

Cryopreservation of Human Spermatozoa by Vitrification vs. Slow Freezing: Canadian Experience

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1. Introduction

Many advances in reproductive medicine in the past five decades have made cryopreservation of human spermatozoa an invaluable tool for the clinical management of infertility and sperm banking. The advent of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with microsurgical sperm handling techniques along with advances in female gamete acquisition have resulted in an increased demand for the cryopreservation of semen and tissue samples, often containing a very limited number of spermatozoa. Sperm cryopreservation also makes it possible for cancer patients to preserve their fertility prior to gonadotoxic chemotherapy or radiation. Applications of sperm banking are not limited to cancer patients but extend to patients undergoing certain types of pelvic or testicular surgeries; those who suffer from degenerative illnesses such as diabetes or multiple sclerosis; spinal cord disease or injury; and persons in occupations where a significant risk of gonadotoxicity prevails. Sperm cryopreservation is also available to men undergoing surgical sterilization such as vasectomy, in the event that children may be desired in the future. Another use for semen cryopreservation is to allow donor semen samples to be quarantined while appropriate screening is performed to prevent the transmission of infectious pathogens during therapeutic donor insemination (TDI).

Although major improvements have been made in sperm cryopreservation, there are many unresolved technical issues. Since freezing protocols differ between types of cells, the ideal conditions for human sperm freezing and thawing need to be perfected. To add more complexity, samples with abnormal semen parameters, such as severe oligospermia or high seminal fluid viscosity, often require unique cryopreservation conditions. For example, the particular cryoprotectants can affect cooling rates. In addition, storage temperature can significantly influence cryopreservation outcome. Liquid nitrogen (LN₂) can offer long-term survival of spermatozoa due to essentially absent metabolic activity, such as chemical reactions, genetic modification or aging of cells (Mazur, 1984). A conventional slow freezing protocol has been in use for many years and very little has changed in terms of

methodology and reagents. While freezing aims to preserve cells it can also easily destroy them if certain precautionary steps are not taken into consideration. During cryopreservation cells and tissue undergo dramatic transformation in chemical and physical characteristics as the temperature drops from +37 to -196°C. The cells can lose up to 95% of their intracellular water. The concentration of solutes increases considerably, triggering the possibility of osmotic shock. Moreover, potential intracellular ice crystallization and mechanical deformation by extracellular ice may cause significant injury leading to cell death. Furthermore, if cells survive freezing, they might sustain additional damage during the thawing process due to osmotic shock, uncontrollable swelling and ice re-crystallization (Woods, et al., 2004).

Recently scientists have begun to re-investigate the utility of ultra rapid freezing in the search for alternative methods of sperm cryopreservation. Slow freezing of sperm utilizes cooling rates of 1-10°C/min, while the rapid freezing, or vitrification, technique allows for cooling rates to reach more than 40-1000°C/min in order to avoid intracellular ice formation. As new techniques are perfected, there is a potential for sperm cryopreservation to greatly improve in the future.

2. Cryopreservation of human spermatozoa

2.1 History of human spermatozoa cryopreservation

Remarkably, the first reference of empirical sperm freezing dates as far back as the late 16th century, but it was only with the discovery in 1937 by Bernstein and Petropavlovski that glycerol can aid spermatozoa in surviving long term freezing, that sperm cryopreservation became practical. Expansion of artificial insemination for the dairy industry led to further important research in the field of cryobiology (E. Isachenko, 2003, as cited in Bernstein & Petropavlovski, Polge et al., 1949). Shortly after these practices were initiated with animals, the first pregnancies were reported in humans after insemination with frozen spermatozoa. The next milestone was the discovery of the possibility to store human spermatozoa in LN₂ at -196°C, resulting in superior recovery rates compared to storage at higher temperatures between -20 and -75°C. After the era of empirical freezing; cryobiology matured to its fundamental stage, focusing on the biophysical and biochemical principals of cryopreservation, further advancing the field (Mazur et al., 1972). A comprehensive review of the historical background of sperm freezing was recently published and is recommended for readers looking for more details (Katkov et al., 2006).

2.2 Biological aspects of freezing

Living cells have an isotonic condition with a melting point of their intracellular water of approximately -0.6°C. When cells are cooled below this standard freezing point, supercooling takes place and remains in a metastable state up to -5°C (Katkov et al., 2006; Mazur et al., 1972). Water crystallization and ice formation begin between -5 and -15°C, beginning with the formation of an ice nucleus (seed crystal) in the extracellular water. This 'nucleation' can be induced at a higher temperature by the planned external facilitation of ice formation, often referred to as "seeding". Prior to that stage, water remains unfrozen inside the cell as the membrane prevents ice crystals from intracellular penetration (Woods et al., 2004). Solutes are excluded from ice formation which results in rising concentrations

of solutes within extracellular water. Due to the permeability of the plasma membrane, this chemical imbalance sets up the diffusion of solutes into the cell, forcing water out of the cell. Cells thus undergo excessive dehydration, losing up to 95% of their intracellular water content. This increases the intracellular concentration of solutes, resulting in denaturation of proteins, pH shifts and potential cell death.

Since velocity of cooling is crucial, inaccurate cooling rates can negatively affect sperm survival, motility, plasma membrane integrity and mitochondrial function (Henry et al., 1993). When cooling is slow enough, there is sufficient time for intracellular water efflux and balanced dehydration. If cooling is too slow, damage may occur due to exposure of cells to high concentrations of intracellular solutes. Extreme cellular dehydration leads to shrinkage of cells below the minimum cell volume necessary to maintain its cytoskeleton, genome-related structures, and ultimately cellular viability (Mazur, 1984). On the other hand, if cooling rates are too fast, external ice can induce intracellular ice formation and potential rupture of the plasma membrane and damage intracellular organelles. In addition, mechanical damage of cells is possible due to of extracellular ice compression and close proximity of frozen cells can result in cellular deformation and membrane damage (Fujikawa & Miura, 1986). In contrast, with ultra rapid cooling, the amount of ice formation is insignificant and the entire cell suspension undergoes vitrification. At this stage water transitions, ice formation slows, molecular diffusion and aging stops, and liquids turn into a glass-like condition (Katkov et al., 2006).

Despite the relative insensitivity of human sperm to freezing, optimal cooling rates are needed to ensure appropriate sperm recovery. Currently, there are two types of slow freezing, either static vapour phase freezing to a certain temperature, or the multistep approach using nonlinear controlled-rate freezers, followed by plunging into LN₂. Most laboratories and sperm banks adapt simple static vapour phase cooling in order to avoid induction of ice nucleation by seeding. For this technique samples are lowered into a vapour phase just above the LN₂ level, allowing them to cool for 15-20 minutes before being plunged into LN₂. Alternately, controlled rate freezers can be used to cryopreserve human semen. Most of these protocols utilize a "no seeding" option where samples are cooled from room temperature to -4°C at the rate of 2°C/min, followed by an increase of the cooling rate to 10°C/min until -100°C is reached, and finally plunging into LN₂ (Morris et al., 1999). In contrast to these slow freezing techniques, single step ultra rapid cooling is used for the vitrification technique.

2.3 Cryoprotective agents (CPAs)

Most cells would not survive cryopreservation without CPAs, which can minimize cryo-injury of cells. CPAs are low molecular weight chemicals that serve to protect spermatozoa from freezing damage or ice crystallization by decreasing the freezing point of materials. There are two categories of CPAs, and they differ in their ability to penetrate the plasma membrane. Firstly, permeating CPAs such as dimethylacetaldehyde; dimethyl sulfoxide, glycerol, glycol, ethylene and methanol, stabilize cell plasma membrane proteins and reduce concentrations of electrolytes (Arakawa et al., 1990). In contrast, nonpermeating CPAs such as albumins, dextrans, egg yolk citrate, hydroxyethyl, polyethylene glycols, polyvinyl pyrrolidone and sucrose, minimize intracellular crystallization by increasing viscosity of the sample. CPAs themselves can be toxic if used at high concentrations and spermatozoa are

vulnerable to osmotic changes induced by these agents (Gao et al., 1993). Despite the use of CPAs, plasma membranes can still be damaged or ruptured due to the initial extensive dehydration followed by cell swelling and osmotic stress. Gradual introduction of CPAs to the cell suspension or stepwise increase in their concentration, with a limited waiting period prior to freezing, is utilized to minimize the potential negative effects of these agents (McGann & Farrant, 1976).

2.4 Biological aspects of thawing

While there are many risk factors associated with freezing of cells, thawing can also dramatically affect survival rates of spermatozoa. When frozen samples are returned to ambient temperature, a reversal of the freezing process takes place. Cells that were frozen by the slow method, are more vulnerable to rapid thawing, due to the fast influx of water into cells causing uncontrollable swelling and osmotic shock (Curry & Watson, 1994). If cells were frozen rapidly, intracellular ice crystals could re-crystallize and form larger crystals during a slow thaw. To minimize toxic effects, CPAs have to be promptly removed from the cell suspension by washing samples in isotonic solution. Therefore, the thawing process and CPAs removal technique utilized must take into account the original method that was used for freezing.

2.5 Cryopreservation of spermatozoa for assisted reproductive techniques (ART)

Sperm cryopreservation is widely used in combination with ART techniques such as intrauterine insemination (IUI), IVF and ICSI. Despite many years of research and the discovery of new CPAs, significant numbers of spermatozoa still do not survive cryopreservation (Morris, 1999). Both freezing and thawing can inflict irreversible injury on a proportion of human spermatozoa, marked by a significant increase in some apoptosis markers (Giraud et al., 2000). Lipid peroxidation can lead to a decrease in sperm velocity, motility, viability, and mitochondrial activity (Mossad et al. 1994; O'Connell et al. 2002). The recovery rates of intact spermatozoa are highly dependant on the pre-freezing sample quality (de Paula et al., 2006). Poor quality semen may be more prone to DNA damage and cell death after cryopreservation than normal semen samples and thus have lower fertilizing capacity (Borges et al., 2007). It has been shown that reactive oxygen species (ROS) production impacts membrane fluidity and the recovery of motile, viable spermatozoa after cryopreservation. As well, semen samples containing leukocytes may have higher DNA fragmentation. In addition, the cryopreservation process can diminish the antioxidant activity of the semen fluid making spermatozoa more susceptible to ROS-induced damage (Lasso et al., 1994). The occurrence of sperm DNA damage may also be associated with the thawing process. A rapid increase in post thaw sperm DNA fragmentation over time has been observed, with the highest rate of fragmentation occurring during the first four hours after thawing (Gosalvez et al., 2009).

Normozoospermic semen samples appear to be more resistant to damage induced by freezing and thawing compared with oligozoospermic or asthenozoospermic samples. It has been reported that motile spermatozoa can be recovered after five refreezing and thawing rounds in normozoospermic samples, but only after two rounds in cases of oligozoospermia (Verza et al., 2009). Spermatozoa of infertile men were also found to be less resistant to damage during cryopreservation compared with spermatozoa from fertile men (Donnelly et

al., 2001). Optimization of both CPAs concentrations and cryopreservation protocols will maximize survival of spermatozoa and thus improve ART outcome.

2.6 Cryopreservation of epididymal and testicular spermatozoa

Couples with male factor infertility represent 30 to 40% of the infertile population. Azoospermia accounts for 10% of cases of confirmed male infertility, and often requires surgical retrieval of spermatozoa. Since the introduction of ICSI, many cases of severe male infertility can now be successfully treated. Cryopreservation of surgically retrieved spermatozoa is a valuable component in the effective management of male infertility, reducing the necessity of repeat surgeries. Diagnostic sperm retrieval prior to IVF has several benefits including the possibility of freezing spermatozoa for future use, or if none are retrieved, initiation of the IVF stimulation cycle can be postponed or avoided. Testicular spermatozoa have been utilized to achieve pregnancy in couples with severe male factor infertility, with reported pregnancy rates similar to ejaculated spermatozoa, according to a meta-analysis study (Nicolopoulos et al., 2004). In the case of obstructive azoospermia, recovery of spermatozoa by aspirations varies from 45 to 97% (Craft et al., 1995; Lania et al., 2006). In cases of non obstructive azoospermia recovery depends on the degree of testicular pathology and varies from 0 to 64% (Schlegel et al., 1997; Hauser et al., 2006). A second or third surgery can increase the chance of complications including hematomas, inflammation, testicular devascularization, fibrosis and permanent testicular damage (Schlegel and Su, 1997). To avoid this, if pregnancy is not achieved during the first ICSI attempt, a repeat of the surgical procedure would not be required if a portion of the surgical specimen has been banked. Cryopreservation of surgically retrieved spermatozoa can also aid the coordination of oocyte retrieval and avoids the pressure of having the urologist available on the day of the ICSI procedure. Usually the number of spermatozoa obtained during a surgical procedure is limited, and in the case of testicular sperm they may not be fully matured. In the future, if no mature spermatozoa are recovered, spermatogonial stem cells or early germs cells could potentially be matured in vitro and used for fertility treatments (Hwang & Lamb, 2010).

There are significant technical challenges for successful cryopreservation of testicular tissue due to its complex structure and intracellular interactions. Different cells of testicular tissue will have dissimilar responses to cryopreservation and require different concentration of CPAs. Freezing larger pieces of tissue is not advisable as it would increase resistance of heat transfer and penetration of CPAs leading to variation in cooling rates within different parts of the tissue. In addition, seminiferous tubules capture liquid and increase chances of ice formation (Woods et al., 2004). To avoid these difficulties, cryopreservation of smaller tissue fragments or mincing tissue prior to freezing has been advocated (Hovatta, 2003).

2.7 Cryopreservation of low number or single spermatozoa

The idea of cryopreservation of low numbers or individual spermatozoa was introduced more than a decade ago (Cohen et al., 1997). While this approach remains very attractive, there are multiple biological and technical issues to overcome. Early attempts to freeze individual spermatozoa were performed by placing them in empty animal or human zona pellucida prefilled with CPAs (Walmsley et al., 1998). Data from these studies suggested lower recovery and fertilization rates with human zona in comparison to hamster, possibly due to the presence of the ZP3 binding protein and induced acrosome reactions when human zona were

used (Cohen et al., 1997). While this method requires special skills, equipment, and is very labour-intensive; live births were reported using both human and hamster zona (Walmsley et al., 1998). Spermatozoa were also injected and frozen within spheres of *Volvox Globator* algae and recovered after thawing using an ICSI needle (Just et al., 2004). While all of these methods appear to be attractive for single spermatozoa cryopreservation, they have a number of limitations. Issues around the use of donor human zona pellucida as well as exposure of human gametes to animal or algae genetic materials present potential risks that restrict the use of such methods for human ART procedures. While in theory zona could be obtained from the female partner of men with severe male infertility, this would be unrealistic in the clinical setting, as it would require IVF egg retrieval and destruction of ooplasm to obtain empty zona pellucidae. An alternative proposal would be to use a non-biological carrier such as non-toxic polysaccharide alginate agarose to cryopreserve small numbers of sperm (Herrler et al., 2006; Isaev et al., 2007). In these studies spermatozoa were mixed with CPAs and added to the alginate before the gelatin stage and then frozen in small bead microspheres. After cryopreservation, they were dissolved in a sodium citrate solution. The residual alginic acid on the sperm membrane can reduce sperm motility with slow freezing (Herrler et al., 2006). Agarose microspheres were also frozen in 0.25 cc straws by vitrification with better recovery rates (Isaev et al., 2007). Another reported method was to divide the sample into several small aliquots of 15–20- μ l and to freeze in 0.2-mm cryopreservation embryo straws cut into smaller sections, sealed on one end (Desai et al., 2004). Conventional and open-pulled straws containing 1 or 5 μ l of sperm suspension frozen by vitrification has also been reported (V. Isachenko et al., 2005). However, individual spermatozoa could not be easily sequestered because of possible adherence to the walls of the straws. ICSI pipettes were suggested as a container to freeze individual spermatozoa by either the slow method or vitrification (AbdelHafez et al., 2009; Sohn et al., 2003). Cryopreservation of sperm in microdroplets containing 1 or 40 μ l of spermatozoa in cryoprotectant placed on a cold surface or directly plunged into liquid nitrogen was also reported (Gil-Salom et al., 2000; Isachenko et al., 2005). Microdroplets covered by mineral oil in a plastic tissue culture dish placed in liquid nitrogen were also used to cryopreserve individual spermatozoa (Quintans et al., 2000; Sereni et al., 2008). A nylon cryoloop first introduced for embryo freezing was successfully used to cryopreserve small volumes of sperm suspension by both slow freezing and vitrification (Nawroth et al., 2002; Schuster et al., 2003; V. Isachenko et al., 2004; Desai et al., 2004). However direct placement of sperm into LN₂ without a container using an 'open system' such as cryoloop or unsealed culture dish increases the risk of cross-contamination and such techniques are discouraged by regulatory agencies such as the FDA and the European Tissue Directive on Sperm.

Overall reported recovery rates of a known number of frozen spermatozoa varied from 59 to 100% with reported survival rates of 8–85% and motility of 0 to 100%. The wide ranges of results depended on patient population, initial quality and number of frozen spermatozoa, as well as the type of cryopreservation device, type of cryoprotectant, and freezing and thawing protocols.

2.8 Sperm packaging and relation to the method of cryopreservation

Storage of frozen samples has to be in suitable freezing containers and at an optimal temperature to ensure long term survival. The packaging containers must meet several criteria. They must: 1) hold freezing temperatures without cracking or leaking, 2) have a large

surface to enable a uniform cooling rate of the sample, 3) have proper heat exchange properties, 4) be easy to label and seal securely and 5) be available in small sterile units. When storage packaging is chosen, the possible risk of microbial or viral contamination must also be considered. The type of packaging also depends on the freezing protocol and sometimes on the quality of the sample. For conventional slow freezing, the two most common types of containers currently used are plastic screw-top vials or straws. Straws can be made of polyethylene terephthalate glycol (PETG) or ionomeric resin (CBS High Security Straws by CryoBioSystem, Paris, France). As described above, a low number or single spermatozoa have been experimentally frozen in empty animal or human zona pellucida, spheres of *Volvox Globator* algae, alginate agarose bead microspheres and ICSI pipettes (AbdelHafez et al., 2009; Herrler et al., 2006; Isaev et al., 2007; Just et al., 2004; Walmsley et al., 1998). For vitrification purposes different types of storing strategies have been suggested. These include: cryoloops, electron microscope copper grids, nylon meshes, open-pulled straws and standard open straws (V. Isachenko et al., 2005).

3. Cryopreservation of human spermatozoa by vitrification

3.1 Background on vitrification of spermatozoa

Vitrification is an alternative method of freezing based on the rapid cooling of water to a glassy state through extreme elevation of viscosity without intracellular ice crystallization (Fahy, 1986; Katkov et al., 2006). The relationship between the size of different cells, particularly, different spermatozoa species, and the ability of cells to be vitrified are discussed in details in the paper by Katkov (Katkov et al., 2006).

The earliest experiments on vitrification from the 1930s was not successful because critical rates of cooling were unachievable at that time. With the use of LN₂ and the discovery of cryoprotectants, however, it became possible to vitrify many types of cells. The five basic ways to achieve vitrification have been described in details by Katkov et al.: equilibrium freezing-out of the bulk of water with the use of CPAs and storage at ultra low temperature; lyophilization using slow freezing to moderately low (-40 °C) followed by secondary drying at +30°C (mostly used in food and pharmaceutical industries); ice-free vitrification at high rates and high concentration of CPAs; ice-free vitrification at very fast rates without permeable agents ("CPAs-free vitrification"); high temperature' vitrification by air/vacuum drying at temperature above 0°C (Katkov et al., 2006).

However, until only recently, vitrification of spermatozoa was unsuccessful, possibly due to high concentrations of permeable CPAs (30-50% compared to 5-7% with slow freezing) and low tolerance of spermatozoa to permeable agents. Even brief exposure to a high concentration of CPAs can lead to toxic and osmotic shock and would be lethal for spermatozoa. One possible strategy to lower the concentration of CPAs could be to increase the speed of cooling and warming temperatures as higher rates of cooling and warming, require lower concentrations of CPAs; these conditions can help eliminate intracellular ice crystallization, and facilitate the formation of a glassy state (Katkov et al., 2006). Another option is to add non-permeable CPAs--such as carbohydrates--to permeable CPAs to minimize osmotic shock by decreasing osmotic pressure and stabilizing the nuclear membrane. Since the intracellular matrix of human spermatozoa contains large amounts of proteins and sugars, they can be successfully frozen in the absence of permeable CPAs using protein- and sugar-rich non-permeable agents (Koshimoto et al., 2000).

Successful vitrification of human spermatozoa was first reported by the Isachenkos' group (Nawroth et al, 2002; E. Isachenko et al, 2003). The high viscosity of the intracellular milieu due to large amounts of proteins, nucleotides and sugars and low water in human spermatozoa content determines the ability of human sperm to be vitrified at relatively low cooling rates (Katkov et al., 2006). It was noted that human spermatozoon is one of the smallest germ cells among mammals, has almost no residual histones and has very compacted DNA (Holt, 2000), which indirectly confirms this hypothesis (see also Chapter by Katkov et al in this Book).

As we mentioned above, the major breakthrough in successful vitrification of *human* spermatozoa without the use of permeable CPAs was reported only recently by the Isachenko group (Nawroth et al., 2002), who actually re-invented the work of the "pioneers" in the 1930-40s mentioned above. The combination of extremely high rates of cooling/warming and utilization of vitrification media containing proteins and polysaccharides made it feasible to avoid de-vitrification during warming without use of toxic CPAs. The same group compared viability, survival rate and sperm DNA damage between slow freezing and vitrification and found that DNA integrity was independent from the mode of cooling and the presence of cryoprotectants in thawed spermatozoa (V. Isachenko et al., 2004). The acrosome reaction, capacitation and mitochondrial activity of spermatozoa were compared vis-a-vis slow freezing and vitrification (E. Isachenko et al., 2008). The group reported that changes in the mitochondrial membrane potentials relate to the type of vitrification media with the best achieved results when both sugar and albumin were added to the media. To achieve high cooling rates the vitrification specimen volume needs to be kept to a minimum. Specially designed freezing carriers such as cryoloops and electron microscope copper grids have been suggested for vitrification of human spermatozoa (E. Isachenko et al., 2003; Nawroth et al., 2002). However, placing drops of semen directly into LN₂ raises the issue of the potential risk of microbial or viral cross contamination during freezing and storage (Katkov, 2002). The development of aseptic techniques of vitrification allowing to freeze 5-10 µl of sperm suspension in open-pulled straws (OPS) or 1-2 µl of sample cut standard straw (CSS) placed inside of insemination straw further advanced the methodology of human sperm vitrification (V. Isachenko et al., 2005). The ultra-high freezing rates utilized for vitrification, via direct plunging of specimens into LN₂, leads to solidification of a solution by an intense increase in viscosity during cooling which avoids water crystallization and damaging ice formation (Katkov et al., 2006).

Most importantly, vitrified spermatozoa were successfully utilized in ICSI treatment with clinical pregnancy resulting in delivery of healthy twins (E. Isachenko et al., 2011). While only a small volume 0.2 to 40 µl of sample suspension was frozen in the past, recently larger amounts of spermatozoa (100 µl) were successfully vitrified using newly developed straw packaging system (SPS) made from cut in half 0.25 ml plastic straw (E. Isachenko et al., 2011). A first live birth was reported following intrauterine insemination of semen vitrified without permeable cryoprotectants from patient with oligoasthenozoospermia making this freezing technique even more attractive in clinical practice (Sanchez et al., 2011).

3.2 Vitrification of human spermatozoa: Canadian experience

Encouraged by the findings of the German group, we have also looked at possibilities to utilize vitrification in our laboratory. We have compared sperm motility, kinetics and DNA

damage between semen samples cryopreserved by standard vapour freezing verses vitrification protocols (Moskovtsev et al., 2011). Semen samples from 11 patients presenting for infertility were washed by density gradient centrifugation and evaluated by Computer-Aided Sperm Analysis (CASA). Subsequently kinematic parameters were assessed as previously described: sperm motility, average path velocity (VAP) curvilinear velocity (VCL), straight-line velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH) (Moskovtsev et al., 2009). However, kinematic parameters are averages of values obtained from analyzing the entire motile fraction of cells in a sample and include absolute (actual) parameters (VAP, VCL, VSL, ALH) and relative (derived) such as LIN. When cryopreserved samples are evaluated after thawing, the CASA-paradox can take place, when despite of deterioration of semen samples after cryopreservation “pseudo-enhancement” of kinematics characteristics is observed (Katkov & Lulat, 2000). Modified Kinematic Parameters (MKP) were calculated as previously described: Kinematic Parameters (KP) x Motility/ 100% To account for this phenomenon, modifications of actual CASA-parameters are recommended and are incorporated into our data (Table 1). (Katkov& Lulat, 2000).

Parameters	Prior to freezing	Post vitrif.	Post vitrif. MKP	Post slow freezing	Post slow MKP	P (vitrif. vs. slow freezing)
Motility (%)	68.0 ± 10.79	25.4 ± 13.6		14.6 ± 10.2		< 0.05
Rapid motility (% of motile)	50.64 ± 4.52	19.45 ± 12.98	4.9 ± 3.3	10.45 ± 8.49	1.5 ± 1.2	< 0.05*
VAP (microns/sec)	60.73 ± 8.93	43.09 ± 14.24	10.9 ± 3.6	38.00 ± 8.57	5.5 ± 1.2	< 0.05
VSL (microns/sec)	47.73 ± 9.39	35.45 ± 13.98	9.0 ± 3.5	31.00 ± 8.27	4.5 ± 1.2	< 0.05
VCL (microns/sec)	96.18 ± 3.68	81.18 ± 21.31	20.6 ± 5.4	68.73 ± 13.01	10.0 ± 1.9	< 0.05
ALH (microns)	4.42 ± 0.79	4.3182 ± 0.80	1.1 ± 0.2	3.67 ± 0.85	0.5 ± 0.1	< 0.05
LIN (ratio of VSL/VCL)	49.91 ± 0.55	42.45 ± 7.43	10.8 ± 1.9	45.91 ± 5.59	6.7 ± 0.8	< 0.05
TUNEL (%)	7.5 ± 5.5	9.6 ± 4.4		9.5 ± 5.1		NS

Note: * Statistically significant P values, when compared between MKP of samples frozen by vitrification vs. slow freezing.

Table 1. Comparison of CASA and TUNEL results between semen samples frozen by vitrification and slow freezing.

Our results indicate that sperm motility was significantly reduced for both types of frozen-thawed samples ($P < 0.03$) (Table 1). Mean motility of vitrified samples was $25.4\% \pm 13.6$ (a decrease of 36.4% compared to samples prior to freezing), which was almost two-fold higher compared to motility of samples frozen by standard slow vapour protocol ($14.6\% \pm 10.2$, decrease of 47.2% compared to samples prior to freezing), ($P < 0.05$). Sperm kinematics such as VCL, VSL, and LIN were not significantly different between the two types of cryopreservation protocols without taking into account CASA- paradox. However, when MKP were calculated, it was revealed that indeed vitrified samples had superior recovery of sperm kinematic parameters in comparison to slow freezing.

Samples for slow vapour freezing were diluted 3:1 with commercial cryoprotectant medium and frozen by standard protocol in CBS. Aliquots of samples for vitrification were diluted 1:1 with a G-IVF medium (Vitrolife, Göteborg, Sweden) supplemented with 0.25M sucrose and 1% of LSPS (Life Global Protein Supplement, IVF Online, Guelph, ON, Canada). We have used 0.5 ml OPS and loaded 5 μ l of vitrified sample in each straw by capillary; OPS were inserted into 0.5 CBS straws and sealed (Figure 1).

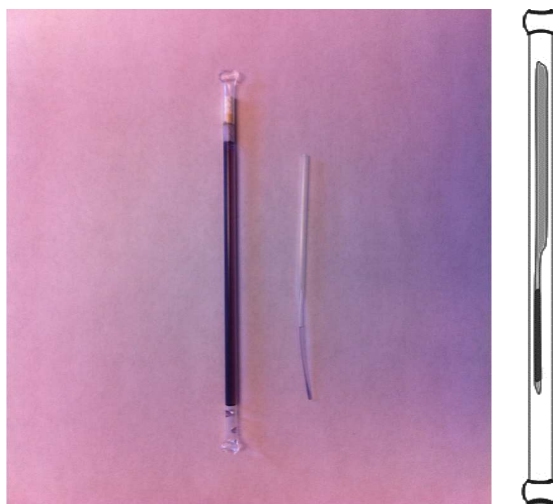
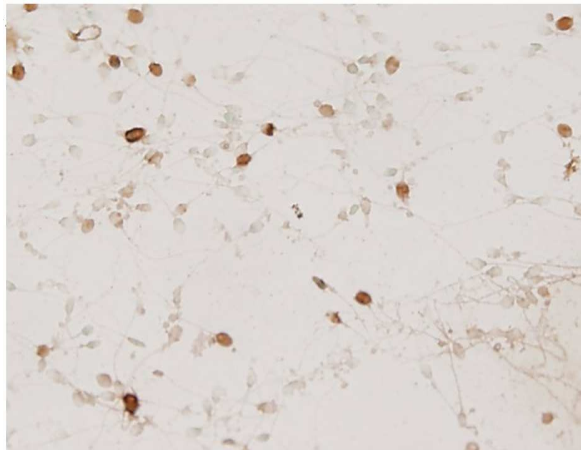


Fig. 1. Comparison of 0.5 ml CBS straw and 0.5 cc OPS straw and schematic of OPS inserted and sealed inside a CBS.

Samples were immediately plunged into LN2 and stored there for several days. For thawing procedure, OPS were rapidly removed from CBS straws, and plunged into 2 ml of the same medium used for vitrification at 37°C for 10 seconds.

We have evaluated the effect of cryopreservation on sperm DNA damage as the subject remains controversial. |While several reports indicate no negative effect of freezing on sperm DNA integrity (Duty et al., 2002; V. Isachenko et al., 2004). others have reported significant negative effect of sperm cryopreservation and DNA damage and chromatin stability (Hammadeh et al., 1999; Said et al., 2010). Significant increase in percentage of DNA fragmentation was associated with an increase in oxidative stress during cryopreservation

(Thomson et al., 2009). A slide-based technique for the assessment of sperm DNA was performed as previously described (TUNEL: TdT-mediated dUTP nick end labelling) (Moskovtsev et al., 2010) (Figure 2).



Note: Brown (TUNEL-positive): damaged DNA; gray-green (TUNEL-negative): undamaged cells.

Fig. 2. Sperm DNA damage assessment by TUNEL assay.

We found statistically significant increase in sperm DNA damage after both methods of sperm freezing ($P < 0.05$). However, the increase in DNA damage was minimal and to a degree probably irrelevant to clinical concerns. No significant differences were observed in sperm DNA damage between slow freezing and vitrification (9.6 ± 4.4 vs. 9.5 ± 5.1).

We can now confirm previous reports that human spermatozoa can be successfully vitrified without the use of potentially toxic cryoprotectants. The vitrification protocol showed significantly better results in preserving motility rates of spermatozoa when compared to slow vapour freezing. No significant differences were observed in post thaw sperm DNA damage in comparison to the standard slow freezing method. While our results are based on the freezing of a small volume of specimens, we are evaluating vitrification of larger volumes of spermatozoa with a proprietary mixture developed in our laboratory in CBS. We have achieved comparable results with both small volume ($5 \mu\text{l}$) and relatively large volume of $200 \mu\text{l}$ semen samples (unpublished data).

4. Sperm banking

4.1 Referring patients to a sperm bank

Human semen cryobanking can be divided into two broad categories: autologous banking for personal fertility preservation and donor sperm banking. Semen banking is useful in many situations and can be considered a safeguard against unforeseen future circumstances. These may include: prior to chemotherapy or radiation therapy; pre- vasectomy; before certain types of pelvic or testicular surgery; in cases of degenerative illnesses such as diabetes or multiple sclerosis, spinal cord disease or injury; high risk occupations or sports;

and preparation for future fertility treatment. Usually several steps are required prior to initiation of semen storage. Most sperm banks only accept patients referred by their physician, however sometimes self referral is possible. Patients have to be screened by collection of blood, urine and semen samples to ensure that their samples will not contaminate others with infectious diseases when placed into storage. Consent for freezing and storage of semen has to be signed and witnessed. It is important that patients have a clear understanding of the process of banking and that they provide clear instructions on who is responsible for the disposition of the specimens in the event of their demise. Similarly, costs associated with banking and long term storage need to be clearly defined and assigned in case the patient becomes incapacitated. When samples are either used for procreation, transported to another facility, or storage is to be discontinued, the patient or designate must complete specific consent forms allowing the bank to comply with their wishes. In most countries it is assumed or stated by law that the semen belongs to the individual who produced the sample, and it is this individual who must sign all consents, unless the sample was designated for donation (e.g., in the case of anonymous sperm donation). If possible, multiple donations are usually recommended to ensure an adequate sperm reserve for future procreation, depending on the quality of the sample, individual circumstances and reason for sperm banking.

4.2 Methods of sperm collection and retrieval

In most cases patients are able to produce an ejaculate by masturbation. Patients are given instructions to have minimum of 2 days and a maximum of 5 days of sexual abstinence, and to collect an entire ejaculate into a sterile specimen container. Condoms, creams or lubricants must not be used during collection as they can interfere with sperm motility and vitality due to the spermicidal properties of many products. Some patients would not be able to produce sample at the sperm bank by masturbation due to psychological, medical reasons or religious restrictions. In this case collection of samples by intercourse using a non-spermicidal condom, often referred to as a "semen collection device", is acceptable. Penile vibratory stimulation (PVS) can be helpful in spinal cord injury patients and those who are unable to produce semen by masturbation or intercourse (Brackett et al., 1998). However samples collected after PVS often exhibit relatively low motility (Hovav et al., 2002). PVS can help to produce a semen sample in most patients with spinal cord injuries (Brackett et al., 1998).

In some situations, collection of a retrograde sample is necessary. Retrograde ejaculation is a condition in which some or all of the sperm are not expelled through the urethra during ejaculation, but because of an incompetent bladder neck, the ejaculate refluxes back into the bladder. Reasons for this problem include organic conditions such as diabetes or multiple sclerosis, or pharmacological effects (eg. alpha adrenergic blocker use for hypertension). The general approach is to neutralize the urine pH and normalize the urine osmolarity by giving the patient sodium bicarbonate to alkalinize his urine. The urine sample is subsequently washed and used for insemination, in-vitro fertilization or cryopreservation. In patients with anejaculation, electro-ejaculation might be necessary to obtain semen, but this usually requires anaesthesia and has the associated risk of rectal injury. Additionally, samples produced by electro-ejaculation tend to be of relatively low quality (Denil et al., 1996).

Surgical sperm retrieval should be the last option in patients who can not produce a sample or patients who are diagnosed with azoospermia. Several methods of sperm retrieval are available depending on the etiology of the problem. To retrieve epididymal spermatozoa in cases of obstruction, percutaneous epididymal sperm aspiration (PESA) can be performed without surgical scrotal exploration, it is repeated easily, and does not require an operating microscope or expertise in microsurgery. Microsurgical epididymal sperm aspiration (MESA) is performed under the operating microscope and general anaesthesia. Individual tubules of the epididymis are isolated and aspirated. Testicular sperm aspiration (TESA) is a needle biopsy of the testicle. It is an office procedure performed under local anaesthesia. Testicular sperm extraction (TESE) is the process of making a small incision and removing a small portion of tissue from the testicle under sedation or local anaesthesia. Microdissection (or sometimes referred to as microscopic or microsurgical) testicular sperm extraction (MicroTESE) is a very rigorous search for sperm under high magnification in cases of azoospermia or extremely low sperm production. MicroTESE is usually performed in the operating room under general anaesthesia utilizing an operating microscope. Cryopreservation of surgically retrieved epididymal and testicular spermatozoa is a valuable component in the effective treatment and management of these patients and it reduces the necessity of repeat surgeries when the initial procedure is unsuccessful or if additional children are desired.

4.3 Long term storage

Proper long term storage is usually achieved by placing specimens in LN₂ freezers, which have been safely used since the 1970s. Some automated systems are available and are capable of LN₂ autofilling, supplied with alarms and data recordings for all activities and are designed to minimize the chances of loss or damage of samples. Despite automation, quality control procedures must be implemented by sperm banks to ensure proper monitoring and safety of samples and staff. The LN₂ itself can be a source of microbial contamination so every available practical step has to be considered to reduce the risk of transmission (Fountain et al., 1997). In general cross-contamination of frozen samples by pathogens are extremely rare, and have not been reported in the setting of sperm banks. It is, however, theoretically possible, as often patients are not fully screened prior to freezing, as in cases of autologous sample cryopreservation or due to time constraints for oncology banking (Clarke, 1999). "Quarantine" tanks are often used to separate samples with pending, unclear laboratory results or unscreened patients. However, contamination is still possible, as released samples that are moved to long term storage could have acquired pathogenic contamination from one of other "pending" samples in the quarantine tank. When samples are cryopreserved for patients with known infections such as HIV or hepatitis B and C carriers, separate tanks for each type of infection are required (Tomlinson & Sakkas, 2000). Cross-contamination can also be avoided by storage of samples in nitrogen vapour. However, in contrast to liquid nitrogen, there are some concerns that vapour has poor heat transfer rates, lower thermal capacity, and significant temperature fluctuation may exist within the vapour (Tomlinson & Saakas, 2000; Wood, 1999). Some older types of vapour storage systems could only guarantee the maintenance of temperature around -100°C and were not acceptable for long term sperm storage. Storage temperatures have to be maintained below -135°C to ensure a glass-like condition of frozen water and for secure long

term storage of semen samples (Clarke, 1999). Newer types of high efficiency LN₂ vapour freezers and others that have a LN₂ "jacket" provide working environments of below -160°C and are more suitable for sperm banks.

4.4 Quality control and quality assurance

Amongst the many government or professional organizations that require periodic inspection of frozen samples, the standards put forward by Health Canada are the strictest (Health Canada, 2000). Rigorous standards of operation are essential for sperm banks. Sperm banks must have specific requirements for screening, processing and quarantine of samples. Licensing is required in some countries and sperm banks are inspected in accordance with existing standards or regulations. While auditing is absolutely necessary, it might pose the risk of exposure of frozen specimens to room temperature while such inventory is performed. Straws thaw more rapidly than vials and can warm up to -80°C within 8 to 15 seconds at room temperature, dramatically increasing the possibility of damaging samples during inventory or verification of samples' identity (Tyler et al., 1996). Clear labelling systems to easily identify and link samples to a specific donor or patient must be in place to enable sample location and for performing inventory. The samples should remain in LN₂ during the duration of inventory performance and the audit must be performed by qualified and skilled staff.

Several facility and equipment-related quality control and risks factors must be considered for cryopreservation and storage of semen. Physical security of bank facilities and proper identification of sample location within freezers is crucial. Equipment must be appropriate and functional, with defined periodic service and maintenance schedules. Staff must be supplied with all necessary personal protective equipment. Adequate supplies of LN₂ gas must be guaranteed and spare LN₂ prefilled tank must be available in case of emergency. All staff involved in handling LN₂ must be properly trained by a certified organization. Standard operating procedures must be developed to clearly describe each step of the process of sample collection, processing, banking and handling. Annual reviews of both proper documentation and LN₂ training must be performed. Temperature of freezers chambers and LN₂ levels in tanks must be monitored on a continuous basis and all data logged in secure databases. Alarm systems and appropriate call procedures must be in place to attend to any emergencies. 24 hour monitoring and response is absolutely essential to safely maintain the integrity of the clinical samples in storage. Storage rooms must be monitored continuously for O₂ levels and staff activity in enclosed spaces must be monitored to avoid hypoxic injury. Backup power generation must be available in the event of a power failure. Each sample designated for storage has to be properly verified, assessed, processed, labelled, frozen and stored. Double-checking the identity of samples at each step is highly recommended. Some banks choose to divide samples from individual patients or couples and store them in different tanks or locations to minimize the risk of total loss of their biologic material (WHO, 2010).

5. Sperm donation

5.1 Applications for sperm donation

In cases of severe male infertility, single or lesbian women, the use of donor sperm is the only approach to address fertility issues (Botchan et al., 2001; Golombok, 2005). Advances in

sperm cryopreservation have created opportunities for many families to achieve pregnancies through therapeutic donor insemination or IVF with donor sperm. Pregnancy rates are estimated to be around 10-12% per unstimulated cycle and can be achieved when at least 5×10^6 progressively motile spermatozoa inseminated into the lower cervical canal on 2-3 occasions during the ovulatory phase of menstrual cycle (Scott et al., 1990). At present, some 30,000 births per year worldwide are attributable to frozen donor sperm inseminations (Mortimer, 2004). While this seems like a large number, it may fall in the future, as the recruitment of sperm donors is increasingly difficult due to complicated and strict regulatory procedures, as well as lack of interest from potential donors.

The screening process for donor sperm is quite rigorous and includes obtaining a complete medical and sexual history, physical examination, psychological assessment and laboratory work-up on blood, urine and semen specimens to screen for pathogens including Hepatitis B, C, Human Immunodeficiency Virus (HIV 1&2), Human T-cell Lymphotropic Virus (HTLV 1&2), *Treponema pallidum* (Syphilis), Cytomegalovirus, *Chlamydia trachomatis* and *Neisseria gonorrhoea*. Sperm banks perform genetic screening for heritable diseases based on the ethnic background of sperm donors (eg. Cystic Fibrosis for Caucasians). Donors must be retested after the required quarantine interval, and specimens may be released only if the results of repeat testing are negative. Specimens can only be used after they have been quarantined for a minimum of 180 days to avoid the risk of HIV transmission. Donor eligibility restrictions apply to employees of sperm banks, poor donors' health or quality of the semen and in some countries by sexual orientation of the donor, as gay or bisexual men are considered at higher risk for HIV and prohibited from being sperm donors in some countries (including Canada and USA). Many countries have age restrictions for sperm donation. The minimum age is usually 18 and the maximum 40 years of age (Health Canada 2000, American Society for Reproductive Medicine (ASRM), 2004)

5.2 Anonymous donors

Semen donors can be classified into two specific groups, anonymous and non-anonymous (known). Currently, with the establishment of many commercial sperm banks and the ability to safely transport samples even between continents, anonymous sperm donation is the method of choice for most recipients. The anonymity of the donor is maintained through the process. This is an important issue to both the recipient and the donor (Ernst et al., 2007). For fully anonymous sperm donation, the recipients would not be known to the donor and the donor offspring would have no future contact with the donor. The sperm donor gives up all legal rights over the biological children conceived from his samples donated to sperm bank. Anonymous donation allows parents, if they wish, to conceal the issue of infertility, or the fact of non-genetic parenting from the offspring. The motivation to hide this information most commonly is driven by pressure from other family members; fear of being rejected by the child or to protect children from the complicated psychosocial matters related to sperm donation. In many Western countries disclosure is encouraged by many counsellors, and if open disclosure is chosen by the parents, it is usually advised to disclose the method of conception to their children at an early age. Non-disclosure by parents of the biological origin of their children is viewed by some as misleading the child and could potentially affect trust between parents and their children, if their origin eventually becomes known to the child (Patrizio et al., 2001). However, it is ultimately the decision of the parents to disclose or not as in adoption cases.

There is some consensus that there should be limits on the number of offspring allowed from a given sperm donor. This is driven by possibility of accidental consanguinity between children from the same sperm donor. For example ASRM recommends a limit of 25 children per population of 800 000 for a single donor, but there are no federal or state laws limiting the number of sperm donation by a donor. In UK the number is limited to 10 different families, but does not apply if a genetically related sibling for an existing child is desired. Some countries limit the number of children to 4 in New Zealand; 5 in China; and 5 to 10 in Australia depending on the region; 25 in the Netherlands (Gong et al., 2009).

Recently, open-identity sperm donors have become available through many sperm banks. These donors have agreed to at least a single contact with any children born through use of their sperm, usually when the child reaches the age of consent (18 years old in most jurisdictions), for those individuals who wish to contact them (Gottlieb et al., 2000; Frith et al., 2007). In some cases audio interviews and pictures are available from these donors.

Two types of anonymous donor samples are usually available through sperm banks, prewashed or unwashed: Prewashed samples are obtained by processing the ejaculate by density gradient centrifugation for seminal fluid removal prior to freezing and can be directly inseminated into the uterine cavity after thawing (Larson et al. 1997). These samples are favoured by doctors' offices without access to an Andrology laboratory for post thaw processing. For processing unwashed samples, density gradient isolation is required to remove contaminants and CPAs after thawing the specimen prior to intrauterine insemination or for IVF. The removal of CPAs has to be performed step-by-step and gradually to minimize osmotic stress on spermatozoa. Drop-wise dilution of the sample with 1:10 sample to sperm wash medium ratio is recommended (Mortimer, 2004).

5.3 Non-anonymous sperm donors

Some donors and recipients choose to arrange donations privately and the donor in this case is known to the recipient(s). The donor may be a family member such as a brother or father or a friend. Most of these donations are done altruistically and acceptable only if all parties are in agreement. All participants involved in the donation process are generally required to attend a separate and a joint counselling session. An initial counselling interview with the donor and his spouse or partner (if applicable) is arranged to discuss the personal, social and legal aspects of donation. The known donor has to meet all requirements to be accepted for donation and undergo the same screening tests and laboratory evaluation as an anonymous donor, including 180 days quarantine for his frozen sample. Proper consent and declaration forms are required to be signed by known semen donor. Furthermore, a child conceived using donated semen is legally deemed to be the child of the recipient(s), and the donor has no legal rights or responsibilities regarding the child. Usually the donor may at any time prior to the use of his semen, vary or revoke his consents. Most clinics require a legal contract with all parties having received independent legal advice. As the use of the third party reproduction such as sperm and egg donation becomes more acceptable in many countries the ethical and legal aspects of these procedures become increasingly important. Issues of the donor's anonymity, financial compensation, religion and cultural acceptance, regulation of donor and prospective parent screening, as well as consideration of the welfare of children conceived with the use of donor sperm are widely discussed in the scientific literature and public media. While guidelines on the use of donated sperm come from

government or professional organizations, they may also be influenced by religious institutions and they vary widely from country to country (Gong, 2009).

6. Social importance and psychological aspects around banking oncology patients, adolescent and young adults

When an individual is diagnosed with cancer almost every aspect of their physical and psychosocial well-being is altered. Quite often in clinical practice, the long term effects of cancer therapy on a patients' ability to have children in the future is not adequately addressed (Thaler-DeMers, et al., 2001). While the priority is to eliminate the cancer and save their life, fertility preservation especially among adolescent or young adults to ensure the potential of procreation with their own gametes after treatment, needs to be considered. Impaired spermatogenesis has been demonstrated before treatment in some patients with malignancies, depending on their location (eg. testicular cancer) or type (eg. Hodgkin's lymphoma) (Rueffer et al., 2001). Current treatment options such as surgery, chemotherapy and/or radiation can impair spermatogenesis and sexual function and lead to temporary or permanent infertility (Magelssen et al., 2006).

The scale of negative effects of cancer treatment on spermatogenesis depend on the specific gonadotoxicity of administered chemotherapeutic agents, number of chemotherapy treatment cycles, radiotherapy field location and dosage, type and stage of the cancer, and age of the patient. Considering combination cancer therapy, uncertainty in individual response to treatment and the large number of confounding variables, it becomes very challenging to assess the risk of iatrogenic infertility in many patients. The ability of cancer survivors to have their own biological offspring is very important for many oncology patients, especially at younger ages (Schover et al., 1999). Advances in early diagnostic investigation and treatments have led to increasing numbers of young cancer survivors. Unfortunately up to 30% of childhood cancer survivors are permanently sterile following cancer treatment (Tournaye et al., 2004). In Canada and the United States, cancer in patients 15 to 29 years of age who can benefit from sperm banking is nearly three times more common than in patients younger than 14 years (Bleyer et al., 2006). Early germ cells, (spermatogonia) are very sensitive to radiation and chemotherapy. Even low doses or a single dose treatment can potentially cause functional impairment of spermatogenesis. With increase in dosage or duration of the treatment, initially spermatocytes get damaged and as treatment progresses spermatids also become damaged. Radiation doses of less than 0.8 Gy can result in oligospermia and doses between 0.8 and 3 Gy can result in azoospermia (Rivkees & Crawford, 1988).

Cryopreservation of semen has changed the reproductive prospects for young patients diagnosed with cancer. Unfortunately, banking services continue to be underutilized since cancer patients and their families are not always informed about the potential fertility risks associated with cancer treatments, or the availability of banking. According to some surveys, less than 20% of patients undergoing chemotherapy or radiation treatment are informed about the adverse effects of such treatment on spermatogenesis or are offered sperm banking for fertility preservation. Cancer patients are usually under huge physiological and time pressure to make cryopreservation decisions while dealing with a life threatening situation. To complicate matters, some young patients are unable to produce semen samples by masturbation. In such cases, PVS or electro-ejaculation under general anaesthetic might be required. Surgical retrieval of testicular tissue may be an option for

prepubertal boys who are not capable of producing mature sperm. Testicular tissue cryopreservation has been reported in boys with cryptorchidism to preserve fertility (Bahadur et al., 2000). Cryopreserved testicular tissues can be autografted to restore reproductive functions; however recurrence of neoplastic process is a concern in oncology patients and such procedures are still considered to be experimental (Hwang & Lamb, 2010). A multi-disciplinary team approach is important to ensure that patients have the opportunity to preserve their fertility potential if they elect to do so.

The posthumous use of semen is an entirely separate and complex ethico-legal subject. The ethical and legal aspects of posthumous assisted reproduction have been recently addressed by the European Society of Human Reproduction and Embryology Task Force on Ethics and Law (ESHRE, 2006).

7. Conclusions

Human spermatozoa can be successfully cryopreserved and utilized. Cryopreservation now plays an essential role in fertility preservation under the following scenarios:

- couples undergoing infertility treatment.
- cancer patients undergoing gonadotoxic chemotherapy or radiation.
- patients undergoing certain types of pelvic or testicular surgeries
- patients suffering from degenerative illnesses such as diabetes or multiple sclerosis; spinal cord disease or injury.
- men undergoing surgical sterilization such as vasectomy
- screening and quarantine of donor semen samples

Normozoospermic semen samples appear to be more tolerant to damage induced by freezing and thawing compared with oligozoospermic or asthenozoospermic samples. Cryopreservation of surgically retrieved epididymal and testicular spermatozoa is challenging, but a valuable component in effective treatment and management of severe male factor infertility. Cryopreservation of low numbers or single spermatozoa has multiple biological and technical aspects yet to be worked out; therefore, further research is required to introduce this technique into clinical practice. During cryopreservation, cells and tissue undergo dramatic transformation in chemical and physical characteristics as temperature drops from +37 to -196°C, thus risking cryoinjury. Velocity of cooling and warming is crucial and inaccurate cooling or thawing rates negatively correlate with sperm survival.

Spermatozoa cannot survive slow freezing without CPAs; CPAs have to be used at low concentrations with minimum exposure as CPAs are toxic and can cause osmotic damage. Gradual, stepwise introduction before freezing and removal of CPAs after thawing is essential. Conventional slow freezing with CPAs can offer cooling rates of 1–10°C/min. Vitrification, currently only an experimental technique, allows for extremely rapid freezing at rates of up to a 1000°C/min. LN₂ can offer long-term survival of spermatozoa due to essentially absent metabolic activity and aging of cells and tissues in the frozen state. Rigorous standards of operation and quality control are essential for sperm banks. Social, psychological, legal and ethical issues surrounding sperm banking are very complex and must be considered in each case.

The vitrification method uses no specially developed cooling program; it does not need permeable cryoprotectants; it is much faster, simpler and cheaper; and it can also provide a

high recovery of motile spermatozoa after warming as effective protection of spermatozoa against cryodamage and helps to avoid many problems relevant to slow freezing such as ice formation; shifts in pH, extensive rehydration and osmotic damage.

Successful vitrification of human spermatozoa without toxic CPAs has been reported now by two independent groups. Moreover, live births were reported after vitrification of semen utilized for intrauterine insemination and IVF with ICSI procedures, making this freezing technique even more attractive in clinical practice.

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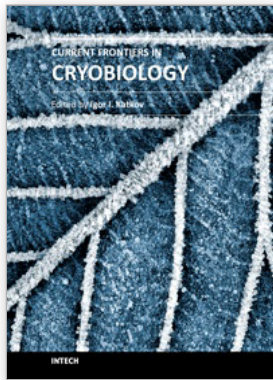
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Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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