Human Reproduction vol.12 no.5 pp.994–1001, 1997

Cryopreservation of single human spermatozoa

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A procedure is described that allows cryopreservation and efficient post-thaw recovery of either a single or a small group of human spermatozoa. This is achieved by injecting them into cell-free human, mouse or hamster zonae pellucidae before the addition of cryoprotectant. The method involves a combination of physical micromanipulation procedures and glycerol-mediated cryoprotection. Zonae were tracked by positioning them in straws between two small air bubbles prior to freezing. Spermatozoa from poor specimens were cryopreserved and their fertilizing ability after thawing was compared with that of fresh spermatozoa from fertile men. Human eggs used for fertilization testing were either 1 day old or in-vitro matured. Only 2% of the frozen zonae were lost and >75% of spermatozoa cryopreserved in this manner were recovered and prepared for intracytoplasmic sperm injection. The feasibility of cryopreserving a single spermatozoon was assessed. Fifteen motile spermatozoa were frozen in 15 zonae, of which 14 were recovered after thawing. Ten were injected into spare eggs, of which eight became fertilized. Spermatozoa recovered mechanically from human zonae fertilized the same proportion of oocytes as fresh fertile control spermatozoa. The recovery and fertilization rates with spermatozoa frozen in animal zonae were 87 and 78% respectively. The fertilization rate was marginally higher ($P < 0.05$) than that for spermatozoa frozen in human zonae, perhaps because the latter may have acrosome reacted more frequently. The zona pellucida appears to be an ideally suited sterile vehicle for storage of single spermatozoa.

Key words: empty zona pellucida/ICSI/micromanipulation/motility recovery/zona pellucida freezing

Introduction

The outlook and treatment for conditions involving adverse human gamete interaction have drastically changed with the advent of intracytoplasmic sperm injection (ICSI) and manipulation of enriched but often poor-quality sperm suspensions, which sometimes yield counts of <10 spermatozoa (Palermo *et al.*, 1995; Silber *et al.*, 1995a). Men who are azoospermic can now be treated using surgical isolation of spermatozoa from their testicles or reproductive tract (Palermo *et al.*, 1992; Craft *et al.*, 1993; Schoysman *et al.*, 1993; Devroey *et al.*, 1995; Silber *et al.*, 1995b). Even men with arrested spermiogenesis can be included, since it is evident that spermatids contain all the necessary elements for decondensation and complete participation at syngamy (Palermo *et al.*, 1994; Fishel *et al.*, 1996). In the near future, even less mature diploid stages such as secondary spermatocytes and possibly spermatogonia may be used for fertilization or zygote reconstitution; this seems a beneficial development, since in some men, spermatogenesis arrests early during meiosis. The in-vitro culture of spermatogonia appears to be possible hypothetically, but clinical application faces major physiological hurdles involving combinations of experimental molecular and cellular strategies that have yet to be developed.

Technical, physiological and genetic problems have already been encountered in the new field of single mature gamete manipulation. The presence of Y-deletions in some azoospermic men and their possible consequences in male offspring have recently caused the Health Council of the Netherlands (1996) to review and change regulations concerning assisted fertilization and ICSI in particular. Another important problem concerns the possible negative consequences of certain diagnostic and therapeutic extraction procedures upon testicular function (Jarow, 1990; Schlegel, 1996). Repeated surgical procedures are not only costly and invasive but, in the case of testicular sperm extraction (TESE), can cause transient and even permanent adverse physiological effects (Schlegel, 1996). Repetition of these procedures can in some cases be avoided by cryopreservation of spermatozoa, but this is only possible when sufficient numbers of functional cells are isolated (Silber *et al.*, 1995a). Although there are anecdotal reports (Patrizio *et al.*, 1995; Podsiadly *et al.*, 1996) describing sperm survival and birth of live offspring after cryopreservation of sperm-rich epididymal and testicular aspirations, conventional sperm freezing cannot work for limited numbers of spermatozoa (Hewitt *et al.*, 1985). However, it is crucial that a sperm freezing method be developed to avoid the necessity for repeated surgical attempts at sperm extraction.

The present paper describes the development of a new approach to spermatozoa cryopreservation which allows freezing and recovery of single spermatozoa. It is believed this procedure can be applied even in the most extreme cases, such as for men who have <100 spermatozoa present in the final suspension obtained for an ICSI attempt. The issue of losing spermatozoa through conventional addition and removal of cryoprotectant in relatively large volumes of media is completely circumvented by insertion of the spermatozoon into an

enclosed porous capsule which can be properly visualized and handled microscopically before and after cryopreservation. The chosen vehicle for this purpose is the zona pellucida following removal of its cellular material. The feasibility of this technique was demonstrated by inserting small groups of spermatozoa, and even single cells, into evacuated rodent and human zonae for cryopreservation and by assessing sperm recovery by fertilization of human oocytes.

Materials and methods

Equipment, culture media and tools

All micromanipulation was performed in HEPES-buffered human serum albumin-supplemented human tubal fluid (HTF; Cohen *et al.*, 1992). Micromanipulation was performed in shallow Falcon 1006 dishes using eight, 5 µl droplets surrounding a 5 µl droplet containing polyvinylpyrrolidone (PVP; Medi-Cult, Copenhagen, Denmark) according to the method of Palermo *et al.* (1992). Two different solutions of PVP were used: (i) a 10% solution for sperm capture and insertion into empty zonae and (ii) a 12% solution for sperm recovery from thawed zonae. The ICSI procedures were performed at 37°C, but all other micromanipulation was performed at room temperature in order to reduce sperm velocity. The procedures were performed under magnification of ×40 using an IX-70 inverted Olympus microscope equipped with Hoffman interference optics and connected to a 14-inch monitor. The microtools for partial zona dissection (PZD), ICSI and zona drilling were prepared as described previously (Cohen *et al.*, 1992; Palermo *et al.*, 1992, 1995). A new tool was developed specifically for extraction of cellular material from the zonae. This tool was pulled from Drummond glass using pulling parameters generally applied to ICSI needles. It was cut on a microforge to have a 15 µm diameter tip and bevelled at a 45° angle without a spike.

Eggs were incubated after ICSI using standard procedures described elsewhere (Cohen *et al.*, 1992; Palermo *et al.*, 1995). Data comparisons were made using χ^2 analysis or Fisher's exact test when applicable.

Source of gametes and embryos

All protocols were approved in 1995 by the Internal Review Board of Saint Barnabas Medical Center, USA. In all, 49 patients undergoing egg retrieval, in-vitro fertilization (IVF) and ICSI consented to donate unused gametes and embryos (discarded biological material). Spermatozoa were obtained from six ICSI patients with severe oligoasthenoteratozoospermia for individual cryopreservation. Empty zonae were obtained from seven patients, and 21 other patients consented to donate unfertilized and immature eggs. Spermatozoa obtained from 15 fertile men whose partners had female-factor-related infertility were used as controls.

Source and preparation of spermatozoa

The sperm suspensions obtained from the six ICSI patients with severe oligoasthenoteratozoospermia had volumes (<50 µl) and counts that would have normally been considered too low or borderline for conventional sperm suspension cryopreservation. Spermatozoa were isolated by centrifugation at 1800 *g* and mini-Percoll as described elsewhere (Ord *et al.*, 1990; Palermo *et al.*, 1995). The sperm suspensions were incubated at 37°C in 5% CO_2 in air prior to isolation and insertion of spermatozoa into empty zonae pellucidae for cryopreservation.

The spermatozoa obtained from men with normal semen analysis were not cryopreserved, and served as controls for thawed spermatozoa from infertile men. These fertile samples were used to determine a maximum rate of fertilization with ICSI using donated spare oocytes, in order to assess and compare the fertilization rates of individually frozen/thawed spermatozoa recovered from empty zonae.

Source and preparation of empty zonae pellucidae

Empty zonae were obtained from multiple sources: (i) pre-fertilization human immature eggs; (ii) post-fertilization ICSI embryos, with abnormal fertilization or development; (iii) pre-fertilization mouse oocytes and (iv) pre-fertilization frozen hamster oocytes.

(i) Five consenting patients provided a total of 18 eggs at the germinal vesicle or metaphase I stage for this research. The cumulus was removed using hyaluronidase and the corona stripped using fine micropipettes (Palermo *et al.*, 1992). Pilot studies had shown that two small incisions in the zonae improved extraction of the egg ooplasm and insertion of the spermatozoa into the evacuated zonae, because this prevented collapse of the zona during suction and excessive inflation when the spermatozoon was inserted (Figure 1a and b).

Holes were made chemically in only three pre-fertilization zonae by releasing acidified Tyrode's solution from a 10 µm open microneedle. Holes in all other zonae were made mechanically by PZD with a spear-shaped closed microneedle. Cytoplasm was extracted using a bevelled 15 µm micropipette (Figure 1a) that was connected in turn to a Narishige IM-6 suction device (Tokyo, Japan) made with thin glass, and equipped with a wide-bore metal plunger, without the use of O-rings. The zona was positioned so that one of the two incisions was situated at the three o'clock position. The bevelled microtool was inserted through the aperture using the sharp edge on the lower end of the bevel. The tool was moved through the oolemma, and the cytoplasm was fully aspirated until the zona was empty. The pipette was occasionally emptied outside the zonae or more medium was sucked up to remove any sticky cytoplasm from the pipette-tip.

(ii) Ten post-fertilization, sperm-free zonae were obtained from two consenting ICSI patients (Figure 1c). The embryos were either activated, digynically fertilized or abnormal. None was considered suitable for transfer or embryo cryopreservation. Incisions were made mechanically using PZD. Cells were removed using the extraction procedure described above.

(iii) Mouse eggs were obtained from superovulated 10–12 week old CB6F1 hybrid females by flushing from the oviduct. Cumulus and corona cells were removed as described elsewhere (Levron *et al.*, 1996). Mechanical PZD was performed to make two incisions. The whole contents of the egg were removed, including the oolemma and polar body, using the extraction procedure described above. As a result of suction, the zona frequently collapsed. When this occurred, the pipette was partially withdrawn with its bevelled aperture facing the zona pellucida and fresh medium was aspirated and gently blown in, to re-inflate the collapsed zona (Figure 1b).

(iv) Frozen hamster eggs were obtained from Charles River Inc. (Wilmington, MA, USA). The eggs were frozen in 1.5 M propylene glycol and mixed in a sucrose solution during thawing. The straws were thawed for 2 min in air, 3 min at 37°C in water, 2 min at room temperature and expelled into HEPES-HTF and incubated for 10 min at room temperature (Quinn *et al.*, 1985). Thawed intact eggs were washed four times in culture medium and the zonae were evacuated using a procedure similar to that described above for mouse zonae. Extraction micropipettes needed changing frequently because of the adhesive nature of the thawed cytoplasm and its response to the extraction process.

Sperm insertion into zonae and cryopreservation

All spermatozoa were released into the 10% PVP solution prior to insertion into empty zonae. If the sperm concentration and/or motility

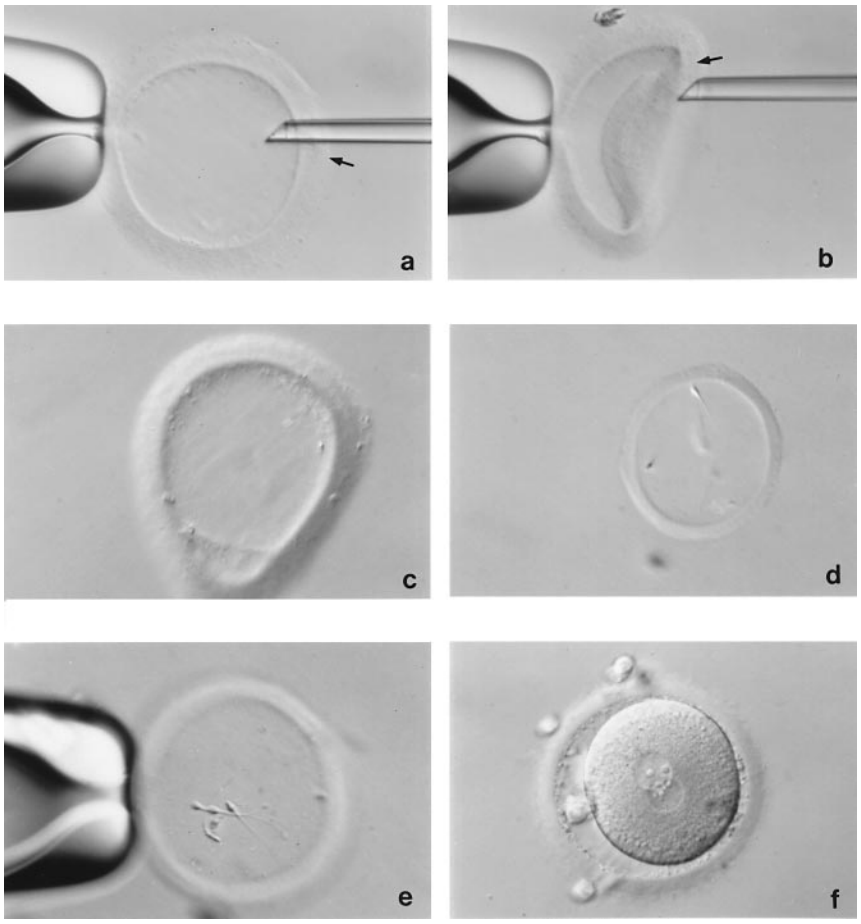

Figure 1. (**a**) Pre-fertilization human zona pellucida with two small incisions, of which one is visible (arrow). The oocyte and polar body were removed using an extraction microtool inserted through one of the incisions. The extraction microtool had a sharp bevel and a diameter of 15 μm. (**b**) Excessive suction may collapse the zona pellucida, which can be re-inflated by inserting small amounts of medium after passing the needle through the hole (arrow). Alternatively, after a few minutes, the zona will fill up spontaneously and recover its original shape. (**c**) Human post-fertilization zona from an intracytoplasmic sperm injection (ICSI) patient from which the abnormal embryo had been removed. A single spermatozoon has been inserted and is located at 3 o'clock. (**d**) Mouse zona pellucida containing two human spermatozoa. (**e**) Aggregation of motile spermatozoa after thawing of a mouse zona pellucida. Five human spermatozoa can be seen, each of which could be isolated with an ICSI needle and prepared for ICSI. Aggregation typically occurs after injection of more than three spermatozoa into the zona pellucida and inhibits motility. (**f**) Normally fertilized in-vitro-matured spare human oocyte following injection with a spermatozoon that had been singly frozen and thawed in an empty hamster zona.

permitted, 1 μl of the sperm suspension was added to the PVP; otherwise, spermatozoa were taken individually from small 2–5 μl droplets of sperm suspension using an ICSI microtool. After addition to PVP, some spermatozoa were immobilized mechanically using the ICSI microtool and then injected into eight evacuated human zonae maintained in HEPES-buffered solution. In all other experimental groups, motile spermatozoa were inserted into empty human or animal zonae (Figure 2). All zonae were maintained in HEPES-buffered HTF at room temperature. The number of spermatozoa inserted into each zona was between one and 15, but only three zonae contained more than five cells (Figure 1c, d and e). Hamster zonae were injected with single spermatozoa. Individual spermatozoa were counted twice and observed during and immediately after insertion and then a third time in order to ensure an exact count prior to cryopreservation. Sperm release from the microtool was performed slowly in order to minimize inflation of the zona. Between three and 15 zonae were used for each experiment.

Injected zonae were then placed in an 8% glycerol solution in phosphate-buffered saline (PBS) supplemented with 3% human serum albumin (Plasmanate, National Hospital Specialties, New Jersey, USA). The zonae were frozen separately in 0.25 ml sterile plastic straws (#ZA475; IMV International, Minneapolis, MN, USA) between two small air bubbles to indicate their position. One end of the straw was closed using sealant PVA powder, while the other end was heat sealed. The freezing procedure was based on a simple standard semen cryopreservation protocol in which the straws were exposed to liquid nitrogen vapour for 120 min or overnight followed by a plunge into liquid nitrogen. They were then kept in liquid nitrogen for at least 48 h. TEST-yolk buffer and other commercially available semen freezing media were not used, as it was suspected that the zonae could be lost or would float on the surface of the viscous solution.

Thawing, sperm recovery and ICSI

Straws containing zonae were thawed in a water bath at 30°C for 30 s. One end of the straw was cut with sterile scissors and a 16-gauge needle of a syringe with raised plunger was inserted. The other end was then cut and the medium expelled as far as the first bubble by gentle depression of the plunger. The straw was inserted into a droplet of medium and the cryopreservation medium containing the zona was slowly expelled until the second bubble was reached. The zona was gently washed four times and pipetted into an ICSI dish containing eight droplets of HEPES-buffered medium surrounding a

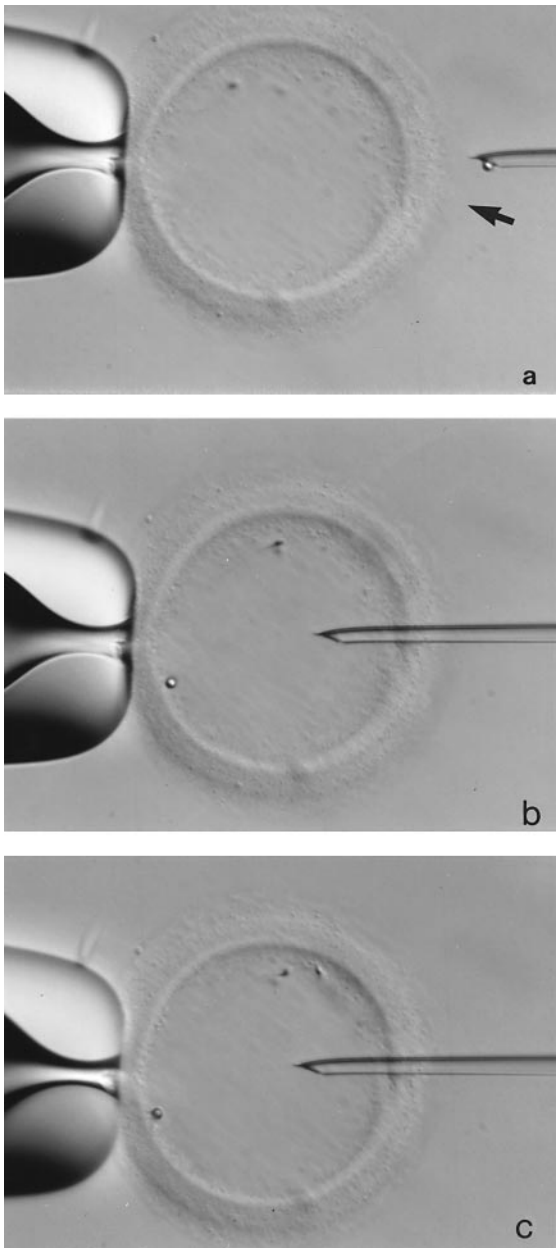

Figure 2. (**a**) Empty pre-fertilization human zona. Arrow indicates side of incision which runs between 3 and 4 o'clock. The intracytoplasmic sperm injection (ICSI) needle has a small oil bubble at the tip and is filled with two spermatozoa in 10% polyvinylpyrrolidone (PVP). (**b**) The oil bubble is at 8 o'clock after expulsion of fluid. The microtool was inserted into the zona through the incision and one spermatozoon was released slowly and is visible at 12 o'clock. (**c**) A second spermatozoon was inserted. Both spermatozoa are now between the 12 and 1 o'clock positions. Note that the needle was preloaded with 12% PVP for sperm extraction from the thawed zona. Tool insertion after thaw is by a similar procedure.

central droplet containing 12% PVP in supplemented intracellular solution.

The zona was positioned using the holding pipette and a PVP-filled ICSI microtool. Spermatozoa were counted and their motility assessed by rotating and rolling the zona. The zona was then positioned so that one of the incisions and a spermatozoon were lined up to allow penetration of the ICSI needle and aspiration of the spermatozoon (mechanical recovery method). Minimum suction was used for this process. Some spermatozoa showed considerable motility within the zona. These cells were aspirated by positioning the needle at the contra-lateral side and applying suction when the spermatozoon passed the needle aperture. All spermatozoa were removed individually and released gently into the PVP solution. Any that remained motile were immobilized using the microneedle.

The mechanical sperm recovery method was used only after two other methods proved less successful. A first attempt used an enzymatic method, in which the zonae were pipetted into a 0.1% pronase solution. An ICSI microtool was inserted into the partially digested zona mass in which the majority of spermatozoa became trapped and were difficult to remove. Some motile cells escaped from the digested zona. A second method involved the use of acidified Tyrode's solution for removal of zona material (Cohen *et al*., 1992). The reduced pH immobilized spermatozoa rapidly, and the digested zona mass again trapped the spermatozoa, which could only be removed by aggressive pipetting with the ICSI tool. Both of these methods were abandoned after use on a total of three zonae.

Recovered spermatozoa were immediately injected into 1 day old metaphase II or in-vitro-matured eggs. Each spermatozoon was released into the egg with the polar body at either the 7, 8 or 11 o'clock position, since this had led to optimal results in our earlier work using fresh eggs (Blake *et al*., 1996). Such positioning is believed to allow close proximity between the injected spermatozoon and the metaphase plate. Injected eggs were washed four times in culture medium and incubated in HTF supplemented with human serum. Control eggs were injected with fresh normal motile spermatozoa after immobilization in PVP solution. Eggs were checked after 20 h for the presence of polar bodies and pronuclei using an IX-70 (Olympus) inverted microscope with ×40 and Hoffman interference optics (Figure 1f). Cleavage of embryos after an additional culture period of 20–28 h was used to determine the success of the method.

Results

General findings and considerations

A total of 50 empty zonae were used for these experiments, of which 28 were from human oocytes or embryos; 194 spermatozoa were selected and injected into the empty zonae in groups of 1–15 for freezing (Figure 2). Only one zona (2%) from a mouse egg was not found upon thawing; consequently, the spermatozoa inside it were lost. It is not surprising that it was a mouse rather than a hamster or human zona which was lost, since both the latter have a larger mass and do not float as much as mouse zonae when pipetted. In all but three human zonae, spermatozoa were removed by inserting an ICSI microtool and applying suction (mechanical method of sperm recovery). Prior to freezing, no spermatozoa were lost through the narrow mechanical PZD incisions made in 25 human zonae. However, when acidified Tyrode's solution was used for drilling in three zonae, 21% of spermatozoa were lost ($P < 0.001$; Table I). The number of spermatozoa lost through holes after thawing ranged from 10 to 37% ($P < 0.05$). The highest loss was again found in the zonae in which larger holes had been made using acidified Tyrode's solution. Sperm loss after thawing through narrow incisions happened occasionally because of inadvertent excess suction applied through the holding pipette. This could be avoided by visualizing both holes prior to micromanipulation and sperm recovery. The rate

Table I. Individual sperm loss before and after freezing through holes in evacuated human zonae after sperm insertion

Type of hole	Source of zona	Type of sperm injected	No. of evacuated zonae	No. of sperm lost/ no. injected (pre-freeze)	No. of sperm lost/ no. frozen (post-thaw)
Low pH	oocyte	motile	3	7/34 (21%)[a,b]	10/27 (37%)[c,d]
Mechanical	oocyte	motile	4	0/20 (0%)[a]	6/20 (30%)[c]
Mechanical	embryo	motile	10	0/30 (0%)[a]	3/30 (10%)[c,d]
Mechanical	oocyte	immobilized	8	0/40 (0%)[a,b]	6/40 (15%)[c]

[a]Difference between mechanical groups combined and low pH; $P < 0.001$.
[b,d]$P < 0.05$.
[c]Difference between mechanical groups combined and low pH; $P < 0.001$.

of sperm loss through the incisions diminished markedly with increased experience.

The number of spermatozoa lost during and after thawing did not vary greatly whether mechanical or chemical methods of recovery were used (Table II). However, in the three chemically digested zonae, spermatozoa often became trapped and immobilized in the remains of the digested glycoprotein matrix. All spermatozoa (8/8) that were recovered by exposure to acidified solution were immobilized without damage, whereas 5/7 spermatozoa recovered following pronase digestion were either partially or completely damaged. Of the least motile spermatozoa, at least three heads suffered pronase digestion, presumably following exposure of susceptible surfaces following acrosome reaction. Tails that had been severely broken during immobilization were also digested by the pronase. No damage occurred when spermatozoa were recovered by the mechanical method. Both mechanical preparation of the zona pellucida by PZD and mechanical sperm insertion and recovery by suction into an ICSI needle were obviously superior to their chemical counterparts and thus were used in all further experiments (Figure 2).

Table II. Loss and damage of individual spermatozoa during thawing and recovery into a microneedle and subsequent immobilization in polyvinylpyrrolidone (PVP)

Sperm recovery procedure	Source of zona	No. of sperm lost during and after thawing	No. of sperm damaged during and after recovery[a]
Zona digestion by pronase	oocyte	3/10	5/7
Zona digestion by reduced pH	oocyte	2/10	8/8
Mechanical removal through microneedle	oocyte	6/20	0/14
Mechanical removal through microneedle	embryo	3/30	0/27

[a]Differences between mechanical and other groups combined; $P < 0.005$.

Motility recovery and general efficiency

The recovery of motility was determined by visualizing spermatozoa for at least 10 s inside the zona pellucida at ×40 immediately following thawing and cryoprotectant removal, but prior to insertion of microtools into the culture media droplets. Some spermatozoa inserted into human zonae appeared trapped within cytoplasmic remnants or crevices inside the glycoprotein matrix, but this occurred less frequently when animal zonae were used. Aggregation of spermatozoa also appeared to inhibit individual motility, but was avoided when three or fewer spermatozoa were inserted per zona (Figure 1c, d and e). Some cells that were motile, but did not show marked velocity, became progressively more motile after aspiration and exposure to medium. Motility recovery rates were defined as the percentage of spermatozoa that were motile, and varied between 65 and 100% (average 82%; see Table III). It is possible that these rates could have been higher, since some motile spermatozoa were lost through holes in the zonae.

The efficiency of the procedures was determined by attempting to isolate individual spermatozoa from the thawed zona using a microtool, placing them in PVP solution and immobilizing them as is usually performed for ICSI. The sperm recovery rate was calculated as the ratio of the number of isolated and immobilized spermatozoa after thawing to the initial number of spermatozoa inserted prior to freezing. The rates varied from 44 to 93% (average 73%). Rates of recovery >80% were obtained in experiments involving rodent or human zonae that were embryonic in origin or in which the spermatozoa were immobilized prior to freezing. Significantly ($P < 0.05$) lower rates (50%) were found in zonae from pre-fertilization human oocytes, but this could have been caused by incomplete removal of the egg contents in one of the experiments. The use of only one to three spermatozoa per zona pellucida appeared optimal. The potential of this cryopreservation method was shown by inserting single spermatozoa into 15 empty hamster zonae, from which all but one of the spermatozoa were recovered.

Fertilizing ability of individually cryopreserved spermatozoa

A total of 103 oocytes donated for research by 21 patients were available for testing the fertilizing ability of the cryopreserved spermatozoa, using ICSI. The oocytes were randomly allocated to fresh control spermatozoa from fertile men ($n = 47$) and thawed spermatozoa from infertile men ($n = 56$). Consequently, only a small proportion of the recovered spermatozoa could be tested due to the shortage of available oocytes. Of the oocytes available, 37 were in-vitro matured, and the remainder were mature 1 day old cells that had not become fertilized after routine ICSI or IVF. The fertilization rates of in-vitro-

Table III. Post-thaw motility and recovery of human spermatozoa individually inserted into and mechanically removed from human, mouse and hamster zonae pellucidae

Type of hole	Source of zona	Species	Sperm condition pre-freeze	No. of evacuated zonae	No. of individual sperm inserted	Motility recovery	Sperm recovery in microneedle[a]
Low pH	oocyte	human	motile	3	34	11/17 (65%)	15/34[b,c] (44%)
Mechanical	oocyte	human	motile	4	20	11/14 (79%)	10/20[d] (50%)
Mechanical	embryo	human	motile	10	30	24/27 (89%)	25/30[b,d] (83%)
Mechanical	oocyte	human	immobilized	8	40	NA	34/40[c,d] (85%)
Mechanical	oocyte	mouse	motile	7	35	22/30 (73%)	25/30[b,d] (83%)
Mechanical	frozen oocyte	hamster	motile	15	15	14/14 (100%)	14/15[c,d] (93%)

[a]After isolation from zona and immobilization in polyvinylpyrrolidone.
[b]$P < 0.005$.
[c]$P < 0.001$.
[d]$P < 0.05$.

Table IV. Fertilization of 1 day old unfertilized metaphase II or in-vitro-matured human eggs by intracytoplasmic sperm injection using recovered spermatozoa that had been individually frozen in evacuated human, mouse or hamster zonae pellucidae

Sperm recovery procedure	Source of zona	Zona pellucida species[a]	Sperm condition pre-freeze	No. of oocytes injected with thawed sperm	No. of eggs fertilized[a]	No. of oocytes injected with fresh sperm	No. of eggs fertilized
Chemical	oocyte	human	motile	12	3	9	4
Mechanical	oocyte	human	motile	16	8	8	5
Mechanical	oocyte	human	immobilized	10	3	9	4
Mechanical	oocyte	mouse	motile	10	7	12	6
Mechanical	frozen oocyte	hamster	motile	8	7	9	6
Total (%)				56	28 (50.0)	47	25 (53.2)

[a]$P < 0.05$ when comparing fertilization rates of all spermatozoa frozen in human zonae with those frozen in animal zonae.

matured and 1 day old oocytes were 51.3 and 51.5% respectively.

The fertilization rates for the fresh and freeze–thawed groups of spermatozoa were 53.2 and 50.0% respectively (Figure 1f). The difference was not statistically significant. Both rates were, however, significantly lower ($P < 0.001$) than the routine fertilization rate (1078/1376, 78.3%) in our ICSI programme established from 98 patients treated during the same period of 3 months, indicating the inferior quality of in-vitro-matured and 1 day old oocytes compared to fresh metaphase II oocytes. Obvious differences in fertilization rates were not found when different cryopreservation procedures were compared (Table IV). Although 3/12 spermatozoa recovered from chemically digested zonae retained their fertilizing capacity, two of these were already damaged when injected and did not have intact tails. It should also be noted that only 3/10 eggs became fertilized after injection of spermatozoa that had been immobilized prior to freezing. The formation of pronuclei in these eggs appeared to be delayed, but this observation may not be significant because of the tiny sample size. A higher proportion of spermatozoa frozen in animal zonae were able to fertilize oocytes (14/18, 78%) compared with spermatozoa frozen in human zonae (14/38, 37%), but the level of statistical significance was low ($P < 0.05$).

Discussion

Our results show that freezing and efficient post-thaw recovery of a single or a few spermatozoa is now possible if they are cryopreserved in evacuated empty human or animal zonae pellucidae. The development of the current method was the result of a limited number of pilot observations and experiments. Spermatozoa were inserted into empty zonae rather than into the perivitelline space of intact eggs or embryos, since oolemma and blastomere membranes become lysed following use of the typical sperm freezing protocols. Complex cryoprotectants typically used for sperm freezing, such as TEST-yolk buffer, were not used because empty zonae have a tendency to float in viscous solutions. Similarly, the zonae were not placed in PVP solution for sperm retrieval after thawing, since this often caused the entire matrix structure to collapse, making sperm removal very difficult.

The method does not use any enzyme, enzyme inhibitor or motility enhancer, and is based entirely on combinations of physical micromanipulation procedures, the general practice of placing embryos allocated to cryopreservation into straws, and the physics of standard semen cryoprotection. It is likely that improvements can be made as far as the freezing protocol is concerned, such as the addition of dithiothreitol (Sawetawan *et al.*, 1993) or small amounts of TEST-yolk or other freezing

buffers. Also, human zonae can be pretreated in order to minimize sperm–ZP3 binding, and consequently the acrosome reaction and motility reduction. Pretreating animal zonae is probably unnecessary, but certain enzyme inhibitors might be used to avoid non-specific interaction between spermatozoa and molecules released from lysed ova.

A solution containing 12% PVP was used rather than 8–10% since it permitted gentle and controlled retrieval of thawed spermatozoa. Zonae with two pre-drilled holes were preferred, since this enabled simultaneous release and absorption of fluids, other than those exchanged by the ICSI and extraction needles. Single spermatozoa frozen in this way did not become lost in the vial or supernatant and did not adhere to debris, plastic or glassware.

More than three quarters of the spermatozoa injected and frozen in empty rodent zonae were recovered for ICSI and retained their fertilizing ability. In the most extreme demonstration of this method, the ratio of zonae to spermatozoa was one-to-one. The high recovery rate with animal zonae is probably because of the reduced chance of binding to zona receptors.

Although the freezing method reported here was performed with spermatozoa from men with extreme oligozoospermia, average motility recovery rates were considerably higher than those generally reported for moderately abnormal semen and were comparable to results from donor semen or from semen from moderately oligozoospermic patients after treatment with a combination of Cryoseeds and dithiothreitol (Bongso *et al.*, 1993; Sawetawan *et al.*, 1993; Verheyen *et al.*, 1993). The use of the zona pellucida as a vehicle avoids the known loss of motility associated with post-thaw dilution and sperm washing seen for frozen donor semen (Verheyen *et al.*, 1993). It is possible that the presence of multiple spermatozoa in a small volume exerts an internally deleterious effect during freezing, thawing and centrifugation, which may be avoided by freezing spermatozoa singly or in very small groups (Aitken and Clarkson, 1988; Mortimer and Mortimer, 1992). The differences between the method reported here and conventional methods of freezing whole semen or sperm preparations do not extend to altered tail characteristics. A proportion of the spermatozoa frozen inside the zona pellucida exhibited tail coiling (Pedersen and Lebech, 1971), but this did not inhibit their ability to fertilize after ICSI.

This method could be used to cryopreserve any unused spermatozoa and even precursor cells, following ICSI, from highly enriched sperm suspensions of surgically treated azoospermic men (Hovatta *et al.*, 1995; Silber *et al.*, 1995a,b; Fishel *et al.*, 1996). Ejaculated spermatozoa from men who are occasionally azoospermic could be selected and effectively aggregrated using stored human or animal zonae, avoiding the need for a surgical extraction procedure or preventing a failed ICSI attempt due to unexpected sperm absence. This method makes it feasible to perform surgical extractions independently of the time and place of egg retrieval. Also, a distinct advantage of this method is that animal zonae, such as those from the mouse and hamster, can be used for storage.

One suitable application of this new freezing protocol would be its use after TESE, since even minor surgical invasion may cause adverse physiological effects, including deterioration of spermatogenic development, temporary inflammation, irreversible atrophy and partial testicular devascularization (Jarow, 1990; Schlegel, 1996). Complications may occur after multiple attempts. The use of in-vitro maturation for spermatogenic stages may be combined with the current freezing technique, since it would avoid the need for simultaneous timing of egg and sperm retrieval (Liu *et al.*, 1996). It remains to be determined whether physiologically immotile or artificially immobilized cells can maintain their fertilizing ability after freezing.

It can be concluded that the zona pellucida is an ideally suited sterile vehicle for storage of single spermatozoa. It can be tracked microscopically by simple manual pipetting during the many steps involved in freezing and thawing and avoids the general inefficiencies associated with conventional semen cryopreservation. Using this method, the cryoprotectant can be added and removed while the spermatozoa remain in the zona capsule, without dilution, centrifugation or Percoll treatment. Clinical application of this procedure to extremely poor sperm specimens will be necessary in order to confirm these findings.

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Received on October 7, 1996; accepted on January 20, 1997