Chapter 1

# The history of sperm cryopreservation

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## Importance of sperm cryopreservation

While there have been several relatively recent comprehensive reviews of mammalian sperm cryopreservation (Watson, 2001; Leibo, 2004), this chapter is not meant to be a review of the literature of mammalian sperm cryopreservation. Instead, we intend to provide an understanding of the history, current status, and potential future direction of mammalian sperm cryobiology. There are many reasons why sperm cryopreservation is important, including: (1) maintenance of genetic diversity in domestic and wild species populations (Wildt, 1992; Critser & Russell, 2000); (2) facilitating the distribution of "genetically superior" domestic species lines; (3) treatment of iatrogenic infertility (Kuczynski *et al.*, 2001; Ranganathan *et al.*, 2002; Tash *et al.*, 2003; Agarwal *et al.*, 2004; Nalesnik *et al.*, 2004); and (4) genetic banking of genetically modified animal models of human health and disease (Critser & Russell, 2000; Knight & Abbott, 2002).

While actual references to sperm cryobiology and cryopreservation date as far back as the 1600s (Sherman, 1964), it was not until the development of artificial insemination (AI) in the late 1950s and early 1960s, when the dairy industry needed longer-term storage methods for bull sperm, that sperm cryopreservation became a major area of scientific investigation. Polge *et al.* (1949) made a pivotal discovery that showed that the use of glycerol (a permeating solute) could provide protection to cells at low temperatures. This is often cited as the defining moment in the establishment of modern sperm cryobiology. However, there is actually a very important body of literature which predates it, some of which established the scientific basis upon which their work was interpreted and further developed (Lovelock, 1953a; Lovelock & Polge, 1954; Menz & Luyet, 1965; Luyet, 1966; MacKenzie & Luyet, 1967a; 1967b; Luyet & Rapatz, 1970; Luyet, 1971; Rasmussen & Luyet, 1972; Rapatz & Luyet, 1973), and some of which was parallel literature that was not incorporated into the main pathway of sperm cryobiology mainly because it was published in Russian (Katkov, 2005).

Based upon this scientific history, the development of "successful" mammalian sperm cryopresveration methods was established. It is critical to realize, however, that even today only a very few mammalian species' sperm can be effectively cryopreserved. Even in those cases, the "success," as measured by postthaw motility, routinely is 50 percent or less than that of the prefreeze motility. Successful cryopreservation varies highly among species, individuals within species, and even within ejaculates of individuals, which is largely attributed to the differences in biophysical characteristics among cell types (Gao *et al.*, 1997; Thurston *et al.*, 2002; Walters *et al.*, 2005).

Sperm Banking: Theory and Practice, eds. Allan A. Pacey and Mathew J. Tomlinson.
Published by Cambridge University Press. 

## History of AI and sperm cryopreservation

It is believed that Lazzaro Spallanzani performed the first successful AI in 1780. Spallanzani inseminated a confined bitch in heat by depositing semen in the uterus with a syringe. This resulted in the birth of three pups 62 days later that resembled the dam as well as the semen donor (Herman, 1981). Other investigators repeated the work of Spallanzani, including John Hunter in 1799, which resulted in a human pregnancy (Herman, 1981). It was not until the 1880s that AI became a means of improving fertility and increasing the number of offspring that one sire could achieve in a lifetime. An English dog breeder between 1884 and 1896 used AI to breed several bitches with one ejaculate (Herman, 1981). At about the same time a French veterinarian demonstrated the effectiveness of AI as a way of improving fertility in the horse as well. Following these initial demonstrations of the effectiveness of AI, horse centers began to spring up all over Europe to collect and extend semen and breed mares (Herman, 1981). The techniques for AI, training of technicians, and improved methods for collection and extending semen continued to increase, and, by 1938, it was reported that some 40 000 mares, 1.2 million cows, and 15 million sheep had been serviced by AI in Russia (Herman, 1981).

In the early 1800s most of the AI work was being conducted in Europe or Russia; however, there had been successful attempts at AI in horses and cattle in the USA as well. In 1907 a calf was born from AI by L.L. Lewis at the Oklahoma Experimental Station (Herman, 1981). After this successful AI attempt in cattle, the use of AI in the USA increased, and, in 1937, the practice was started in the dairy herd at the University of Missouri, and, subsequently at other agricultural universities such as Cornell, Minnesota, Nebraska, Wisconsin, and Tennessee (Herman, 1981). In 1939 the American Society of Animal Production (ASAP) was founded by investigators from several universities to discuss and develop protocols for semen collection, evaluation, and preservation from domestic animals, and in particular, the bull (Foote, 1998). It was believed that with the continued development of AI, the dairy industry in the USA could start progressive breeding programs and begin advancing the industry from a genetic perspective. Research programs across the country began investigating sperm biology in terms of collection, equipment, evaluation of sperm morphology, and composition of extenders for both sperm biology and fundamental cryobiology (Foote, 1998).

### Cryopreservation of bull sperm

The development of cryopreservation protocols for the bull to be used for AI in the dairy industry began in the 1950s. Bratton *et al.* (1955) demonstrated in field trials that bovine sperm frozen to −79 °C and packed on dry ice could still yield high fertility. Several different media formulations were then investigated in terms of their ability to maintain a high level of motility postthaw at reduced temperatures, and these were termed "extenders." Early in extender development it was found that lipids in egg yolk could protect bull sperm from "cold shock," the sensitivity of cells to reduced temperatures (Watson & Martin, 1973; Watson, 1975; Watson & Martin, 1975; Foote, 1998). The lipid work, in combination with the discovery of the cryoprotective properties of glycerol, aided the development of freezing extenders for cryopreservation of bull sperm. The sum of these discoveries yielded the Tris-egg yolk–glycerol method for freezing bull sperm, which has now become a standard (Foote, 1970; Watson & Martin, 1973; Watson, 1975; Watson & Martin, 1975).

## Cryopreservation of boar sperm

Preservation of boar sperm was developed in the 1970s; however, the method used was different than that for other species. Specifically, Pursel & Johnson (1975) developed a "pellet" method for successful freezing of boar sperm. First, samples were cooled to 5°C at a rate of 0.22°C/min. At this temperature, cooled media containing extender and glycerol were added. After the addition of glycerol, aliquots of the samples were placed directly on a block of dry ice (−79°C) and then plunged into liquid nitrogen (−196°C). This pellet method was relatively effective in terms of postthaw motility, but a major drawback was the inability to label the pellets individually and the difficulty involved with shipment of the samples. In recent years, other methods have been developed such as "maxi" (5 mL) and "mini" (0.25 or 0.5 cc) straws, which allow individual identification and ability to ship domestically and internationally (Bwanga *et al.*, 1990, 1991).

## Cryopreservation of mouse sperm

G.L. Rappatz's unpublished and undated work is believed to be the first successful attempt to produce live mice from frozen sperm. In a review by Graham *et al.* (1978) Rappatz's work was discussed as part of a final progress report. However, cryopreservation of mouse sperm was not studied intensively until three independent groups reported successful cryopreservation essentially simultaneously (Okuyama *et al.*, 1990; Tada *et al.*, 1990; Yokoyama *et al.*, 1990). Laboratories across the world have repeated these findings and reported acceptable postthaw viability, but most success has been limited to sperm samples from hybrid or mixed genetic backgrounds (Bath, 2003). Sperm cryopreservation protocols for inbred strains (e.g. C57BL/6) have only been marginally successful due to their extreme sensitivity to the multiple procedural steps involved, such as pipetting, centrifugation, addition and removal of cryoprotective agents (CPAs), and hypothermia (Willoughby *et al.*, 1996; Katkov & Mazur, 1998, 1999; Agca *et al.*, 2002).

There are known differences in terms of fertility and cryosurvival between genotypes of mice (Pomp & Eisen, 1990; Rall *et al.*, 2000) as well as other species such as the rat (Rall *et al.*, 2000) and pig (Thurston *et al.*, 2002). It has been shown that the efficiency of in vitro fertilization (IVF) using fresh sperm is highly variable between genetic backgrounds; for example the fertilization rates (two-cell development) for an inbred line (BALB/c) are lower than fertilization rates for a hybrid line (B6C3F1) (Kawase *et al.*, 2004). Kawase *et al.*, (2004) also reported a difference in the fertilization rates between BALB/c (26 percent) and C57BL/6 (85 percent) for IVF with fresh sperm. The fertilization rates for conventional IVF were even further reduced with frozen–thawed sperm from B6C3F1 males (Kawase *et al.*, 2004). Frozen–thawed sperm from an inbred strain used in a conventional IVF system usually results in very low efficiency largely owing to the lack of motility and cellular damage that occurs during cryopreservation. Futhermore, Nishizono *et al.* (2004) reported that frozen–thawed C57BL/6 sperm had abnormal mitochondrial morphology. In addition, Willoughby *et al.* (1996) reported a significant difference between ICR and B6C3F1 for the percentage of sperm maintaining functional mitochondria following hypo- and hyperosmotic exposure. Nishizono *et al.* (2004) also reported a strain difference between inbred mice, C57BL/6J, and DBA/2N in cryopreservation-induced cellular injury. In the C57BL/6J sperm 83.7 percent of the sperm were damaged compared with DBA/2N (10 percent).

## Cryopreservation of human sperm

The glycerol work of Polge *et al.* (1949) laid the foundation for cryopreservation of human sperm. In 1953 Sherman and Bunge froze human sperm equilibrated with 10 percent glycerol on dry ice with a 67 percent survival rate. Shortly thereafter Sherman and Bunge (1953) reported three pregnancies with the use of AI in combination with frozen–thawed sperm. After this report of pregnancies following insemination with frozen–thawed cells, many groups began working toward cryopreservation as a treatment for infertility. Sherman continued to pursue the improvement of human sperm preservation by investigating various cryopreservation protocols along with freeze-drying techniques (Sherman, 1954, 1963). One of Sherman's discoveries was that storing human sperm at liquid nitrogen ($LN_2$) temperature (−196°C) was superior to storage at −75°C. In addition, no loss of motility was observed when the sperm were stored in $LN_2$ for one year; however, there was a decline in motility after storage at −75°C (Sherman, 1963). Prior to 1964 all pregnancies were produced from short-term storage of sperm; however, Perloff *et al.* (1964) reported pregnancies from insemination with frozen–thawed sperm stored for one to 5.5 months.

## History of fundamental cryobiology

Before outlining the history of fundamental cryobiology we must first understand what fundamental cryobiology is and why it falls into a separate category. Cryobiology has functioned for centuries as an observational science. From at least 1787 the effects of cold temperatures on cells have been investigated empirically (Luyet & Gehenio, 1940). Before Polge *et al.* (1949) made their accidental discovery of glycerol as a cryoprotectant (a fortuitous empirical observation), the nascent field had been limited to descriptions of cellular and tissue behavior of many different cell types at subphysiologic temperatures. These empirical studies, however, formed a basis for a theory of the causes of freezing success or failure. The discovery of CPAs opened the doors to a new type of cryobiological study because new variables had been identified that could be used in the optimization of cryopreservation protocols.

In comparison with classic empirical cryobiology, fundamental cryobiology is the quantitative study of the biophysical and biochemical phenomena that occur during cryobiological procedures. These include the transmembrane fluxes associated with the addition and removal of CPAs, the change in chemical potentials during cooling and warming, both intracellular and extracellular ice formation, the effects of cooling and warming rates and storage temperatures, heat transfer in solutions and tissues, and, most importantly, the optimization of cryobiological procedures in conjunction with this information. Because, paradoxically, freezing is used both with the goal of preserving cells and of selectively destroying them, we have been careful to define fundamental cryobiology in the most general context. In the context of this chapter, however, we may be more specific: fundamental cryobiology attempts to quantify the biophysical phenomena that occur during freezing and thawing in order to use this information to develop theoretically optimized cryopreservation protocols. In fact, we will further limit our discussion to the simplest case of single cells frozen and thawed in suspension (e.g. sperm), and we will try to provide both a theoretic and historic background to this area of cryobiology.

With a proper definition in place we can see that fundamental cryobiology covers an enormous range of topics in science, from physics and chemistry to engineering and mathematics, and it is this amalgamation of sciences that appeals to many investigators

from these diverse fields. The first quantitative studies of the biophysical effects of cryopreservation and the use of this knowledge to predict more optimal cryopreservation protocols were explained in Mazur (1963). In his manuscript Mazur examined the connection between a cell's permeability to water and solutes and the freezing rates at which it might be effectively frozen. Mazur notes that, with the knowledge of the biophysical parameters of the cellular membrane, we may significantly narrow the range of empirical research needed to optimize freezing protocols. This reduction in empirical research is perhaps the most significant advantage of quantitative approaches.

In essence, with his paper Mazur formed the basis for much of the work that we call "fundamental cryobiology" today. For example, presently there is a large canon of literature devoted to determining the membrane permeability characteristics of cell types with the purpose of improving cryobiological protocols. These experiments typically determine membrane permeability coefficients by calculating cell volume as a function of time. Devices to determine these coefficients have included stopped flow apparatus (Liu *et al.*, 2002), electronic particle counters such as Coulter Counters (Gilmore *et al.*, 1998), microscope stages designed to allow the perfusion of fluids while keeping the cells in place (either with a porous membrane or by holding with a pipette) (Gao *et al.*, 1996), and several others (McGrath, 1997). These data are then fit with one of several transmembrane flux models to determine the membrane transport coefficients for a specific cell type (see Kleinhans (1998) for a review). Armed with this knowledge, computer simulation can quickly determine which protocols are most likely to provide a successful cryopreservation scheme. We will discuss this in greater detail below. Interestingly, as with many aspects of cryobiology and especially fundamental cryobiology, much research had been done on various cell types before the application of this knowledge to cryopreservation (Jacobs, 1932; Staverman, 1951).

The survival of cells with respect to cooling rate is almost always described in an inverted "U" shape. This may be observed experimentally for many cell types (see Figure 1.1). Mazur's 1962 paper explained quite well that the most probable cause for the second half of the inverted "U" (i.e. higher cooling rates), came from the supercooling (cooling of the solute below its freezing point) induced by cooling at a rate that prevented the cell from maintaining an equilibrium between the intracellular and extracellular milieu. As the cell and the surrounding media cool below freezing, pure water is precipitated out of the extracellular media as ice. This causes an increase in extracellular concentration, which, at slow enough cooling rates, causes the cell to lose water and increase intracellular concentration. When cooling rates are too rapid, water cannot leave the cell quickly enough to maintain equilibrium with the external medium and supercools. Since the probability of ice formation increases with the degree of supercooling (a topic we will discuss in further detail below), the faster the cooling rate the more likely ice will form inside the cell, a major factor of cell death in cryopreservation procedures.

There is considerable debate about the mechanism of damage during slow cooling. Based on the original work of Lovelock (1953a, 1953b), Levitt (1962), and Karow and Webb (1965), Meryman (1968) ascribed the damage of slow cooling to the effects of salt concentrations on human red blood cells and had determined that the membrane became porous to these molecules at extremely high salt concentrations (which are often reached at subzero temperatures). Upon warming the salt molecules were trapped in the membrane, causing a difference in osmotic pressure, an influx of water, and the probable lysis of the cell. Pegg and Diaper (1988) attribute slow cooling injury to decreases in cell volume during slow freezing. In 1972, Mazur and coworkers formalized his explanation of cell damage

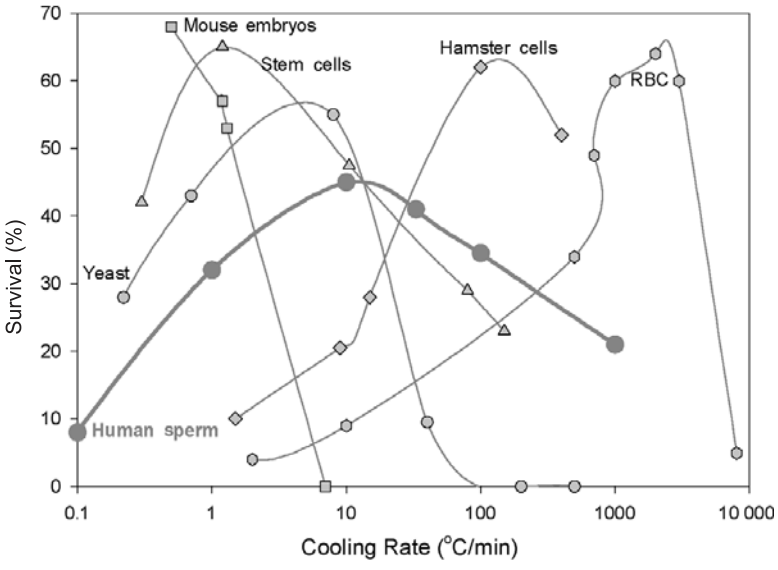


**Figure 1.1** Comparison of cryopreservation survival of various cell types as a function of the cooling rate. Note that all cell types shown here demonstrate an inverted "U"-shaped curve, indicating that an ideal cooling rate exists for these cell types. Redrawn and modeled from Mazur (1976).

during freezing and thawing and defined his "two-factor hypothesis" (Mazur *et al.*, 1972). In this manuscript Mazur explained damage at relatively slow cooling rates by the long-term exposure of cells to very high concentrations of non-permeating salts, and damage at relatively high cooling rates by the amount of supercooling that occurs. Regardless of their explanation, both of these damages could be mitigated by the addition of permeating solutes such as glycerol or dimethyl sulfoxide (DMSO). These permeating cryoprotective agents, discovered by Polge *et al.* (1949), had been in use for 25 years, but Mazur's seminal paper gave a much-needed foundation for the development of new cryopreservation protocols and allowed the description of an ideal freezing protocol: cooling just slow enough to avoid more than two degrees of supercooling. After this, a major goal of fundamental cryobiology was to characterize the permeability of the membrane to permeating cryoprotectants, and then use this information to maximize the cooling rate without causing supercooling.

Typically, cryobiologists have dealt with linear cooling rates, most likely because these are the easiest to repeat, calculate in a differential equation, and approximate with cooling apparatus, but the natural cooling rates of objects exposed to low temperatures are exponential (Luyet & Rapatz, 1970). Although there have been several reports of success using non-linear cooling rates (Morris *et al.*, 1999), there was no firm basis for the theory of optimizing non-linear cooling rates until very recently. In 2004, Woelders and Chaveiro (2004) published a theoretical development of non-linear cooling rates developed by fixing the amount of supercooling at two degrees. This allowed them to calculate the cooling rates needed to achieve this fixed amount of supercooling. This, in theory, should be the fastest, "safe," slow cooling rate, but there are many caveats. First, the permeability of the cell to water and solutes must be known at all subzero temperatures. This is not a trivial measurement, and only recently was a method for subzero water permeability measurement published (Devireddy *et al.*, 1998). Additionally, as we will discuss shortly, the probability

of ice formation is a function of both supercooling and temperature. Thus there are several variables not accounted for in this model.

Fundamental cryobiology was further advanced in the 1990s by Toner's intracellular ice formation model (Karlsson *et al.*, 1995). This model took into account the temperature, the degree of supercooling, and the cell's inherent ice-nucleating properties to predict a "probability of intracellular ice formation" ($P_{IIF}$). This $P_{IIF}$ could then be used in conjunction with the Mazur two-factor hypothesis to develop more complicated freezing protocols such as a two-step or three-step protocol where cells were cooled relatively rapidly to a subzero temperature, allowed to equilibrate or partially equilibrate at that temperature, and then cooled rapidly to a plunging temperature (Karlsson *et al.*, 1995; Liu *et al.*, 2000). These protocols allowed investigators to avoid the potentially damaging effects of slowly cooling to zero °C.

Until now, we have mainly discussed the development of "slow" or "equilibrium" cooling protocols. Vitrification, on the other hand, has always been the goal of cryobiologists. Essentially, at high enough cooling rates (>1000°C/min) the extracellular solution does not crystallize, and instead forms an amorphous glass, which is called "vitrification." This vitrification of the solution is associated with little if any cell damage, and is the most appealing cryopreservation protocol because it is so fast (compare this with a standard 1–10°C/min protocol), and has the potential to require no expensive equipment (i.e. plunge the sample directly into $LN_2$). The downside to vitrification procedures is that although isotonic saline is theoretically vitrifiable, the cooling rates needed to achieve vitrification are on the order of $10^5$–$10^6$°C/min. Alternatively, much work has been done investigating the vitrification properties of many different solutions (Fahy *et al.*, 2004). At higher concentrations, greater than four or five molar, typical CPAs such as glycerol or propylene glycol become vitrifiable at cooling rates of the order of $10^3$°C/min.

Unfortunately, as appealing as vitrification seems, there are several downsides. First, the cooling rates are difficult to achieve. In order to achieve ultra-rapid cooling rates, the surface area to volume ratio of the cell suspension (i.e. the container that holds the cells and their suspending solute) must be very high. This has been achieved by use of a "cryo-loop" (Lane *et al.*, 1999). The idea of the cryo-loop is to have a very thin film of solution that maximizes the surface exposed to liquid nitrogen. Alternatively, very thin straws also have a very high surface to volume ratio and have been used successfully to vitrify oocytes (Ramezani *et al.*, 2005). The second problem is achieving the high molar concentrations (4–5M) of permeating cryoprotectants. Many cell types have limits to which they can shrink or swell without significant damage. These limits are called "osmotic tolerance limits" and are cell type and species (sometimes even interspecies) dependent (Walters *et al.*, 2005). For example, Gilmore *et al.* (1998) established that, when boar sperm swell above 103 percent of their isosmotic volume or shrink below 97 percent of their isosmotic volume, their motility significantly declines. Because the addition of CPAs causes cells to shrink and then re-swell, there is a possibility of adding CPAs in such a way as to cause significant damage to the cell. This consideration is also important while removing CPAs after thawing. Therefore CPA addition and removal protocols, especially in the context of vitrification, often consist of multiple steps. For example, in order to add 1M glycerol to boar sperm, the sperm must first be equilibrated with 0.3M for six seconds, followed by a 0.6M step for another six seconds, a 0.95M step for six seconds, and finally a 1M step. In order to reach remotely vitrifiable concentrations (4M), we would have to perform at least eight steps (see Figure 1.2).

With all of this as background, we will conclude by outlining the typical fundamental cryobiological experiment in the following steps (examples of these experiments may be

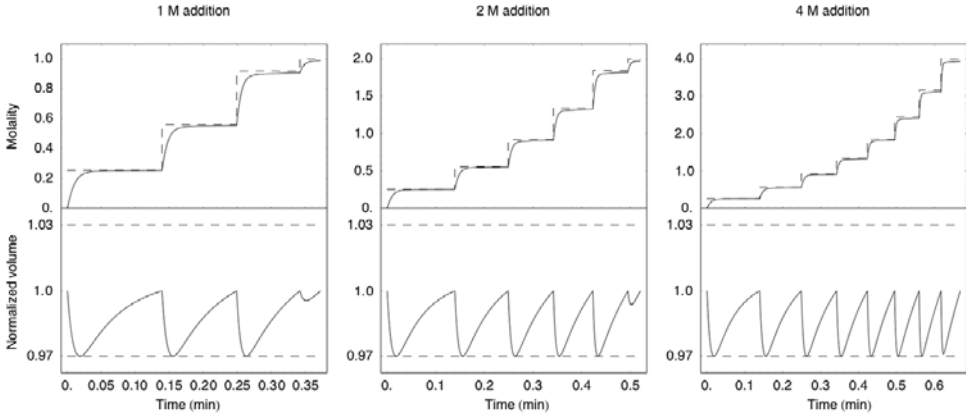


**Figure 1.2** Plot of volume and molarity versus time for the addition of glycerol to boar sperm. The dashed lines indicate osmotic tolerance limits (OTLs) and extracellular molality in their respective rows and the solid lines indicate normalized cell volume and intracellular concentration, respectively.

found in Gilmore *et al.*, 1996, 1998; Pfaff *et al.*, 1998; Liu *et al.*, 2000; Woods *et al.*, 2000; Agca *et al.*, 2002; Chaveiro *et al.*, 2004). First, cells are exposed to various anisosmotic salt solutions and their subsequent volume is measured. The reciprocal of concentration versus normalized volume is plotted. This is to establish that cell volume corresponds linearly with a change in osmotic pressure (i.e. they behave as linear or ideal osmometers, the Boyle–Van't Hoff relationship). The intercept of the regression (toward a theoretical infinite concentration) predicts the osmotically inactive fraction of the cell, which is the cell membrane, organelles, proteins, and bound water that will not cross the plasma membrane. This establishes the osmotically inactive portion of the cell (often referred to as $V_b$). In the case of mammalian sperm this value is usually between 60 and 70 percent of the isosmotic volume (Gilmore *et al.*, 1996; Karow & Critser, 1997; Guthrie *et al.*, 2002).

The next step is to establish the biophysical parameters of solute and water permeability, usually indicated as $P_s$ and $L_p$, respectively, at several temperatures. Because sperm have an enormous surface area to volume ratio, their permeability is very high relative to other cell types. This poses a significant challenge to the researcher attempting to determine these parameters. This challenge has been surmounted in two different ways. The first and most common has been the use of the Coulter Counter, which records electric pulses proportional to cell volume. This allows the real-time measurement of thousands of cells exposed to various solutions. The second method is the use of a stopped flow apparatus that takes advantage of the relationship between average population volume and light refraction. The advantage of the stopped flow apparatus is its temporal resolution. The disadvantage of the stopped flow apparatus is that the measurement of volume is indirect.

If the experiment were to stop here, we would have enough information to predict both an optimal CPA addition and removal protocol and a probable optimal cooling rate. Alternatively, we can also establish the parameters of the intracellular ice formation model of Karlsson *et al.* (1993). This is done by monitoring cells under a microscope while cooling and noting the percentage of cells which freeze intracellularly at a given temperature and degree of supercooling. After these parameters are established a theoretically optimized protocol can be developed which obeys the fundamental principles outlined in Mazur *et al.* (1972): the optimal freezing protocol cools the cell the fastest while avoiding a degree of supercooling associated with a high probability of ice formation.

Although there is a large amount of work involved in establishing these cryobiological parameters, the work is quite significantly less than that which would be involved in developing a cryopreservation protocol entirely empirically, given the wide variation in cryopreservation protocols. Fundamental cryobiology can give investigators a much narrower band of possibility for empirical experimentation, saving valuable time and resources.

## Current state of the art in sperm cryopreservation

Successful sperm cryopreservation requires maintaining the postthaw structural and functional integrity. However, to maintain functional integrity, the compartments of the sperm need to be fully protected so that frozen–thawed sperm can undergo normal fertilization under either in vitro or in vivo conditions. Differences in the various sperm compartments: we expect that each compartment (i.e. acrosome, flagella, midpiece) of sperm will be affected by cryopreservation differently. While motility may be protected at a high level, acrosome integrity may be severely damaged under a similar physical alteration such as osmotic stress (Willoughby *et al.*, 1996; Agca *et al.*, 2002; Guthrie *et al.*, 2002; Walters *et al.*, 2005). Although the effects of an entire cryopreservation procedure on acrosome integrity have been investigated to some extent (Okada *et al.*, 2001; Hollinshead *et al.*, 2003), the specific effects of anisosmotic stress on acrosome integrity have not been previously investigated. Previous investigations have shown that mammalian sperm have a very broad range of osmotic tolerance, as assessed by plasma membrane integrity and motility following exposure to various anisosmotic conditions (Willoughby *et al.*, 1996; Songsasen & Leibo, 1997; Agca *et al.*, 2002).

The methods of cryopreservation of bull sperm have not changed much in the past few years. Recently, there has been some work on the addition of cholesterol to the membrane of bull sperm prior to cryopreservation. Work by Purdy and Graham (2004) has shown that the addition of cholesterol to the membrane improves postthaw motility. In addition, cholesterol added to the membrane did not inhibit fertility, the ability of the sperm to undergo the capacitation, or acrosome reaction. Work is also being done to improve the methodology for cryopreservation; however, it appears currently that the methods are equal to, but not improved over, current systems.

Currently, methods are being developed to freeze boar sperm by alterations of the freezing medium composition such as the addition of antioxidants (Funahashi & Sano, 2005), various forms of packaging the semen for cryopreservation, and freeze-drying. Recently, there has been an effort to investigate the effects of reactive oxygen species on cryopreservation of boar sperm by the addition of antioxidants to the extender prior to freezing. In addition to antioxidants, work is being conducted to determine the effects of the vessel (0.5 cc and 5 mL straws, bags, cryovials) used to freeze boar sperm. In the swine industry, producers will limit the use of frozen–thawed semen if they have to thaw 10–15 0.5 cc straws to achieve the desired AI dose. However, if the producer can thaw one flatpack (containing 5 mL of sperm) and dilute to achieve an AI dose, frozen–thawed boar sperm will have a huge impact on the industry.

An alternative method of preserving pig sperm is freeze-drying, which has the advantage of room temperature storage, but freeze-dried sperm must be used in combination with intra cytoplasmic sperm injection (ICSI), as a high percentage of motility is lost (Kwon *et al.*, 2004). Currently, the biggest drawback to freeze-dried sperm is the low evidence of ICSI-derived offspring in the pig. However, because of the appeal of convenient storage there are many groups currently working in this direction.

In the mouse, several alterative methods to traditional cryopreservation protocols have been investigated, such as freeze-drying (Kawase *et al.*, 2005) and cryopreservation without cryoprotectants (Van Thuan *et al.*, 2005). One reason for the large number of methods to freeze mouse sperm is the development and success of many assisted reproductive technologies such as IVF and ICSI. More importantly, the development of ICSI in the mouse has increased the use of frozen–thawed sperm because the postthaw motility does not have to be good. Several groups have begun to gain an understanding of the biophysical and biochemical characteristics of mammalian sperm, which is critical to the development and optimization of strain-specific cryopreservation protocols. Mathematical modeling of these protocols may then be used to determine the optimal addition and removal of CPAs as well as cooling and warming rates for mouse sperm. Fundamental cryobiological properties associated with osmotic changes such as the membrane permeability to water ($L_p$) and cryoprotectants ($P_s$), their activation energies ($E_a$), and the sperm's osmotic tolerance limits (OTL) (Willoughby *et al.*, 1996) are critical to the development of cryopreservation protocols. Walters *et al.* (2005) investigated the OTLs of mouse sperm from various genetic backgrounds for maintenance of motility, plasma membrane, and acrosomal integrity, and found that maintenance of motility was affected by the genetic background; however, plasma membrane and acrosomal integrity were not. With the OTLs as well as some of the other cryobiological parameters of mouse sperm from different genetic backgrounds we can begin to provide the fundamental basis for the development of species and even strain-specific cryopreservation protocols.

Cryopreservation of human sperm has been developed for many different reasons, including development of assisted reproductive technologies such as intracytoplasmic sperm injection, "fertility insurance" for potential illness-induced infertility early in childhood, and azoospermic patients. As an example of "fertility insurance," Horne *et al.* (2004) have reported the birth of a live offspring utilizing IVF/ICSI with sperm that had been cryopreserved for 21 years (see Chapter 8). The postthaw motility of human sperm can range from 20 percent to 50 percent (Sbracia *et al.*, 1997). This loss of motility of human sperm after cryopreservation is believed to be caused by several factors, including diminished integrity of the membranes and cryodamage to the membranes of the intracellular compartments, which then affects energy metabolism and synthesis. Early work by Critser *et al.* (1987a, 1987b) also showed that loss of motility is largely caused by the addition and removal of CPAs, which cause the sperm to shrink and swell beyond their OTLs. Additionally, there has been work investigating the addition of components such as hyaluronan, a polysaccharide which mediates cell locomotion (Laurent, 1987) to the postthaw medium to increase postthaw motility. Sbracia *et al.* (1997) added sodium hyaluronate to the postthaw medium, which improved postthaw motility at 30 minutes postthaw. However, 24 hours after thawing there was no difference in motility between control and treated sperm.

## Applications of sperm cryopreservation

### Human semen cryopreservation for iatrogenic infertility

Over the last few decades there have been many improvements to human medicine that have greatly increased the indications for sperm banking. This will be discussed in more detail in later chapters of this book. However, briefly, the many advances achieved in cancer therapies for men of pediatric, adolescent, and reproductive age have led to increases in