15 Chapter

Male Germ Cell Cryobanking

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INTRODUCTION

While the idea of freezing human male gametes has been the subject of experimentation since the late 1700's, it was not until 50 years ago that technology was developed to the point where human sperm could be frozen and later thawed in such a way that they could fertilize an egg and initiate development. In the past several decades the technology of cryopreservation, or maintaining life in a frozen state, has advanced considerably. With the use of modern techniques, cryopreservation of sperm to preserve an individual's ability to reproduce has become successful, safe and widely available.

The first reported observations of the effects of low temperatures on spermatozoa were recorded by Lazaro Spallanzani in 1776, and the first to discuss the possible uses of sperm banks was the Italian Paolo Mantegazza. He wrote the following in 1866: "It might even be that a husband who has died on a battle-field can fecundate his own wife after he has been reduced to a corpse and produce legitimate children after his death".[1] However, sperm cryopreservation did not become a realistic proposition until the discovery in 1949 of the potent cryoprotective properties of glycerol.[2] The first human births resulting from artificial insemination of cryopreserved semen were reported by Bunge and Sherman in 1953.[3] Since that time, many children have been born as a result of this rather simple procedure of assisted reproduction. Theoretical considerations suggested that long-term cryostorage would require the use of temperatures lower than –130°C, the glassy-transition temperature below which icecrystal growth is inhibited.[4] Consequently, liquid nitrogen (–196°C) storage became the standard very early in the history of sperm banking.[5]

Nowadays, human semen cryopreservation is a routine technique performed extensively in fertility clinics and hospitals worldwide. Sperm cryopreservation may provide the opportunity for future fertility in a variety of situations. Although semen cryopreservation has proven to be very valuable, the quality of frozen sperm is highly affected during the process.

GENERAL ASPECTS OF SPERM CRYOPRESERVATION

A lack of correlation between the motility and fertilizing ability of frozen sperm is generally accepted. Sublethal damages occurring within the sperm cell are thought to be responsible for this difference.[6] Living cells that undergo cryopreservation are subjected to two major factors that are responsible for cryoinjury in a sequential manner, low temperature and crystallization of intracellular and extracellular water. These factors have deleterious effects on the sperm plasma membrane, causing changes in lipid composition and location.[7-9] These insults to the sperm membrane are in turn responsible for cell leakage of many intracellular components, resulting in reduced sperm metabolic activities. The end result is the elimination of cytoplasmic and membrane-bound proteins and enzymes from the sperm cell.[10-13] These cryoinjuries include loss of membrane fluidity and integrity,[14-16] oxidative stress leading to lipid peroxidation,[17,18] DNA fragmentation[19-21] and cytoskeleton modifications.[22] The freezing process also causes

disruption of cold-sensitive microtubule-containing structures such as the meiotic spindle.[23-27] However, Donnely et al demonstrated that sperm frozen unprepared from seminal fluid appear to be more resistant to freezing damage than are frozen sperm prepared by either Percoll density centrifugation or a direct swim-up procedure followed by freezing in seminal plasma.

In fact, although progressive motility is significantly greater in fresh prepared sperm compared with fresh unprocessed semen, prepared sperm suffer a greater decrease in progressive motility than raw semen after freezing. In addition, progressive motility is significantly improved by preparation of thawed semen, although the sperm velocity does not change. Progressive motility may be significantly improved by freezing prepared sperm in seminal plasma, although values are still significantly lower than those of fresh samples. This is again due to the presence of seminal plasma because the high content of polyunsaturated fatty acids and lack of repair mechanisms of human sperm make them particularly sensitive to free-radical assault.[28] Further improvements can be achieved by selecting out the subpopulation of sperm with best motility and DNA integrity and freezing these sperm in seminal plasma, making this the optimal procedure. Therefore, freezing sperm in seminal plasma improves motility and DNA integrity after thawing.[28]

Cryopreservation of human sperm remains an essential tool for the preservation of male fertility. Freezing of sperm before initiation of treatment provides the patient a type of "fertility insurance" and may allow him to father his own children through the use of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). On the other hand, human spermatozoa have unusual cryobiological behavior, and improvements in their survival have not been achieved by the standard cryobiological approaches. In fact, despite continuous methodological optimization, the process of cryopreservation and thawing leads to an activation of apoptotic signal transduction in a certain percentage of the cryopreserved spermatozoa, probably contributing to the reduction of the fertilizing capacity.[29-31]

As far as sperm membrane damage is concerned, the susceptibility of the sperm of other mammal species to cryodamage during the freezing process appears to be related to a high ratio of saturated vs. unsaturated fatty acids, together with low cholesterol content.[32] Interestingly, human sperm membranes have unusually high cholesterol contents, and these high levels are known to stabilize membranes during cooling.[33]

According to Morris, et al viability on thawing does not appear to correlate with conventional theories of cellular freezing injury, suggesting that other factors

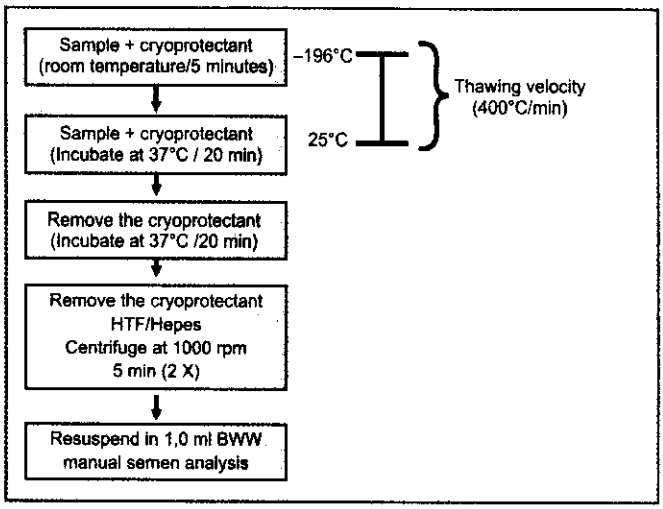

Fig. 15.1: Process of semen thawing

determine viability following freezing and thawing of human spermatozoa. Improved cryopreservation methods may be developed by specifically manipulating the manner in which cells experience physical changes instead of imposing a linear temperature reduction. Treatments that followed a chosen non-linear concentration profile, referred to as 'controlled concentration," allowed recovery of almost all the cells that were motile before freezing[34] (Fig. 15.1).

EFFECT OF STORAGE TEMPERATURE ON SPERM CRYOPRESERVATION

Storage in liquid nitrogen at a temperature of –196°C (Figs 15.2 and 15.3), is the standard method for preservation of human sperm.[2] A number of studies have documented that improvements in cooling technique and the use of

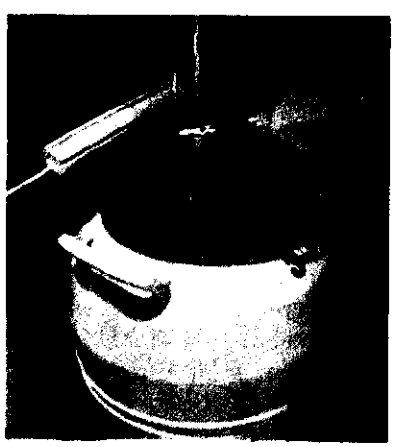

Fig. 15.2: Semen sample premixed with semen freezing media being plunged in liquid nitrogen after equiliberation in vapor phase for 20 minutes

Fig. 15.3: Liquid nitrogen storage can. Liquid nitrogen vapors are depicted

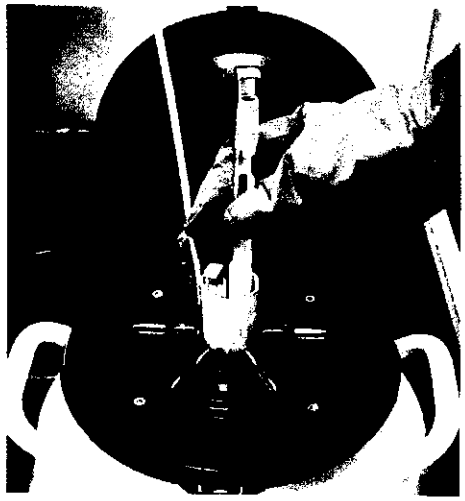

Fig. 15.4: Sample being storaged

Fig. 15.5: Semen sample at –196°C being retrieved for thawing and insemination

improved cryopreservatives, as well as thawing at 37°C in a water bath, improve post-thaw sperm quality.[3] It appears, however, that the loss of sperm motility with the cryopreservation process does not increase with prolonged periods of cryopreservation, making long-term storage feasible. In fact, a report that the semen of six donors was stored for 28 years suggests that human sperm may be stored virtually indefinitely if it is kept under liquid nitrogen. This is pertinent information for clinicians referring pubescent boys and young men for sperm banking before chemotherapy. Some of these young men may require sperm storage for long periods (years).[35-38]

Sperm cryopreservation in liquid nitrogen at –196°C has become standard protocol in most andrology facilities. Because this technique is acceptable virtually everywere, little consideration has been given to the potential effectiveness of short- or long-term storage of sperm at higher temperatures. Unfortunately, not every laboratory has consistent access to liquid nitrogen cryopreservation facilities. Trummer et al showed that sperm storage at –70°C is linked with a greater loss of sperm motility than is storage at –196°C. The reduction in motility noted at one week, however, was fairly modest. Unfortunately, the loss of sperm motility increased dramatically after three months of storage. These results indicate that short-term storage of sperm at –70°C may be a viable option if liquid nitrogen storage facilities are not available (Figs 15.4 and 15.5). Long-term storage at this temperature (70°C), however, appears to be linked with an unacceptable reduction in sperm motility.[39]

Even though the standard protocol of sperm storage at –196°C is widely used in almost every center, the technical aspects of freezing and thawing sperm and preparing cryopreservation media (Fig. 15.6) have been refined over the years.[40-43] Various methods of cryopreservation have been evaluated for their effects on sperm motility.[44-46] However, the methods for freezing and thawing semen that optimize motility recovery have not been firmly established. In addition, the optimum rate of temperature drop during freezing remains controversial.[44,45] The flash-freezing technique in which the sample is plunged directly into liquid nitrogen produces sperm recovery rates that are comparable to those seen with computer-controlled, slow-staged freezing.[46] In addition, a variety of cryoprotectants are available to protect sperm from the negative effects of the cryopreservation process.

The advantages of the fast-freezing and slow-staged cooling methods have long been debated. Studies have

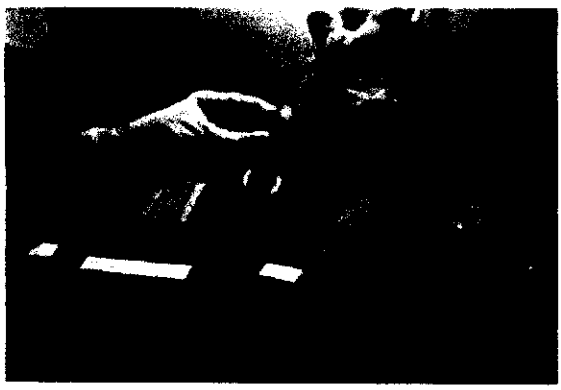

Fig. 15.6: Semen freezing media being added to the semen sample before freezing process

reported results in favor of both the fast-freezing method[47] and the slow-staged cooling method.[48-50] A recent study showed that there was no difference in sperm quality preservation when semen samples are frozen by fast-freezing techniques or by slow, controlled freezing methods in either liquid nitrogen or vapor-phase nitrogen.[51]

SPERM CRYOPRESERVATION IN SPECIFIC SITUATIONS

Obstructive Azoospermia

Obstructive azoospermia is caused by several different etiologic processes. Ejaculatory duct obstruction, vasectomy, post-infection obstruction and congenital bilateral absence of the vas deferens are some of the major causes of obstruction. An important approach in the management of patients with obstructive azoospermia is retrieval of sperm for various assisted reproductive technology (ART) procedures through microepididymal sperm aspiration (MESA), testicular sperm extraction (TESE) and percutaneus epididymal sperm aspiration (PESA).

In general, spermatozoa from epididymis are considered more mature than the testicular spermatozoa and provide a higher pregnancy rate.[52] Often, retrieval of spermatozoa from the epididymis is not possible. In some cases the epididymis is absent. Testicular sperm extraction is the modality of choice in the management of non-reconstructable obstruction of the excurrent duct system, when epididymal sperm aspiration is not available or is unsuccessful. A large number of spermatozoa can be extracted from the epididymis or testicular tissues of patients with azoospermia of obstructive etiology. In fact, pregnancy outcome in obstructive azoospermia using these spermatozoa is higher than in non-obstructive azoospermia.[53] Only a small portion of these spermatozoa are needed for IVF/ICSI techniques, and the remaining tissue can be divided into several aliquots and cryopreserved for subsequent use.

After carrying out a TESE procedure, the testicular tissue can be divided into several aliquots and cryopreserved. This would favor multiple ICSI cycles,[54] thereby avoiding additional surgical biopsies to retrieve spermatozoa in the future.[55] Generally, the time from biopsy to processing and freezing is within one to one and a half hours; however, a recent case report showed pregnancy even with the interval between the biopsy and the testicular tissue cryopreservation as long as 15 hours. Therefore, for many programs, the use of cryopreserved testicular tissue avoid the need for fresh testicular tissue at the time of ICSI.

Non-obstructive Azoospermia

In contrast to obstructive azoospermia, where viable sperm can easily be retrieved from the frozen specimens, the impaired quality of the testicular tissue present in non-obstructive azoospermic (NOA) patients does not allow for cryopreservation and later use for ICSI in all cases. As has been demonstrated for ejaculated sperm, a significant decrease in sperm motility and viability by freezing and thawing also occur for testicular sperm.[56] Even in a program with low-restrictive criteria for patient allocation and cryopreservation of testicular sperm, diagnostic testicular sperm retrieval followed by cryopreservation may be the procedure of choice. To counteract the reasonable risk of not finding sperm or finding only immotile sperm, scheduling fresh surgery as a backup or counseling the couple for donor sperm as a backup is recommended. Using totally immotile sperm after thawing should be discouraged on the basis of the present data.[57]

Patients with Cancer

Recent advances in the diagnosis and treatment of malignant diseases has brought into focus certain quality of life issues, such as the problem of infertility.[58] The impact of these problems is magnified in malignant diseases that predominantly affect patients in the reproductive age group. An increasing number of people are being successfully treated for cancer, and for those with an expectation of long-term survival the late effects of treatment are of concern. In the past, cancer survivors tended to be most concerned about disease recurrence and treatment side effects. As survival rates have increased, however, patients are now also concerned about qualityoflife issues such as preserving fertility potential.[59]

Depending on the underlying disease, the age of the oncological patient, the type of therapeutic agent used to

treat the cancer, the cumulative doses used and the duration of the treatment, between 10 and 100% of surviving cancer patients will show reduced semen parameters after their cure. An average of 15 to 30% of cured cancer patients remain sterile in the long-term.[60]

According to a study by Spermon et al, before the cancer was diagnosed, 79 (66%) of 120 couples who attempted to conceive succeeded within one year. After treatment, 38 (43%) of 88 couples conceived within one year. Seven couples used cryopreserved sperm to conceive a child after treatment. The different treatment modalities do not significantly influence the outcome of patients' desire for children. Congenital malformations were recorded in approximately 4% of the children born before or after treatment. Therefore, although the majority of patients with testicular cancer have fulfilled their desire with regard to children, fathering a child after treatment seems to be more difficult compared with the case in the general population. Predicting which patient will have fertility problems after treatment is not possible; therefore, cryopreservation should be offered to every testicular cancer patient. An increased risk for congenital malformations has not been observed among these patients.[61]

In many cancer subjects, sperm quality is already reduced before receiving any treatment. Studies have shown that semen parameters may be diminished in adolescent male patients with cancer, suggesting that sperm cryopreservation during potentially sterilizing treatment should be considered.[62-64] At present, sperm banking remains the only proven method, although hormonal manipulation to enhance recovery of spermatogenesis and cryopreservation of testicular germ cells are possibilities for the future.[5,65-67] According to Hallak et al, patients with testicular cancer, leukemia and lymphoma had the worst seminal quality compared to other cancer groups.[68,69] Patients with malignant diseases in general have lower total motile sperm count and motility compared to normal semen donors. Furthermore, and of utmost importance, the authors concluded that pre-freeze or post-thaw semen quality in cancer patients is not affected by the type of disease. According to Gandini et al, the recovery of spermatogenesis after chemotherapy or radiotherapy in their group of testicular cancer patients studied was not a function of pre-therapy sperm parameter quality.[65] Therefore, cryopreservation of semen should be offered to cancer patients regardless of the type of disease.[5,70-72] According to Zapzalka et al, most oncologists (74%) are unaware of recent advances in reproductive technology in which only a few sperm are needed for successful in vitro fertilization with ICSI.[73] This lack of awareness may be contributing to underutilization of sperm cryopreservation by male cancer patients. Coincidentally, the number of patients that oncologists estimated who actually cryoperserve sperm is also very small (27%). This leads to speculation that if more oncologists knew of the existence of ICSI, the percentage of patients who cryopreserve sperm might increase.

According to Schover et al, 19% of the 283 survivors of cancer from the Cleveland Clinic tumor registry had significant anxiety that their cancer treatment could negatively affect their children's future, and only 57% received information from their health care providers about infertility after cancer.[74]

Other reproductive concerns are discussed even less often. For instance, patients with cancer may have chromosomal abnormalities in the malignant cell of origin, and an increased frequency of human sperm chromosomal abnormalities after radiotherapy has been reported. Reassuringly, however, studies have shown that children born after completion of cancer treatment have no increased risk for chromosomal abnormalities or birth defects before or after treatment.

Intraoperative Sperm Harvesting during a Vasectomy Reversal and Cryopreservation

The availability of ICSI has encouraged some surgeons to offer cryopreservation of sperm that may be harvested during vasectomy reversals. Cryopreservation of sperm during vasoepididymostomy is especially important because of a reported 35% rate of azoospermia after microsurgical vasoepididymostomy.[75] Other investigators have reported that motile sperm were present in the intraoperative vasal and epididymal fluid in 35% of 603 vasectomy reversals.[76] However, performance of a vasal or epididymal anastomosis should be prioritized over sperm harvesting during vasectomy reversals. To harvest motile sperm, the surgeon should perform the reversal at the location farthest from the testicle where intact sperm are present, regardless of their motility. Before sperm harvesting and cryopreservation are performed, the patient and his partner should assess the cost effectiveness of and their ability to afford IVF/ICSI. When harvesting sperm during vasectomy reversals, surgeons must alert laboratory personnel to cryopreserve small aliquots of sperm that are appropriate for later use with ICSI rather than larger aliquots for either vaginal or intrauterine insemination or IVF without ICSI.[77,78]

Prior to the Absence of the Partner

If it is anticipated that the male partner will be unavailable during the optimal time for the procedure, a semen sample can be cryopreserved prior to his departure as a

backup in the event he is prevented from providing a sample at the time of the procedure. While this may not be optimal, it can often keep the time and finances invested in a procedure from being wasted due to unforeseen circumstances.[79]

ASSISTED REPRODUCTION

Since the introduction of a method for freezing human semen, the indications for sperm cryobanking have been greatly expanded by recent breakthroughs in assisted reproduction, for the patient's own future use and for donor banking.[5] However, the freeze-thaw process has been shown to be related to a variable loss of sperm quality.[80]

Although the adverse effect of freezing and thawing upon sperm quality is even more pronounced in cases of poor semen quality, with the advent of ICSI only a small number of motile spermatozoa are required for a successful fertilization.[81,82] In fact, ICSI can be performed with fresh and cryopreserved spermatozoa from ejaculated semen from patients with oligoasthenoteratozoospermia (OAT) or from spermatozoa extracted from the epididymis or testis in cases of obstructive or non-obstructive azoospermia.[83]

It is well known that intrauterine insemination with cryopreserved sperm results in a lower pregnancy rate compared to fresh sperm, but many studies have been showing that fertilization and pregnancy rates for ICSI using cryopreserved spermatozoa are similar to those achieved with freshly obtained sperm.[84-88] Even though there is no doubt that cryopreserved spermatozoa can be used to fertilize oocytes by ICSI, more studies comparing the efficacy of ICSI with either fresh or cryopreserved ejaculated spermatozoa from infertile patients should be performed. Also, the majority of studies comparing fresh and cryopreserved sperm have shown results from sperm that were surgically retrieved.[85-88] Borges et al, demonstrated that when the semen sample had decreased motility, the fertilization rate was higher with fresh sperm than with cryopreserved sperm. However, the implantation, pregnancy and miscarriage rates were similar. This finding corroborates the idea that the cryopreservation process may cause more damage to patients with asthenozoospermia than patients with normal semen analysis or oligozoospermia. In fact, it is postulated by the authors that the lower normal fertilization rate detected in men with asthenozoospermia compared to normozoospermic or oligozoospermic men could be because the semen with low motility may have abnormalities in the sperm even before the cryopreservation, and the damage caused by the cryopreservation could be much higher than detected in other sperm characteristics.[83]

According to a study published by Schmidt et al, following antineoplastic treatment, 43% of the men had motile spermatozoa in the ejaculate, but 57% were azoospermic. A total of 151 ART cycles were performed [55 intrauterine insemination (IUI), 82 ICSI and 14 ICSI–frozen embryo replacement (FER)]. The clinical pregnancy rate per cycle was 14.8% after IUI, 38.6% after ICSI and 25% after ICSI–FER. The corresponding delivery rates were 11.1, 30.5 and 21%, respectively. Cryopreserved semen was used in 58% of the pregnancies. The delivery rate per cycle was similar after using fresh or cryopreserved spermatozoa. Therefore, male cancer survivors have a good chance of fathering a child by using either fresh ejaculated sperm or cryopreserved sperm.[89] Recently, Agarwal et al (2004) reported the outcome of ART in 29 male cancer survivors all using cryopreserved semen. A total of 87 cycles were performed with a mean pregnancy rate of 18.3% per cycle (7% after IUI, 23% after IVF and 37% after ICSI).[66]

Male cancer patients should be encouraged to freeze numerous sperm samples even when sperm count and motility are poor.[90] In these cases, ICSI is a powerful technique compared with intrauterine injection since thawed sperm samples with poor parameters can produce relatively high fertilization rates resulting in normal pregnancies and deliveries. The possibility to repeat treatments even in the face of a limited number of sperm samples appears to be of utmost importance. Cancer patients should be informed that there currently is no available evidence for increased incidence of congenital abnormalities in children.

In recent years, some physicians have raised doubts regarding the justification and necessity of providing the facilities for banking spermatozoa before cancer treatment because of the relatively small number of men who used it following completion of treatment and, consequently, the small number of children born as a result of cryopreserved spermatozoa. Indeed, it seems that 5 to 15% of the patients who bank their semen before treatment return for fertility purposes. These findings are due to several reasons: short period of original disease, anxiety about potential risks for the children, uncertainty about their long-term health and recovery or waiting for possible recovery of gonadal function. Hallak et al, surveyed 56 patients at Cleveland Clinic who requested discontinuation of sperm storage. They concluded that most patients decided to discontinue sperm banking because either they regained fertility or had improved semen quality.[91] Even if properly counseled, not all patients eventually will bank semen before their treatment. One study reported that only 42% of appropriately counseled patients did bank their semen to counter sterility,[92] while another recent study reported a value of 54%.[93]

The Cleveland Clinic survey revealed that 76% of childless patients wanted children in the future.[94] Moreover, approximately 80% of patients viewed themselves positively as actual or potential parents. Addressing these issues immediately upon diagnosis helps young cancer patients and their families to face the disease and cope with treatment in a more optimistic light. Currently, all male cancer patients of reproductive age who will have treatment that may affect testicular function and who may desire children in the future should cryopreserve sperm before the initiation of therapy. It is vital, therefore, to keep records of patients having post-cancer infertility treatment and to monitor the children born as a consequence of these treatments.

While many oncologists now tend to use less gonadotoxic treatments, semen cryopreservation should always be offered to each cancer patient since recovery of spermatogenesis cannot be guaranteed for the individual patient. Other considerations include inter-individual variances in response to treatment and the possibility of changing the therapeutic regimen from one with limited gonadotoxicity to a more gonadotoxic therapy due to treatment failure.

PERSPECTIVES

Although still purely experimental at this stage, testicular stem cell transplantation may provide an adequate solution to preserve the progenitive capacity of pre-pubertal boys. Although it is still surrounded by complex ethical issues, cryobanking of testicular tissue from pre-pubertal boys may now be considered an acceptable strategy, analogous to cryobanking of ovarian cortex in young girls. However, in contrast to girls, stem cells in boys are the target of storage, which represents an important difference in terms of potential future applications for preserving fertility.

CONCLUSION

Reproductive physicians play important roles in helping to preserve the reproductive capacities of young cancer patients. First of all, the reproductive physicians are involved in developing and using procedures to preserve sperm and gonadal tissue before treatment. Secondly, fertility specialists will assist cancer survivors in using preserved gametes and tissue or in providing other assistance in reproduction. The fact that the patient has just been diagnosed with cancer or survived the acute or extended phase of coping with cancer distinguishes the cancer patient from other fertility patients. Variations in type of cancer, time available to onset of treatment, age, partner status, type and dosage of chemotherapy, and the risk of sterility with a given treatment regimen require that each case have its own treatment strategy. Consultation with the patient's oncologist often is essential. A key issue at the time of cancer treatment is whether obtaining gametes or gonadal tissue for storage and later use is medically feasible. Questions about the patient's health and prognosis also will arise when the patient is deciding later whether to reproduce.

APPENDIX: CRYOPRESERVATION OF HUMAN SPERMATOZOA

- Equipment
- Microcell counting chamber (Conception Technologies, San Diego, Calif.)
- 5 µl Eppendorf Pipette
- Aliquot mixer
- Vortex
- –20°C freezer
- LN_2 container with racks
- Sterile specimen container
- Sterile 15 ml centrifuge tubes with caps
- Sterile serological pipettes (1 ml, 2 ml, and 5 ml capacity)
- Sterile Nunc cryovials (1 ml and 2 ml capacity)
- Colored cryomarkers
- Test tube racks (for 15 ml test tubes)
- Cryovial racks
- Stainless steel canes for cryovials
- Plastic cryosleeves
- Cryogloves
- Latex gloves
- 37°C incubator
- LN_2 from supplier
- Eosin-nigrosin stain
- Microslide
- Coverslips
- Sperm washing media
- Makler chamber (MidAtlantic Diagnostics, Mt. Laurel, N.J.)
- Reagents
- Freezing medium (Test yolk buffer with glycerol; TYB-G; Irvine Scientific, Santa Ana, Calif.).
- Sperm washing media (HTF; Enhance –W, Conception Technologies).

Procedure (Aspiration from Surgery)

Technical note: Sterile techniques should be used throughout specimen processing. Gloves are mandatory for all procedures dealing with body fluids. Latex, however, may be toxic to sperm. Therefore, care should be taken to prevent contami-

nation of the specimen with latex or talc. Vinyl gloves are an available alternative.

1 Cryopreservation worksheet labeled with the patient name, diagnosis and LN_2 container and rack. If there is more than one specimen, have a worksheet for each specimen.
2 Every time a laboratory is notified that an aspirate is ready, take one bottle of frozen test yolk media and put into the 37 °C incubator to thaw.
3. A technologist will go to the surgery room to retrieve the specimen(s). Keep the vials warm in your hands.
4. Normally, two to three specimens will arrive from surgery. Make sure patient name and specimen number are marked on the vials.
5. Using sterile technique, measure the volume of each specimen and record on the appropriate cryopreservation worksheet.
6. Centrifuge the specimen in the original container for 5 minutes at 1600 rpm.
7. Label three (3) sterile 15 ml conical centrifuge tubes with the patient's name, specimen number.
8. Transfer the supernatant of specimen 1, specimen 2 and specimen 3 if there is one into one supernatant tube. Both (or all three) supernatants are now combined into one tube.
9. Add 0.5 ml HTF (sperm washing media) to each pellet to resuspend. Mix gently.
10. Transfer a drop of each specimen into a pre-labeled conical cup for semen analysis.
11. Perform the following on each specimen aspirate: Regular manual semen analysis, Endtz test (leukocytospermia test), eosin-nigrosin stain procedure, Tygerberg's strict criteria for morphology evaluation.
12. Always notify the surgeon if no motile sperm are found on the wet prep.
13. Within 1 hour of specimen collection, add an aliquot of freezing medium equal to 25% of the resuspended aspirate volume to the centrifuge tube with a sterile pipette.
    Note: Since the resuspended volume for each aspirate is 0.5 ml, divide 0.5 by 4 to obtain 0.13. Therefore, add 0.13 mL of freeze media four (4) times to the specimen.
14. Gently rock the specimen(s) with the freezing media for 5 minutes on an aliquot mixer.
15. Repeat steps 13 and 14 three (3) times or until the volume of freezing media added is equal to the specimen volume in step 9.
16. Centrifuge the tube labeled "supernatant" for 5 minutes at 1600 rpm.
17. Remove the supernatant from the "supernatant" tube. Resuspend the pellet with 0.5 ml of HTF (sperm washing media). Mix gently. Remove one drop for a semen analysis.
    Note: If no motile sperm are found in the "supernatant" tube, do not start freezing. If motile sperm are found, freeze as in steps 13 to 15.

    During the mixing steps above, use appropriately colored cryomarkers to label 2 ml cryovials and canes. The volume added to the vial should not exceed 1.8 ml per vial.
18. Label an additional 1.0 ml cryovial for each specimen. This will contain a leftover aliquot of the cryodiluted specimen to be assessed for cryosurvival 24 hours after freezing in LN_2.
19. A visual inspection should therefore be made of the cryodiluted specimen for motility. A manual motility can be done using a Microcell chamber or a Makler chamber and a Nikon two-phase microscope. The percent motility should be documented on the cryopreservation worksheet under cryodilution motility.
20. Distribute the well-mixed, cryodiluted semen into pre-labeled vials using a 1 or 2 ml sterile serological pipette. Add at least 0.2 ml to the smaller 1.0 ml cryovial.
21. Place labeled vials into a plastic freezing rack along with canes and cryosleeves and place in a -20° C freezer for 8 minutes. Do not open the freezer under any circumstances during this incubation.
    Note: Exposure to freezing conditions should occur within 1.5 hours of specimen collection.
22. After the 8-minute incubation, remove the rack and canes from the 20° C freezer. Place a maximum of 2 cryovials into bottom slots of canes upside down. Put into cryosleeves.
23. After a minimum 2 hours incubation in liquid nitrogen vapors, turn cases upside down, immersing them into liquid nitrogen.
24. After a minimum of 24 hours in liquid nitrogen, thaw the aliquot in the 1.0 ml cryovial.
25. Using cryogloves, remove cane containing the vial and snap it out. Loosen the cap and place in the 37° C incubator for 20 minutes.
26. Mix the vial well and analyze manually.
27. Record the cryosurvival rate in the appropriate area of the cryopreservation worksheet.
28. Assess cryosurvival using the formula:
    % motility of post-thaw specimen
    % motility of pre-freeze specimen

Procedure (Semen from Ejaculate)

Technical note: Sterile techniques should be used throughout specimen processing. Gloves are mandatory for all procedures dealing with body fluids. Latex, however, may be toxic to sperm. Therefore, care should be taken to prevent contamination of the specimen with latex or talc. Vinyl gloves are an available alternative.

1. Cryopreservation worksheet labeled with the patient name, diagnosis and LN_2 container and rack. If there is more than one specimen, have a worksheet for each specimen.
2. Using sterile technique, measure the volume of each specimen and record on the appropriate cryopreservation worksheet.

3. Perform the following on the semen sample: Regular manual semen analysis, Endtz test (leukocytospermia test), eosin-nigrosin stain procedure, Tygerberg's strict criteria for morphology evaluation..
4. Within 1 hour of specimen collection, add an aliquot of freezing medium equal to 25% of the resuspended aspirate volume to the centrifuge tube with a sterile pipette.
5. Note: Since the resuspended volume for each aspirate is 0.5 ml, divide 0.5 by 4 to obtain 0.13. Therefore, add 0.13 ml of freeze media four (4) times to the specimen.
6. Gently rock the specimen(s) with the freezing media for 5 minutes on an aliquot mixer.
7. Repeat step 4 three (3) times or until the volume of freezing media added is equal to the specimen volume in step 2.
8. During the mixing steps above, use appropriately colored cryomarkers to label 2 ml cryovials and canes. The volume added to the vial should not exceed 1.8 ml per vial.
9. Label an additional 1.0 ml cryovial for each specimen. These will container a leftover aliquot of the cryodiluted specimen to be assessed for cryosyrvival 24 hours after freezing in LN_2.
10. A visual inspection should therefore be made of the cryodiluted specimen for motility. A manual motility can be done using a Microcell chamber or a Makler chamber and a Nikon two-phase microscope. The percent motility should be documented on the cryopreservation worksheet under cryodilution motility.
11. Distribute the well-mixed, cryodiluted semen into pre-labeled vials using a 1 or 2 ml sterile serological pipette. Add at least 0.2 ml to the smaller 1.0 ml cryovial.
12. Place labeled vials into a plastic freezing rack along with canes and cryosleeves and place in a –20° C freezer for 8 minutes. Do not open the freezer under any circumstances during this incubation.
13. Note: Exposure to freezing conditions should occur within 1.5 hours of specimen collection.
14. After the 8-minute incubation, remove the rack and canes from the 20° C freezer. Place a maximum of 2 cryovials into bottom slots of canes upside down. Put into cryosleeves.
15. After a minimum 2 hours incubation in liquid nitrogen vapors, turn cases upside down, immerging them into liquid nitrogen.
16. After a minimum of 24 hours in liquid nitrogen, thaw the aliquot in the 1.0 ml cryovial.
17. Using cryogloves, remove cane containing the vial and snap it out. Loosen the cap and place in the 37°C incubator for 20 minutes.
18. Mix the vial well and analyze manually.
19. Record the cryosurvival rate in the appropriate area of the cryopreservation worksheet.
20. Assess cryosurvival using the formula:
    % motility of post-thaw specimen
    % motility of pre-freeze specimen.

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