Recovery, preparation, storage and utilization of spermatozoa for fertility preservation in cancer patients and sub-fertile men

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Abstract Sperm cryopreservation is an important part of an infertility program for patients undergoing infertility treatments, fertility assurance for vasectomy cases, and for fertility preservation due to cancer or other medical conditions. With recent developments in reproductive technology, even men with severely impaired sperm parameters can benefit from cryopreservation as procedures such as intra-cytoplasmic sperm injection (ICSI) require only a few sperm to achieve fertilization and pregnancy. The increasing success of cancer treatment and concerted efforts to ensure quality of life after successful treatment have placed great emphasis on the need to preserve the reproductive capability of young men. It is a highly effective method of protecting male fertility potential, and involves collection, freezing, and long-term storage of sperm. Based on the etiological condition of the patients, sperm can be collected by ejaculation or by surgical retrieval from epididymis or testes. The option to bank sperm should be offered systematically to all patients who may benefit. However, this is not a standard of practice yet; it may be overlooked due to lack of physician awareness regarding the need for fertility preservation and the effectiveness of this option, and/or overestimating the limitations of poor baseline sperm quality leading physician to view cryopreservation as futile. Failure to offer cryopreservation ignores the only possible reproductive option available to certain patients.

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Sperm banking & cryopreservation
Cryopreservation of human semen is an important procedure used regularly for different purposes, including donor insemination and the preservation of gametes in patients undergoing gonadotoxic treatment. Conception can be difficult even for some fertile couples for a variety of reasons and cryopreservation may be beneficial for them. Sperm banking allows men to protect their future fertility. There is a limited use of semen cryopreservation by physicians in the majority of IVF programs. This leads to an inability to improve care for patients that could benefit from this technology (AbdelHafez et al. 2009). Physicians have certain doubts about the justification of banking sperm before chemotherapy. Few patients (less than 5-10%) who bank sperm before cancer treatment return for infertility treatments, and about half of these patients have successful outcomes. Recovery of spermatogenesis, death, and anxiety regarding ART, financial considerations, and uncertainty about long term prognosis are just a few reasons why patients who cryopreserve do not return for infertility treatment (Houvitz et al. 2008). Men suffering with cancer, azoospermia, and other infertility problems have the option to cryopreserve their sperm. However, cryopreservation has the ability to impair sperm motility, vitality, and acrosome integrity (Esteves et al. 2000a) . There is continuous research involving improvement of the various methods of cryopreservation and cryoprotectants.

A. Relevance to modern medicine/ and today’s world
Advanced assisted reproductive techniques help millions of people suffering from sexual dysfunction, cancer and those undergoing gonadal surgery. These techniques have
given these people a chance to realize their fertility potential. The sperm banks provide extended storage that allows sufficient time to perform screening on the donor. The developments made in intra-cytoplasmic sperm injection (ICSI) and gamete isolation and maturation have promoted interest in cryopreservation of gonadal tissue which appears highly promising for fertility treatments. Cryopreservation has numerous advantages but also significant challenges make it an active area of research (Bagchi et al. 2008). The changing attitude towards sexuality has achieved a wider social phenomenon that has changed the behavior as well as ideas concerning reproduction and associated techniques (Mori 2008).

B. Need for increased awareness by Oncologists:

Oncologists need to know about the regional availability of gamete cryopreservation facilities. Physicians also need to be willing to discuss the issue of cryopreservation with the patient and his family. In the era of ICSI when only a few sperm are needed to achieve fertilization and pregnancy, even men with severely impaired sperm parameters will benefit from sperm cryopreservation and should be encouraged to do so (Houvitz et al. 2008). There is limited use of cryopreservation by urologists and gynaecologists in the majority of IVF programs. There is a lack of information regarding the effectiveness of gamete cryopreservation and a lack of agreement on the best universal method. Unfortunately, this is a missed opportunity to improve care for patients that could benefit from this technology.

C. Need for increased awareness among patients

There is a lack of education/counselling by the health care professionals (Hallak et al. 1999a). It is of crucial importance that all newly diagnosed male cancer patients be advised to cryopreserve their sperm at the earliest stage and most importantly before starting treatment. Although many cancer patients have poor pre-treatment semen quality, most have suitable sperm for freezing with good expectations for sperm survival. All young males 12 years of age or older should be offered the opportunity to bank their sperm prior to administration of any treatment which likely have adverse effect on the spermatogenesis process (Bonetti et al. 2009). Semen cryopreservation should be performed before cancer treatment begins, and it is preferable that multiple samples are preserved. All males of reproductive age should consider banking semen samples before undergoing any type of chemotherapy or radiation therapy, and physicians should always provide them with the education they need to decide for or against cryopreservation.

Sperm banking can be a difficult subject to discuss with young patients and their parents because of sensitive topics such as developing sexuality, the grief associated with facing infertility as a side effect, and masturbation as a means of collecting a sample, but it is still very important to preserve the reproductive future of the patient if possible (Menon et al. 2009a). This study suggested that the majority of physicians and about half of the patients preferred to have initial discussions about sperm banking without the patient’s parents present (de Vries et al. 2009). Semen cryopreservation is the standard of care for these individuals. Failure to offer this option ignores the patient’s only reproductive option (Houvitz et al. 2008).

Indications of sperm banking

A. Couples who can benefit from sperm banking

Couples undergoing fertility treatment can benefit from cryopreservation if the male partner is not available at the time of the ovulation process. The success rates with cryopreserved sperm for intra-uterine insemination (IUI) and ICSI cycles are similar to those with fresh sperm. The recommendation is to have the male partners with oligoasthenozoospermia to bank multiple samples so that the samples can be used for repeat cycles in case of failed cycles of IUI or ICSI.

i. Absent male partner

Fertile couples may use sperm cryopreservation for a number of reasons. If either the male or female partner is often absent, for example, when travelling for business, the couple may find conception difficult. It may be hard for the couple to
coincide intercourse with ovulation. Cryopreservation of sperm may be convenient for the couple, allowing the woman to receive the male’s sperm in a clinical setting when she is ovulating.

**ii. Participating in ART**
About 12% of couples are unable to conceive after one year of unprotected intercourse and are therefore considered infertile (Eisenberg et al. 2009). About 30-40% of these couples cannot conceive because the male partner has infertility issues, and 10% of male factor infertility is caused by azoospermia. In the most severe cases of male infertility, couples may decide to use a sperm donor (AbdelHafez et al. 2009).

**B. Cancer patients**
The various gonadotoxic treatments can have differential effects on spermatogenesis and sperm quality in patients with diverse types of cancers. A European study observed that the incidence of testicular cancer has doubled in the past 20 years, and the incidence of germline testicular cancers increased drastically from 1967 to 1987, as well as 1987 to 1996 (Lacerda et al. 2009). Between 15-30% of male patients undergoing gonadotoxic treatments do not regain their fertility (Menon et al. 2009a). Approximately 90% of men with testicular cancer ranked fertility as an important issue after cancer treatment (Bonetti et al. 2009). Both male and female cancer survivors report a large degree of stress regarding their own ability to reproduce (de Vries et al. 2009), which can have a high impact on one’s quality of life. Each year, approximately 1.3 million patients are diagnosed with cancer in the United States, with an average 5-year survival rate of 60%, resulting in about 9.8 million cancer survivors. Today, the cure rate for testicular cancer, Hodgkin’s disease, lymphoma, and leukaemia can be as high as 90% (Bonetti et al. 2009).

Patients receiving radiotherapy are at high risk for developing infertility, and cancer surgery can reduce sperm concentration and cause erectile dysfunction or dry ejaculation. Full recovery can be achieved in the majority of these patients; so many recent efforts have been concentrated on reducing the morbidity associated with gonadotoxic treatments (Hallak et al. 1999b). Hodgkin’s disease, testicular cancer, leukaemia, and non-Hodgkin’s lymphoma are the most common malignancies seen in the male reproductive-age group (Hourvitz et al. 2008). Analysis of covariance of semen parameters showed the greatest impairment in prostate cancer patients. Testicular cancer patients had relatively lower sperm counts but relatively good motility. Men with lymphomas and other systemic (non-reproductive) cancers have relatively normal semen parameters. Testicular cancer (Audrins et al. 1999; Berthelsen 1984; Botchan et al. 1997b) and lymphoma (Botchan et al. 1997a, Chapman et al. 1981) have been associated with impaired sperm quality.

Padron et al. (1997) have shown similar semen quality in men with Hodgkin’s disease, leukaemia, and testicular cancer. Several of the most common malignancies in men of reproductive age have good long-term survival rates, for example, testicular cancer and Hodgkin’s disease (Howell and Shalet 2001). However, antineoplastic therapy is associated with significant morbidity, and testicular dysfunction is among the most common long-term side effects of cytotoxic chemotherapy in men.

The degree to which testicular function is affected is dose and agent dependent (Palermo et al. 1992). Alkylation agents (e.g., cyclophosphamide and busulfan) and ionizing radiation frequently induce azoospermia, rendering the patient infertile. Another major reason to freeze sperm before treatment is the concern for potential chromosomal aberrations in sperm exposed to chemotherapy (Lass et al. 2001). Although no increase in malformation rate has been reported in children born to patients who have had chemotherapy or radiotherapy, the available data and follow-up are still limited and these children should be closely monitored. Semen parameters in patients with lymphomas and all other systemic malignancies were associated with better semen profile than in patients with testicular or prostate carcinoma (Hourvitz et al. 2008). After receiving cancer treatment, 77.8% of patients became azoospermic,
which emphasizes the importance of early cryopreservation.

Infertility is a major sequel of cancer and/or its therapy. The quality of spermatozoa in men diagnosed with cancer is suboptimal, even prior to the initiation of chemo/radiotherapy. Lower sperm quality in patients with testicular malignancy can be explained by the fact that genital tumours exert local negative effects. Sperm concentration was significantly lower in patients with testicular malignancy compared to those with systemic malignancy and healthy proven fertile donors \((p<0.001, \ p<0.05, \) respectively). Motility was found to be significantly lower in patients with testicular and systemic malignancy compared to healthy proven fertile donors \((p<0.001: \) Williams et al. 2009). In a study with 2680 subjects the average age of men with testicular cancer was 29.9 years prior to therapy that signifies the importance of cryopreservation in this target population. Men with testicular cancer usually have a lower sperm count and motility due to the tumour growth. Infertility is clearly associated with therapy, and chemotherapy doses (Schrader et al. 2001). The majority of patients develop azoospermia 12 weeks after beginning of chemotherapy.

Chemotherapy targets cells outside the G0 phase, destroying proliferating spermatogonias (Bonetti et al. 2009). The majority of chemotherapy patients develop azoospermia during treatment and it is difficult to predict if and when spermatogenesis will recover. This tends to be dose dependent; while patients receiving low doses of these agents may recover spermatogenesis within 12 weeks of completing chemotherapy, more than 50% of patients receive high doses which might contribute to the 15-30% of all patients who remain sterile in the long term. It is estimated that up to 15% of male patients will already be azoospermic before undergoing any type of treatment. Semen cryopreservation should be performed before cancer treatment begins, and it is preferable that multiple samples are preserved (Bonetti et al. 2009). Patients who are most at risk are those who undergo a treatment modality encompassing successive multiple toxicity, like bone marrow transplantation (de Vries et al. 2009).

Currently there is a high incidence of malignant testicular tumors, lymphatic, and hematopoietic tissue origin tumors among men of reproductive age (Crha et al. 2009). The high remission rates have caused an increase in recent efforts to reduce the morbidity associated with gonadotoxic treatments (Hallak et al. 1999b). Radiotherapy patients are at high risk for developing infertility, and cancer surgery can reduce sperm concentration, cause erectile dysfunction or dry ejaculation (Bonetti et al. 2009).

C. Before prostate/testicular surgery/biopsy

The testicular tissue can be cryopreserved at the time of surgery for future use in treatment of infertility. Mature spermatozoa extracted from frozen thawed testicular biopsies have been used in ICSI (Bagchi et al. 2008). A study was conducted in patients with prostate, bladder or kidney cancer to find out whether these cancers were an independent predictor of the patients wish for sperm banking before any surgical or nonsurgical therapy which may have a potential adverse effect on male fertility. The study findings showed that having the therapy non-germ cell urological cancer (NGCUC) was independently associated with the wish for cryopreservation (Salonia et al. 2009).

D. Severe oligozoospermia or low quality sperm

Azoospermia may occur in some healthy men, and natural fertilization may be impossible for them. However, cryopreservation and IVF may allow these men to father children. The recovery of viable sperms is comparatively low with less than 60% of cells retaining motility on thawing. This loss of viability becomes a major issue in case of oligozoospermic samples. For these cases there is a growing need to store low numbers of sperm by developing improved freezing techniques. The use of frozen-thawed testicular biopsies in ICSI is very helpful in patients with obstructive azoospermia with normal spermatogenesis. (Bagchi et al. 2008).
E. Ejaculatory dysfunction
Erectile dysfunction and anxiety issues may prevent a couple from successfully conceiving, so cryopreservation and IUI can allow these couples to conceive in a clinical setting.

F. Prior to vasectomy
Indications for sperm banking other than for infertile men are collectively included under the term “special storage” which includes storage as insurance before vasectomy (pre-vasectomy storage) and storage before treatment that could damage sperm production, such as cytotoxic therapy or radiation therapy (medical storage) (Audrins et al. 1999). A patient undergoing vasectomy is offered sperm banking prior to vasectomy. The issue to consider is sperm freezing and storage (cryopreservation) as a reasonable alternative to vasectomy reversal in the unlikely event that the individual wishes to father children in the future. The reversal of vasectomy procedure is expensive and requires hospitalization and utilizes time. It is therefore far more cost-effective to cryopreserve semen before the vasectomy, which then can be stored indefinitely at little cost (Audrins et al. 1999). The availability of ICSI technology has made cryopreservation of sperm during vasectomy reversals possible.

G. Men with high-risk occupations
The exposure to environmental factors air pollution, pesticides, phthalates and PCB has been reported to affect semen quality (Jurewicz et al. 2009). The occupations related to the use of chemicals, pesticides, etc are considered high-risk and men engaged in these high risk work areas should be offered sperm banking as an option.

H. Issues related to sperm donation
Cryopreservation is an important tool that preserves fertility for men with cancer and is akin to providing these individuals “fertility insurance”. Patients are concerned about the quality of life issues related to cancer treatment and want to preserve the sperm for future fertility. However the cancer patients may have difficulty in providing semen samples due to privacy issues or ejaculatory problems or if they are an adolescent. The various issues related to sperm donation are discussed below.

i. Privacy
Sperm donation raises a number of ethical questions for the sperm donors. Since decades there have been questions raised on anonymous sperm donation. Donors usually have no legal or financial responsibility for the child. But there are exceptions to the case as seen from time to time in many legal situations. The ethical dilemma arises about the responsibility of the child and also in certain cases where there is a need to find out about genetic conditions.

ii. Difficulty providing semen sample
Reasons could be anejaculation, psychogenic ejaculation, spinal cord injury, retrograde-ejaculation etc. The prevalence of premature ejaculation varies from 8 to 30% for all age groups (Corona et al. 2010).

I. Cryptorchidism and genetic disorders
With the help of cryopreservation germ cells are harvested at an earlier stage of development for further maturation in vitro. Over the past decade, developments in microsurgical techniques and advances in ART allowed more than 50% of patients with Klinefelter’s syndrome (KS) to have their own children through the combination of microsurgical testicular sperm extraction (TESE) and use of freshly retrieved sperm for IVF. However, this technique requires an expensive surgical procedure and hormonal stimulation of a female partner despite the uncertainty of sperm recovery from testis.

Surgical sperm retrieval
Surgical sperm retrieval techniques are applied for azoospermia cases which have either obstructive or non-obstructive. Percutaneous epididymal sperm aspiration (PESA) and microsurgical epididymal sperm aspiration (MESA) are used for obstructive case, while testicular sperm aspiration (TESA) and testicular sperm extraction (TESE) are applied for non-obstructive cases.

A. Epididymal sperm retrieval techniques
Epididymal sperm can be retrieved by two methods of percutaneous epididymal sperm...
aspiration and microsurgical epididymal sperm aspiration.

i. Percutaneous epididymal sperm aspiration (PESA)
PESA is performed without surgical scrotal exploration. It does not require an operating microscope or expertise in microsurgery. To perform, a butterfly needle (attached to a 10-20 ml syringe) is inserted into the caput epididymis and tip of the needle is gradually moved within the epididymis until clear or opalescent fluid seen in the needle tubing. The procedure is repeated until adequate amount of epididymal fluid is retrieved (Craft et al. 1995). The aspirate is then flushed into a sterile tube before sending to lab for evaluation and processing.

ii. Microsurgical epididymal sperm aspiration (MESA)
It is performed under anesthesia. After opening the tunica vaginalis and exposing the epididymis, single epididymal tubules are identified under operating microscope. The tubules are then punctured and the effluent aspirated into an aspiration device (syringe). Sequential aspirations are performed until optimal quality sperm are obtained (Schlegel et al. 1994; Tournaye et al. 1994). Best quality sperm are found in the proximal epididymis close to the testis. The aspirates are then sent to lab for evaluation and processing.

B. Testicular sperm retrieval techniques
It has been shown that mature spermatozoa can be found in only part of the testes of non-obstructive azospermic man. In examining the testes of infertile men, Levin (1979) found a mixed histological pattern of germinal cell aplasia and minute foci (local) spermatogenesis. A similar histology of side by side presence of different patterns of focal spermatogenesis and Sertoli-cell syndrome was observed in non-obstructive azospermic men (Devroye et al. 1995; Gil-Salom et al. 1995). Therefore, the performance of multiple focal testicular sperm retrieval was recommended to ensure the presence of sperm in testicular sample (Hauser et al. 1998). The most common methods for retrieving the testicular sperm are testicular sperm aspiration (TESA: needle/fine needle aspiration), and open testicular biopsy (testicular sperm extraction TESE). Recently, however optical loupe magnification TESE (microdissection TESE) was applied in retrieving sperm from non-obstructive azoospermia cases (Mulhall et al. 2005). All procedures are performed under anesthesia (general, or local). Generally, the scrotum is opened via a median raphe incision and all layers are cut until there is full exposure of the testis.

i. Testicular Needle aspiration
Testicular needle aspiration was initially used for diagnostic purposes and later to establish the likelihood of sperm retrieval for testicular ICSI cases on the day of oocyte retrieval (Fasouliotis et al. 2002; Turek et al. 1999). Different techniques have been described with variations in the needle diameter and the number of testicular punctures (Donoso et al. 2007). Lewin et al. (1996) successfully performed testicular fine needle aspiration (TFNA) to retrieve sperm from a patient with maturation arrest and elevated gonadotrophins.

Testicular needle aspiration is usually performed in 3 different locations: in the centre of the testis and in the upper and lower poles; with the aim of aspirating testicular tissue from the depth of the testis. Upon exposure of testes, the needle is inserted into the centre of the testis and negative pressure is applied. While maintaining negative pressure, the needle is partially withdrawn and inserted again at different angles. The sampling is performed using a needle biopsy gun that enabled a controlled and accurate sampling as well as the creation and maintenance of a substantial negative pressure during the procedure. A separate 20-ml syringe containing 0.5 ml of culture medium and an 18 gauge needle are used for each sample. The aspirated samples are transferred immediately to the laboratory for sperm search and isolation (Hauser et al. 2006).

ii. Testicular sperm extraction (TESE)
For TESE, the tunica albuginea is incised transversely at 3 locations of centre, upper, and lower poles in each testis. The testis is then gently squeezed and the protruding tissues are excised, each weighing approximately 50 mg. The biopsy material is placed in culture medium and transferred
immediately to the laboratory for sperm cell isolation (Hauser et al. 2006).

Microdissection TESE is an advanced version of TESE that applies microsurgical techniques for sperm retrieval. In this technique, individual seminiferous tubules can be seen under the surgical microscope allowing the identification of active spermatogenesis sites (Schlegel 1999). This strategy could facilitate the removal of smaller amounts of testicular tissue, which becomes crucial in testicular atrophy cases and minimize the chance of vascular injury as vascular regions of the tunica albuginea during the opening could be identified (Donoso et al. 2007).

C. Laboratory preparation of TESE and TESA sample

Upon receipt of the biopsy tissues, they are shredded into small pieces with sterile 25 gauge needle or fine scissors. The presence of spermatozoa is assessed using an inverted microscope. The effluents as well as the shredded biopsy tissue can be centrifuged and the pellet re-suspended in culture medium, incubated in few droplets under mineral oil or ~1 h prior to selection of spermatozoa. Motile spermatozoa usually migrate to the edge of the drop (Craft and Tsirigotis 1995; Nijs et al. 1996). Alternatively, original shredded specimen can be incubated in droplets under the oil without centrifugation prior to selection of sperm. The selected spermatozoa can be washed in PVP droplet before proceeding to injection into oocytes.

FACTORS PREVENTING INDIVIDUALS FROM SPERM BANKING

A qualitative study on 20 cancer survivors and 18 health care professionals conducted in-depth interviews to examine their perspectives on factors that facilitate or hinder sperm banking. The data collected was analyzed using a mixed approach and a three-step process of data reduction, data display and conclusion drawing and verification. The study reports several factors that have an impact on Sperm Banking. The findings in the study recommended that effective promotion of sperm banking involves adequate communication regarding the severity and risk of infertility, assessment of the importance of having children, emphasis on the benefits of banking and addressing possible obstacles such as cost, misconceptions or cultural and other factors (Achille et al. 2006).

It is very surprising to see that in spite of a high risk of developing infertility and the available option of sperm banking, still very few patients go ahead with banking of their sperm (Achille et al. 2006).

A. Priority

Sperm banking is not usually a priority for patients that have already completed their family, for those that do not want to have children, and for those patients who think that they maybe delaying their cancer treatment by selecting the option of sperm banking. Some of the patients are too young to understand the impact of gonadotoxic therapy or surgery (Achille et al. 2006).

B. Cost

A presumed high cost for the procedure is a major factor inhibiting patients from electing to bank sperm (Bonetti et al. 2009). The cost of banking of sperm is not covered by the insurance companies and may be a very important factor for rejection of banking by low income patients.

C. Time interval

The urgency to start chemotherapy as soon as cancer diagnosed is one of the major factors that prevents a number of patients to go for sperm banking. The risk for infertility following treatment for cancer is higher in adolescent males. Therefore treatment exposures for them should be carefully assessed before initiating therapy. Fertility preservation by sperm banking should be part of the initial treatment discussion with family and the patient (Hobbie et al. 2009).

D. Lack of information

A study was done to survey oncologists in different practice settings to determine their knowledge, attitudes, and practices regarding referring patients to bank sperm before cancer treatment. The study found that ninety-one percent of respondents agreed that sperm banking should be offered to all men at risk of infertility as a result of cancer treatment. Forty-eight percent of physicians did not mention the option to less than a quarter of eligible men.
The reasons for lack of discussion and non-dissemination of information by physicians on sperm banking were lack of time for the discussion, perceived high cost, and lack of convenient facilities. HIV-positive men, patients with a poor prognosis, or having aggressive tumors would be less likely to be offered sperm banking (Schover et al. 2002). The staff at the oncologist’s office can educate the patient thus reducing the amount of physician time spent in explaining sperm banking. Self help books can be provided to patients that discuss cancer, male infertility, and sperm banking. The sperm banks are very easily found on various websites like www.sperm-banks.com. The availability of home kits for sperm collection and express shipping to the sperm bank makes it more convenient for the patients to collect the sample in the privacy of their homes (Schover et al. 2002). Patients who have been properly advised about the high risk of infertility associated with chemotherapy regimens for testicular cancer or Hodgkin’s disease are more likely to utilize sperm banking (Achille et al. 2006; Schover et al. 2002).

Another study observed that only 47% of oncologists always or often refer their patients to an REI or infertility specialist. The knowledge about banking is high among oncologists, but still there are a few questions regarding fertility and cancer that are not well answered such as the ability to determine an individual’s risk. Future efforts are required to include additional training for health care providers regarding this. The limited amount of time to discuss the cancer diagnosis and treatment plan as well as to deal with the psychosocial issues of a newly diagnosed patient is a major barrier. Therefore it is essential that nurses or social workers be trained to initiate discussions about sperm banking. (Quinn et al. 2009).

**E. Religious or ethical concerns about sperm banking**

The practice of sperm donation is opposed by many religions and hence the option of sperm banking is not accepted by some infertile couples due to religious reasons. There is strong influence exerted on the civil authorities in the field of reproduction and artificial reproduction by many religious authorities.

Most countries require a mandatory formal written consent for sperm donation. Many ethical issues are raised regarding the rights and obligations of the mother, the husband, and the child. Ethical questions arise about duties and rights of the parent toward a child who is not their biological offspring (Meirow and Schenker 1997).

**F. Psychosocial issues with sperm banking – anxiety and emotional stress**

It is a life crisis situation for many young individuals affected by cancer. The cancer diagnosis and the threat of infertility both cause a tremendous stress on these individuals (Tschudin and Bitzer 2009). Schover et al. (1999) have elucidated several causative factors for psychological stress in cancer patients such as distress related to future infertility, concern about quality of life issues, patients with distress due to inheritable cancer and lack of knowledge regarding various risk factors associated with the gonadotoxic treatment (Schover 1999; Tschudin and Bitzer 2009). These factors need to be acknowledged by health care professionals and utilized in proper care and treatment of these patients.

**Screening of client depositors prior to sperm banking**

Both male and female cancer survivors report a large degree of stress regarding their ability to reproduce, and its high impact on quality of life (de Vries et al. 2009). Approximately 90% of men with testicular cancer ranked fertility as an important issue after cancer treatment (Bonetti et al. 2009). 15-30% of male patients undergoing gonadotoxic treatments do not regain fertility later (Menon et al. 2009b), so it is crucial to discuss sperm banking with all males of reproductive age. Physicians need to be aware of the availability of gamete cryopreservation facilities and be willing to discuss this issue with patients. Surveys show that the lack of timely information is the most common reason for not banking sperm (de Vries et al. 2009).

Screening is done in order to reduce the risk of passing on sexually transmissible infections through insemination. The patient is tested for sexually transmissible infections. Some banks also get an HIV risk
assessments; a personal and family health history form; a medical examination (which must include a genital examination for indications of sexually transmitted infections); and blood testing to identify blood type and Rh factor. These examinations must be completed no more than twelve months prior to the first storage appointment.

The blood tests include HIV-1/2, HTLV-1/2, hepatitis B, hepatitis C, syphilis, and in some cases CMV. Genetic Testing of the client sperm depositor or sperm donor is required by certain states such as New York.

**TECHNIQUES OF CRYOPRESERVATION**

**A. Preparation and selection mechanisms prior to banking**

Swim up and density gradient are the two widely used methods for sperm wash and processing. Sperm washing techniques separate ejaculated spermatozoa from the seminal environment, and eliminate dead spermatozoa along with exfoliated epithelial cells, cellular debris, leukocytes, and amorphous material (Berger et al. 1985). The swim-up method involves centrifugation of a semen sample into pellets followed by covering with culture medium; allowing the spermatozoa with better motion characteristics and motility to swim up into the culture medium to be selected for cryopreservation (Esteves et al. 2000b). In comparison to untreated specimens, swim up cryopreserved sperm have been shown to exhibit faster velocity and progression, higher percentages of intact acrosomes, increased ability to undergo acrosome reaction and, better performance in the sperm penetration assay after thawing (Esteves et al. 2000b; Russell and Rogers 1987).

Density gradient centrifugation comprised of either continuous (Bolton and Braude 1984) or discontinuous (Pousette et al. 1986). In continuous gradients, there is a gradual increase in density from the top of the gradient to its bottom, whereas there is a clear boundary between each layer in discontinuous gradient method. The ejaculate is placed on top of the density gradient, and centrifuged for 15–30 minutes. During this procedure, highly motile spermatozoa move actively in the direction of the sedimentation gradient and penetrate the boundary quicker than poorly motile or immotile sperm, enriching the soft pellet at the bottom with highly motile sperm.

The most widely used gradient substance for assisted reproduction has been the polyvinylpyrrolidone (PVP) - coated silica particles called Pecoll. It was withdrawn from the market because of the risk of contaminations with endotoxins (Andersen and Grinsted 1997; De Vos et al. 1997; Scott and Smith 1997), possible membrane alteration (Arcidiacono et al. 1983; Strehler et al. 1998) and inflammatory responses induced by the insemination of sperm contaminated with Pecoll.

The replacement products for Percoll® are Nycodenz (Nyegaard & Co., Oslo, Norway), IxaPrep® (Medicult, Copenhagen, Denmark), SilSelect® (FertiPro N.V., Beernem, Belgium), PureSperm® (NidaCon Laboratories AB, Gothenburg, Sweden) or Isolate® (Irvine Scientific, Santa Ana, CA, USA). These replacements were found to produce populations of highly motile spermatozoa with better yields and survival than either swim-up or Percoll® gradients from oligozoospermic and asthenozoospermic semen samples (Gellert-Mortimer et al. 1988; Mortimer 1994). Compared to the swim-up procedure, Percoll® replacement seems to be superior in zona-free hamster egg penetration test (Serafini et al. 1990). Thus, this technique has clearly great potential in the preparation of motile spermatozoa from poor quality semen.

Magnetic-activated cell sorting (MACS) is another preparation technique that has been shown to select motile, viable, morphologically normal spermatozoa displaying higher cryosurvival rates and subsequent fertilization potential (Grunewald et al. 2001; Grunewald et al. 2006; Said et al. 2005). MACS uses annexin microbeads, a phospholipids-binding protein with a high affinity for phosphatidylserine (PS)- an early marker of apoptosis, to label and remove apoptotic spermatozoa (Said et al. 2008). Although this method may offer more advantages over the other techniques by selecting genetically intact, non-apoptotic sperm it is still experimental.
B. Slow Freezing
Slow freezing is based on the principle of dehydration, where equilibration is achieved by combination of low cryoprotectant concentrations and slow rate of cooling, allowing dehydration to occur during cooling. It is the method of choice for cryopreservation of human spermatozoa. In this method, semen samples (raw and washed) are diluted by drop-wise addition of the freezing medium with continuous uniform mixing to a final ratio of 1:1 (volume-to-volume). They are then loaded into straws or transferred into cryovials before exposure to −20 °C for 15-30 minutes, followed by exposure to −79 °C for another 15-30 minutes before placing in liquid nitrogen for storage (WHO 5th Ed.). This slow rate can be achieved by using a programmable freezer or manually in liquid nitrogen vapour.

Various techniques have been proposed for freezing of the sperm for surgically retrieved cases where a few sperm are retrieved. One approach is to inject isolated sperm cells into a hamster zona pellucida which is then placed in a straw between two air bubbles to facilitate location of the cells after thawing. (Cohen et al. 1997; Hsieh et al. 2000). Others suggested the freezing of the minute sample under a layer of paraffin oil with glycerol (Craft and Tsirigotis 1995). Romero et al. (1996) described a frozen “testicular pill” composed of a mixture of some sperm and testicular tissue.

C. Vitrification
Vitrification is preservation at extremely low temperatures without freezing. Freezing involves ice crystal formation, which damages delicate organelles. Vitrification instead involves the formation of a glassy or amorphous solid state which, unlike freezing, is not intrinsically damaging even to the most complicated living systems. In this process the ice formation during cooling is inhibited by high concentration of viscous solutions which produce glass-like state at low temperatures. However, because spermatozoa are of small size and contain little cytoplasm or water they are sensitive to cryoprotectant (Gilmore et al. 1997; Mazur et al. 1981).

Recently a new technique of ice- and cryoprotectant-free cryopreservation (vitrification) was developed by direct plunging of a sperm suspension into liquid nitrogen (Isachenko et al. 2003; Nawroth et al. 2002). After storage, warming is achieved by direct melting of the frozen suspension. This is a simple, straightforward approach preserves the motility and fertilizing ability of the spermatozoa. The improved results over the conventional slow freezing (ice-equilibrium) may be attributed to omitting permeable cryoprotectants, thus preventing the lethal effects of osmotic shock (Isachenko et al. 2004). Vitrification of sperm is relatively new technique and it is not standardized yet to be implemented clinically.

Advantages and disadvantages of various cryoprotectants
Since Polge & Rowson (Polge 1952) first successful report of glycerol as cryoprotectant for bull spermatozoa cryopreservation, there have been many reports on the cryopreservation of spermatozoa of various species including horse (Nishikawa 1975), pig (Pursel and Johnson 1975), sheep (Colas 1975), dog (Seager and Fletcher 1973), rabbit (Fox 1961), and man (Bunge and Sherman 1953). It is the most widely used and successful cryoprotectant for human sperm. A final concentration of 7.5% has been shown to be an optimal concentration of glycerol for freezing solution. Egg yolk on the other hand, which is not a cryoprotectant itself and often used in combination with glycerol, seems to confer improved sperm plasma membrane fluidity, resulting in improvement in cryo-survival (Hallak et al. 2000).

Prins and Weidel 1986 compared eight different cryopreservatives, concluding sperm frozen with egg yolk buffer demonstrated the highest post-thaw survival. Mahadevan and Trounson (1983) developed a modified Tyrode’s medium containing 7.5% glycerol referred to as Human Sperm Preservation Medium (HSPM), demonstrating high pregnancy rate in comparison with egg yolk-citrate-glycerol medium with no difference in post-thaw motility and viability.

In comparing three cryopreservatives of TEST yolk, glycerol, and HSPM, Centola et
e. (1992) demonstrated that HSPM had the best recovery in regards to concentration and motility, while glycerol had better recovery rate of progressive velocity than TEST yolk. These data suggest that HSPM is a superior cryopreservative based on post-thaw recovery of motile sperm, confirming earlier report of Mahadevan and Trounson (1983).

**The effect of cryopreservation on sperm characteristics**

**A. DNA stability**

Semen cryopreservation has been reported to induce DNA damage. The exact mechanism of the effect on DNA integrity is not known. It has been proposed to be induced by causing a rise in oxidative stress in the semen. Stimulation of caspases and apoptotic mechanisms has been proposed by some literature reports. This mechanism has been refuted by other reports where addition of caspase inhibitors to the cryopreservation medium did not prevent apoptosis. The addition of the caspase inhibitors had no significant effect on the post-thaw motility.

Cryopreservation can cause and exacerbate DNA fragmentation in spermatozoa (Thomson et al. 2009). Some studies indicate that cryopreservation can increase inappropriate chromatin condensation in human sperm (Yildiz et al. 2007). DNA integrity can be determined by several methods. These methods include TUNEL, a direct measure of DNA damage, and Comet, an electrophoresis assay that evaluates how well DNA is packed within the nucleus (Bakos et al. 2008; Yildiz et al. 2007).

Sperm chromatic structure assay (SCSA) is another method which measures the extent of DNA denaturation (Kobayashi et al. 2001). This method allows one to decide whether the DNA within a sperm cell is structurally intact and normal or abnormal. Oxidative damage caused by reactive oxygen species is suspected to play a role in cryo-injury to sperm DNA. Antioxidants such as genistein (a plant-derived phyto-estrogen) and those found in native semen protect sperm from oxidative stress and lipid peroxidation, and thereby reduce DNA fragmentation (Thomson et al. 2009). Increase in the activation of the intrinsic apoptotic cascade might result from insults to structural integrity of sperm that occurs during cryopreservation, but are most likely not responsible for DNA damage in sperm.

**B. Acrosomal integrity**

Zona-free hamster oocyte (ZFHO) penetration assay evaluates the ability of a sperm population to capacitate, acrosome react, bind and penetrate the membrane of an oocyte lacking a zona pellucida. When acrosomal stimulants such as TEST-yolk buffer are used to treat the sperm, the ZFHO penetration assay correlates well with IVF success. Poor results are expressed when low percentage of oocytes is penetrated. Poor results indicate that a male patient is unlikely to impregnate his partner without the use of ICSI.

The acrosome is an organelle which facilitates the passage of the spermatozoa through the zona pellucida of the oocyte just prior to fertilization. Studies have shown that acrosome is affected by cryopreservation more severely than any other organelle (Ozkavukcu et al. 2008). Acrosome is characterized by a fragile membrane which is susceptible to changes in osmolarity and physical or chemical conditions, which are extreme in case of cryopreservation of gametes. Cryopreservation of sperm can lead to acrosomal abnormalities, such as cracks or peelings, due to low temperatures which can increase cytoplasmic Ca\(^{2+}\) levels, capacitation-like reactions, ionic leakage, and exocytosis of acrosomal content (Ozkavukcu et al. 2008).

**C. Motility and viability**

The cryopreservation survival rate (%) is calculated by dividing post-thaw motility by pre-freeze motility and multiplied by 100. A study reports on ART outcomes which analyzed 118 male cancer survivors undergoing 169 IVF-ICSI cycles (Hourvitz et al. 2008). Analysis of covariance, showed that type of cancer was significantly associated with post-thaw semen concentration (P<0.0001) and with total motile sperm count (P<0.005). All semen parameters were found significantly better in case of lymphomas as compared to all other systemic malignancies.

Furthermore, the effect of cancer type on post-thaw semen parameters was not
influenced by the cryopreservation storage interval and the age of the patient at diagnosis. Post-thaw motility is reduced in cryopreserved semen samples, and depends on the pre-freeze motility of the semen sample (Ozkavukcu et al. 2008). Most studies indicate that viability and motility, the more important sperm parameters determining independent fertilization capacity, are reduced by 50% between the pre-freeze and post-thaw semen samples. It is likely that much (but not all) of the reduced motility is a direct result of reduced viability caused by damage to cell membrane of the sperm when they are frozen. Organelle defects may also have some role in the loss of motility. In addition, reactive oxygen species can be formed during both freezing and thawing processes, leading to decreased motility through peroxidation of the plasma lipid membrane. However, seminal plasma contains innate antioxidants. This provides a rationale for using neat semen during freezing (Smith et al. 1996).

D. Fertilization capacity
There was no correlation reported between cancer type and outcomes such as pregnancy or delivery rates (Houri vitz et al. 2008). Both viability and motility decrease with cryopreservation in samples from both healthy and ill individuals. Sperm morphology can change with cryopreservation, leading to lower motility, and less potential for fertilization. Fertilization capacity is impacted negatively because capacitation and the acrosome reaction can be inhibited when cryopreservation damages the membrane around the sperm head. Pentoxifylline treatment significantly increases the pre-freezing sperm motility amplitude of the lateral head displacement and the frequency of spontaneous acrosome reactions (Schmidt et al. 2004).

This study found that the positive effects of pentoxifylline may be attributed to its pentoxifylline intercepting ROS and causing increase of intracellular cAMP. Cryopreservation of spermatozoa may have decreased function due to reactive oxidative species, acrosomal dysfunction as well as cellular changes that mark the spermatozoa for apoptosis. Pentoxifylline was found to have antioxidant properties that allow for the stabilization of the acrosomal membrane and maintain the spontaneous acrosomal reaction. Pentoxifylline has beneficial effects of spermatozoa prior to cryopreservation and is proposed to improve the fertilization ability of cryopreserved spermatozoa.

Reasons for non utilisation of banked samples
The 95% of banked sperm samples are not utilized. The common reasons for the non-utilization could be death of the patient, patients changed their mind regarding having more children, and financial constraints. Audrins et al. (1999) showed in a study that very low number of men returned to use their stored sperm samples. In this study 256 men who cryopreserved their semen before undergoing vasectomy, only 4 men returned to use them with the aim of achieving a pregnancy. The interval between storage and use ranged from 11 months to 10 years. And 258 men who cryopreserved their semen before chemotherapy and/or radiation therapy, eighteen men returned to use their stored semen.

The duration of storage since the diagnosis of their disease in the men who continued to store ranged from 1 month to 16 years and the interval between storage and use of the cryopreserved sperm ranged from 1 month to 7 years (Audrins et al. 1999).

Art outcomes with banked semen specimens
Cryopreservation of sperm provides a readily available sperm and allows flexible coordination with female partner to undergo ART treatment cycle. While post-thaw semen quality is often not suitable for IUI, however IVF-ICSI allows even the poorest quality sperm to fertilize oocytes. Kelleher et al. (2001) reported 29 pregnancies in 64 men who underwent 85 ART cycles using frozen sperm (35 IUI, 28 IVF cycles, and 22 IVF-ICSI cycles). The success rates of IVF and ICSI treatments using cryopreserved semen was comparable with fresh semen; with average pregnancy rate of 54% ranging from 33% and 73% (vanCasteren 2008). Currently, there is limited data on ART
treatment outcome of cryopreserved sperm from male cancer survivors (Tournaire et al. 2004). Only 18 of 258 patients who cryopreserved their semen prior to chemotherapy used their frozen sperm for ART resulting in six pregnancies (Audrins et al. 1999).

Hourvitz et al. (2008) described the ART outcome in 118 male cancer survivors undergoing 169 IVF-ICSI cycles, the largest series of couples treated with IVF-ICSI using cryopreserved sperm stored before cancer therapy. They reported clinical pregnancy rate of 56.8%, which is comparable to the average pregnancy rate achieved with other male-factor patients in their center. However, Lass et al. (1998) reported lower pregnancy rates. Only six out of 231 cancer patients with cryopreserved sperm returned for infertility treatment after chemotherapy; two couples achieved pregnancy after IUI, one couple after IVF, and two couples after ICSI. Schmidt et al. (2004) reported a total of 151 ART cycles with clinical pregnancy rate of 14.8% after IUI and 38.6% following ICSI.

A recent review on TESE studies described a mean successful rate of 52% (Colpi et al. 2005). In cases associated with cryptorchidism a significantly higher success rate than unexplained non-obstructive azoospermia (NOA) have been reported (Raman and Schlegel 2003; Vernaeve et al. 2004). Earlier studies comparing ICSI outcomes in fresh vs. frozen-thawed cycles in patients with NOA of all degrees of severity demonstrated that pregnancy rates were similar (Ben-Yosef et al. 1999). Hauser et al. (2005) evaluated the outcome of fresh and frozen TESE in the most difficult subgroup of NOA patients, those with very few, and sometimes exclusively immotile sperm (severe hypospermatogenesis). Their results indicated that pregnancies can be achieved at similar rates as with fresh testicular sperm, even when motility is lost during the cryopreservation process. The initial lack of motility correlated with a significant reduction in fertilization rates and with similar magnitude for both the fresh and frozen-thawed cycles; suggesting that post-thaw loss of motility should be considered differently than primary lack of motility of fresh sperm. Motile sperm cells that lost motility during the freezing-thawing process might still be viable and their fertilizing capacity might be preserved. This capacity may be better than the fertilizing capacity of sperm cells that are primarily immotile when retrieved from the testes (Hauser et al. 2005)

Challenges of sperm banking
There are various ethical, legal and technical challenges associated with the banking of semen samples. Crawshaw et al. (2004) has enumerated 5 challenges of sperm banking dealing with young cancer patients. These are: attributes of professionals, skills of professionals, consent issues, issues relating to the effects of the process on the young men, and follow up services. This study also showed difficulties in building and maintaining an adequate knowledge and skills base in this field, and lack of appropriate training. Challenges also arise in regards to what to be done with stored specimen in the event of the patient's death. Clear and precise instructions regarding the posthumous use of stored gametes or gonadal tissue taken from the patient along with informed consent should be recognized (Robertson 2005). The health care policy makers are also faced with challenges in allocating resources and making sperm banking accessible and affordable to people.

The sperm banks are faced with challenges of cross contamination of samples, as well as providing efficient quality control. It is a requirement for sperm banks to be accredited by regulatory bodies and to be in compliance with the current good tissue practice regulations enforced by them. The adherence to the good tissue practice guidelines will prompt many of the challenges of tissue banking.

Conclusion
Based on the etiological condition of the patients sperm can be collected by ejaculation or by surgical retrieval from epididymis or testes. The sperm can be used as fresh or frozen for latter use depending on the situation. Swim up and density gradients are the two most widely used methods for sperm processing to separate motile sperm from seminal plasma, dead sperm and other cells. Sperm cryopreservation is an important part of an infertility program for patients undergoing
infertility treatments, fertility assurance for vasectomy cases, and for fertility preservation in cancer patients before starting chemotherapy or radiotherapy.

Chemotherapy and radiation therapy or a combination of both has gonadotoxic effects that lead to impairment of sperm quality resulting in infertility. Fertility preservation options should be discussed with patients at an early stage of treatment planning for cancer. The reported usage rates of patients coming back after cancer treatment and utilizing their cryopreserved samples are low varying between 5-10%. Although usage rates are low, at least half of the couples can benefit from getting pregnant with ART techniques utilizing cryopreserved sperm. Continuing research needs to focus on further improving cryopreservation protocols. There is a need for guidance, training and support, and availability of appropriate information system at national level for different health and social care sectors that are involved in treating cancer patients and preserving their fertility by sperm banking. These sectors need to be well organized to face all of the challenges of sperm banking services from diagnosis of the patient to eventual discharge from the health system (Crawshaw et al. 2004). Fertility preservation of younger cancer patients also requires coordinated efforts and attention by oncologists and fertility specialists. They have to be aware of all legal and ethical issues and also what to do with posthumous stored specimen after the death of patient (Robertson 2005).

Reference
Utilization of spermatozoa for fertility preservation


Padron OF, Brackett NL, Sharma RK, Lynne CM, Thomas AJ, Agarwal A. Seminal


