Evaluation of Human Sperm Function After Repeated Freezing and Thawing

ENOKA BANDULARATNE AND ARIFF BONGSO

From the Department of Obstetrics and Gynaecology, National University Hospital, Kent Ridge, Singapore.

ABSTRACT: Sperm storage via freezing has been useful for men who have difficulty masturbating during assisted reproductive technology (ART) programs and before impotency caused by chemotherapy, vasectomy, and other procedures. Studies were undertaken to evaluate the extent of cryoinjury to sperm after repeated freezing and thawing. The results showed that normozoospermic and oligozoospermic sperm survived after 3 repeated freeze-thaw cycles. The inclusion of seminal plasma did not seem to protect human sperm during freezing and thawing. There were no significant differences in recovery percentages for motile, vital, and morphologically normal sperm between slow and rapid freezing methods in thaws 1, 2, and 3 of normozoospermic and oligozoospermic unwashed (u), washed (w), and washed + seminal plasma (ws) samples. However, there were significant percentage drops in the recovery of motile and vital sperm between each thaw (ie, first to second thaw, and second to third thaw) using both slow and rapid freezing for u, w, and ws samples ($P < .01$). There were also no significant differences in percentage recovery of motile, vital, and morphologically normal sperm between u, w, and ws samples during thaws 1 to 3 in the normozoospermic and oligozoospermic groups. Sperm were capable of fertilizing hamster oocytes microinjected with single sperms after 3 freeze-thaw cycles as evidenced by the formation of 2 distinct pronuclei and 2 polar bodies in 22.2% and 17.2% of normozoospermic and oligozoospermic samples, respectively. The numbers of normal vital motile sperm after 3 serial freeze-thaw cycles are adequate for bringing about fertilization via intracytoplasmic sperm injection in ART programs. Thus, leftover washed sperm in laboratories that perform in vitro fertilization can be frozen, thawed, and refrozen several times without loss of the sperms’ ability to fertilize. This approach has tremendous benefits for men who have difficulty producing sperm and for those with low and declining sperm counts.

Key words: Freezing methods, HICSI, seminal plasma, normozoospermia, oligozoospermia.

J Androl 2002;23:242–249

The robust nature of sperm has enabled them to be used in medically assisted conception. Sperm cryopreservation has been important for men who have difficulty masturbating during donor and assisted reproductive technology (ART) programs, and before chemotherapy or radiation therapy, vasectomy, and other procedures leading to impotency (Glander et al, 1989; Mossad et al, 1994; Tomlinson and Barratt, 1999). It is interesting that the male gamete can be stored in liquid nitrogen for indefinite periods, often without compromising its ability to be used for the treatment of infertility. Protocols for human sperm storage have been simplified by using glycerol as a cryoprotectant, together with rapid and slow cooling programmable and nonprogrammable methods. (Mortimer, 1990; Avery, 1999). Conventionally, semen samples are frozen fresh in the presence of seminal plasma, a nutrient medium such as egg-yolk-citrate, or human serum albumin (HSA) and glycerol. Such cryopreservation protocols for the male gamete have also contributed to the conservation of endangered species and the improvement of genetic merit in the livestock industry. The advent of intracytoplasmic sperm injection (ICSI) has shown that a single human sperm can be microinjected into an oocyte, bring about fertilization and embryogenesis, and when transferred to the uterus, results in the birth of a baby (Palermo et al, 1992). Recently, it was shown that a single human sperm could be successfully frozen inside an empty human oocyte (ie, ooplasm removed and zona pellucida intact). Thaw survival of the single sperm could be used for ICSI, thus helping men with hopelessly poor sperm counts father their own children. Insertion of sperm into empty zonae was carried out either by conventional microinjection (Cohen et al, 1997) or with the use of a laser beam (Montag et al, 1999). All these methods imply that sperm cryopreservation is an important and useful tool in human reproduction.

In human ART programs semen is usually washed by a variety of methods to remove the seminal plasma prior to in vitro fertilization (IVF) or ICSI, because it interferes with fertilization and contains contaminants. In most ART laboratories washed sperm are discarded following an ICSI or IVF cycle. If the washed sperm can be refrozen, it removes the need for men to produce further samples during subsequent ART cycles and will be a good alternative for those men who have difficulty producing semen. Stress has been shown to have a significant influ-
ence on semen quality and production in men enrolled in ART programs (Harrison et al, 1987; Kentenich, 1989; Boivin et al, 1998; Clarke, 1999). Also, sperm survival is known to significantly decrease after the freeze-thaw process. Thus, men with severe oligozoospermia and men presenting with systemic disease such as testicular cancer often have impaired sperm quality and quantity to begin with, and recovery of even a few sperm for ICSI after serial freeze-thaw cycles will be beneficial (Rofeim et al, 2001).

It was recently shown that mouse sperm that become nonviable through repeated freezing and thawing can fertilize mouse oocytes, with subsequent embryonic development following ICSI (Ahmadi and Ng, 1999). Because similar work has not been carried out in humans, studies were undertaken to evaluate human sperm function after repeated freezing and thawing in liquid nitrogen in the presence or absence of seminal plasma, using different freezing protocols.

**Materials and Methods**

**Experiment 1**

Experiment 1 consisted of an evaluation of sperm variables after repeated freezing and thawing, and an investigation of the protective effects of seminal plasma during freezing and thawing.

**Material**—A total of 40 sperm samples (20 normozoospermic and 20 oligozoospermic) were donated for this experiment by patients enrolled in the andrology clinic of the National University Hospital, Singapore.

**Sperm Assessment**—Sperm samples before freezing and after thawing were evaluated for sperm concentration and motility using a Makler chamber (Sefi Medical Instruments, Haifa, Israel), for morphology using a smear stained with eosin-nigrosin (Sigma Chemical Company, St Louis, Mo), and for vitality using the eosin-nigrosin smear and hypo-osmotic swelling test (HOST; Jeyendran et al, 1984). All assessments were made according to World Health Organization (1999) guidelines. For the HOST, a hypo-osmotic solution of 0.735 g sodium citrate dihydrate and 1.351 g fructose were dissolved in 100 mL distilled water. Sperm suspensions (2 × 10^6 sperm/mL culture medium) were added to this hyposmotic solution at a ratio of 1:10 (vol/vol). The mixture remained at 37°C for 30 minutes, following which a drop of sperm suspension was examined on a glass slide covered with a coverslip at 40× magnification using phase contrast optics. The number of curled swollen sperm tails were counted per 100 sperm. Because freezing induces the curling of sperm tails even in the absence of a hypo-osmotic swelling solution, the percentage of sperm that were swollen before HOST were also examined. These values were subtracted from the experimental values when calculating recovery percentages for vital sperm after HOST.

**Separation of Seminal Plasma, and Sperm Freezing and Thawing**—Two-milliliter sperm samples were resuspended in twice the volume (4 mL) of culture medium (Ferticult Flushing Medium, FertiPro, Brussels, Belgium) and centrifuged at 300 × g for 10 minutes. Ferticult is a bicarbonated medium composed of salts, glucose, pyruvate, lactate, insulin, and FSA. The supernatant (5.5 mL containing the seminal plasma) was frozen at −20°C for later use in the freezing experiments. The sperm pellet was resuspended up to 0.5 mL. Washed and unwashed samples (controls) were cryopreserved using a commercially prepared cryoprotectant medium consisting of 15% glycerol and 0.4% HSA (SpermFreeze, FertiPro). The cryoprotectant medium was added drop by drop with gentle swirling movements after each addition. After 15 minutes of equilibration the samples were loaded into 0.5-mL straws (IVF, Paris, France) and then frozen using 2 freezing protocols.

The rapid freezing protocol was carried out in a wide-mouthed dewar (height 70 cm, diameter 40 cm; MVE, Paris, France) that was prepared by pouring 2 L of liquid nitrogen (up to a level of 9 cm) 8 to 12 hours before freezing, according to a protocol described by Mortimer et al (1989). The straws were placed horizontally in a stainless steel wire tray and allowed to cool from room temperature (25°C) to 4°C for 15 minutes, then from 4°C to −20°C for 15 minutes, and then quickly transferred to the prepared dewar. The stainless steel wire tray containing the straws was first lowered to 35 cm (−60°C) above the level of liquid nitrogen and cooled for 15 minutes, then lowered to 15 cm (−125°C) above the level of liquid nitrogen and cooled for 15 minutes, followed by direct plunging into liquid nitrogen (−196°C). The frozen straws were stored in liquid nitrogen storage dewars until further use.

Slow freezing was performed through a programmed, slow machine–freezing method. The straws were placed vertically in the freezing chamber of a semiprogrammable freezing machine (Planer Products Ltd, London, United Kingdom). Freezing was carried out according to the Norfolk (Norfolk, Va) freezing protocol. The samples were cooled from room temperature (25°C) to −4°C at a rate of 10°C/min, from −4°C to −90°C at 0.5°C/min, and finally from −90°C to −130°C at 15°C/min, and then plunged directly into liquid nitrogen.

Straws were thawed on a laboratory bench at room temperature for 15 to 30 minutes. Small aliquots of each thawed sample were evaluated for sperm concentration, motility, vitality, and morphology. The remaining thawed samples were divided into 2 groups (experimental and control). To the experimental group, frozen-thawed seminal plasma (prepared and frozen previously at 2 mL semen diluted in 4 mL culture medium) was added in the ratio of 1:1 (vol/vol), whereas the samples from the control group were resuspended in flushing medium (1:1 [vol/vol]) without seminal plasma. Both groups of samples were refrozen at the same time, and thawed and evaluated using the same volumes of culture medium for controls and experimental samples. This sequence of freezing, thawing, and evaluation was repeated a total of 3 times.

**Experiment 2**

In experiment 2, the ability of human sperm to fertilize was evaluated after ICSI into zona-intact hamster oocytes (HICS).

**Superovulation of Female Hamsters**—Golden hamsters were superovulated by intraperitoneal injections of 30 IU of pregnant mare serum gonadotropin (PMSG; Folligon, Intervet Labs, Box-
meier, Holland) and 30 IU human chorionic gonadotropin (hCG; Chorulon, Intervet Labs) 56 hours apart. The hCG was administered 16 to 18 hours before killing by ether narcotization followed by cervical dislocation. The uterine horns were dissected out into warm (37°C) pre-equilibrated culture medium (Ferticult) and the cumulus-oocyte complexes were teased out using 26-gauge, 1½-inch hypodermic needles. The masses were picked up with a sterile Pasteur pipette and washed 4 times in 100-µL droplets of 1% hyaluronidase (Sigma) for less than 1 minute until the oocytes were freed from the cumulus and corona cells. The oocytes were washed further in Ferticult-IVF medium and incubated until further use.

Preparation of Unfrozen Sperm Samples for Hamster Oocyte–Human ICSI (Controls)—A total of 8 semen samples (4 normozoospermic and 4 oligozoospermic) were used as controls in experiment 2. Two milliliters of fresh semen was resuspended in 4 mL of Ferticult Flushing Medium and then centrifuged at 300 × g for 10 minutes. The supernatant was decanted, leaving 0.5 mL of a sperm suspension. This sperm suspension was adjusted to 1 × 10^6 sperm/mL with Ferticult-IVF medium. The prepared sperm suspensions were left in an incubator (Heraeus, Germany) at 37°C in 5% CO₂ in air until microinjection.

Preparation of Frozen-Thawed Sperm for Hamster Oocyte–Human ICSI (Experimental)—Ten normozoospermic and 10 oligozoospermic semen samples were frozen by the same manual rapid method according to the protocols used in experiment 1. For each of the normozoospermic and oligozoospermic groups there were 2 subgroups of 5 samples each in which straws were thawed after the first and third freezings. There was no subgroup for the second thaw-freezing because the results of the beginning and end points (thaws 1 and 3) were adequate to evaluate the results of sperm fertilizability via HICSI. The straws were cut at either end and emptied into 5-mL tubes (Falcon). Three milliliters of Ferticult-IVF medium was added to the sperm suspension, resuspended, and centrifuged for 10 minutes at 300 × g. The supernatant was discarded, leaving behind 0.25 mL of the pellet. The resuspended pellet was adjusted to 1 × 10^6 sperm/mL and assessed for motility. For oligozoospermic samples, at the third thaw, when sperm numbers were low, the entire pellet was used and diluted accordingly. The prepared sperm suspensions were left in an incubator (Heraeus, Germany) at 37°C in 5% CO₂ in air until microinjection.

Intracytoplasmic Injection of Frozen-Thawed Human Sperm Into Hamster Oocytes—ICSI of human sperm into hamster oocytes received institutional and ethical approval for this project based on the conditions that this procedure can be used only as an assay, and that after fertilization, pronuclear oocytes would be fixed and no further development would be encouraged. A total of 28 different semen samples were used for this experiment, of which 20 were freeze-thawed (first and third thaw) for the main experiment (5 normozoospermic samples for thaw 1, 5 normozoospermic samples for thaw 3, 5 oligozoospermic samples for thaw 1, and 5 oligozoospermic samples for thaw 3). Four unfrozen normozoospermic sperm samples and 4 unfrozen oligozoospermic samples served as controls. ICSI was performed on a heated stage on an Olympus inverted microscope fitted with micromanipulator arms and joysticks (Narishige, Japan). All necessary culture dishes were prepared and labeled beforehand using warm pre-equilibrated culture medium under oil. Three types of dishes were prepared: one set was used for ICSI, a second set was used post-ICSI, and a third set served as growth medium dishes. The ICSI dishes consisted of 5-µL droplets of Ferticult Flushing Medium and one flattened 4-µL droplet of ICSI 100 (polivinyl pyrrolidone [PVP], Scandinavian IVF Science, Göteborg, Sweden) under oil. The post-ICSI and growth medium dishes consisted of 100-µL and 5-µL droplets of Ferticult-IVF under oil, respectively. All dishes were left to equilibrate at 37°C in 5% CO₂ in air until use.

The microinjection pipettes (Cooks, Australia) were aligned parallel to each other on the micromanipulator. Ten to 5 minutes before oocytes were loaded into the ICSI dish, a 1-µL droplet of sperm suspension was added to the PVP. The dish was left in the incubator at 37°C in 5% CO₂ in air. Then, oocytes were loaded into the droplets of the ICSI dish. While lowering the sperm microinjection pipette into the sperm droplet, a morphologically normal-appearing motile spermatozoon was selected from the available group of motile sperm, immobilized, and aspirated into the pipette. The holding pipette was lowered into an egg droplet, and an oocyte was held by suction with its polar body at the 6 o’clock or 12 o’clock position. The oocyte was aligned in such a way that it was touching the base of the dish and was on the same plane as the sperm microinjection pipette carrying the single human sperm. The sperm was placed at the tip of the pipette by suction, the pipette tip was passed through the zona pellucida, the oolemma was broken, some of the ooplasm was drawn into the tip of the sperm pipette, and finally, both sperm and ooplasm were injected into the center of the oocyte. The sperm microinjection pipette was then gently withdrawn out of the oocyte and the oocyte was released into the medium. The remaining oocytes were injected using the same procedure. Finally, the oocytes were washed in Ferticult-IVF medium and transferred to the growth medium droplets for monitoring of fertilization.

Fertilization Checks—The injected oocytes were checked for fertilization 16 hours later using Hoffman optics. Only those oocytes showing 2 distinct pronuclei and 2 distinct polar bodies were considered fertilized.

Statistical Analysis

Fertilization rates were calculated and statistical analysis was performed using the SPSS 10.0 statistical package (SPSS, Chicago, IL). For experiment 1, repeated measure analysis of variance with the Tukey test was used. For experiment 2 the Student’s t test and the Fisher exact test were used because only 2 values were compared.

Results

In experiment 1, sperm variables were evaluated after repeated freezing and thawing, and the protective effects of seminal plasma during the freeze-thaw process was investigated.

The mean ± SD prefreeze sperm concentration (× 10⁶), motility (%), vitality (%), and morphology (% normal
Table 1. Motility, vitality, and morphology recovery percentages of thawed normozoospermic sperm samples after slow and rapid freezing

<table>
<thead>
<tr>
<th>Sperm Parameters</th>
<th>Slow</th>
<th>Rapid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thaw 1</td>
<td>Thaw 2</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>Unwashed</td>
<td>53.7 ± 3.0</td>
</tr>
<tr>
<td>Washed</td>
<td>68.9 ± 21.4</td>
<td>20.7 ± 12.3</td>
</tr>
<tr>
<td>Washed + seminal plasma</td>
<td>68.9 ± 21.4</td>
<td>16.4 ± 14.2</td>
</tr>
<tr>
<td>Vitality (%)</td>
<td>Unwashed</td>
<td>46.6 ± 17.9</td>
</tr>
<tr>
<td>Washed</td>
<td>52.5 ± 13.1</td>
<td>27.8 ± 9.5</td>
</tr>
<tr>
<td>Washed + seminal plasma</td>
<td>52.5 ± 13.1</td>
<td>29.0 ± 15.1</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>Unwashed</td>
<td>92.6 ± 12.0</td>
</tr>
<tr>
<td>Washed</td>
<td>81.7 ± 15.1</td>
<td>68.8 ± 26.5</td>
</tr>
<tr>
<td>Washed + seminal plasma</td>
<td>82.8 ± 13.4</td>
<td>70.4 ± 17.5</td>
</tr>
</tbody>
</table>

ANOVA with the Tukey test; a,b/c/d/e/f/g/h/i/j,k/l/m,n/o/p,q/r/s/t/u/v,w/x/y,z/a not significant.

The mean ± SD motility, vitality, and morphology recovery percentages of the 20 normozoospermic samples frozen by the programmable slow-machine and rapid non-machine freezing methods are shown in Table 1. There were no significant differences in recovery rates for motility, vitality, and morphology between the slow and rapid freezing methods for thaws 1, 2, and 3 for unwashed (u), washed (w), and washed + seminal plasma (ws) samples. There were significant drops in the percentage recovery of motile sperm between each thaw (ie, thaws 1 to 2, and thaws 2 to 3) in the slow freezing samples for all 3 categories of semen (u, w, and ws) (P < .01). The same was true for the rapid freezing group (P < .01). Significant drops in percentage recovery of vital sperm were also observed between thaws 1, 2, and 3 in the slow and rapid freezing groups for u, w, and ws samples (P < .05). However, there were no significant differences in morphology recovery percentages between thaws 1, 2, and 3 for both slow and rapid freezing of u, w, and ws samples. Also, no significant differences were observed in percentage recovery of motile, vital, and morphologically normal sperm between u, w, and ws samples for thaws 1, 2, and 3 in the normozoospermic and oligozoospermic groups.

The mean ± SD motility, vitality, and morphology recovery percentages of the 20 oligozoospermic samples frozen by slow and rapid methods are summarized in Table 2. In the oligozoospermic group there were no significant differences in the recovery rates for motility, vitality, and morphology between the slow and rapid freezing methods for thaws 1, 2, and 3 for u, w, and ws samples. The significant trends between thaws for motility and vitality and the nonsignificant trends between u, w, and ws samples were similar to those of the normozoospermic group.

Thus, to summarize, even though there were drops in the percentage recovery of motile and vital sperm after 3 freeze-thaw cycles for u and w normozoospermic and oligozoospermic samples, the numbers of such sperm at the end of 3 freeze-thaw cycles are adequate to bring about fertilization via ICSI.

The inclusion of seminal plasma did not appear to exert any statistically significant improvement in the percentage recovery of motile, vital, and morphologically normal sperm for both freezing methods for normozoospermic and oligozoospermic samples.

The mean ± SD vitality percentages were similar and not significantly different between HOST and eosin-nigrosin methods for oligozoospermic prefreeze samples for u, w, and ws samples (Table 3). However, after the third freeze-thaw cycle, vitality recovery percentages were significantly higher for HOST compared to eosin-nigrosin treatment for u, w, and ws samples (P < .05).
Unwashed seminal plasma, and compared 2 freezing methods on 2 types of sperm samples (normozoospermic and oligozoospermic) after 3 repeated freeze-thaw cycles. The ability of sperm to fertilize after thawing was further evaluated with the hamster oocyte-human intracytoplasmic sperm microinjection (HICSI) assay (Lanzendorf et al, 1988).

The fact that both normozoospermic and oligozoospermic spermatozoa had the ability to fertilize after 3 freeze-thaw cycles shows the tremendous advantages and clinical implications these results will have in assisted conception. Stress during assisted reproduction cycles has been shown to lead to a significant drop in the quality and concentration of sperm (Harrison et al, 1987; Kenzenich et al, 1992; Boivin et al, 1998). Salvaging the washed sperm that remain (and are usually discarded) after an IVF or ICSI cycle will overcome this disadvantage, especially if a man is unable to produce sperm in future cycles.

The results of the present study confirm the work of Polcz et al (1998), that human sperm can survive after repeated freezing and thawing. However, the methodolo-

**Discussion**

Polcz and his colleagues (1998) demonstrated that human spermatozoa were able to withstand 5 freeze-thaw cycles and maintain their motility and vitality, although significant reductions were seen in these parameters. However, because those researchers did not study sperm function after repeated freezing and thawing, it is not known whether such spermatozoa retained their fertile attributes even after being subjected to the damaging effects of repeated freezing and thawing. The present study evaluated sperm function after thaw and the protective effects of seminal plasma, and compared 2 freezing methods on 2 types of sperm samples (normozoospermic and oligozoospermic) after 3 repeated freeze-thaw cycles. The ability of sperm to fertilize after thawing was further evaluated with the hamster oocyte-human intracytoplasmic sperm microinjection (HICSI) assay (Lanzendorf et al, 1988).

Intracytoplasmic Injection of Frozen-Thawed Human Sperm Into Zona-Intact Hamster Oocytes

The fertilization rates after microinjection of hamster oocytes with frozen-thawed human sperm are shown in Table 4. The mean fertilization rates for thaw 1 and thaw 3 normozoospermic and oligozoospermic sperm samples were slightly less than those of their controls, but not significantly different. Fertilization rates between thaw 1 and thaw 3 were also not significantly different for both normozoospermic and oligozoospermic samples.

**Table 2. Motility, vitality, and morphology recovery percentages of thawed oligozoospermic sperm samples after slow and rapid freezing**

<table>
<thead>
<tr>
<th>Sperm Parameters</th>
<th>Slow</th>
<th>Rapid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thaw 1</td>
<td>Thaw 2</td>
</tr>
<tr>
<td>Unwashed</td>
<td>28.0 ± 15.4a</td>
<td>11.7 ± 4.5b</td>
</tr>
<tr>
<td>Washed</td>
<td>36.8 ± 17.7a</td>
<td>14.3 ± 6.8a</td>
</tr>
<tr>
<td>Washed + seminal plasma</td>
<td>36.8 ± 17.7a</td>
<td>11.0 ± 8.5b</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unwashed</td>
<td>49.1 ± 7.5a</td>
<td>25.6 ± 13.0a</td>
</tr>
<tr>
<td>Washed</td>
<td>49.1 ± 7.5a</td>
<td>24.2 ± 9.6c</td>
</tr>
<tr>
<td>Washed + seminal plasma</td>
<td>49.1 ± 7.5a</td>
<td>24.2 ± 9.6c</td>
</tr>
<tr>
<td><strong>Vitality</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unwashed</td>
<td>80.2 ± 42.1a</td>
<td>80.4 ± 44.6a</td>
</tr>
<tr>
<td>Washed</td>
<td>76.8 ± 41.7a</td>
<td>73.5 ± 40.4a</td>
</tr>
<tr>
<td>Washed + seminal plasma</td>
<td>76.8 ± 41.7a</td>
<td>74.8 ± 37.3a</td>
</tr>
</tbody>
</table>

ANOVA with the Tukey test; a,b,c,e,g not significant; b,d,f,h,i,j P < .05.

**Table 3. HOST and eosin-nigrosin vitality parameters in oligozoospermic prefreeze sperm and sperm after the third freeze-thaw cycle**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sperm Vitality (Mean ± SD%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HOST</td>
</tr>
<tr>
<td></td>
<td>Prefreeze 3</td>
</tr>
<tr>
<td>Unwashed</td>
<td>43.3 ± 6.4a</td>
</tr>
<tr>
<td>Washed</td>
<td>38.9 ± 5.6a</td>
</tr>
<tr>
<td>Washed + seminal plasma</td>
<td>38.9 ± 5.6a</td>
</tr>
</tbody>
</table>

* A total of 8 sperm samples were used in this experiment. The total count and motility for unwashed samples and washed samples were 16.4 ± 2.7 × 10^6 and 50.9% ± 7.2% and 18 ± 6.2 × 10^6 and 49.7% ± 17.7%, respectively.

ANOVA with the Tukey test; a,b,c,g not significant; b,d,f,h,i,j P < .05.
Repetitive Freeze-Thaw of Human Sperm

Table 4. Intracytoplasmic sperm injection of first and third thaw normozoospermic and oligozoospermic human sperm into hamster oocytes

<table>
<thead>
<tr>
<th>Sperm Patients</th>
<th>Motility (Mean ± SD%)</th>
<th>Number of Oocytes Injected</th>
<th>Number of Oocytes Fertilized</th>
<th>Fertilization Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normozoospermic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thaw 1</td>
<td>5</td>
<td>11.6 ± 11.2</td>
<td>43</td>
<td>9</td>
</tr>
<tr>
<td>Thaw 3</td>
<td>5</td>
<td>4.3 ± 1.8</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>Fresh unfrozen (controls)</td>
<td>4</td>
<td>46.3 ± 5.3</td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td>Oligozoospermic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thaw 1</td>
<td>5</td>
<td>7.4 ± 3.2</td>
<td>52</td>
<td>9</td>
</tr>
<tr>
<td>Thaw 3</td>
<td>5</td>
<td>4.7 ± 2.4</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td>Fresh unfrozen (controls)</td>
<td>4</td>
<td>52.5 ± 3.5</td>
<td>24</td>
<td>6</td>
</tr>
</tbody>
</table>

Student’s t test and Fisher exact test; *not significant; **not significant.

The recovery percentages for motility, vitality, and morphology were not significantly different between thaws and between normozoospermic and oligozoospermic samples. This shows that regardless of the freezing method used, if the sperm number is low (<20 × 10⁶/mL), motility is also reduced significantly with thawing, which is similar to samples with high sperm numbers (>20 × 10⁶/mL). Thus, if sperm samples are of good quality but low in counts, it may be advisable to pool several ejaculates from the same male before freezing so as to optimize the number of viable sperm after freezing. The number of motile and viable sperm recovered after the third freeze-thaw cycle are adequate to bring about fertilization and generate embryos by ICSI in ART programs.

In most good conventional sperm freezing programs in which fresh unwashed normozoospermic sperm are frozen for the first time, usually no more than 50% of motile sperm are recovered after the first thaw (Mortimer, 1990). In this study too, 47.4% ± 15.4% to 53.7% ± 23.0% of motile sperm were recovered (Table 1), confirming the reliability of the freezing protocols used. It is interesting, however, that the washed samples generated higher motile recovery percentages at first thaws (67.1% ± 21.7% to 68.9% ± 21.4%), although this was not statistically significant (Table 1). It is therefore more advantageous to freeze washed sperm samples without the seminal plasma.

HOST values showed that live sperm, even though reduced in number to just a few after the third freeze-thaw cycle, could still be picked up and used immediately for...
ICSIs. The results confirm that a combination of HOST on repeatedly frozen, washed human sperm is a useful protocol for salvaging viable sperm for fertilization via ICSI in men who are unable to readily produce sperm or for those with hopelessly poor sperm counts. Successful fertilization with ICSI using HOST-treated unfrozen sperm has been previously demonstrated (Bourn et al, 1995, Casper et al, 1996, Liu et al, 1997, Ved et al, 1997).

The results of HICSI in the present study suggests that human sperm frozen and thawed up to 3 times are still viable because normal pronuclear oocytes (2PN) with 2 polar bodies were produced when third-thaw sperm were microinjected into zona-intact hamster oocytes. Only those oocytes that showed fertilization with 2 distinct pronuclei and 2 distinct polar bodies were included in the results. Oocytes with decondensed sperm heads were observed, and if these had been included they would have raised the fertilization percentages. Because of the subjectivity of recognizing sperm decondensation, the presence of 2 pronuclei with 2 polar bodies observed with Hoffman optics was taken as a more reliable indication of fertilization.

Fertilization rates between normozoospermic and oligozoospermic sperm were not significantly different, although they were slightly lower in oligozoospermic samples. The results confirmed that despite cryoinjury to sperm after repeated freeze-thaw cycles, sperm are still fertile through ICSI, and when HICSI was performed, fertilization rates did not differ, regardless of the number of thaw cycles and quality of sperm.

Ahmadi et al (1996) showed that no significant differences were observed in fertilization rates between patients with male factor infertility (pronuclear + sperm decondensation = 28%) and severe male factor infertility (pronuclear + sperm decondensation = 21.7%). Pronuclear formation and sperm head decondensation was observed 18 hours postinjection in those studies. Gvakharia et al (2000) used the HICSI assay as a tool for improving the proficiency of technical staff. Those researchers reported sperm head decondensation rates in pregnancy-proven donors and oligospermic sperm as 62% and 54.2%, respectively, 5 to 7 hours after ICSI. They also showed no statistical difference between fresh and frozen-thawed sperm decondensation rates (42% to 100%) when hamster ICSI was performed. Thus, frozen sperm thawed for the first time can fertilize human oocytes to an extent equal or slightly lower to that of fresh, unprocessed spermatozoa. The results of these workers also confirm the reliability of the HICSI assay for evaluating reacted or nonreacted fresh or frozen human sperm. Based on the observations of these reports and the present study, it appears that HICSI, HOST, and rapid and slow freezing methods are reliable protocols for freezing and assessing human sperm. To summarize, even though there were drops in the percentage recovery of motile and vital sperm after 3 freeze-thaw cycles for unwashed and washed normozoospermic and oligozoospermic samples, the numbers of such sperm at the end of 3 freeze-thaw cycles will be adequate to bring about fertilization via ICSI. These protocols will be of enormous value from a logistical and practical sense in an ART setting. More work is required to evaluate them, however, in actual clinical trials on ART patients.

Acknowledgments

The authors thank Dr Dong Fang of the National University Medical Institute for statistical analysis, and Ms Asma Bevi for secretarial assistance.

References


Ahmadi A, Ng SC. Developmental capacity of damaged spermatozoa. Hum Reprod. 1999;14:2279–2285.


Clarke GN. Sperm cryopreservation: is there a significant risk of cross-contamination? Hum Reprod. 1999;14:2941–2943.


Jeyendran RS, Van der Ven HH, Perez-Palaez M. Development of an assay to assess the functional integrity of the human sperm membrane...


