8

Cryopreservation of Testicular Tissue

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1. Introduction

Immediate use of freshly collected testis tissue in diagnosis or in reproductive technologies is not always possible or desirable. Therefore, the ability to properly preserve the tissue for varying intervals is an essential step for maximizing the use of the source tissue. Preservation of gametes and gonads is a topic of interest in reproductive biomedicine. Other chapters in this book have elegantly covered current knowledge on the cryopreservation of sperm, oocytes, and early embryos as well as ovarian tissue, among other cells and tissues. However, the main objective of this chapter is to provide a focused discussion of the importance, methodology, potential applications, and limitations for applying cryopreservation to testicular tissue.

Cryopreservation of human testis tissue obtained by biopsy can be used as a potential future source of sperm. For adult cancer survivors whose only source of sperm is the testis parenchyma, cryopreservation of testis biopsies may be the only option remaining if they prefer to father their own biological progeny. This will require detection of sperm in frozen-thawed cell suspensions of testis tissues for use in intra-cytoplasmic sperm injection (ICSI). More importantly, cryopreservation of immature testis biopsies can offer a unique alternative for prepubertal boys undergoing gonadotoxic cancer treatments, whose only future source of spermatogenesis (*i.e.*, spermatogonial stem cells) is at risk. These strategies can also be applied to genetic preservation of endangered species/breeds through the cryopreservation of testis tissue from young animals that die prior to reaching maturity. Restoring the developmental potential of testis tissue after cryopreservation may also provide insight into proper banking of other immature tissues.

The effects of cryoprotectant concentration and cooling rate are not similar among tissues or species. Therefore, we will discuss the basis for a number of successfully applied strategies and workable protocols that have been used to effectively cryopreserve testis tissue in various species.

In summary, this chapter provides an overview of the current literature and contributions by the author and colleagues on cryopreservation of testicular tissue and its potential applications in experimental and clinical settings in reproduction medicine.

1.1 Developmental changes in the structure of testis tissue

In mammals at birth, all organs/tissues required for sustaining life display functional competence and histological similarity to those in mature individuals. Reproductive tissues,

on the other hand, attain maturity much later and only when other bodily requirements of parenthood are also in place. Therefore, in discussion of testis tissue cryopreservation, the developmental stage of the tissue is an important factor to be considered. For instance, for cryopreservation of testis tissue from an immature individual, the differing tissue texture and need for maintaining its future developmental potential are to be taken into account.

Embryonic development of the testis begins when the SRY gene in a genetic male is expressed, driving the transformation of an indifferent early gonad to a testis. This in turn causes differentiation of Sertoli cells to enclose the fetal germ cells, to mark the differentiation of primordial germ cells into gonocytes, and results in the formation of seminiferous cords. In humans, this process begins at 7-9 wk gestation (Wilhelm et al., 2007) and is immediately followed by differentiation of fetal Leydig cells, located in the interstitial spaces between the seminiferous cords, to allow production of testosterone thus causing masculinization of the foetus (Scott et al., 2009).

In early postnatal humans and most domestic species, the testis still contains interstitial tissue and seminiferous cords, with gonocytes as the only type of germ cells present (Franca et al., 2000). Initially, gonocytes reside in the centre of the seminiferous cords (**Fig. 1A**), but they gradually migrate toward the periphery of the cords and remain in close contact with Sertoli cells and peritubular myoid cells at the basement membrane to form the stem cell niche (Pelliniemi, 1975; Van Straaten & Wensing, 1977). Gonocytes eventually give rise to spermatogonial stem cells (SSCs), which have the ability to both self-renew and give rise to differentiating germ cells. Postnatal development of the testis also involves proliferation and maturation of Sertoli cells to transform testicular cords into seminiferous tubules (containing a lumen), followed by sequential division and differentiation of germ cells to generate sperm (**Fig. 1B**) (Hughes & Varley, 1980; Ryu et al., 2004). Therefore, SSCs form the foundation of spermatogenesis and are responsible for a lifetime supply of sperm.

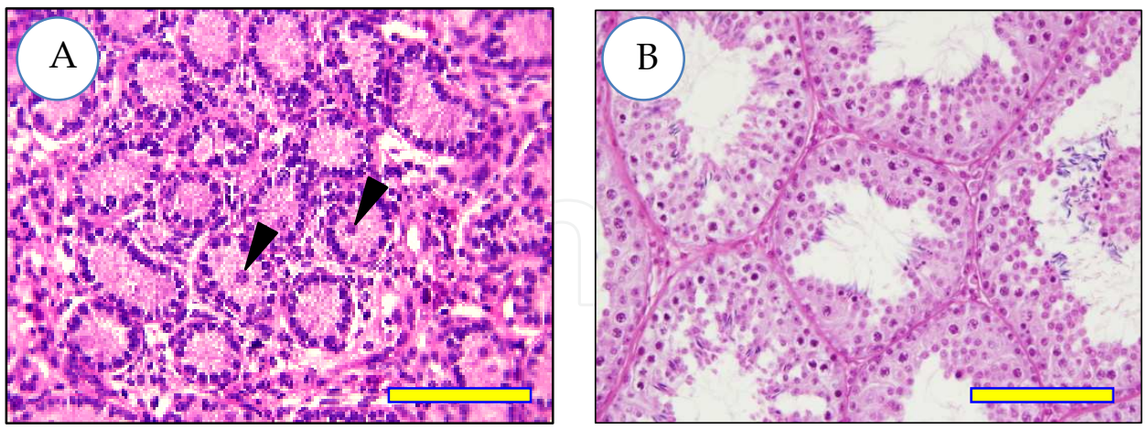

Fig. 1. Histological differences between an immature and a mature testis tissue. In the immature testis (**A**), seminiferous cords contain only one type of germ cells - gonocytes (arrow heads). In the mature testis (**B**), on the other hand, seminiferous tubules are much larger in diameter, contain a lumen, and a repertoire of germ cell types. The composition and extent of the interstitial tissue also changes over development. These differences may affect the response of the tissue to a given cryopreservation protocol even within the same donor species. Scale bar = 100 mg. Images modified from Abbasi & Honaramooz (2011).

As highlighted in **Figure 1**, the cellular composition of a typical mature testis is quite different from that of an immature testis; for instance, the latter hosts a considerably higher number of differentiating germ cells, known to be more sensitive to manipulations and temperature changes (Franca et al., 2000; Frankenhuis et al., 1981). Consequently, the tissue composition of the testis changes during development and proportionally larger volumes of the mature testis are occupied by the seminiferous tubules. Therefore, the developmental state of the testis affects the tissue composition and has important implications for its cryopreservation.

2. Rationale for preserving testis tissue from human and animal donors

Preservation of testicular tissue could be pursued for multiple reasons. An estimated 1 in 650 children will be diagnosed with malignancies by age 16, of which 80% will be cured (Stiller et al., 2006). However, irreversible gonadotoxic insult of chemo/radio-therapy remains a major concern in the use of these life-saving treatments, which render about 20% of boys sterile in the long term, likely as a result of the loss of spermatogonial stem cells (Apperley & Reddy, 1995; Naysmith et al., 1998). With improved treatments, the proportion of childhood cancer survivors is expected to increase, posing an even greater challenge for reproductive medicine and oncologist practitioners in the decades to come. A routine strategy to offer preservation of future fertility for adult men undergoing sterilizing cytotoxic treatments is to freeze semen samples; however, some men may be azoospermic at the time of cancer diagnosis. More critically, in pre-adolescent boys, collection of sperm is not possible because spermatogenesis has not yet started. In such cases, cryopreservation of testicular biopsies collected prior to the start of the treatment may provide a potential source for future use in emerging reproductive technologies.

In animal conservation, preventing the permanent loss of a male's potential contribution to the genetic variability of a rare or endangered species/breed is feasible through the collection of sperm before or even shortly after death by retrieval from the ejaculate, epididymis, or testes, which is then cryopreserved for future use in assisted reproduction (Gañán et al., 2009; Kishikawa et al., 1999; Martínez et al., 2008; Maksudov et al., 2009). Preservation of sperm, however, is not an option when young offspring die prior to reaching sexual maturity. Cloning has been used for a number of species and especially where the goal has been to produce a genetically exact replica of an individual animal. However, development of cloning for a new species is technically demanding and costly but, more importantly, does not immediately provide the genetic diversity that would otherwise be offered by gametes. In such cases, cryopreservation of testicular tissue can again provide an alternative strategy for *ex situ* generation of sperm from these neonatal/immature animals for use in reproductive technologies (Abbasi & Honaramooz, 2011).

3. Methodology for cryopreservation of testicular tissue

A number of cryogenic strategies have been developed to serve as a means to maintain functional properties of the preserved cells and tissues. Apparently, the first successful cryopreservation of cells was carried out by accidental freezing of fowl sperm in diluents containing glycerol (Polge et al., 1949). Later, cryopreservation of bull sperm using glycerol (Polge & Lovelock, 1952; Smith, 1961), set the stage for revolutionizing the bovine artificial

insemination industry. At about the same time, cryopreservation of unfertilized oocytes was also studied following exposure to glycerol and low temperatures (Smith, 1952). After initial success with *in vitro* embryo manipulation in the 1950s (McLaren & Biggers, 1958), research involving embryo freezing intensified. Many methods have now been developed for embryo cryopreservation and, since the 1980s, some have become routine procedures (Whittingham et al., 1972; Whittingham, 1977; Wilmut, 1972). Cryopreservation of mature oocytes has also been achieved (Fabbri et al., 2001; Porcu, 2001; Porcu et al., 1997), with high survival rates and development of normal pregnancies after *in vitro* fertilization (IVF).

Cryopreservation of structurally intact tissues in certain situations is more desirable than cryopreservation of isolated cells. This is especially important for complex tissues in which preservation of the target cells' functionality depends on that of other cell types present within the tissue. In case of testicular tissue, not only germ cells but also the intra-tubular supporting - Sertoli - cells as well as androgen producing interstitial - Leydig - cells are of particular interest. However, this requires devising suitable freezing protocols to maintain the existing relationship among different compartments of the tissue.

The first gonadal tissue to be successfully cryopreserved was ovarian tissue, using exposure to glycerol, resulting in preservation of cell viability and normal function after being autografted back into the animals (Deanesly, 1954; Green et al., 1956; Parkes, 1958). Subsequent reports of live rat offspring, sheep ovarian cyclic function, and pregnancy after grafting cryopreserved ovaries represented important steps in demonstrating the feasibility of this approach (Gosden et al., 1994; Parrot, 1960). Restoration of spermatogenesis was then obtained after cryopreserved testis cells were transplanted into recipient testes (Avarbock et al., 1996; Brinster & Nagano, 1998; Ogawa et al., 1999).

Cryopreservation of testicular tissue to be used as tissue *per se*, however, was not widely considered, perhaps due to lack of its potential applications. This need changed when we and others were first to show that cryopreservation of immature testis tissue prior to its xenografting can be done so as to maintain its potential for development of complete spermatogenesis (Honaramooz et al., 2002a; Schlatt et al., 2002). In a short period of time since then, major advances in cryopreservation of testicular tissue have opened new possibilities for preservation of male fertility in animals and humans. More recently, induction of complete spermatogenesis *in vitro* has further highlighted the importance of applying cryopreservation to testicular tissue for future applications. Overall, major advances have been made in the cryopreservation of reproductive tissues. The following sections review the primary contributing factors to be considered for optimal cryopreservation.

3.1 Biophysics of cryopreservation

A clear understanding of biophysical behaviour of cells at the time of freezing and exposure to different cryoprotectants is critical in providing conditions to improve the cell structural and functional potential after freezing-thawing. During slow rate of cooling, extracellular ice crystal formation begins with the presence of a nucleation site in the extracellular medium. Because ice is pure crystalline water, the extracellular space becomes hypertonic due to the removal of water as ice crystals develop. Intracellular water, therefore, moves outward across the cell membrane due to the differential osmotic gradient, and cells dehydrate and shrink. This is the opportunity when certain cryoprotective compounds come into play,

permeating the cells and protecting them against high solute concentration or ice crystal damage. Because various cryoprotectant agents (CPAs) permeate different cell types at varying rates, it is of benefit to understand the biophysics of cryopreservation to minimize damage (Fuller & Paynter, 2004; Pegg, 2007).

3.2 Freezing injuries

Two main rival theories have been proposed to explain cell damages due to freezing. One emphasizes the direct and primarily mechanical damage to live cells by ice crystals puncturing through the cell membranes, and the other highlights the secondary effects of ice formation via osmotic changes. Perhaps, both mechanisms are important and what is recently agreed upon is that for individual cells, for example those in suspensions, intracellular freezing is very hazardous, while the extracellular ice may not be as harmful (Pegg, 2007). Unlike cell suspensions, the cellular organization and structural composition of the tissue may be seriously affected by cryogenic damage through widespread extracellular ice formation (Hunt et al., 1982; Taylor & Pegg, 1983). Ice formation within a tissue, initiated in the extracellular space, leads to an osmotic gradient across the cell membranes, causing intracellular water to move toward the concentrated extracellular space surrounding the cells (Bagchi et al., 2008; Fuller, 2004). Due to the differential destructive effects of extracellular ice formation between cell suspensions and complex tissues, conventional approaches to cryopreservation of cells, even testis cells for instance, may not necessarily be suitable for multicellular tissues such as the testis tissue. Optimal cooling rates for various cell and tissue types have been shown to differ and be directly associated with the degree of water permeability of cell membranes at different temperatures during freezing (Leibo et al., 1970; Mazur, 1990; Pegg, 2007).

When extracellular ice formation causes elevated solvent concentrations, it leads to cell dehydration; prolonged exposure to which can permanently damage cell membranes and destabilize proteins (Fuller, 2004). However, short exposure of cells to optimized concentrations of hypertonic media before freezing might protect them from retention of supercooled water within cells and subsequent crystallization during freezing (Fuller, 2004). When cooling is faster than optimal, intracellular ice formation could occur due to inadequate time for water to follow the osmotic gradient across the cell membrane (Fuller, 2004; Fuller & Paynter, 2004; Pegg, 2007). The osmotic tolerance of cells is another critical factor to be considered during addition and removal of different cryoprotectants. Physical destruction, subsequent organelle disruption, and functional damage are some of the known consequences of ice crystal formation (Mazur, 2004).

3.3 Protection mechanism and toxicity of cryoprotectants

Sufficient concentration of cryoprotectants could minimize ice crystallization and/or promote amorphous solidification (vitrification). Glycerol was introduced as a CPA in 1949 (Polge et al., 1949) and, a decade later, cryoprotective properties of dimethyl sulfoxide (DMSO) were also reported (Lovelock & Bishop, 1959). These two cryoprotectants have mainly been used since then as classic cryoprotective additives, although many other CPAs have been introduced. Permeating CPAs, such as DMSO, glycerol, methanol, propanediol, ethylene glycol, and dimethyl acetaldehyde, as well as non-permeating CPAs, including sucrose, dextran, albumin, polyvinyl pyrollidone, and hydroxyethyl starch, have also been shown to afford effective cryoprotection (Bagchi et al., 2008; Fuller, 2004).

Cryoprotective agents are known to act through different pathways to protect cells against freezing injuries. This includes modulation of hydrogen bonding and interaction with water molecules, which give CPAs solubility and high permeability across cell membranes (Fuller, 2004). As a second mechanism, CPAs may provide a salt-buffering effect. During freezing, cells experience osmotic dehydration and shrinkage; therefore, the addition of CPAs into the cells maintains salt dilution. Basically, the CPA replaces water in cells, which dilutes the intracellular salts and prevents intracellular crystal formation. The amount of CPAs and water that permeates into the cells depends on the concentration of permeable solutes and the final cell volume. The properties of CPAs and those of cell membranes will influence the degree of cryoprotection for different cell types (Fuller, 2004; Fuller & Paynter, 2004). A third potential pathway is the stabilization of biomembrane critical macromolecules. Under normal conditions, water stabilizes the membrane bilayers. Loss of water during cryopreservation may disrupt normal membrane permeability and damage the membrane itself. The CPAs stabilize proteins as well as phospholipid bilayers of cell membranes and help to protect the membrane against freezing and dehydration stresses (Crowe, et al., 1990). Studies have collectively demonstrated that CPAs, including DMSO and disaccharide sugars such as sucrose and trehalose, may electrostatically interact with membrane phospholipids to provide stabilization (Anchordoguy et al., 1987; Rudolph & Crowe, 1985). The fourth mechanism by which CPAs protect the cells and tissue is through scavenging oxygen free radicals and preventing oxidative stress to the cells (Fuller, 2004). CPAs block the action of unstable intermediate products, such as oxygen free radicals, by binding their hydrogen atoms to them (Benson, 2004; Fleck et al., 2000). The fifth possible pathway for the protective effects of CPAs is the inhibition of nucleation, through which ice formation occurs in the media. During cooling, initial heterogeneous nucleation sites, such as small particles, change in shape and increase in size within media, eventually reaching a stage that forms ice crystals. Alternatively, induced nucleation could be beneficial to provide consistent extracellular crystallization. This phenomenon is the basis for “seeding”, which induces nucleation onto supercooled media enabling proper cryopreservation (Fuller, 2004). Seeding can be achieved by clamping the side of vials or straws with a forceps cooled in liquid nitrogen to stimulate local ice growth in the solutions. Intracellular nucleation can also be lethal or damaging for cells and tissues. Some CPAs, such as DMSO or glycerol, inhibit nucleation by increasing the high viscosity of intracellular water (Fuller, 2004). Non-permeating CPAs, on the other hand, increase and promote cellular dehydration by increasing the extracellular solute concentration thereby reducing intracellular crystallization (Bagchi et al., 2008).

Despite the protective potential of CPAs, a side effect of their addition is cytotoxicity. Tissue tolerance to CPAs is limited and overexposure may cause damage (Pegg, 2002); however, measuring this toxicity is difficult to precisely assess (Fuller, 2004). Cytotoxicity is further exacerbated by increasing CPA concentrations during ice formation. Optimizing the freezing rate as well as the addition or removal of CPAs could reduce their toxicity (Pegg, 2002).

3.4 Choice of cryopreservation strategies

For cryopreservation of testicular tissue, two popular strategies are slow freezing and vitrification. These techniques differ mainly in the concentration of CPAs used.

Cryopreservation of cells within intact tissues is obviously more demanding than for cells within suspensions. Theoretical differences include heterogeneity of cells, slower rates of solute diffusion, and heat exchange through the mass of a complex tissue. However, judging from evidence from other tissue types, if a sufficient concentration of CPAs is provided, finding a proper cooling rate can yield high survival for different cell types within the tissue (Pegg, 2007). Critical factors for effective cryopreservation, such as cell permeability to water or CPA and subsequent osmotic changes, are directly affected by the rate of cooling (Mazur, 1990). Therefore, finding the optimal cryopreservation protocol for testicular tissue of a particular species/maturational state depends on the application of a proper concentration of the cryoprotectant with a suitable cooling rate.

Slow (controlled) freezing is considered the conventional method for cryopreservation of testicular tissue, in which the CPA is used at low concentrations (usually 0.5 to 2 M) to minimize both cell damage and CPA toxicity. During slow freezing (*e.g.*, -1°C/min), the CPA is given a chance to slow down the formation of extracellular ice crystals (and prevent the intracellular ones) but especially to moderate the indirect solution effects as freezing proceeds. However, prolonged exposure to CPA before completion of cryopreservation can also cause cell toxicity (Fuller, 2004). On the other hand, if the cell is cooled more rapidly, then water will not leave the cells fast enough to avoid intracellular freezing, which is very damaging to the cells (Pegg, 2007). Using automated systems, freezing curves (**Fig. 2**) can be customized to maximize cell viability after cryopreservation of the tissue.

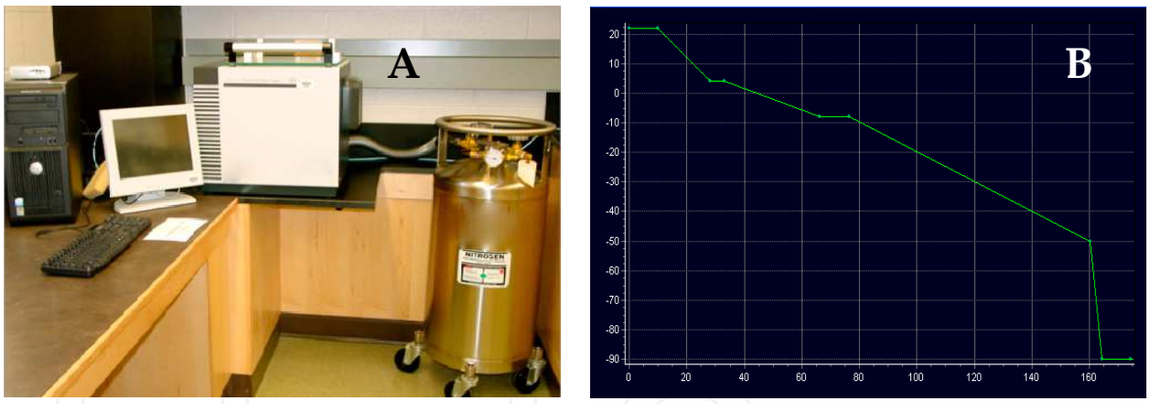

Fig. 2. A programmable automated freezing system. Although requiring larger capital investments, automated cell/tissue freezing systems (**A**), consisting of a freezing chamber attached to a computer and a liquid nitrogen tank, allow customization of the freezing curve (**B**) to achieve pre-defined temperatures (Y-axis) for desired lengths of time (X-axis), in an accurate and consistent manner.

As indicated earlier, the formation of extracellular ice, which may not pose a problem for freezing of cell suspensions, is likely the main problem for tissues. Therefore, an alternative route to avoid ice crystal formation and solute damage within the tissue is to avoid ice crystal formation altogether using transformation of aqueous milieu of the cell/tissue to the amorphous character of a glassy state, known as vitrification. Vitrification is a cryopreservation method in which ice crystal formation is prevented because the cells or tissues are exposed to very high concentrations of CPAs (*e.g.*, 5 to 8 M) and undergo ultra rapid freezing rates (*e.g.*, up to -2500°C/min) (Fuller, 2004; Pegg, 2002, 2007). However, this

approach is compromised by the cytotoxic effects of CPAs at such high concentration, especially with increased exposure times (Fuller, 2004; Fuller & Paynter, 2004). For small volumes of cell suspension, CPA concentrations can be reduced somewhat by using very rapid cooling and warming rates. However, especially with increasing size and complexity of the tissue, the limits of temperature exchange rates are more restricted, hence the use of very high concentrations of CPAs are unavoidable (Pegg, 2007). To overcome this problem, the use of a combination of CPAs to improve vitrification while reducing toxicity has been suggested. Proper media may include disaccharides, such as sucrose or trehalose, and proteins or polymers (Kasai & Mukaida, 2004; Sutton, 1992). The optimal CPA concentrations and exposure times to prevent toxicity must be specifically considered for each tissue type. (Fuller & Paynter, 2004; Pegg, 2007). We have used a solid-surface vitrification method to minimize the volume surrounding the tissue pieces, while avoiding liquid nitrogen (LN_2) vapour formation and preventing direct contact with LN_2 to prevent potential contamination (**Fig. 3**, Abrishami et al., 2010a).

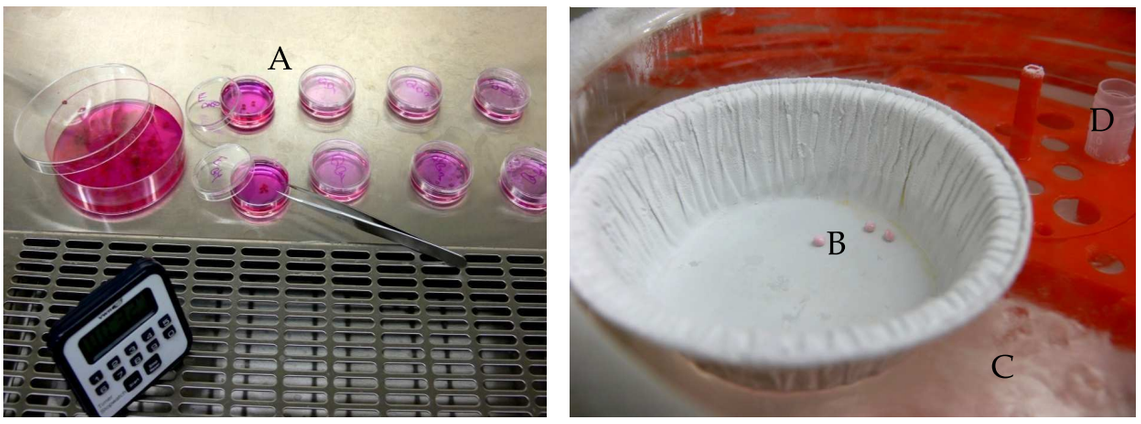

Fig. 3. Solid-surface vitrification procedure for testicular tissue fragments. After exposure of testis tissue fragments to differing concentrations of vitrification solutions for varying lengths of time (**A**), testis tissue fragments are placed on a sterile aluminum boat (**B**) floating on liquid nitrogen (**C**), then transferred into cooled cryovials (**D**) followed by plunging into liquid nitrogen (images modified from Abrishami, 2009).

3.5 Thawing methods

Whether freezing is permitted (conventional cryopreservation) or prevented (vitrification), the CPA that has reached the internal compartments of a multicellular system must diffuse back through numerous membranes in the tissue, with each acting as a barrier. Therefore, optimal thawing and CPA removal procedures are also critical factors for cell/tissue survival after freezing (Bagchi et al., 2008). Earlier studies pointed out that consistent cooling and thawing rates (slow-freezing followed by slow-thawing, or fast-freezing followed by fast-thawing) can improve cell/tissue survival after cryopreservation (Whittingham et al., 1972). Moreover, extreme osmotic changes during CPA removal might damage the cells by extensive cell shrinkage or swelling associated with the rapid movement of water into the cell as compared to the slower movement of the CPA out of the cell. However, a limited amount of water replacement is needed to restore osmotic equilibrium and physiologic cell volume (Pegg, 2007).

3.6 Post-thawing analysis

For successful cryopreservation of a complex vascularized tissue, such as testis tissue, the majority of essential cells need to be viable for the tissue to survive and retain its function. However, there is not yet a comprehensive and universally applied method for post-thawing analysis of cryopreserved testis tissue; subsequently, multiple approaches have been used to assess tissue/cell viability and extent of cryogenic injuries. These approaches commonly include histopathological examination of tissue sections for morphological changes. Using light microscopy, for instance, such objective criteria as seminiferous cord/tubular diameter or cell density within tubule cross sections can be measured, or semi-quantitative morphometric analyses applied to subjectively score such criteria as health or integrity of tissue compartments (Abrishami et al., 2010a; Curaba et al., 2011; Milazzo et al., 2008; Travers, et al., 2011). Transmitted electron microscopy, although not widely used, can be invaluable in the examination of subcellular components most likely to be affected by testis tissue cryopreservation, including cytoplasm integrity, nuclear membrane, and various organelles (Keros et al., 2007). Other valuable morphological analyses may include assessment of cell-specific changes, for example, using double-staining of proliferation markers (*e.g.,* Ki67) and MAGE-AH, vimentin, or CD34 for identification of spermatogonia, Sertoli cells, or peritubular cells, respectively (Keros et al., 2007; Wyns, et al., 2007).

A quantitative measure of tissue damage due to cytotoxicity after cryopreservation can be achieved through lactate dehydrogenase release assays (Curaba et al., 2011) or through viability assessment of dissociated cells after digestion of frozen-thawed tissues using Trypan blue exclusion assays or the various cell viability kits using a flow cytometer analyzer (Abrishami et al., 2010a; Gouk et al., 2011). Assessment of apoptosis, using for instance, caspase-3 (Wyns et al. 2008), or TUNEL assay for detection of DNA fragmentation provides insight into the extent of cell damage (Milazzo, et al., 2008). Detection of phophatidylserine translocation from the inner to the outer layer of the plasma membrane, using fluorescent-labelled Annexin V, also allows more targeted assessment of apoptotic-associated changes within the cryopreserved testis tissue (Milazzo et al., 2008).

Having merely high cell survival rates or lacking visible damage does not guarantee functional preservation of the tissue as a whole. A thorough post-thawing analysis should include a form of testing for the functionality of the cryopreserved tissue. Post-thawing *in vitro* organotypic culture of the cryopreserved testis tissue has allowed assessment of its survival in the short-term (Curaba et al 2011; Keros et al., 2007) and measurement of its hormone release into culture media (Gouk et al., 2011). Perhaps more robust examination is provided by grafting, where the survival and developmental competence (both in terms of germ cell differentiation and androgen release) of the cryopreserved tissue *in vivo* as grafts allows a longer-term functional assessment (Abrishami et al., 2010a; Jahnukainen et al., 2007; Wyns et al., 2007).

3.7 Effects of tissue size

To offer cryoprotection, the CPAs need to diffuse rapidly in and out of the tissue; therefore, the size of testis tissue samples undergoing cryopreservation can be an important intuitive consideration. The results of studies differ depending not only with respect to the donor species but also potentially on the protocols employed. For instance, while cryopreservation of immature rat testis using similar procedures demonstrated better results for 7.5 mg pieces than 15 mg pieces (Travers et al., 2011), cryopreservation of immature mouse testis using

whole testes with punctured tunica albuginea was deemed more suitable than using whole testes with intact tunica, whole testes without tunica, or testis halves (Gouk et al., 2011). Mouse testes have considerably less connective tissue content than most other species; therefore, tissue fragment size is especially a concern for testis tissues from species with higher interstitial tissue density. For cryopreservation of (cryptorchid) testes from prepubertal boys, fragments sizes of 2-9 mm^3 were used successfully (Wyns et al., 2007). We also reported that immature porcine testis tissues undergoing the same cryopreservation treatments were not affected by the original size of the testis tissue fragment (5, 15, 20, or 30 mg) (Abrishami et al., 2010a). Although not used for cryopreservation, no effect of tissue sample size was observed for one-wk old piglet testes (as intact or fragments of 100 or 30 mg) when used for hypothermic preservation for 6 days (Yang et al., 2010). It remains to be seen if whole human testes can be cryopreserved as has been accomplished for whole ovaries (Courbiere et al., 2006; Jadoul et al., 2007; Martinez-Madrid et al., 2007).

4. Applications of testis cryopreservation for new reproductive technologies

Given that properly cryopreserved testis biopsies can last decades in liquid nitrogen and that most prepubertal cancer patient boys donating biopsies may not need to resort to assisted reproductive technologies for a couple of decades, it is advisable that cryopreservation of testicular biopsies be offered to such patients in a hope that our ability to use such tissues will be further improved and the options expanded in the coming years.

A number of potential applications already exist for the use of cryopreserved testicular tissue in experimental and clinical settings in reproduction medicine/science. Such technologies allow retrieval of existing sperm from mature donor samples and, more importantly, offer hope for production of sperm in samples of cryopreserved testis immature testis. If the preserved testis tissue contains endogenous spermatogenesis (*e.g.*, from obstructive azoospermic adult patients), it can be used to extract sperm, elongated spermatids, or even round spermatids to be used for fertilization of oocytes through ICSI (Rosenlund et al., 1998; Schrader et al., 2002; Gianaroli et al., 1999; Tesarik et al., 2000; Schoysman et al., 1999).

If preserved testis samples are obtained from neonatal/immature donors, they can still be used to induce spermatogenesis through the following approaches.

4.1 Germ cell transplantation

The technique for germ cell transplantation has allowed (re)establishment of spermatogenesis after introduction of donor testis cell suspensions into the seminiferous tubules of infertile recipient testes. Once deposited in the tubular lumen, donor SSCs are recognized by the host Sertoli cells and allowed passage to the stem cell niche, where new colonies of spermatogenesis can begin and expand. This approach has allowed production of sufficient numbers of sperm to allow infertile recipient mice to sire donor-derived progeny (Avarbock & Brinster, 1994; Brinster & Zimmermann, 1994). Later, the capability of cryopreserved mouse testis cells after transplantation into recipient testes to start spermatogenesis was also confirmed (Avarbock et al., 1996; Brinster & Nagano, 1998; Ogawa et al., 1999). While heterologous transplantation of human germ cells into recipient mice did not lead to completion of spermatogenesis (Nagano et al., 2002), the transfer technique has been tested

using human testes (Schlatt et al., 1999; Brook et al., 2001). Although autologous/homologous transplantation of germ cells for humans is currently considered purely experimental, one possibility for prepubertal human testis samples taken and frozen prior to treatments is to isolate testis cells and transfer them back to the individual. As a major problem with this approach is the risk of reseeding a systemic cancer, solutions to this (e.g., soring out tumour cells) and other safety issues are under investigation.

We have expanded the technique for germ cell transplantation into farm animals (**Fig. 4**), showed the feasibility of SSC engraftment in unrelated recipient individuals (of the same species) without a need for immune-suppression, and further demonstrated the applicability of the approach through donor-derived sperm production by the recipients and birth of progeny carrying the donor characteristics (Honaramooz et al., 2002b 2003a, 2003b; Honaramooz & Yang, 2011). Therefore, although experimental at this stage, the approach may offer promise in salvaging genetic material from cryopreserved testicular tissue from immature endangered species.

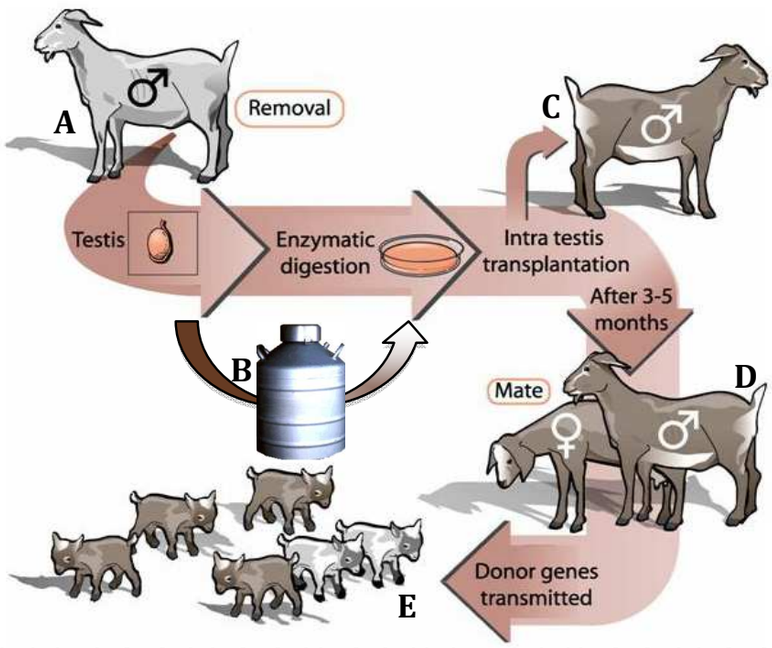

Fig. 4. Schematic overview of germ cell transplantation from a donor male into the testes of a recipient. The testes are collected from a donor animal (**A**), which could theoretically include post-mortem testis recovery from a recently deceased juvenile individual of an endangered species. The testis tissue could be cryopreserved (**B**) until conditions for its use are in place. At the time of transplantation, a single-cell suspension is prepared and the cells are infused into the seminiferous tubules of a recipient animal (**C**). Mating of the recipient (**D**) produces progeny (**E**), some of which will carry the donor genome (image modified from Honaramooz et al., 2003b).

4.2 Testis tissue (xeno)grafting

Another potential strategy for the use of cryopreserved testis tissue is represented by testis tissue xenografting. Grafting of both fresh and cryopreserved testis tissue fragments from

donors of different species under the back skin of recipient mice results in the production of functional sperm (Honaramooz et al., 2002a). The approach has especially been successful using neonatal/immature donors (**Fig. 5**), from laboratory animals to domestic animals, primates, and even humans (Honaramooz et al., 2002a, 2004, 2008; Schlatt et al., 2002; Oatley et al., 2004; Snedaker et al., 2004; Rathi et al., 2005, 2006; Arregui et al., 2008; Abrishami et al., 2010b).

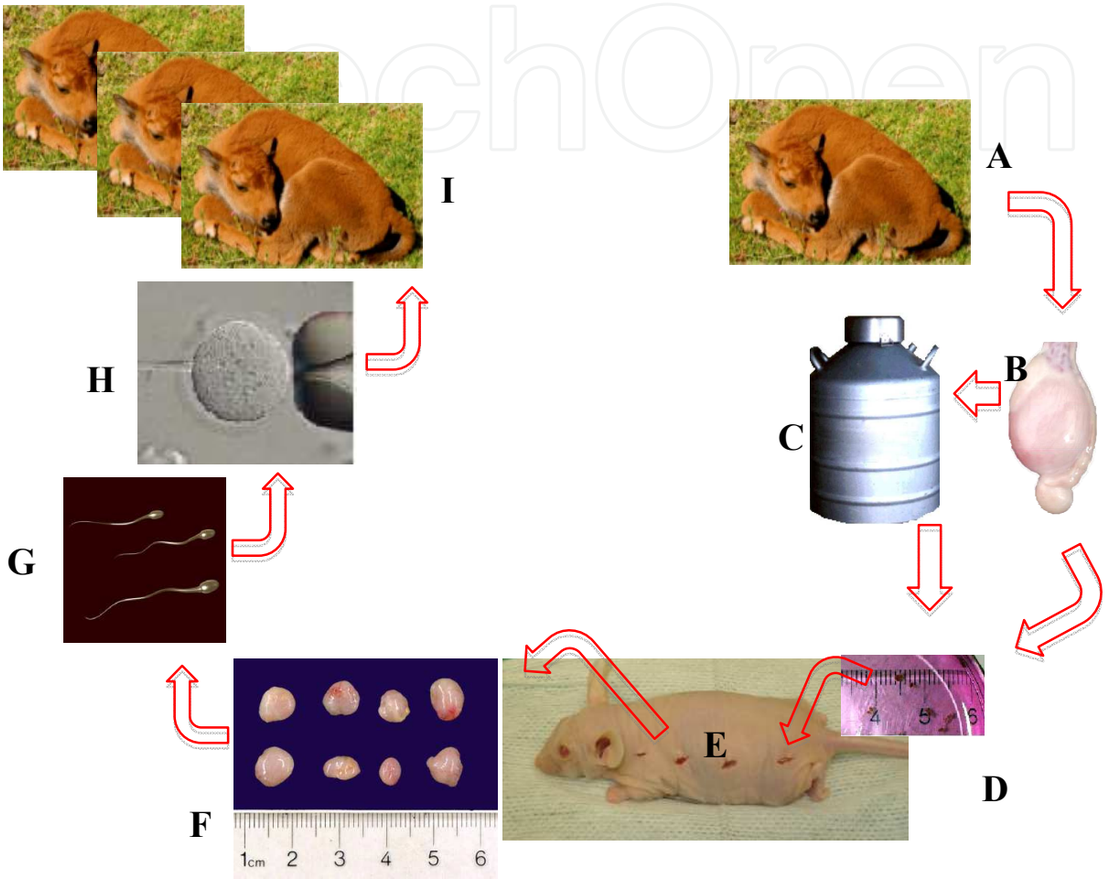

Fig. 5. Schematic representation of testis tissue (xeno)grafting from an immature donor individual into the back skin of a host mouse. The testes are collected from a donor animal (**A**), which could include post-mortem testis recovery from a recently deceased newborn animal of an endangered species. The testis tissue (**B**) could be cryopreserved (**C**) until grafting. At the time of grafting, tissue fragments of ~0.5 mm^3 (**D**) are prepared and the fragments are grafted subcutaneously into an immunodeficient host mouse (**E**). When given enough time, the grafts can grow in size (**F**) and undergo development, leading to the production of complete spermatogenesis, including fertilization-competent sperm (**G**). The sperm can then be extracted from the grafts and used in intracytoplasmic sperm injection (ICSI) (**H**), which after embryo transfer can potentially lead to birth of progeny (**I**).

The sperm recovered from such grafts, including those from primates, have been shown to be fertilization competent after ICSI (Honaramooz et al., 2002a, 2004, 2008), leading to the birth of healthy progeny (Schlatt et al., 2003; Nakai et al., 2010). We recently showed that testes recovered post-mortem from newborn bison calves, as a model for closely-related rare

or endangered ungulates can be used for this application, and when allowed to develop in the host mouse, lead to full spermatogenesis (Abbasi & Honaramooz, 2011). Therefore, testis tissue xenografting can be used as unique solution for genetic conservation of immature males by producing sperm from these otherwise resource-less donors in xenografts, followed by extraction and cryopreservation of sperm for future use in ICSI (**Fig. 5**).

However, xenografting of human gonadal tissues into animals to harvest the resultant gametes for use in IVF for humans is prohibited in Canada, and possibly in other countries, due to the potentially serious risk of animal viral transmission or contamination with animal genetics. Nevertheless, the promising results from animal research suggest a potential hope for future use of cryopreserved testis biopsies from pre-adolescent boys to be grafted back to the individual; whether this technique can be used to produce viable sperm for future use from prepubertal boys undergoing gonadotoxic treatments remains to be determined. However, the same safety risks as for autologous germ cell transplantation exist and require addressing before such an option can be offered clinically.

4.3 *In vitro* maturation of germ cells

In theory, cryopreserved testicular tissues can also be used for *in vitro* induction of differentiated germ cells and ideally production of sperm or spermatids to be used for ICSI. If successful, this approach can circumvent the potential risk of reintroducing cancer cells into post-recovery patients. Many labs have experimented with the idea, and some have had success with maturation of later stages of human spermatogenesis (but not from SSCs), including live births (Tesarki et al., 1999). Availability of a culture system to support complete *in vitro* spermatogenesis from the SSC stage was, however, elusive until very recently when it was reported that all spermatogenic lineage cells including fertilization-competent sperm could be produced from neonatal mouse testes maintained exclusively in a culture system (Shinohara et al., 2011). This is a very promising step, indicating that similar results may be achievable in future using immature human testis biopsies.

5. Current trends in testis tissue cryopreservation

Since the first reports of successful germ cell transplantation and xenografting of testis tissue raised new interest in this field, several promising cryopreservation protocols have been introduced. Perhaps not surprisingly, the results differed and at times conflicted depending on the tissue donor species/developmental stage. These first reports of cryopreservation of pig and mouse testis tissues were based on DMSO-based slow freezing protocols originally developed for isolated testis cells or for ovarian tissue, respectively (Honaramooz et al., 2002a; Schlatt et al., 2002). Later, other detailed studies comparing multiple protocols showed high cell viability with programmed slow-freezing of immature mouse testis tissue using 1.5M DMSO as a cryoprotectant (Milazzo et al., 2008; Traverse et al., 2011). DMSO has also been found to be a more suitable cryoprotective agent than ethylene glycol for immature mouse and rat testis tissue (Goossens et al., 2008; Jezek, 2001). Shinohara et al. (2002) reported the birth of mouse offspring from sperm retrieved from cryopreserved pre-pubertal testis tissue with DMSO after transplantation under tunica albuginea of the recipient testes (Shinohara et al., 2002). Similar results were obtained using primate testis tissue, where 1.4M (but not 0.7M) DMSO was able to protect some of the developmental potential of grafts from rhesus monkeys (Jahnukainen et al., 2007) but the 0.7M DMSO protocol was successful for cryopreservation of

human testis tissue (Wyns et al., 2007) at one age/developmental stage but not others (Wyns et al., 2008; Keros et al., 2005, 2007). Somewhat different from reports in other species, and after an extensive study of several strategies for cryopreservation of immature testis tissue, we concluded that glycerol was a better cryoprotectant for pig tissues (Abrishami et al., 2010a). These results suggest that each species and donor developmental age may need a different cryopreservation protocol, with a concomitant need to adjust the concentration of cryoprotectant or even adopt different cryoprotectants. These differences may be related to testicular architecture, morphology, or lipid composition.

In a first report of immature testis tissue vitrification, we also showed maintenance of cell viability and developmental potential to actively (re)establish complete spermatogenesis after xenografting into immunodeficient mice (Abrishami et al., 2010a). Recently, similar or much higher cell viability results were obtained using immature mouse testis tissue with vitrification compared with conventional slow freezing (Gouk et al., 2011; Curaba et al., 2011). With proper tissue handling, and the use of an appropriate choice of final cryoprotectant exposure, vitrification can provide preferential conditions for tissue freezing with proven superior results in restoration of immature testis tissue. Vitrification also does not require the extensive laboratory equipment commonly used for programmed slow freezing; however, direct plunging of tissues into liquid nitrogen, a common procedure in routine vitrification, poses a greater risk of contamination. The solid-surface vitrification of testis tissue (**Fig. 3**) is an easy, safe, and applicable cryopreservation technique for the preservation of tissue structural integrity and developmental potential.

6. Conclusion

Although cryopreservation of isolated testis cells has been successfully achieved for animals and humans, only in the past 10 years has intense attention been paid to cryopreservation techniques aimed at maintaining the developmental potential of structurally intact testis tissue. Cryopreservation of testis tissue theoretically offers a practical method when other techniques such as cryopreservation of ejaculated sperm are not available or applicable. Preservation of testis tissue has many applications, including conservation of fertility for prepubertal boys undergoing gonadotoxic cancer therapies. Ovarian and testicular toxicity are the inevitable long-term consequences of certain therapeutic oncological regimens, leading to premature fertility failure or sterility in cancer patients. Cryopreservation of gonadal cells or tissue before high-dose gonadotoxic chemo- and radio-therapy may therefore be considered in a comprehensive treatment and recovery plan. This could provide an alternative method for preserving the fertility potential of prepubertal boys with cancer or azoospermic men, as spermatogenesis is not completed in these patients. Although successful gamete and gonadal tissue restoration could have major impact on the enhancement of fertility preservation, serious ethical implications associated with collection and preservation of human gametes and gonadal tissues have yet to be resolved. Salvaging the genetic potential of immature endangered and valuable animals through banking of gonadal tissue is also a subject of clinical significance in animal reproduction and conservation. Optimal cryoconservation methods could also be combined with transplantation, xenografting, or culturing techniques to overcome some of the complications in the biodiversity crisis of rare or endangered species. In fact, experimental methods for the generation of fertility-competent gametes from cryopreserved ovarian or testis tissues have paved the way for future clinical use in human patients. Therefore,

experimental conservation of gonadal tissue and cells by cryopreservation can serve as a platform for further evaluation of the potential for long-term storage.

Many challenges are associated with the optimal maintenance of tissue structure and the subsequent functional restoration of cryopreserved samples. It is intuitively known that optimal cryopreservation requires refinement of freezing and thawing rates, osmotic conditions, choice and concentration of cryoprotectants, and equilibration times in cryoprotective solutions. Indeed, improvement of all aspects of freezing techniques will ensure survival rates of tissue structure and subsequent functional restoration of cryopreserved cells within those tissues. Several studies have examined cryopreservation of testis cell suspensions or tissue fragments using glycerol, ethylene glycol, DMSO, or propanediol. In most cases, analyses of the cryopreserved samples lacked functional assessments of the preserved testicular cells/tissues. We now know that even if many cells of a multicellular system survive freezing and thawing, preservation of all functional compartments of the tissue is not guaranteed. Merely maintaining the physical characteristics of the cryopreserved testis tissue is not adequate, and an efficient approach to overcome the deficiencies in developmental (re)establishment of spermatogenesis is also required.

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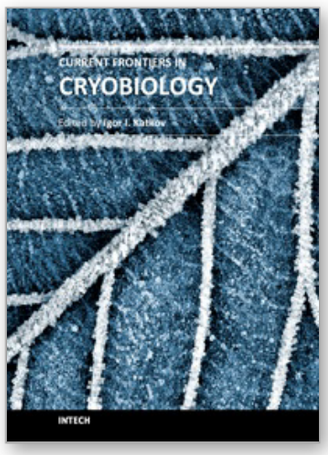

Current Frontiers in Cryobiology

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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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