Maintenance of the Fertilizing Ability in Capacitated Mouse Spermatozoa

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Abstract. Capacitated mouse spermatozoa were stored for 24 to 72 h under the following conditions: 4C in air, 24C in air, 24C in 5% CO₂ in air, or 37C in 5% CO₂ in air. None or few of the oocytes inseminated with the capacitated spermatozoa stored for 24 h at 37C in 5% CO₂ in air or 4C in air were fertilized. Fertilization rates of oocytes inseminated with capacitated spermatozoa stored for 24 h at 24C in 5% CO₂ in air and 24C in air were 46% and 37%, respectively. When capacitated spermatozoa were stored at high concentrations, over 2×10^4 cells/ μ l, 87% of the oocytes were fertilized. Seventy-one percent of the fertilized eggs developed to term after transfer into the oviduct of recipients. These results indicate that capacitated mouse spermatozoa stored for 24 h at 24C can fertilize ova that subsequently develop normally through pre-and post-implantation stages.

Key words: Mouse, Spermatozoa, Capacitation, *In vitro* fertilization.

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Numerous reports have been published on *in vitro* fertilization (IVF) in mice since the first success using uterine sperm was reported in 1968 [1]. Mouse cauda epididymal spermatozoa can be capacitated by an incubation for one hour at 37C in 5% CO₂ in air [2]. If incubation under this condition exceeds 8 h, fertility of the capacitated spermatozoa declines [3]. Furthermore, no spermatozoa can penetrate the zona pellucida after incubation for 16–18 h [3]. It is known that the fertility of capacitated human spermatozoa incubated at 37C may decrease within one day, although motility can be observed after 3 days [4–5]. No report has been published on maintaining the fertility of capacitated spermatozoa in other mammals including mice.

Long-term storage of viable spermatozoa at refrigerator or room temperature in IVF or culture medium has implications for transporting

spermatozoa to distant laboratories plus a tremendous advantage for large scale production with unique spermatozoa such as transgenic or mutant mice. In this study, we examined how long capacitated mouse spermatozoa could retain fertility under various conditions *in vitro*.

Materials and Methods

Mature female and male mice of the Jcl: ICR strain (CLEA, Japan) were used in this experiment. Methods for IVF in this study were the same as described by Toyoda *et al.* [6]. TYH medium [7] for IVF and storage of the spermatozoa and Whitten's medium [8] supplemented with 100 μ M EDTA [9–11] for culture of fertilized eggs were equilibrated under paraffin oil (Art 7162, Merck) at 37C in 5% CO₂ in air overnight. IVF and embryo culture were performed at 37C in 5% CO₂ in air.

Females were superovulated by injection with 5 i.u. of PMSG (Serotropin, Teikokuzoki, Japan)

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and 5 i.u. of hCG (Puberogen, Sankyo, Japan) 48 h apart. Their oocytes were obtained 15–16 h after hCG injection. Spermatozoa were collected from the cauda epididymis and suspended in 400 μ l of TYH medium covered with paraffin oil. After incubation for 10 min at 37°C in 5% CO₂ in air, the sperm suspension was divided into 3 aliquots, 100 μ l each, in 3 dishes (3001, Falcon) covered with paraffin oil equilibrated at 37°C in 5% CO₂ in air. Capacitation of spermatozoa was induced by preincubation for 1–1.5 h at 37°C in 5% CO₂ in air [2]. The first IVF was performed by adding a small volume of the preincubated sperm suspension to the medium containing eggs. The final concentration of spermatozoa was adjusted to 150 cells/ μ l.

After the first IVF, the preincubated sperm suspensions were stored for 24 to 72 h under the following conditions: 4°C in air, 24°C in air, 24°C in 5% CO₂ in air, or 37°C in 5% CO₂ in air. In the second IVF, the stored spermatozoa were inseminated at a final concentration of 150 or 750 cells/ μ l after an additional incubation at 37°C in 5% CO₂ in air for 1–1.5 h. Calculation of sperm numbers was performed using a sperm counting chamber (Kitazato, Japan).

Six hours after insemination the oocytes were observed, and those with second polar bodies and both male and female pronuclei were recognized as fertilized eggs. Fertilized eggs were cultured in Whitten's medium supplemented with 100 μ M EDTA after washing twice.

Some fertilized eggs at the pronuclear stage were transferred into the oviduct of pseudopregnant recipients to assess their developmental ability after implantation. Pseudopregnancy was induced by mating with sterility proven, vasectomized

males. After anesthetizing the recipient with 50 mg/kg body weight of sodium pentobarbital (Nembutal, Abbot Laboratories) and exposing the ovary through dorsal incision, 6 fertilized eggs were introduced into each oviduct via the fimbria using a fine pipette that was made of a disposable micropipette (100 μ l, Drummond USA) and attached to a mouth piece. The narrow part of the pipette was about 3 cm in length and 100–200 μ m in internal diameter. The pipette was first filled with medium by capillary action. A small amount of air was taken up, then medium, and then a second air bubble. This manipulation was carried out several times over. Finally, the fertilized eggs were drawn up in a minimal volume of medium, followed by a last air bubble. The ovary, oviduct, and proximal end of the uterus were held outside the body by means of a serafine clip attached to the fat pad above the ovary. The bursa, which surrounded an ovary and an oviduct, was torn with two watchmaker's forceps. An edge of the bursa near the infundibulum was picked up with fine forceps, and then the pipette containing the eggs was inserted down the opening to the ampulla and blown until both air bubbles 2 and 3 entered the ampulla. Embryo transfer was carried out as quickly and gently as possible.

The data were analyzed statistically by the chi square test.

Results and Discussion

The results of the first and second IVF are shown in Table 1. More than 80% of oocytes inseminated with the preincubated spermatozoa

Table 1. Results of *in vitro* fertilization using capacitated spermatozoa stored for 24 h

Storage conditions	No. of eggs fertilized/no. of eggs examined (%)		
	Before storage	After storage	
	150 ¹⁾	150 ¹⁾	750 ¹⁾
37 C, 5% CO ₂ in air	162/174(93) ^a	0/169(0) ^b	1/201(1) ^b
24 C, 5% CO ₂ in air	88/110(80) ^a	43/94(46) ^a	51/77(66) ^a
24 C in air	628/696(90) ^a	222/593(37) ^a	252/399(63) ^a
4 C in air	163/186(88) ^a	7/173(5) ^b	0/156(0) ^b

1) Final sperm concentration at insemination (cells/ μ l). Capacitation was induced by preincubation for 1–1.5 h at 37°C, 5% CO₂ in air. Values with different superscripts are significantly different in the same column at P<0.05.

were fertilized in the first IVF. This result indicated that the majority of spermatozoa obtained capacitation after preincubation for 1–1.5 h at 37C in 5% CO₂ in air. In the second IVF, no oocytes were fertilized after insemination with the preincubated spermatozoa stored for 24 h at 37C in 5% CO₂ in air. When the oocytes were inseminated with 5 times the volume of the spermatozoa, 750 cells/ μ l, only one out of 201 eggs was fertilized. These results confirmed the previous observation of Toyoda [3]. After storage under this condition, the spermatozoa showed poor motility. Consumption of ATP might cause this decline in motility.

Fertilization rates of oocytes inseminated with preincubated spermatozoa stored at 24C in 5% CO₂ in air at a final concentration of 150 and 750 cells/ μ l were 46% (43/94) and 66% (51/77), respectively. When preincubated spermatozoa were stored at 24C in air, 37% (222/593) and 63% (252/399) of the oocytes were fertilized by spermatozoa at final concentrations of 150 and 750 cells/ μ l, respectively. Media color changes indicated an increase in pH during incubation at 24C in air. These results indicated that different

oxygen concentrations and/or pH did not influence the fertility of these spermatozoa. Under both 24 C conditions, an increase in the concentration of spermatozoa at insemination showed significantly higher fertilization rates. Thus, the number of spermatozoa capable of fertilization may be decreased by these storage conditions.

The fertilization rate of oocytes inseminated with spermatozoa stored at 4C in air was 5% (7/137). Although a marked reduction in motility was reported in refrigerated human spermatozoa, some of the apparently "immotile" spermatozoa became motile again after they were warmed to 37C [4]. However, it has not been examined whether capacitated human spermatozoa become motile again. Exposure of capacitated mouse spermatozoa to refrigerator temperatures caused a significant and irreversible decrease in viability (cold shock). No spermatozoa became motile again before or after warming at 37C in 5% CO₂ in air in this experiment. It has been concluded that cold shock damage is associated with lipid phase transitions in cell membranes [12].

Because the range of fertilization rates was

Table 2. Effect of sperm concentration on the fertility of capacitated spermatozoa stored for 24 h at 24 C in air

Sperm concentration at storage ($\times 10^4/\mu$ l)	No. of eggs fertilized/no. of eggs examined (%)		
	Before storage	After storage	
	150 ¹⁾	150 ¹⁾	750 ¹⁾
1.3	38/38(100)	0/39(0)	10/30(33)
1.4	77/81(95)	18/87(21)	50/58(86)
1.5	50/54(93)	2/36(6)	—
1.6	31/32(97)	4/84(4)	—
1.7	33/35(94)	13/20(65)	13/40(33)
1.9	48/58(83)	0/50(0)	3/35(9)
2.0	47/48(98)	8/52(15)	30/51(59)
Subtotal	324/346(94)	44/368(12)	106/214(50)
2.4	67/73(92)	46/54(85)	54/59(92)
2.5	115/139(83)	36/37(97)	—
2.7	58/59(98)	15/45(33)	23/41(56)
3.4	19/20(95)	36/41(88)	16/31(52)
4.1	45/59(76)	45/48(94)	53/54(98)
Subtotal	304/350(87)	178/225(79)	146/185(79)
Total	628/696(90)	222/593(37)	252/399(63)

1) Final sperm concentration at insemination (cells/ μ l).

Capacitation was induced by preincubation for 1–1.5 h at 37 C, 5% CO₂ in air.

0–98% after storage for 24 h at 24°C in air, we analyzed the effect of storing low and high concentrations of the sperm suspensions. (Table 2). When the spermatozoa were stored at a low concentration, below 2×10^4 cells/ μ l, the mean fertilization rates were 12% and 50% at a final concentration of 150 and 750 cells/ μ l, respectively. On the other hand, a significantly higher rate was observed when the spermatozoa were stored at high concentrations, over 2×10^4 cells/ μ l. When the spermatozoa were inseminated at final concentrations of 150 and 750 cells/ μ l, both of the mean fertilization rates were 79%. After high concentration storage at 24°C in air for 24 h in IVF media, the fertility of capacitated mouse spermatozoa was significantly higher than that of low concentration

Fig. 1. Result of *in vitro* fertilization using capacitated spermatozoa stored at 24°C in air.

Table 4. Results of embryo transfer of fertilized eggs inseminated with capacitated spermatozoa stored for 24 h at 24°C in air

No. of eggs transferred	No. of pregnant/ no. of recipients	No. (%) of live young
24	2/2	17(71)

The eggs were transferred into the oviducts of pseudopregnant recipients.

spermatozoa in the majority of cases. Capacitated spermatozoa should be stored at a high concentration in this condition.

Maintenance of fertility after prolonged storage at 24°C in air is shown in Fig. 1. The fertility of capacitated spermatozoa declined gradually; the spermatozoa could fertilize eggs after storage for 2 days.

As shown in Table 3, preimplantation development of the fertilized eggs showed good results. The high rate of development into blastocysts suggests that polyspermic fertilization did not occur, even with 750 sperm/ μ l final concentration.

Finally, to confirm the developmental ability of the fertilized eggs that were inseminated with the preincubated spermatozoa stored for 24 h at 24°C in air, the eggs were transferred into the oviduct of pseudopregnant recipients. Seventeen out of 24 fertilized eggs developed into newborns after embryo transfer (Table 4). These results indicate that when capacitated spermatozoa stored for 24 h at 24°C are used to fertilize ova, normal pre- and post-implantation embryonic development occurs.

Table 3. Preimplantation development of fertilized eggs inseminated with capacitated spermatozoa stored for 24 h

Storage conditions	No. of eggs developed to blastocyst ¹⁾ /no. of eggs cultured (%)		
	Before storage		After storage
	150 ²⁾	150 ²⁾	750 ²⁾
37°C, 5% CO ₂ in air	54/56(96) ^a	—	0/1(0) ^b
24°C, 5% CO ₂ in air	74/79(94) ^a	40/45(89) ^a	37/45(82) ^a
24°C in air	292/316(92) ^a	131/157(83) ^a	165/200(83) ^a
4°C in air	53/57(93) ^a	0/7(0) ^b	—

1) 96 h after insemination.

2) Final sperm concentration at insemination (cells/ μ l).

Capacitation was induced by preincubation for 1–1.5 h at 37°C, 5% CO₂ in air.

Values with different superscripts are significantly different in the same column at $P < 0.05$.

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