

Cryopreservation of a Small Number of Fresh Human Testicular Spermatozoa and Testicular Spermatozoa Cultured In Vitro for 3 Days in an Empty Zona Pellucida

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ABSTRACT: It is known that the motility of human testicular sperm can be improved when they are cultured in vitro for a few days. The purpose of this study was to determine whether it is better to freeze human testicular spermatozoa on the day of biopsy (fresh) or after they were cultured for 3 days. A modified, single-sperm freezing technique was used in this study. The study consisted of two parts: (1) ejaculated spermatozoa were used to examine the influence of different concentrations of glycerol and synthetic serum substitute (SSS) on the survival rate after cryopreservation, and (2) the survival rates between cryopreserved fresh testicular spermatozoa (Group 1) and testicular spermatozoa that were cultured for 3 days before freezing (Group 2) were compared. Empty zonae pellucidae were obtained from mouse eggs. Five to 10 motile spermatozoa were selected and injected into an empty zona pellucida. For freezing, the zona pellucida with spermatozoa was transferred into a HEPES-buff-

ered human tubal fluid containing different concentrations of glycerol and kept at room temperature for 10 to 15 minutes, and then loaded into a 0.25-ml-plastic straw. The straws were exposed to liquid nitrogen vapor for 2 hours and then plunged into liquid nitrogen. For thawing, the straws were taken out of liquid nitrogen and placed into a 37°C waterbath for 25 to 30 seconds. There was no statistically significant difference in survival rates between 3% and 10% SSS with different glycerol concentrations. There was no statistically significant difference in the survival rates of spermatozoa between Group 1 and Group 2 after cryopreservation. It appears that in vitro culture of testicular spermatozoa before freezing does not increase survival rate.

Key words: Single-sperm freezing, spermatozoa cryopreservation. *J Androl* 2000;21:409–413

Cryopreservation of spermatozoa is routinely used in the assisted reproductive laboratory. Different cryopreservation methods have been shown to provide enough good-quality spermatozoa for in vitro insemination or intracytoplasmic sperm injection (ICSI) (Cohen et al, 1981; Weidel and Prins, 1987; Morales et al, 1991; Perez-Sanchez et al, 1994; Nagy et al, 1995; Grizard et al, 1999; Yogev et al, 1999). Because the use of frozen and then thawed human spermatozoa can yield similar fertilization and pregnancy rates, ICSI with frozen-thawed spermatozoa has also become a routine clinical procedure for the treatment of infertile patients with azoospermia (Romero et al, 1996; Gil-Salom et al, 1996; Fischer et al, 1996; Liu et al, 1997a; Friedler et al, 1997; Oates et al, 1997; Dohle et al, 1998; Madgar et al, 1998; Prins et al., 1999). Obviously, the main advantage of cryopreservation of testicular spermatozoa is to avoid repeated testicular biopsy for patients who need more treatment cycles. Motility

changes in human spermatozoa have previously been noticed after they were cultured in vitro for a few days (Liu et al, 1996, 1997a; Edirisinghe et al, 1996). To determine whether the spermatozoon survival rate can be increased by cryopreservation, spermatozoa were first cultured for a few days, a modified, single-sperm freezing procedure described by Cohen et al (1997) was used in this study. By using this method, it is possible to precisely examine the viability of spermatozoa after cryopreservation. The aim of this study was to compare survival rates after cryopreservation between fresh testicular spermatozoa and testicular spermatozoa that were first cultured for 3 days.

Materials and Methods

Prior to cryopreservation of testicular spermatozoa, one study, using the same cryopreservation method as for testicular spermatozoa, was carried out with ejaculated spermatozoa to examine the influence of different concentrations of glycerol and synthetic serum substitute (SSS) on the survival rate of spermatozoa.

Preparation of Spermatozoa

Ejaculated semen was obtained from 4 patients who were evaluated for infertility and underwent semen analysis in our center. Their semen had normal characteristics according to the World

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Health Organization criteria (WHO, 1992). Semen was washed twice in HEPES-buffered human tubal fluid medium (H-HTF) (Irvine Scientific, Santa Ana, Calif) containing 3% synthetic serum substitute (SSS; Irvine). Motile spermatozoa were obtained after the swim-up procedure. The sperm concentration was $13 \pm 4.6 \times 10^6/\text{mL}$ (mean \pm standard deviation) after swim-up. Motile spermatozoa were then randomly picked up, injected into empty zonae, and frozen in different groups.

Spermatozoa obtained from patients who underwent testicular biopsies for ICSI were used in this study. The preparation of spermatozoa was similar to that reported by Liu et al (1995). Briefly, fresh human testicular tissue was surgically obtained from patients with obstructive or nonobstructive azoospermia. The testicular tissue was placed in a Petri dish containing about 5 mL H-HTF, then shredded with microscopic glass slides under a stereomicroscope at 40 \times magnification. The contents of the Petri dish were transferred to a 15-mL Falcon tube. Before centrifugation, the larger debris of testicular tissue was removed from the tube and the tube containing the sperm suspension was centrifuged for 5 minutes at 300 \times g. After centrifugation, the supernatant was removed and the pellet was resuspended in about 100 μL of H-HTF.

In Vitro Culture of Testicular Spermatozoa

The method of culturing sperm obtained from testicular biopsy was the same as previously described (Liu et al, 1997a). Briefly, 5 μL of sperm suspension was added to a 30- to 40- μL droplet of HTF medium containing 3% SSS under mineral oil (M-8410; Sigma Chemical Company, St Louis, Mo) and cultured in an incubator at 37°C in an atmosphere of 5% CO₂.

Preparation of Empty Zonae Pellucida

The cryopreservation of a single spermatozoan was modified from the report by Cohen et al (1997). Empty zonae pellucida were obtained from mouse eggs that had been collected from 7-week-old F₁ female mice (C57B1 \times CBA/Ca). The superovulation procedure was the same as described before (Liu et al, 1993). At 18 hours after injection of human chorionic gonadotropin, eggs were collected in H-HTF. Cumulus-enclosed eggs were incubated in H-HTF containing 0.5% hyaluronidase (Sigma, Type IV) for 3 minutes and the cumulus cells were then removed by using a glass pipette. The ooplasm and polar body of mouse eggs were removed using micromanipulation with a micropipette (10–12 μm in diameter). The empty zona pellucida was transferred into H-HTF containing glycerol for about 10 minutes before the spermatozoa were injected.

Injection of Spermatozoa Into Empty Zona Pellucida and Cryopreservation Procedure

Single motile spermatozoans were selected and injected into an empty zona pellucida. Five to 10 spermatozoa were injected into each zona pellucida, which was then transferred into H-HTF that contained different concentrations of glycerol and kept at room temperature for 10 minutes. The zona pellucida with spermatozoa was then loaded into a 0.25-mL plastic straw. The opening of the straw was sealed using heated forceps. The straws were exposed to liquid nitrogen vapor for 2 hours and then plunged into liquid nitrogen.

To thaw, the straws were taken out of liquid nitrogen and placed into a 37°C waterbath for 25 to 30 seconds, and the contents of the straw was transferred into H-HTF. The number of motile spermatozoa was recorded.

To cryopreserve spermatozoa obtained from testicular biopsy, the freezing and thawing procedures were the same for ejaculated semen except that an H-HTF medium containing 8% glycerol and 3% SSS was used. For each patient, part of the fresh testicular spermatozoa was frozen about 2 hours after biopsy (Group 1). The other part was cultured for 3 days and then cryopreserved (Group 2). The viability of nonmotile spermatozoa was examined using the Eosin Y staining method (WHO, 1992). Briefly, after thawing, the number of motile spermatozoa was first counted. Zona pellucida with spermatozoa were then placed in an Eosin Y solution (0.5% wt/vol) for 1 to 2 minutes, then rinsed in droplets of H-HTF. The spermatozoa were evaluated under an inverted microscope (Nikon Diaphot, Tokyo, Japan) at 400 \times magnification. Unstained spermatozoa were considered live. Stained spermatozoa (red) were considered dead. Motile spermatozoa and nonmotile but viable spermatozoa were considered as having survived after cryopreservation.

Statistical Analysis

A Chi-square test was used to compare the survival rates between different concentrations of glycerol and SSS, and between Group 1 and Group 2 of testicular spermatozoa. Probabilities of less than 5% were considered significant.

Results

When spermatozoa were cryopreserved in 6%, 8%, 10%, 12%, and 14% glycerol in H-HTF containing 3% SSS, the survival rates were 51%, 61%, 60%, 58%, and 53%, respectively. When spermatozoa were cryopreserved in 6%, 8%, 10%, 12%, and 14% glycerol in H-HTF containing 10% SSS, the survival rates were 44%, 54%, 48%, 48%, and 47%, respectively. There was no statistically significant difference in survival rates between 3% and 10% SSS with different glycerol concentrations. A few zonae pellucida were not recovered after thawing. All spermatozoa from recovered zonae pellucida remained inside (Table 1).

Spermatozoa obtained through testicular biopsy were obtained from 9 patients with azoospermia; 6 had obstructive azoospermia, 3 had nonobstructive azoospermia. Because the H-HTF containing 3% SSS and 8% glycerol exhibited a slightly higher survival rate, it was used to freeze the spermatozoa. The survival rates (motile + nonmotile viable spermatozoa) after cryopreservation were 77% in Group 1 and 74% in Group 2, showing no statistically significant difference. All frozen zonae pellucida and spermatozoa were recovered after thawing (Table 2).

Table 1. The results of the influence of different concentrations of glycerol and Synthetic Serum Substitute (SSS) on the survival rate of ejaculated spermatozoa after cryopreservation in an empty zona pellucida

	H-HTF# Containing 3% SSS and 5 Different Concentrations of Glycerol, %					H-HTF# Containing 10% SSS and 5 Different Concentrations of Glycerol, %				
	6	8	10	12	14	6	8	10	12	14
Zonae pellucididae frozen, n	20	22	22	21	20	17	19	19	18	18
Sperm frozen, n	119	132	150	130	120	107	125	124	126	120
Zonae pellucididae recovered, n	19	22	21	21	20	17	18	19	18	18
Sperm recovered, n	111	132	142	130	120	107	118	124	126	120
Motile spermatozoa after thawing, n	57 (51)*†	81 (61)*†	85 (60)*\$	76 (58)*	64 (53)*¶	47 (44)*†	64 (54)*‡	59 (48)*\$	60 (48)*	59 (47)*¶

*†‡§¶|| P > .05 by χ^2 test.
 # H-HTF indicates HEPES-buffered human tubal fluid medium.

Discussion

The results demonstrate that a similar survival rate was obtained when fresh testicular spermatozoa or testicular spermatozoa that were first cultured for 3 days were cryopreserved in an empty zona pellucida. It appears that the technique is simple, and different concentrations of glycerol can be used for cryopreservation in an empty zona pellucida. Glycerol has been used as a cryoprotectant for freezing spermatozoa since 1949 (Polge et al, 1949). It has been widely used in assisted fertilization programs to cryopreserve human semen, testicular spermatozoa, and blastocysts. Some studies have shown that glycerol should be used together with yolk or an other buffer media to cryopreserve spermatozoa (Thachil and Jewett, 1981; Jeyendran et al, 1984; Centola et al, 1992). However, there were also reports that glycerol alone gave similar results to glycerol with yolk buffer (Cohen et al, 1981; Phillip et al, 1983; Critser et al, 1988). Nevertheless, there is no question that glycerol does minimize sperm damage during the freezing and thawing process. In our study, yolk buffer was not added to the freezing medium. Glycerol only was used in this study because an empty zona will float on the surface of the yolk buffer solution, and yolk buffer affects its visibility, thus, it was difficult to find empty zonae in yolk buffer after freezing. Therefore, we do not know whether the addition of yolk buffer to the cryopreservation solution may or may not further improve the results.

As we know, survival, motility, and function of spermatozoa can be damaged by freezing. Our results also indicate that the concentration of glycerol is not critical for freezing ejaculated spermatozoa because the number of motile spermatozoa was not different in 5 different concentrations of glycerol (Table 1); thus, it is feasible to use a lower concentration of glycerol for cryopreservation. As suggested by Critser et al (1988), a lower concentration of glycerol may minimize its toxicity and improve human sperm survival rates after cryopreservation. Our results also reveal no statistically significant difference in survival rates between 3% and 10% SSS with different glycerol concentrations (Table 1). Ten percent of SSS did not provide more protection to spermatozoa against freezing; therefore, it is not necessary to use a higher concentration of SSS in the freezing medium.

It has been observed that the motility of testicular spermatozoa can be improved by culturing them in vitro for a few days (Liu et al, 1996, 1997a; Edirisinghe et al, 1996). Initially, we believed that testicular spermatozoa with better motility (after being cultured for 3 days) would be more tolerant of freezing than those that exhibited poor motility (fresh testicular spermatozoa). However, the results clearly show that there is no difference

Table 2. Results of cryopreservation of testicular spermatozoa in HEPES-buffered human tubal fluid medium containing 3% Synthetic Serum Substitute and 8% glycerol

	Cryopreservation of Testicular Spermatozoa	
	Fresh (Group 1)	Cultured 3 Days Before Freezing (Group 2)
Zonae pellucidae frozen, n	45	42
Sperm frozen, n	370	334
Zonae pellucidae recovered, n	45	42
Sperm recovered, n	370	334
Motile spermatozoa after thawing, n (%)	213 (58)*	187 (56)*
Nonmotile spermatozoa after thawing, n	157	147
Viable sperm, n (%)	71 (19)†	59 (18)†
Nonviable sperm, n (%)	86 (23)‡	88 (26)‡

* † ‡ $P > .05$. χ^2 test.

in survival rate after cryopreservation whether testicular spermatozoa are fresh or cultured for a few days. Therefore, the beneficial effect on survival rate of an in vitro culturing of testicular spermatozoa prior to cryopreservation may be limited, and culturing does not offer better protection of the sperm that are already motile. However, an in vitro culturing of spermatozoa before cryopreservation does not adversely effect survival rate. As indicated from our results, sperm survival rates showed no statistically significant differences between the 2 groups; so we may now suggest that either testicular biopsy and cryopreservation of testicular spermatozoa can be carried out on the same day of oocyte retrieval and ICSI, or that testicular biopsy and cryopreservation of spermatozoa can be carried out 3 to 4 days before retrieval of oocytes and ICSI. In the latter case, a testicular biopsy could be done a few days before an ICSI procedure; part of the sperm can be kept for in vitro culturing and used for ICSI, and the remainder can be frozen for possible later use.

Compared with the use of a laser to create a hole for single-sperm freezing reported by Montag et al (1999), the advantage of direct injection of spermatozoa into an empty zona pellucida using an injection pipette is that the loss or escape of motile spermatozoa from the zona pellucida rarely occurs. The zona pellucida containing spermatozoa can be easily recovered after thawing, and more than 50% of motile spermatozoa can be obtained after freezing and thawing. Although some of the spermatozoa became nonmotile after freezing and thawing, others were still viable. It is known that nonmotile but viable spermatozoa can still be used for ICSI without adversely affecting fertilization and pregnancy rates (Casper et al, 1996; Liu et al, 1997b; Ved et al, 1997). If the numbers of motile and nonmotile viable spermatozoa were added, approximately 75% of cryopreserved spermatozoa can be used for ICSI. Therefore, the method described here may be useful to cryopreserve sperm in patients with oligospermia and in those with nonobstructive azoospermia because sperm can be easily found after thawing by using this procedure and less time is needed to find sufficient

spermatozoa for ICSI. However, for patients with normal sperm parameters in an in vitro fertilization program, we do not believe it is necessary to use this method for cryopreservation because there will always be sufficient spermatozoa for insemination or ICSI after cryopreservation.

In conclusion, an in vitro culturing of spermatozoa before freezing does not increase survival rate.

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