Fertility of Mouse Spermatozoa from Cauda Epididymis Preserved in Paraffin Oil at 4°C

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Abstract: *Fertile spermatozoa can be obtained from cauda epididymis after short term preservation at 4°C in paraffin oil. We examined the preservability of fertility under these conditions. Of ICR oocytes, 89% and 69% were fertilized in vitro with ICR and C57BL/6J spermatozoa from cauda epididymis stored in paraffin oil at 4°C for 24 h, respectively. Almost all of these fertilized oocytes developed to the blastocyst stage. The fertility of spermatozoa decreased noticeably with prolonged storage. From the stock stored for 96 h, the percentage of fertilized oocytes from ICR and C57BL/6J mice was only 13% and 1%, respectively. C57BL/6J zygotes which fertilized with either H or F gene disrupted spermatozoa previously stored for 16 h were implanted into individual oviducts. Fifty percent and 41% of these transferred zygotes developed into young, respectively. This study shows that mouse spermatozoa can be simply stored for 24 h within the intact cauda epididymis maintained at 4°C in paraffin oil; this procedure can facilitate the transportation of spermatozoa for this length of time.*

Key words: *Spermatozoa, Preservation, Cauda epididymis, Mouse.*

Preservation of gametes and embryos is important for the storage and transportation of gene resources. It has recently become possible to cryopreserve mouse spermatozoa in most strains of mice that are capable of fertilization of oocytes at high frequency [1–7]. Transgenes can therefore now be preserved in paternal gametes which require much less manipulation than storage in embryos.

The use of liquid nitrogen during transportation of gametes can be cumbersome and requires special equipment. We recently reported a simple method for the transportation of fertile mouse paternal gametes which were stored at room temperature and able to fertilize eggs at 48 h after preservation [8]. A recently published abstract also showed that frozen-thawed spermatozoa from the cauda epididymis of a Japanese monkey (*Macaca fuscata*) stored at 5°C for 24 h fertilized the related oocytes [9]. We examined the optimum time for storage in relation to the maintenance of fertility.

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Materials and Methods

Mature mice, either males from two gene disrupted strains, named H and F, or both sexes from ICR (CLEA, Japan) and C57BL/6J (CLEA, Japan), were used in this study. Methods for *in vitro* fertilization were the same as described by Toyoda *et al.* [10]. TYH medium [11] for *in vitro* fertilization and Whitten's medium [12] supplemented with 100 μM EDTA [13–15] for the culture of fertilized eggs were equilibrated overnight at 37°C under paraffin oil (Art 7162, Merck) in 5% CO_2 in air. *In vitro* fertilization and embryo cultures were also performed at 37°C in 5% CO_2 in air.

ICR and C57BL/6J females were superovulated with 5 IU of PMSG (Serotropin; Teikoku Hormone Mfg. Co., Japan) and 5 IU of hCG (Puberogen; Sankyo, Japan) injected 48 h apart. Their oocytes were obtained 15–16 h after hCG injection. The males were killed and their cauda epididymides were excised, placed into 15 ml centrifuge tubes (2096, Falcon) filled with approximately 15 ml of paraffin oil, cooled gradually from 24°C to 4°C (cooling rate; −1°C/min) and stored at 4°C for time periods ranging from 24 h to 120 h.

Cauda epididymides of H and F knockout mice were excised and stored in the same manner as described above for approximately 16 h. Spermatozoa were then collected from each cauda epididymis and suspended in 200 μl of TYH medium covered with paraffin oil. After preincubation for capacitation of the spermatozoa, *in vitro* fertilization was performed by adding a small volume of the sperm suspension to the medium containing the oocytes. Spermatozoa were adjusted to concentra-

Table 1. *In vitro* fertilization with spermatozoa collected from cauda epididymis stored at 4°C in paraffin oil

Strain		Strage time (h)	No. of eggs fertilized/ No. of eggs examined (%)	No. of embryos developed to Blastocysts/No. of embryos cultured (%)
Male	Female			
ICR	ICR	0	106/112 (95)[a]	11/12 (92)[a]
		24	73/ 82 (89)[a]	N.D.
		48	61/120 (51)[b]	N.D.
		72	32/127 (25)[c]	32/32 (100)[a]
		96	16/127 (13)[c,d]	N.D.
		120	2/ 64 (3)[d]	N.D.
C57BL/6J	ICR	0	258/327 (79)[a]	241/258 (93)[a]
		24	132/191 (69)[a]	125/132 (95)[a]
		48	38/239 (16)[b]	30/ 38 (79)[b]
		72	14/232 (6)[b,c,d]	6/ 14 (43)[b]
		96	2/154 (1)[c,d]	N.D.
		120	0/173 (0)[d]	—

Values with different superscripts are significantly different at $p<0.05$. The data were analyzed statistically by the Tukey-type test for nonparametric multiple comparisons.

Table 2. Effect of sperm concentration at insemination on the fertility of ICR spermatozoa from cauda epididymis stored at 4°C in paraffin oil

Strage time (h)	No. of eggs fertilized/No. of eggs examined (%)	
	Final sperm concentration at insemination (cells/μl)	
	150	750
72	21/98 (21)[a]	36/66 (55)[b]
96	3/39 (8)[a]	7/42 (17)[a]
120	2/40 (5)[a]	5/27 (19)[a]

Values with different superscripts are significantly different at $p<0.05$ when comparing concentrations of either 150 or 750 cells/μl at the same storage time. The data were analyzed statistically by the Tukey-type test for nonparametric multiple comparisons.

tions of either 150 or 750 cells/μl to analyze the effect of the final sperm concentration. Six h after insemination, the oocytes with a second polar body and both male and female pronuclei were recognized as fertilized eggs. After washing twice with Whitten's medium, the fertilized oocytes were cultured in the same fresh medium supplemented with 100 μM EDTA. The eggs fertilized with the spermatozoa from the knockout mice were transferred into oviducts of ICR recipients at the pronuclear or 2-cell stage on 0.5 day post-coitum as described previously [16].

Results

After preincubation of spermatozoa stored at 4°C, head-to-head aggregation was evident. Motility of spermatozoa that were free of agglutination, regardless of the storage period, was similar to that of the non-stored control. As shown in Table 1, 89% (73/82) and 69% (132/191) of oocytes were fertilized by ICR and C57BL/6J spermatozoa stored for 24 h, respectively. Almost all of these fertilized eggs developed to the blastocyst stage *in vitro*. Subsequent prolongation of storage significantly decreased fertilization rates of oocytes by spermatozoa derived from either strain of mouse.

In each of the respective storage times tested, significantly different *in vitro* fertilization rates for ICR oocytes were observed when comparing cauda epididymal spermatozoa from either ICR or C57BL/6J mice. The fertilization rates for oocytes inseminated with C57BL/6J spermatozoa were noticeably reduced after 48 h storage (Table 1).

Table 3. *In vitro* fertilization with spermatozoa collected from cauda epididymis stored in paraffin oil at 4°C for 16 h and subsequent embryo transfer in mice

Strain		No. of eggs fertilized/ No. of eggs examined (%)	No. of pregnant/ No. of recipients (%)	No. of implantations/ No. of embryos transferred (%)	No. of newborns/ No. of embryos transferred (%)
Male	Female				
H	C57BL/6J	324/393 (82)	23/23 (100)	193/275 (70)	137/275 (50)
F	C57BL/6J	120/231 (52)	7/ 7 (100)	N.D.	50/120 (41)

To analyze the effect of the final sperm concentration at the time of insemination (Table 2), ICR spermatozoa stored for between 72 and 120 h were inseminated at final concentrations of either 150 or 750 cells/μl. Oocytes inseminated at the 5 times higher concentration exhibited increased fertilization rates of approximately 2–3 fold. These were statistically significant at 72 h after storage ($P<0.05$).

To examine *in vivo* development of embryos derived from oocytes and stored spermatozoa, C57BL/6J oocytes were inseminated with H or F gene disrupted mice spermatozoa collected from stored cauda epididymis (as described in the methods) for 16 h during transportation from other laboratories. Fertilization at 6 h after insemination with H and F spermatozoa occurred at frequency of 82% (324/393) and 52% (120/231), respectively. Subsequent embryo transfer of these respective strains developed into newborns with 50% (137/275) and 41% (50/120) success (Table 3). These mutated locus/loci were transmitted to progeny according to the Mendelian law inheritance characteristics (data not shown).

Discussion

Mouse spermatozoa mature and are maintained at a high concentration in the cauda epididymis. In the present study, 89% of eggs were fertilized when the oocytes were inseminated with spermatozoa from cauda epididymis stored in oil at 4°C for 24 h. Our previous study showed that ICR capacitated spermatozoa, stored at a concentration of below 2×10^4 cells/μl (in air at 24°C for 24 h), fertilized only 12% of oocytes. In contrast, the same report showed a significantly higher rate (79%) of fertilization when the concentration (under similar storage conditions) was higher than 2×10^4 cells/μl [8]. Taken together these studies suggest that a higher concentration of spermatozoa under the same conditions of storage contributed to the high rates of fertilization observed.

In previous studies, we stored capacitated spermatozoa for 24 h in culture medium at 4°C but found very low (0–5%) rates of fertilization. In these previous studies, most spermatozoa did not regain motility and were irreversibly impotent after exposure to 4°C [8]. This so called cold shock damage can be associated with lipid phase transition in cell membranes [17], but spermatozoa from cauda epididymis stored for 24 h at 4°C were motile and fertilized eggs at a high frequency after preincubation (Table 1). With prolonged storage at 4°C, increasing numbers of immotile spermatozoa were observed but remaining spermatozoa exhibited a high motility, similar to the non-stored control, even after storage for 120 h. The fertilizing ability of these spermatozoa, however, significantly decreased with prolonged storage, even when used at 5 times the volume. Zona opening methods such as zona drilling [18] and/or partial zona dissection [19] may, however, be effective in increasing their fertilization rate.

Embryo transfer experiments revealed that C57BL/6J eggs, fertilized with gene disrupted spermatozoa collected from stored cauda epididymis, were able to develop to term. These results clearly indicate that storage of cauda epididymis at a low temperature in oil (SCELTO) is a simple and useful means of transporting genetic materials via spermatozoa. As *in vitro* fertilization (IVF) and subsequent embryo transfer (ET) are effective and provide a simple alternative to cesarean operation on infected or conventional mice [20], a combination of IVF-ET and SCELTO can be made available to many laboratories for transportation, cleaning and mass production of mice at low cost.

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