

## Current concepts and future trends in male germ cell cryobanking

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## 1. ABSTRACT

While the idea of freezing human male gametes has been experimented since the late 1700's, it was not until 50 years ago that human sperm were 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. Freezing human male gametes has been tried since the late 1700's. However, it was not until 50 years ago that human sperm was successfully frozen in a way that retained its ability to 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 preserving the ability of sperm to maintain its fertilizing potential.

## 2. INTRODUCTION

The first reported observations of the effects of low temperatures on spermatozoa were performed by Lazaro Spallanzani in 1776, and the first to discuss the possible uses of sperm banks was the Italian Paolo Mantegazza, who wrote the following sentence 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 longterm cryostorage would require the use of temperatures

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lower than  $-130^{\circ}\text{C}$ , the glassy-transition temperature, below which ice-crystal growth is inhibited (4). Consequently, liquid nitrogen ( $-196^{\circ}\text{C}$ ) storage became the standard very early in the history of sperm banking (5).

Nowadays, human semen cryopreservation is an extensively performed routine technique 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.

### 3. GENERAL ASPECTS OF SPERM CRYOPRESERVATION

It is generally accepted that the motility and fertilizing ability of frozen sperm do not correlate each other and sublethal damages occurring within the sperm cell are thought to be responsible for this difference (6). Generally, living cells that undergo cryopreservation are subjected to two major factors 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 in the form of changes in lipid composition and location (7–9). The insults to the sperm membrane are in turn responsible for cell leakage of many intracellular components resulting in reduced sperm metabolic activities. Therefore, cytoplasmic and membrane-bound proteins and enzymes, as well as other components, are eliminated from the sperm cell (10–13). Cryoinjuries also 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). In addition, the freezing process causes disruption of cold-sensitive microtubule containing structures such as the meiotic spindle (23–27). Interestingly, Donnelly *et al.*, demonstrated that sperm frozen unprepared from seminal fluid appears to be more resistant to freezing damage than frozen prepared sperm either by Percoll density centrifugation or a direct swim-up procedure and frozen 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. Progressive motility may be significantly preserved by freezing prepared sperm in seminal plasma, although values are still significantly lower than that of the fresh samples. This is again due to the presence of seminal plasma since human sperm are particularly sensitive to free-radical assault due to their high content of polyunsaturated fatty acids and lack of repair mechanisms (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 maintains motility and DNA integrity after thawing (28).

In general, cryopreservation of human sperm remains an essential tool for the preservation of male

fertility. Freezing of sperm before initiation of treatment provides to patients a type of “fertility insurance” and may allow them to father their own children through the use assisted reproductive techniques (ART). On the other hand, human spermatozoa have unusual cryobiological behaviour and improvements in their survival have not been achieved by the standard approaches of cryobiology. In fact, despite continuous methodological optimization, the process of cryopreservation and thawing leads to an activation of apoptosis signal transduction in a certain amount of the cryopreserved spermatozoa probably contributing to the reduction of the fertilizing capacity (29–31). In fact, the mitochondria are preferentially susceptible to apoptotic stimuli due to their compartmentalization within the midpiece region (32,33)

As regards sperm membrane damage, the susceptibility of spermatozoa from other mammalian 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 (34). Interestingly, human sperm membranes have unusually high cholesterol contents, and these high levels are known to stabilize membranes during cooling (35). Recently, a study showed that higher cholesterol content does not appear to protect sperm against cryodamage. Conversely, calcium equilibrium appears to be essential for a good post-thaw recovery. Although further experiments are needed to improve post-thaw recovery, some calcium chelators (i.e. EDTA) or determinate phospholipids, which would prevent calcium flux into the sperm, may be added to the sperm freezing medium. Although ICSI may be used for men with severe sperm pathologies and poor quality of post-thaw sperm, there are some specific cases where an improvement in the freezing methods are essential, such as in management of sperm donor samples (36).

Sperm membrane integrity becomes impaired with translocation of phosphatidylserine from the inner to the outer leaflet of the sperm plasma membrane (37,38). This translocation is considered one of the early signs of terminal phase of apoptosis (39). The specific binding of annexin V to phosphatidylserine can be used for detection (40) and magnetic separation of spermatozoa with disturbed plasma membrane (41).

According to Paasch *et al.*, sperm freezing showed a significant overall activation of caspases with a decrease in mitochondrial membrane potential. Both mature and immature sperm fractions had significant activation of caspases followed by a decreased number of sperm with intact mitochondrial membrane potential after cryopreservation. In addition, cryopreservation and thawing induces caspase activation in mature annexin V-positive in ejaculated human spermatozoa as well as in immature annexin V-positive and annexin V-negative sperm. Cells without susceptibility to apoptosis activation can thus be separated by magnetic field by magnetic cell sorting (MACS) technique, which may prove to be of clinical relevance (42). In fact, the separation of a distinctive population of nonapoptotic spermatozoa with

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intact membranes may optimize the cryopreservation–thawing outcome. Magnetic-activated cell sorting using annexin-V microbeads enhances sperm motility and cryosurvival rates following cryopreservation (43). Moreover, MACS using annexin V microbeads enhances the percentage of spermatozoa with intact transmembrane mitochondrial potential and mitochondrial integrity survival rates following cryopreservation (33).

The change in the plasma membrane selective permeability caused by cold shock induces the loss of many components from the sperm cell resulting in reduced metabolic activities. During this process, cytoplasmic and membrane - bound proteins are lost (44-46). Recently a decrease in P25b and P34H (fertility markers) has been shown after human sperm cryopreservation (47). However, this decrease in P34H detection is most likely a consequence of cryopreservation induced damage to the sperm plasma membrane rather than total cell damage. Due to the fact that sperm deficient in P34H are unable to bind to the egg's zona pellucida (47), lower levels of P34H observed in cryopreserved semen may correlate with the loss of fertility after freezing–thawing of human sperm.

According to Morris *et al.*, viability after thawing does not appear to correlate with conventional theories of cellular freezing injury, which indicates that other factors determine viability following freezing and thawing. Improved methods of cryopreservation may be developed by specifically manipulating the manner in which cells experience physical changes instead of imposing a linear temperature reduction. Treatments which followed a chosen non-linear concentration profile, referred to as 'controlled concentration' allowed recovery of almost all the cells which were motile before freezing (48).

### 4. EFFECT OF STORAGE TEMPERATURE ON SPERM CRYOPRESERVATION

The standard method for preservation of human sperm is storage in liquid nitrogen at a temperature of -196°C (2). A number of studies have documented that improvements in cooling technique and the use of improved cryopreservatives, as well as thawing at 37°C in a water bath, improve postthaw 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, it has been reported that the semen of six donors was stored for 28 years suggesting that it may be possible to store human sperm virtually indefinitely if it is kept under liquid nitrogen. This is a pertinent information for clinicians to refer pubescent boys and young men for sperm banking before chemotherapy. Since some of these young men may require sperm storage for long periods (49-52).

Sperm cryopreservation in liquid nitrogen at -196°C has become standard protocol in most andrology facilities. Because this technique is pretty much acceptable everywhere, little consideration has been given to the

potential effectiveness of short - or longterm storage of sperm at higher temperatures. Unfortunately, consistent access to liquid nitrogen cryopreservation facilities may not be always feasible. 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 1 week, however, was fairly modest. Unfortunately, the loss of sperm motility increased dramatically after 3 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. Long-term storage at this temperature (70°C), however, appears to be linked with an unacceptable reduction in sperm motility (53).

Even though the standard protocol of sperm storage at -196°C is widely used, the technical aspects of freezing and thawing sperm and preparing cryopreservation media have been refined over the years (54–57). Various methods of cryopreservation have been evaluated for their effects on sperm motility (58-60). 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 (54,58,59). 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 (60). In addition, a variety of cryoprotectants are available to protect sperm from the negative effects of the cryopreservation process. Many andrology laboratories use TES and Tris yolk buffer (TYB; Irvine Scientific, Santa Ana, CA), other media commonly used are the following: Sperm Freezing Medium (Medi-Cult, Copenhagen, Denmark) and Enhance Sperm Freeze (Conception Technologies, San Diego, CA). A recent study by Nallella *et al.*, showed that TES and Tris yolk buffer is most effective at protecting sperm from the negative effects of the cryopreservation process (61). This may be due to the presence of egg yolk along with glycerol.

The advantages of the fast-freezing and slow-staged cooling methods have long been debated. Studies have reported results in favor of both the fast-freezing method (62) and the slow-staged cooling method (63-65). A recent study showed that there was no difference in sperm quality preservation when semen samples are frozen by fast-freezing technique or by slow, controlled freezing method, either in liquid nitrogen or vapor-phase nitrogen (66).

Another study showed that freezing spermatozoa in alginate microcapsules resulted in an increase of immobilized spermatozoa by 18.3%, compared with the standard protocol. The method of cryopreservation of small amounts of spermatozoa is a feasible and easy method that does not demand special laboratory equipment. Because microcapsules are dissolved under microscopic control, a 100% recovery rate of spermatozoa is estimated. Furthermore, microencapsulation of spermatozoa excludes the possibility of contamination with foreign material,

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either spermatozoa or genetic material. With microcapsules, the recovered spermatozoa can be divided into samples and cryopreserved separately for consecutive ICSI procedures. This gives the opportunity to collect spermatozoa, independently from oocyte recovery and ICSI, and assures the availability of spermatozoa on the day of ICSI (67).

### 5. CRYOPRESERVATION OF HUMAN SPERMATOZOA WITHIN OOCYTE EMPTY ZONA

Men with azoospermia can now be treated through the surgical isolation of spermatozoa using microsurgical epididymal sperm aspiration (MESA) or testicular sperm extraction (TESE) (68). However, multiple testicular operations are not only costly but may lead to adverse physiologic effects and possible testicular failure (69). The need to repeat these procedures can be avoided by the use of sperm cryopreservation (70). Cohen *et al.* were the first to demonstrate that the zona pellucida (ZP) is an ideal vehicle for sperm cryopreservation. After removal of the cellular material from oocytes or embryos, the empty ZP provides a suitable vehicle for preserving the few spermatozoa that can be obtained from patients with severe male infertility (71). Cryopreservation in ZP avoids the loss of sperm that occurs with the dilution and washing of sperm during conventional cryopreservation procedures (72). The rate of recovery of motile sperm after cryopreservation in ZP is higher than after conventional cryopreservation with cryoseeds and dithiothreitol (72-74). With cryopreservation in ZP, TESE or MESA could be performed without regard to the timing of egg retrieval (75).

### 6. SPERM CRYOPRESERVATION IN SPECIFIC SITUATIONS

#### 6.1. Obstructive azoospermia

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

In general, spermatozoa from epididymis are considered more mature than the testicular spermatozoa and provide a higher pregnancy rate (76). Often it is not possible to retrieve spermatozoa from the epididymis. In some cases there might be a complete absence of epididymis. 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 unsuccessful. It is possible to extract a large number of spermatozoa 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 nonobstructive azoospermia (77). 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 (78), thereby avoiding further surgical procedures to retrieve spermatozoa in the future. (79) Generally, the time from biopsy to processing and freezing is within 1 and 1.5 hours, however, a recent case report showed pregnancy even with the interval between the biopsy and the testicular tissue cryopreservation up to 15 hours. Therefore, for many programs, the use of cryopreserved testicular tissue abolishes the need for fresh testicular tissue at the time of ICSI (51).

#### 6.2. Nonobstructive 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 nonobstructive 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 occurred for testicular sperm (80). This implies that cases with extremely low numbers of sperm retrieved can hardly be considered candidates for cryopreservation.

Even in a programme 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. In order to counteract the reasonable risk of not finding sperm or only immotile sperm, scheduling fresh surgery as back-up or counselling the couple for donor sperm as back-up is recommended. The use of totally immotile sperm after thawing should be discouraged on the basis of the present data (81).

#### 6.3. 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 (82,83). The impact of these problems is magnified in malignant diseases that predominantly affect patients in the reproductive age group. 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, 10–100% of surviving cancer patients will show reduced semen parameters after their cure. An average of 15–30% of cured cancer patients remain sterile in the long term (84).

The degree of testicular damage is drug specific and dose related. Ninety-seven percent of men with Hodgkin's disease (HD) who were treated with MOPP (mechlorethamine, vincristine, procarbazine and prednisone) became azoospermic compared with 54% of those treated with ABVD (adriamycin, bleomycin,

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vinblastine and dacarbazine). Only 14% of those in the MOPP group recovered spermatogenesis compared with 100% in the ABVD group (85). Men with testicular germ cell cancer can expect irreversible impairment of the gonadal function at cumulative cisplatin doses 400 mg/m<sup>2</sup> (86). In the case of irradiation to the adult male testis, permanent azoospermia can be induced by doses in excess of 400 cGy, whereas recovery of spermatogenesis is seen at doses of 300 cGy or below (87).

According to a study by Spermon *et al.*, before the cancer being diagnosed, 79 (66%) of 120 couples who attempted to conceive succeeded within 1 year. After treatment, 38 (43%) of 88 couples conceived within 1 year. Seven couples used cryopreserved sperm to conceive a child after treatment. The different treatment modalities does not significantly influence the outcome of patients' wish for children. Congenital malformations were recorded in approximately 4% of the children born before or after treatment. Therefore, it seems to be more difficult to father a child after treatment compared with the case in the general population. Because it is not possible to predict which patient will have fertility problems after treatment, cryopreservation should be offered to every testicular cancer patient. In addition, an increased risk for congenital malformations is not observed (88).

It is recommended that all male cancer patients up to age 55 (at least or according to institutional criteria) be referred for sperm cryopreservation. The age of 55 years is the maximum recommended age for freezing sperm according to the European Association of Urology guidelines (89). Further deterioration has been observed following cancer treatment; sperm quality following cancer treatment depends on many factors: initial sperm characteristics, type of cancer, and therapeutic approach. Unfortunately, it is impossible to predict who will have normal spermatogenesis and who will become azoospermic.

In many cancer subjects, sperm quality is already reduced before receiving any treatment. Studies have shown that semen quality in adolescent male patients with cancer may be diminished and sperm cryopreservation during potentially sterilizing treatment should be considered (90-92). At present, sperm banking remains the only proven method, however hormonal manipulation to enhance recovery of spermatogenesis and cryopreservation of testicular germ cells are possibilities for the future (5,93-95). According to Hallak *et al.*, patients with testicular cancer, leukemia and lymphoma had the worst seminal quality compared to other cancer groups (96,97). According to Gandini *et al.*, the recovery of spermatogenesis after chemotherapy or radiotherapy in their group of testicular cancer patients studied was not a feature of pre-therapy sperm parameter quality (93). Therefore, cryopreservation of semen should be offered to cancer patients irrespective of the type of the disease (5,98-100).

The intersection of cancer and reproduction raises ethical issues for both cancer and fertility specialists,

including issues of experimental vs. established therapies, the ability of minors to give consent, the welfare of expected children, and posthumous reproduction. In some respects, cancer-related infertility is not markedly different than other types of infertility. However, the context of cancer gives rise to issues of patient and offspring welfare that do not arise in other infertility settings. Reproductive physicians play important roles in helping to preserve the reproductive capacities of young cancer patients. First, they are involved in developing and using procedures to preserve gametes, embryos, and gonadal tissue before treatment. Second, fertility specialists will assist cancer survivors in using preserved gametes and tissue or in providing other assistance in reproduction (101).

Men who once had little or no chance of establishing a pregnancy through assisted techniques now have fertility rates approaching that of a couple undergoing standard *in vitro* fertilization (IVF) when there is no male infertility involved. However, according to Zapzalka *et al.*, most of the oncologists (74%) are unaware of recent advances in reproductive technology in which only a few sperm are needed for successful *in vitro* fertilization with intracytoplasmic sperm injection (ICSI) (102). This lack of awareness may be contributing to under utilization of sperm cryopreservation by male cancer patients. Coincidentally, the number of patients that the oncologists estimated to actually cryopreserve 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 Foundation tumor registry had significant anxiety that their cancer treatment could affect negatively their children's future, and only 57% received information from their health care providers about infertility after cancer (103).

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. However, reassuringly, 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.

### 6.4. Intraoperative sperm harvesting during a vasectomy reversal and cryopreservation

The availability of ICSI technique 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 (104). Other investigators have reported that motile sperm were present in the intraoperative vasal and epididymal fluid in 35% of 603 vasectomy reversals (105). However, performance of a vasal or epididymal anastomosis should be prioritized over sperm harvesting during vasectomy

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reversals. The surgeon should perform the reversal at the location farthest from the testicle where intact sperm, regardless of their motility, are present, rather than closer to the testicle in order to harvest motile sperm. 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 (106,107).

### 6.5. Prior to the absence of the partner

If it is anticipated that the male partner will be unavailable during the optimal time for the ART procedure, it is possible to cryopreserve a semen sample 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 (108).

## 7. 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 one's own future use and donor banking (5). However, it has been shown that the process of freezing and thawing is related with a variable loss of sperm quality (109).

Although the adverse effect of freezing and thawing is even more pronounced in cases of poor semen quality, with the advent of ICSI only a small number of motile spermatozoa is required for a successful fertilization (110-111). In fact, ICSI can be performed with fresh and cryopreserved spermatozoa from ejaculated semen from patients with oligoasthenoatozoospermia (OAT) or from spermatozoa extracted from the epididymis or testis in cases of obstructive or non-obstructive azoospermia (112).

It is well known that intrauterine insemination with cryopreserved sperm results in a lower pregnancy rate compared to fresh sperm, however many studies have showed that fertilization and pregnancy rates of ICSI using cryopreserved spermatozoa are similar of freshly obtained sperm (113-117). It is to be noted that the majority of studies comparing fresh and cryopreserved sperm have shown results from sperm surgically retrieved (114-117). Borges *et al.*, demonstrated that when the semen sample had motility decreased, the fertilization rate is higher with fresh sperm than with cryopreserved sperm. However, the implantation and pregnancy rates miscarriage rates are 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 fertilization rate detected in

men with asthenozoospermia compared to normozoospermic or oligozoospermic men could be due to abnormalities in the sperm even before cryopreservation and subsequently the damage caused by the cryopreservation would be much higher (112).

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 intra-uterine insemination (IUI), 82 ICSI and 14 ICSI-frozen embryo replacement (FER)). Their 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%. 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 (118). 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) (94).

Male cancer patients should be encouraged to freeze numerous sperm samples even when sperm count and motility are poor (119). In these cases, ICSI is a powerful technique compared with intrauterine insemination since thawed sperm samples with poor parameters can produce relatively high fertilization rates resulting in normal pregnancies and deliveries. The possibility to repeat treatments with limited number of sperm appears to be of utmost importance.

There are concerns that men with cancer who initiate a pregnancy either before or after treatment may have children who are at an increased risk for congenital anomalies. However, Hansen *et al.* studied the rate of congenital abnormalities in children born to fathers with cancer before treatment and observed a congenital malformation rate of 3.8%, which is comparable to the general population (120). Redman *et al.* found no congenital abnormalities in three children who were born using cryopreserved semen from patients with Hodgkin disease (121). Thus, cancer patients should be informed that, currently, there 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 5-15% of the patients who banked 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 and uncertainty about

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their long-term health and recovery or waiting for possible recovery of gonadal function. Hallak *et al.*, surveyed 56 patients who requested to discontinue storing of spermatozoa; most patients decided to discontinue sperm banking because either they regained fertility or had improved semen quality (122). Even if properly counselled, not all patients will eventually bank semen before their treatment. One study reported that only 42% of appropriately counselled patients did bank their semen to counter sterility (123), while another recent study reported a value of 54% (124).

The above-mentioned survey revealed that, of those childless, 76% wanted children in the future (125). Moreover, approximately 80% of the 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 way. 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. Therefore, it is vital 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, because of important interindividual variances or because a therapeutic regimen may be started with limited gonadotoxicity, but eventually a more gonadotoxic therapy may be indicated because of treatment failure.

## 8. SPERM CRYOPRESERVATION PRE-PUBERTAL IN BOYS

Another point of interest is the fertility preservation of pre-pubertal boys with malignant disease. Spermatozoa can be obtained by masturbation from about the age of the 14 years. To preserve fertility, it is necessary to determine the risk of fertility impairment before instituting cancer therapy. Predicting the impact of treatment on reproductive function in individual children based on expected exposures is notoriously unreliable. Current tools, both biochemical and biophysical, are unsuitable for assessing actual reproductive impacts in prepubertal and peripubertal children. Even when pubertal onset and progression is apparently normal, the integrity of gametes may have been compromised.

Cryopreservation of semen and subsequent IVF is the only standard option for postpubertal males, and spermarche is the watershed around which options for boys are defined. Spermarche typically is an early to mid-pubertal event and occurs before the ability to achieve ejaculation (126). In the mature adolescent, semen is usually obtained by masturbation, with electrostimulation or vibratory stimulation as alternatives (the latter two may

be applicable in peripubertal boys). However, the rate at which viable samples are obtained is highly variable. These adolescents are often sick as well as embarrassed and uncomfortable. One study of 62 attempts by adolescents to bank sperm before therapy resulted in totally normal semen in only four (127). Adolescents may be more successful if unaccompanied by parents (92,128).

Testicular tissue cryopreservation could be an important technique for fertility preservation in pre-pubertal boys, who do not yet have sperm in the ejaculate and who are scheduled to undergo gonadotoxic treatment (129). If the boy already has spermatozoa in his semen, it is best to freeze sperm (92,130). Cryopreservation of testicular cell suspension has been proposed as an alternative method suitable for patients with azoospermia and young patients who are not yet producing sperm (131). Testicular biopsies for fertility preservation are therefore ethically justified (132). However, malignancy recurrence prevention is an important prerequisite for any clinical application of testicular stem cell transplantation. Therefore, the storage of pre-pubertal testicular tissue is currently emerging as a potential solution (92,129,133-135). After being cured, the frozen-thawed tissue may theoretically be transplanted (133,135), xenotransplanted (136,137) or matured *in vitro* (138,139). So far, the testicular stem cell transplantation has provided the most promising results in animal models, mostly murine models. Also, ethically, autologous testicular stem cell transplantation may be more acceptable than xenotransplantation strategies.

For pre-pubertal boys, when full spermatogenesis is not yet ongoing, it is essential to store the spermatogonia and the neighbouring cells as undamaged integrated tissue (140,141). Preservation of Sertoli cells and cell-to-cell contacts in testicular tissue have proved to be important for subsequent maturation of spermatogonia (142). A study by Kliesch *et al.* demonstrated that adolescent patients, aged 14–17 years, are good candidates for semen banking (130). In a large series, Bahadur *et al.*, reported that 86% of 238 boys of post-pubertal age up to 19 years old could produce a semen sample for cryostorage (92). In four out of the remaining 33 boys, eventually sperm recovered from a urine sample was cryopreserved (92). A study involving 45 male adolescents showed that for 20 of them (44.5%) semen cryopreservation was not performed because they were not judged as being mature enough to deliver a semen sample by masturbation. Another four boys failed to deliver a semen sample because of masturbation problems and, finally, for two boys semen was collected by alternative methods such as penile vibrostimulation or electroejaculation, both performed under general anaesthesia (143). Electroejaculation in adolescents may be an alternative to masturbation in order to obtain semen for cryostorage (144,145).

It is important to have ethics approval and strong motivation for all procedures included in programmes for fertility preservation in young patients (100,132,146,147). Children and their parents should be informed about the

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experimental stage of the study. However, it is important to inform all patients facing infertility as a side effect of gonadotoxic chemo- and radio-therapies about the options available to preserve their future fertility (148-150).

Another interesting situation about cryopreservation is the one involving boys with cryptorchidism. Boys with cryptorchidism often face fertility problems in adult life despite having orchiopexy performed at a very young age. During this operation, a biopsy of the testis is normally taken in order to evaluate their infertility potential and the presence of malignant cells. Evaluating biopsies from 11 testes (eight boys), one fresh and two cryopreserved pieces were cultured for 2 weeks and prepared for histology. The morphology of the fresh and frozen-thawed samples were similar, with well-preserved seminiferous tubules and interstitial cells. A similar picture appeared after 2 weeks of culture, but a few of the cultured biopsies contained small necrotic areas. The presence of spermatogonia was verified by c-kit-positive immunostaining. Production of testosterone and inhibin B (ng/mm<sup>3</sup> testis tissue) in the frozen-thawed pieces was on average similar to that of the fresh samples (151). Therefore, it appears that intact testicular tissue from young boys with non-descended testes tolerates cryopreservation with surviving spermatogonia and without significant loss of the ability to produce testis-specific hormones *in vitro*. It may be an option to freeze part of the testis biopsy, which is routinely removed during the operation for cryptorchidism, for fertility preservation in adult life.

## 9. 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. Also, even though 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, boys' stem cells are the target of storage, which represents an important difference in terms of potential future applications for preserving fertility.

## 10. CONCLUSIONS

Reproductive physicians play important roles in helping to preserve the reproductive capacities of cancer patients. First, 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 has its own treatment strategy. Consultation with the patient's oncologist often is essential. A key issue at the time of treatment of the cancer is whether it is medically feasible to obtain gametes or gonadal tissue for storage and later use. Questions about the patient's health and prognosis will also arise when the patient is deciding later whether to reproduce.

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