ANIMAL EXPERIMENTATION

Effects of Preservation of Mouse Spermatozoa in Electrolyte-Free Solution at 4°C on the Outcome of Mouse In Vitro Fertilization

SONG QUAN,¹ SHUJI YAMANO,^{1,2} HISAYO NAKASAKA,¹ KENJI HINOKIO,¹ KOJI NAGAGAWA,¹ and TOSHIHIRO AONO¹

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Purpose: The aim was to assess the fertilizing capacity of spermatozoa cool-preserved in electrolyte-free (EF) solution.

Methods: Mouse spermatozoa were cool-preserved in EF solution and the acrosomal status of the spermatozoa was compared before and after preservation using chlortetracycline stain. Mouse oocytes were inseminated by spermatozoa cool-preserved in EF solution for 2, 4, or 7 days and fertilization and blastocyst rates were evaluated.

Results: Acrosomal status of spermatozoa cool-preserved in EF solution was not different from spermatozoa before preservation, but the capacitated and acrosome-reacted spermatozoa significantly increased after reinitiation. Cool-preservation in EF solution for up to 4 days did not affect fertilization rate. Blastocyst rate of embryos derived from spermatozoa cool-preserved for 4 or 7 days in EF solution was significantly lower than that of embryos derived from fresh spermatozoa.

Conclusions: Mouse spermatozoa cool-preserved in EF solution possesses as much fertilizing capacity as fresh spermatozoa. However, prolonged preservation affects the embryonic development.

KEY WORDS: Acrosome reaction; capacitation; cool preservation; electrolyte-free solution; spermatozoa.

INTRODUCTION

Cooling has been reported to induce capacitation-like changes in mouse and boar spermatozoa (1, 2). When semen was stored at 5°C for 24 or 48 hr in TEST-yolk buffer, the spermatozoa showed higher motility and were associated with high fertilization rate compared with fresh spermatozoa in artificial insemination of husband and in vitro fertilization-embryo transfer (IVF-ET) (3–5). Cool-preservation in TEST-yolk buffer might induce capacitation and acrosome reaction, resulting in a high fertilization rate. However, a recent report suggested that capacitation is related to destabilization of sperm cell membrane and leads sperm to cell death (6). Indeed, a sharp and significant decrease in sperm motility was noted in human spermatozoa placed in a refrigerator for 3 days (7). In contrast, more than 40% human spermatozoa could survive at 4°C in electrolyte-free (EF) solution for 2 weeks (8, 9). Viability and function of human spermatozoa cool-preserved in EF solution were superior to those of spermatozoa preserved in NaCl solution, KCl solution, or Tris (TEST)-yolk buffer for 2 weeks (10). These facts suggest that cooling may not affect acrosomal status of spermatozoa preserved in EF solution. If spermatozoa cool-preserved in EF solution has the ability to capacitate, undergo acrosome reaction, and fertilize oocytes and embryos derived from the spermatozoa have normal developing potential, then cool-preservation of human spermatozoa may be adapted for clinical use.

In this article, whether cool-preserved spermatozoa in EF solution had normal fertilizing capacity and

¹ Department of Obstetrics and Gynecology, School of Medicine, the University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan.

² To whom correspondence should be addressed.

embryos derived from the spermatozoa had normal developing potential were investigated.

MATERIALS AND METHODS

Source and Preparations of Semen

B6C3F1 hybrid male mice of 6–10 weeks old were killed to obtain epididymal spermatozoa from cauda epididymitis. Sperm pellets from cauda epididymitis were suspended in 0.6 ml of human tubal fluid (HTF) medium with 0.4% bovine serum albumin (BSA) (Sigma Chemical Co, St. Louis, MO). The sperm suspension was divided into three aliquots. The first aliquot of the suspension was stored in a CO₂ incubator at 37°C. The second aliquot of the suspension was prepared depending on the protocol described by Saito et al. (8), i.e., the suspension was laid on the EF three-layer discontinuous Percoll gradient solutions such as 1 ml of 66% (vol/vol) Percoll (Sigma Chemical Co, St. Louis, MO) diluted by 0.33 M glucose with 0.5% BSA, 4 ml of 44 % (vol/vol) Percoll diluted by 0.33 M glucose with 1.5% BSA, and 3 ml of 22% (vol/vol) Percoll diluted by 0.33 M glucose with 2.5% BSA, and were centrifuged at $400 \times g$ for 20 min. After centrifugation, spermatozoa resuspended in 2 ml of 0.33 M glucose solution containing 3% BSA solution (EF solution) and were centrifuged again at $400 \times g$ for 5 min. The sperm pellet was suspended again with 0.2 ml of EF solution and the spermatozoa in EF solution was stored in a refrigerator (4°C) for up to 14 days. The third aliquot of the sperm suspension was centrifuged at $400 \times g$ for 5 min and suspended in 0.2 ml modified HTF (mHTF) (Sigma Chemical Co, St. Louis, MO) solution with 0.4% BSA and stored at 4°C.

Reinitiation Procedure of Cool-Preserved Spermatozoa

Spermatozoa cool-preserved in EF or mHTF solution for 2, 4, 7, and 14 days were washed with 2 ml HTF containing 0.4% BSA and centrifuged at $400 \times g$ for 5 min. After centrifugation, spermatozoa were resuspended in 0.2 ml HTF with 0.4% BSA and incubated at 37°C under 5% CO₂ in air for 1 hr (reinitiation). Percent motility and percent progressive motility of spermatozoa stored in HTF solution at 37°C under 5% CO₂ in air, and spermatozoa cool-preserved in mHTF or EF solution after reinitiation were assessed at the 2nd, 4th, 7th, and the 14th day of preservation

according to World Health Organization's (WHO) standards (11). Here, progressive spermatozoa were defined as the sum of rapid progressive motile spermatozoa and slow or sluggish progressive motile spermatozoa.

Chlortetracycline Staining of Spermatozoa

Spermatozoa cool-preserved in mHTF or EF solution for 2 days were stained by chlortetracycline (CTC) (Sigma Chemical Co, St. Louis, MO) as follows. Forty-five microliter of 750 μ mole CTC solution (130 M NaCl/liter, 5 M cysteine, 20 M Tris-HCL, final pH 7.8) was mixed with an equal volume of sperm suspension and spermatozoa were then fixed by adding 8 μ l of 12.5% (wt/vol) paraformaldehyde (Wako Pure Chemical Industries, Osaka) in 0.5 M Tris-HCl buffer (pH 7.4). After mixing well, 10 μ l of fixed sperm suspension was put on a clean slide with 8 μ l of antifading agent-JNA medium (12). The slide was covered with a coverslip and was sealed with colorless nail varnish. In each sample at least 100 spermatozoa were observed under Olympus BHS microscope equipped with epifluorescence optics (IMT-2-31, Olympus Diaphot, Tokyo) in which the Hg excitation beam was passed through a 405-nm band pass filter and CTC fluorescence emission was observed through an RKP 455 beam-splitting mirror. According to criteria described by Fraser et al. (13) spermatozoa was classified as follows: "F" pattern—uncapacitated; "B" pattern—capacitated (acrosome-intact); "AR" pattern—acrosome-reacted.

Mouse In Vitro Fertilization: Source of Oocytes

B6C3F1 hybrid female mice of 6–10 weeks old (Charles River Laboratory, Yokohama) were induced to superovulation by intraperitoneal injections of 10 IU pregnant mare's serum gonadotropin (PMSG) (Biogenesis, Poole) and 10 IU human chorionic gonadotropin (hCG) (Mochida Pharmaceutical Co, Tokyo) given in turn 48 hr apart. The mice were dissected 14–16 hr after the hCG injection and oocytes were retrieved from their oviducts.

Fifteen to twenty mature oocytes were transferred to a spot containing 100 μ l human tubal fluid (HTF) with 0.4% BSA under liquid paraffin (Wako Pure Chemical Industries, Osaka). Insemination was performed by adding fresh spermatozoa (the control group) or spermatozoa cool-preserved in EF solution spermatozoa for 2, 4, or 7 days into a spot to adjust final concentration of motile sperm approximately 5

$\times 10^6/\text{ml}$ in each group, and the mixture was incubated at 37°C under 5% CO_2 in air for 5–6 hr. After incubation, oocytes were washed and transferred into new spots of $100\ \mu\text{l}$ HTF with 0.4% BSA and were incubated at 37°C under 5% CO_2 in air for 90–91 hr. During incubation, medium was changed every day. Fertilization rate was calculated as follows: fertilization rate = number of 2-cell embryos/number of oocytes inseminated $\times 100$. Developmental stages of mouse embryos also were assessed and recorded 24, 48, 72, and 96 hr after the insemination.

Statistical Analysis

Data were analyzed using Student's *t*-test and χ^2 test. A *P* value of <0.05 was regarded as significant.

RESULTS

Percent Motility and Percent Progressive Motility

Percent motility and percent progressive motility of spermatozoa stored in HTF with 0.4% BSA at 37°C in 5% CO_2 in air quickly decreased in 24 hr and the spermatozoa lost their motility in 48 hr. In five replicate experiments, spermatozoa cool-preserved in EF solution exhibited significantly higher motility and progressive motility than those of spermatozoa cool-preserved in mHTF ($P < 0.01$) (Fig. 1 and Fig. 2).

Fig. 1. Changes in percent motility of mouse spermatozoa preserved under various conditions. Mouse epididymal spermatozoa were cultured in HTF with 0.4% BSA at 37°C under 5% CO_2 in air or preserved in mHTF or EF solution at 4°C within 2 weeks. Percent motility of spermatozoa preserved in mHTF or EF solution was examined after reinitiation. Data represent means $\pm$ SD; * $P < 0.01$.

Fig. 2. Changes in percent progressive motility of mouse spermatozoa preserved under various conditions. Mouse epididymal spermatozoa were cultured in HTF with 0.4% BSA at 37°C under 5% CO_2 in air or preserved in mHTF or EF solution at 4°C within 2 weeks. Percent progressive motility of spermatozoa preserved in mHTF or EF solution was examined after reinitiation. Data represent means $\pm$ SD; * $P < 0.05$; ** $P < 0.01$.

Capacitation and Acrosome Reaction State by CTC Stain

In spermatozoa cool-preserved in mHTF, the percentage of spermatozoa showing “F” pattern significantly decreased and the percentage of spermatozoa showing “B” and “AR” pattern significantly increased compared to those of spermatozoa before preservation (“F” pattern: 45.2 ± 5.8 vs. $88.0 \pm 3.5\%$; “B” pattern: 32.4 ± 5.7 vs. $9.2 \pm 3.0\%$; “AR” pattern: 22.4 ± 3.4 vs. $2.8 \pm 1.1\%$, respectively, $P < 0.01$). However, the percentage of “F”, “B”, and “AR” patterns in spermatozoa cool-preserved in EF solution before reinitiation were not different from those in spermatozoa before preservation (“F” pattern: 88.0 ± 3.5 vs. $82.8 \pm 8.4\%$; “B” pattern: 9.2 ± 3.0 vs. $11.0 \pm 5.2\%$; “AR” pattern: 2.8 ± 1.1 vs. $6.2 \pm 3.6\%$, respectively, $P > 0.05$). Spermatozoa preserved in EF solution exhibited significantly higher percentages of “B” and “AR” patterns after reinitiation than those before reinitiation ($P < 0.01$) (Fig. 3 and Fig. 4).

Fertilizing and Developing Ability

Spermatozoa cool-preserved in EF solution for 2 or 4 days maintained as normal a fertilizing ability as fresh spermatozoa. However, when oocytes were inseminated by spermatozoa cool-preserved in EF solution for 7 days, the fertilization rate significantly decreased compared to that of the control group ($P <$

Fig. 3. CTC fluorescent staining patterns of original spermatozoa (HTF group), spermatozoa preserved in mHTF (mHTF group) or EF solution (EF solution group) at 4°C for 2 days. Data are presented as means $\pm$ SD; $n = 5$ replicates; * $P < 0.01$; the mHTF group vs. the HTF group and the EF solution group.

0.01). The blastocyst rates of embryos derived from spermatozoa cool-preserved in EF solution for 4 or 7 days were significantly lower than that of the control group ($P < 0.05$, $P < 0.01$, respectively), although the blastocyst rate of embryos derived from spermatozoa cool-preserved in EF solution for 2 days was comparable with that of the control group (Table I).

DISCUSSION

The effect of cooling on capacitation and acrosome reaction status of mouse spermatozoa preserved in EF

Fig. 4. CTC fluorescent staining patterns of mouse spermatozoa preserved in EF solution at 4°C for 2 days before and after reinitiation. Data are presented as means $\pm$ SD, $n = 5$ replicates; * $P < 0.01$; after reinitiation compared with before reinitiation.

solution using fluorescent probe CTC assay was investigated. Cooling did not increase the percentages of "B" and "AR" patterns in spermatozoa preserved in EF solution. However, the percentages of "B" and "AR" patterns in spermatozoa cool-preserved in EF solution significantly increased after reinitiation. These results showed that spermatozoa cool-preserved in EF solution could restore capacitation as well as acrosome reaction. Recently, cooling has been reported to induce capacitation-like changes of spermatozoa in mouse and boar (1, 2). It is well accepted that some noncovalent proteins are lost during capacitation and some structural proteins and intramembranous particles are modified (14, 15). Fuller and Whittingham (1) suggested that similar changes are brought about during cooling and warming. Furthermore, Watson (16) suggested that the cooling process induced unstable membrane, thus bypassing the usual mechanisms of capacitation. However, our results clearly showed that cooling alone did not induce capacitation-like changes when mouse spermatozoa were preserved at 4°C in EF solution. EF solution does not contain millimolar level of calcium, although BSA added in the EF solution might contain a little calcium. Fraser et al. (17, 18) reported that the minimum amount of calcium was needed for capacitation. Harrison (6) reported that bicarbonate that was absent in EF solution induced destabilization of sperm membrane and was essential for successful fertilization. Capacitation-like changes induced by cooling may require the presence of calcium and/or bicarbonate.

Fuller and Whittingham (1) reported that fertilization, implantation, and fetal development were not affected by use of sperm cooled slowly or rapidly to 4°C in phosphate-buffered saline. Spermatozoa preserved in EF solution at 4°C for 2 or 4 days could sustain normal fertilizing capacity similar to fresh spermatozoa capacitated in vitro. More than one third of embryos derived from spermatozoa cool-preserved in EF solution for 2 or 4 days developed to blastocyst stage. However, blastocyst rates of embryos derived from spermatozoa cool-preserved for 4 days were significantly lower than that of the control group. It is considered that cool-preservation of spermatozoa in EF solution has limitations on preservation period. Prolonging cool-preservation time might cause some occult injuries of spermatozoa. The mechanism on losing the developmental capacity of embryos derived from spermatozoa cool-preserved in EF solution remains to be clarified. Further studies are needed before application of cool-preservation in EF solution for assisted reproductive technology.

Table I. Effects of Cool-Preservation of Mouse Spermatozoa in Electrolyte-Free Solution on the Fertilization of Oocytes and Development of Embryos

Preservation period (days)	No. of inseminated oocytes	No. of 2-cell embryos (% of inseminated oocytes)	No. of 8-cell to morulae (% of 2-cell embryos)	No. of blastocysts (% of 2-cell embryos)
0	225	179 (79.6) ^a	124 (69.3)	87 (48.6)
2	252	199 (79.0)	130 (65.3)	86 (43.2)
4	238	198 (83.2)	119 (60.1)	76 (38.4*)
7	239	125 (52.3***)	25 (20.0**)	4 (0.3**)

^a Values in parentheses are percentages.

* Four days vs. control: $P < 0.05$.

** Seven days vs. control: $P < 0.0001$.

*** Seven days vs. control: $P < 0.05$.

We conclude that spermatozoa preserved in EF solution have the ability to capacitate and undergo acrosome reaction, and spermatozoa cool-preserved in EF solution for 2 or 4 days possesses fertilizing capability virtually the same as fresh spermatozoa. However, it has adverse effects on the developmental capacity of mouse, if the preservation period is longer.

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