservation of testicular spermatozoa.

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Materials and Methods

Mice

C57BL/6-GFP transgenic mice [TgN(acro/act-EGFP)OsbC3-N01-FJ002] were kindly provided by Dr. M Okabe (Osaka University, Osaka, Japan) [2, 3]. BDF1, C57BL/6 and ICR mice were purchased from SLC (Hamamatsu, Japan). All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the RIKEN Kobe Institute.

Preparation of frozen testicular sections

Frozen testicular sections were prepared from the testes of adult BDF1 and C57BL/6-GFP transgenic mice. Briefly, the testis was removed and embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan). The specimen was then frozen at –30 C and sectioned to a thickness of 25 μm using a cryostat. The sections were mounted on glass slides (10 sections per slide), placed in slide cases and stored at –30 or –80 C for 1 year. To prepare a testicular cell suspension, the entire glass slide was incubated with potassium-rich buffer (nucleus isolation medium: NIM; 123 mM KCl, 2.6 mM NaCl, 7.8 mM NaH_2PO_4, 1.4 mM KH_2PO_4, 3 mM EDTA, pH 7.2 adjusted using 1 M KOH) [4] at 4 C for 2 min. The sections were washed by gently pipetting with approximately 1 ml of NIM using a micropipette. The detached cells were centrifuged at 2,300 × *g* for 2 min at 4 C and then resuspended in NIM. The testicular cell suspensions were kept at 4 C until further use.

Intracytoplasmic sperm injection (ICSI)

Superovulation was induced in BDF1 and C57BL/6 females by

an injection of 5 IU equine chorionic gonadotropin (eCG) followed by an injection of 5 IU human chorionic gonadotropin (hCG) 48 h later. At 14 h post-hCG injection, the cumulus-oocyte complexes (COCs) were collected from the oviducts. The oocytes were freed from the cumulus cells by adding 0.1% bovine testicular hyaluronidase (ICN Biochemicals, Costa Mesa, CA, USA) to the COC-containing medium. After the cumulus cells were dissociated, the oocytes were rinsed twice with Chatot, Ziomet and Bavister (CZB) medium [5]. Approximately 2 μl of the sperm suspension were mixed with a drop (approximately 10 μl) of HEPES-human tubal fluid (HTF) medium containing 10% (w/v) polyvinylpyrrolidone (Irvine Scientific, Santa Ana, CA, USA). The sperm head was separated from the tail by applying several piezo pulses to the neck region, and the head was then injected into the oocyte using the method described by Kimura and Yanagimachi (1995) [6]. The testicular cell suspension was replaced every 30 min during the ICSI experiment, as we observed that the developmental ability of ICSI embryos using frozen-thawed sperm is reduced if the sperm are suspended in PVP solution for a long period of time. The oocytes that survived ICSI were incubated in CZB medium at 37 C under an atmosphere of 5% CO_2. When the embryos reached the 2-cell stage, they were transferred to the oviducts of 0.5-dpc pseudopregnant ICR females.

Statistical analysis

Fisher's exact probability test and the Student's *t*-test were performed for Tables 1 and 2, respectively. $P<0.05$ was considered statistically significant.

Results and Discussion

Our previous study indicated that testicular spermatozoa prepared from 25-μm frozen testicular sections are more effective for ICSI than those prepared from 10-μm sections [1]; therefore, we assessed the function of testicular spermatozoa prepared from 25-μm sections mounted on glass slides (Fig. 1A and B). As shown in Fig. 1C and D, we succeeded in obtaining testicular cell suspensions containing testicular spermatozoa from frozen testicular sections (BDF1 background) preserved at either –30 or –80 C for 1 year. The testicular spermatozoa were then injected into BDF1 oocytes using the standard procedure for ICSI. Approximately 90% of the oocytes formed pronuclei after the injection (Table 1). The pronuclei formation rates did not differ according to the preservation temperature (–30 *vs.* –80 C; Table 1), and the rates were similar to those of testicular spermatozoa preserved for a short period of time [1]. These results indicate that the oocyte activating factor(s) were preserved in frozen testicular sections for up to 1 year.

After culturing the ICSI embryos for 24 h, approximately 70% of the embryos reached the 2-cell stage when using testicular spermatozoa preserved at both –30 and –80 C (Table 1). The *in vitro* developmental rates did not differ from our previous report [1], in which the same experiments were performed using testicular spermatozoa preserved at –30 C for a short period of time. Additionally, no significant difference was found in the comparison with the freshly isolated testicular spermatozoa (Table 1). The

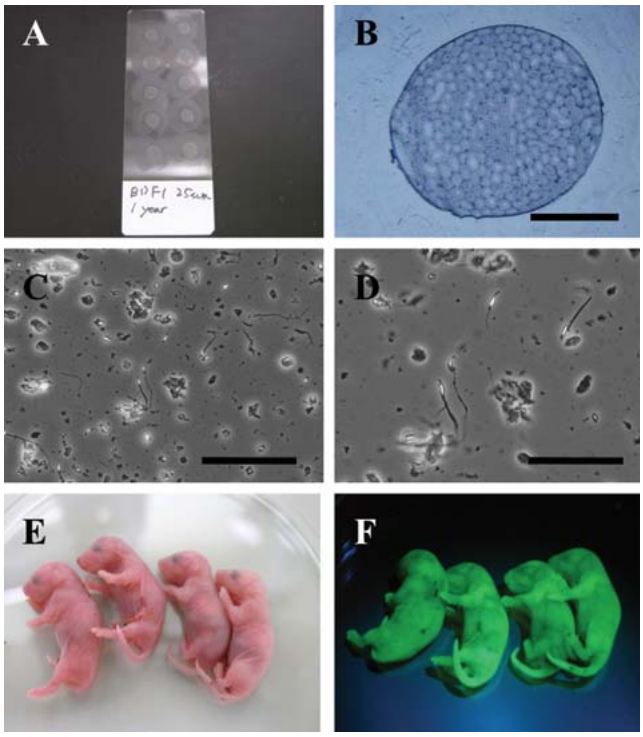

Fig. 1. Preparation of testicular spermatozoa from frozen testicular sections and production of progeny. (A) Frozen testicular sections mounted on a glass slide preserved for 1 year at –30 C. (B) Stereomicroscopic observation of frozen testicular sections. (C) Testicular cell suspension prepared from a frozen testicular section. (D) A higher magnification of (C). (E, F) Newborn C57BL/6 GFP transgenic mice derived from testicular spermatozoa preserved as frozen testicular sections at –30 C for 1 year. (F) Expression of the GFP transgene was confirmed under an excitation light source. Scale bar: 2 mm (B), 100 μm (C), 50 μm (D).

embryos were then transferred into pseudopregnant females to assess developmental ability. Approximately 10% of the zygotes developed to term (Table 1). No obvious abnormalities were found in the resulting progeny in terms of body weight, placenta weight or sex ratio (Table 2). These results indicate that testicular spermatozoa can be preserved as frozen testicular sections for at least 1 year at –30 or –80 C.

Although we generated progeny from preserved testicular spermatozoa, it remained unclear whether this technique would be feasible for large-scale preservation, especially in large animal facilities. In such a setting, it is important to be able to produce progeny even from inbred strains; ICSI, however, has previously been ineffective in producing inbred mice [7, 8]. To determine whether an inbred mouse strain can be recovered from testicular spermatozoa preserved for a long period of time, we prepared testicular spermatozoa from C57BL/6 mice carrying the green fluorescent protein (GFP) transgene, which were preserved as testicular frozen sections at –30 C for 1 year, and then injected them into C57BL/6 oocytes. Pronuclear formation and rate of cleavage were comparable to those of testicular spermatozoa using BDF1

Table 1. ICSI using testicular spermatozoa from frozen testicular sections preserved for 1 year at –30 or –80 C

Genetic background*	Preservation temperature	No. of oocytes that survived injection	No. of oocytes that formed pronuclei (%)	No. of embryos reaching the 2-cell stage (%)	No. of transferred	No. of progeny (%)
BDF1	–30 C	174	167 (95.9)	117 (70.0)	117	15 (12.8)[a]
BDF1	–80 C	151	140 (92.7)	108 (77.1)	108	10 (9.3)[a]
C57BL/6-Tg	–30 C	70	65 (92.9)	48 (73.8)	32	4 (12.5)[c]
	Total	395	372 (94.2)	273 (73.4)	257	29 (11.3)[a,b]
BDF1[#]	Non-preserved control	81	75 (92.6)	69 (92.0)	69	29 (42.0)[a']
C57BL/6[#]	Non-preserved control	123	113 (91.9)	106 (93.8)	90	22 (24.4)[b',c']

All testicular spermatozoa were prepared from frozen testicular sections preserved for 1 year. *Testicular spermatozoa prepared from BDF1 and C57BL/6-Tg were injected into BDF1 and C57BL/6 oocytes, respectively. [#]Data are from our previous report [12] in which ICSI using testicular spermatozoa was performed by the same person (H.O.) as in this study; these data were analyzed using Fisher's exact probability test to determine the significance of the difference ($P<0.05$) between non-preserved controls and the data obtained in this study. [a,a'; b,b'] $P<0.05$. [c,c'] $P=0.32$.

Table 2. Characterization of newborn mice produced from oocytes fertilized with testicular spermatozoa preserved as frozen testicular sections for 1 year

Genetic background*	Preservation temperature	Body weight (n)	Placenta weight (n)	Sex ratio (M : F)[#]
BDF2	–30 C	1.39 ± 0.18 (15)	0.16 ± 0.04 (15)	8:7
BDF2	–80 C	1.43 ± 0.13 (10)	0.17 ± 0.03 (10)	6:4
C57BL/6	–30 C	1.42 ± 0.11 (4)	0.17 ± 0.02 (4)	1:3
Non-preserved control**	–	1.43 ± 0.07 (15)	0.15 ± 0.03 (15)	7:8

*The progeny derived from BDF1 oocytes fertilized with BDF1 testicular spermatozoa were designated BDF2. [#]The sex ratio is shown as Male (M):Female (F). **Freshly isolated testicular spermatozoa from C57BL/6 were injected into BDF1 oocytes. No significant differences were found between the body and placenta weights of progeny obtained in this study and those of non-preserved controls ($P>0.05$, Student's *t*-test).

mice (Table 1). In addition, the birth rate of normal progeny expressing GFP was similar to that observed in experiments using BDF1 mice (Table 1). Transmission of the GFP transgene was observed in all progeny (Fig. 1E and F), as preserved testicular sections were prepared from transgenic mice homozygous for GFP. Therefore, our technique would also be useful for recovering a mouse colony from an inbred mouse strain with a transgene.

In the present study, we examined whether testicular spermatozoa can be preserved as frozen testicular sections for the long term. Our results clearly indicated the testicular spermatozoa retained the functional ability to produce progeny via ICSI after being preserved as frozen testicular sections for 1 year. The success rate of this study (average 11.3%) was comparable to our previous study (approximately 14%), in which frozen testicular sections were preserved for 3 months [1]. Moreover, we succeeded in recovering the C57BL/6 mouse line (Fig. 1E, F and Table 1), which is a standard mouse line widely used for analysis of mutant or transgenic mice. Therefore, our technique may prove useful as an alternative or backup system for the standard procedures currently used in animal facilities.

Typically, male gametes are cryopreserved in liquid nitrogen (–196 C) to maintain sperm function over the long term [9]. However, a recent study indicated that sperm can be preserved as testicular tissue at –20 C for 15 years [10], suggesting that long-term preservation of male gametes is possible at higher temperatures. Consistent with these previous results, we did not observe any difference in the function of testicular spermatozoa preserved at –30 versus –80 C. This suggests that higher temperatures do not cause the loss of function of testicular spermatozoa over a 1-year period. An important issue regarding our technique is how long testicular spermatozoa retain their functional ability in testicular frozen sections. Because it is difficult to obtain frozen testicular sections preserved for longer terms, other methods should be considered to clarify this issue. Accelerated degradation kinetics is one possibility, and has been used to estimate the maximum storage period of freeze-dried mouse spermatozoa [11]. This kind of assessment would support the reliability of our technique.

Although we succeeded in producing progeny from testicular spermatozoa preserved as frozen testicular sections, the success rate with this procedure was relatively low (11.3%, Table 1) compared with freshly isolated testicular spermatozoa [12] (24–42%, Table 1, $P<0.05$) and frozen-thawed testicular spermatozoa (approximately 20% [10]). This inefficiency could be caused by mechanical damage from sectioning in addition to damage from freeze-thawing. To reduce damage from sectioning, it may be effective to preserve thicker frozen testicular sections. The appropriate thickness of frozen testicular sections should be determined in a future study.

In this study, we used testicular spermatozoa preserved as frozen testicular sections rather than epididymal sperm preserved as epid-

idymal sections. Although we do not know which is the most effective tissue for preserving male gametes, a previous report indicates that epididymal sperm are sensitive to freezing when preserved as epididymal tissue [10]. This suggests that preservation of testicular sections is more effective than preservation of epididymal sections, given the possibility that unknown damage may have occurred during our procedure.

Acknowledgments

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