Chapter 11
Gene Banking: The Freezing Strategy

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Abstract Genetic resource banking (GRB) or ex situ conservation in livestock species is the storage of genetic material for breeding purposes. Genes are present, with few exceptions, in every cell within an organism but only germ cells have the ability to transmit this information from one individual to another. Germplasm banking is the main tool for directing and enhancing this genetic flow in intensive farming, as it is for preserving the genetic diversity of a livestock population. The only current technique for the storage of germ cells is freezing them in liquid nitrogen (cryobanking). Traits in the breeding goals of a genetic program are retained in this way from the moment of insemination; otherwise, these genetic resources would be lost. Sperm from boars displaying high genetic values can be preserved following either rapid or slow freezing, as it is explained in this chapter together with a complete introduction to the legal issues of animal cryobanking. A new way of sperm preservation in pigs is also discussed.

11.1 Introduction

11.1.1 Freezing Living Cells

Stopping biological time while maintaining viability is the main objective of freezing strategies, thus permitting long-term storage of cells. Whereas some organisms have evolved to cryobiosis, that is, entering into a state of lethargy in response to decreased temperatures (Lubzens et al. 2010), most isolated cells are not capable of this unless they are manipulated to withstand freezing. These manipulation

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strategies are applied in a wide range of disciplines, from ecology to medicine (Miller et al. 2009). Preservation of germ cells or even embryos is a powerful tool in biomedical research (Mazur et al. 2008) and the ultimate resource of some critically endangered species (Watson and Holt 2001), whereas preserving organs, blood cellular elements or other tissue and cell types assures a continuous stock in hospitals (Fuller and Grout 1991; Chian and Quinn 2010). Moreover, freezing is the strategy of choice in most laboratories that maintain transgenic cell lines with biomedical aims (Baust and Baust 2007).

Regardless of the applications of freezing, all cells share similar behavior at low temperatures. Cells are preferably stored indefinitely in liquid nitrogen at −196 °C instead of being kept in an ultralow freezer at −80 °C. This preference is due to the higher stability of ice below −132 °C, which is the glass transition temperature of water (Rapatz and Luyet 1959). Also, apoptosis may occur when temperature fluctuates around −132 °C or when cells are stored at −80 °C (Lopaczynski et al. 2002). The metabolic activity of enzymes is only possible in an aqueous medium so when water crystallizes no reaction to maintain cellular processes is achievable and cells enter into a state of lethargy. This state is natural in cells that have evolved to resist desiccation, but most cells lack the ability to survive long periods of starvation even if no energy is demanded.

Freezing is a phase change in which a liquid turns into a solid. The cytosol of living cells is a fluid mainly constituted by water (the solvent), and by molecules (the solutes). Freezing the solvent poses a problem for the cell integrity. In a fluid, ice expands like a wave from a focus whose temperature is near freezing point and from which the first ice crystal appears (nucleation) and propagates (Zhmakin 2009). Because the cell membrane diffuses water ice can access the cytosol, shrinking organelles and membranes as if it was a blade. At the same time, the cell meets with another challenge: the expansion of crystallization progressively densifies the solution where the cell is suspended causing the water to flow out of the cell due to osmosis, which are the so-called "solution effects" (Mazur 1963; Dayong and Critser 2000). This is a complex physical event (Han and Bischof 2004) that basically consists of cell dehydration and membrane collapse beyond a critical water loss point.

The amount of water loss depends on factors that affect the water permeability of the cell. Although it is not the scope of this chapter to study in depth the physics of water flow, it is interesting to recall research by Muldrew and McGann (1994) to understand how the osmotic rupture hypothesis of intracellular freezing injury works. Several experimental approaches are described in equations that define the complex dependence relationship among the parameters driving osmosis: pressure P (N m^{-2}) on the membrane due to water flow, temperature T (K), surface area of the cell A (m^2), diffusion coefficient of water within the hydrophobic region of the bilayer D_w (m^2 s^{-1}), water flow J_w (molecules s^{-1}), width of the hydrophobic region of the membrane Δ_x (m), water permeation coefficient of the membrane Lp (μm^3 $\mu m^{-2}{\cdot}min^{-1}{\cdot}atm^{-1}$), isotonic volume V_{iso} (μm^3), osmotically inactive fraction of cell volume V_d (x V_{iso}), etc.

The essential point to protect a cell during freezing is to ensure that the expansion of ice does not damage the cell structure and that osmotic shock is prevented

after the solute concentration increases due to a drop in water content. The cooling rate is critical for the control of both events (Mazur 1984). Moreover, freezing protocols also require the use of cryoprotectants as substitutes for conventional extenders and, in most cases, these are harmful at high concentrations, so a balance between benefits and damage must be established. Needless to say that the success of freezing will not only depend on our protocols but also on the nature of the cell we are dealing with.

An optimal cooling rate must fit the complex osmotic properties of living cells. The Two-Factor Hypothesis of freezing injury, put forward by Mazur et al. (1972), states that with too fast a cooling rate water in the cell has no time to flow out and intracellular ice is formed. On the contrary, with too slow a cooling rate water flows out of the cell so that ice crystal formation is prevented, but the cell is dehydrated and exposed to pH changes and to cryoprotectant toxicity for too long. Thus, the osmotic response of cells is considered to be a determining factor of cell resistance. Meryman (1971) proposes that cells can be dehydrated to a minimal volume (osmotically inactive volume, V_d) beyond which a cell cannot respond to osmotic pressure and the plasma membrane breaks. In boar sperm, the osmotically inactive volume is achieved under 210 mOsm Kg^{-1} (Gilmore et al. 1996; Curry et al. 2000). The optimal cooling curve differs depending on cell types but in most cases it consists of different rates following the physical changes in the extracellular solute concentration, and in the permeability of the cell membrane through decreasing temperatures (Leibo and Mazur 1971; Morris et al. 1999).

Not only the cooling rate but also the addition of cryoprotectants seek to avoid either dehydration or large ice crystal formation through modeling some of the parameters described by Muldrew and McGann (1994). Afterwards, the cell can be safely stored in liquid nitrogen if no irreversible lesion has occurred during freezing. But there is another challenge to overcome before the sample is used: the thawing process (Mazur 1963). The return of cells to physiological conditions when they are being thawed is accompanied by mechanical and osmotic stress, and thus an optimal thawing rate is also mandatory to maintain our cells viable. During thawing, water flows into the cell because there is an abrupt decrease in extracellular concentration due to ice melting. Cells are exposed again to osmotic pressure, their survival depending on the mechanical response of the membrane to volume increase (Rodríguez 2005). The toxicity of the cryoprotectant is not a common problem during thawing, since cells are further diluted in the thawing medium. Rapid warming rates (>1,000 °C min^{-1}) are generally applied to prevent enlargement of ice crystals when temperature rises above 0 °C.

11.1.2 Why Freeze Gametes? Germplasm Banking in Swine

In the livestock industry, the efficient transmission of genetic characters is managed through artificial insemination (AI) or through transfer of viable embryos

after in vitro fertilization (IVF). The latter procedure, although crucial for the swine industry and far beyond for human xenotransplantation, is in the experimental phase in pigs due to the high rates of polyspermy and the poor quality of IVF-derived pig embryos (Koo et al. 2005; Coy et al. 2008; Lloyd et al. 2009; Isom et al. 2011). On the other hand, AI is routinely performed to inseminate a group of sows with semen from one boar, thus spreading the genes from one individual to the maximum number of offspring (See Chap. 12). Swine farms develop exhaustive selection programs to obtain new lines of boars of commercial interest that improve the characteristics of established breeds. However, the genetic erosion makes it difficult to maintain these traits from one generation to another and the solution is to create frozen germplasm banks (also named cryobanking, gene banking or ex-situ preservation) for stabilizing the new characters.

A germplasm bank is a collection of germ cells, which contain the genetic resources of an organism (namely germinal tissues, seeds, pollen, embryos, oocytes or sperm). Although in first experiments scientists used ice for freezing, the introduction of liquid nitrogen in 1938 permitted indefinite storage of viable cells (Jahnel 1938; Fuller et al. 2004).

In the case of pigs, some constraints exist in the implementation of frozen-thawed (FT) sperm. Still today, the sensitivity of some boar ejaculates to cold-shock, the success of long-term extenders, the cost of FT sperm doses, and the timing accuracy required for this kind of AI have restrained the expansion of cryopreservation in the swine market and its use is still limited to special breeding programs. The availability of genetic material in a frozen state, however, provides full-time access to high-value genetic resources for reconstituting populations, introducing genetic variability, improving rates of genetic progress and profitability, developing new breeds, supplying periods of low production and for increasing sanitary control and safety exchange of samples across long distances, among others. These features satisfy the food demand in developed countries and help alleviate food deficiencies in developing ones (FAO 2007).

The Food and Agriculture Organization (FAO) urges the introduction of gene banks or biological resource centers in developing countries to counteract the decrease in farm animal diversity that follows both an excess of outcrossing and inbreeding practices. Cattle, rabbits, horses, and pigs are, in this order, the livestock species that have the highest proportions of breeds at risk (FAO 2009). The cost of frozen samples is relative taking into account that it is calculated as a long-term inversion and so investments are recouped. Compared to the budget for the maintenance of living populations or in situ preservation, the FAO indicates that, in some cases, it is less costly (FAO 2007, 2011; Groeneveld et al. 2008).

Cryopreservation of animal sperm has been carried out since the 1950s when the first cattle cryobanks were set up, but the first swine offspring obtained from frozen semen was achieved in 1970 (Polge et al. 1970). Swine germplasm banks can be found worldwide nowadays, both from private and public funding, and most of these are registered in databases like FABISnet (FABISnet 2011).

11.1.3 Legislation on Animal Germplasm Banking

Gene banking is still a relatively new concern in policy, and legislation does not entirely cover all aspects of animal genetic resources (AnGR) management that stakeholders might encounter. Despite recent efforts, the management of AnGR is still behind the legal achievements of crop banking, which runs under the policies of the Governing Body of the International Treaty on Plant Genetic Resources for Food and Agriculture. Even though there is an incomplete framework, the responsible of the gene bank must be aware of the national and international policies currently regulating this activity.

To put some light on the subject, there exist international organisations that directly refer to AnGR cryobanking (e.g. Rare Breeds International, RBI) (Hiemstra 2011) and almost 40 percent of countries have developed some kind of regulations for AnGR trading (Boettcher and Akin 2010). Most regulations are intended for wild animal populations or for domestic breeds with interest in developing countries, but they have also become a roadmap for swine cryobanking.

A good global framework for the management of AnGR was developed in September 2007 at the first International Technical Conference on AnGR for Food and Agriculture held in Interlaken, Switzerland (FAO 2007). The FAO presented the Global Plan of Action for AnGR that was adopted by the 109 participating countries. The strategic areas have yet to be implemented in different regions worldwide (Hoffman and Scherf 2010) and have been updated in the Guidelines for the cryoconservation of AnGR presented at the FAO conference in Rome (FAO 2011). The latter is a thorough comprehensive manual including recommendations and guidance on all aspects of cryopreservation practices in different animals, including pigs. It also contains a list of suggestions (rather than legislation itself) for better germplasm administration, and a chapter dedicated to financial issues.

The global Convention of Biological Diversity (CBD) also provides tools for germplasm banking management. The convention started in 1993 with the objectives of conserving biological diversity and assuring sustainable use and fair utilization of genetic resources. Two main protocols have arisen from the different meetings of the CBD: the Cartagena protocol (CBD 2000), intended to enhance biosafety, and the Nagoya protocol (CBD 2010), specific for genetic resources. The latter formalized a handbook of legal requirements for access to genetic resources and benefit-sharing (IISD 2007). Although most of the information addresses negotiation practices for exchanging crop genetic resources, it is also a tool for managing AnGR. These Guidelines provide voluntary guidance on ABS practice for companies, researchers, communities, and governments to comply with the CBD Bonn Guidelines (CBD 2002).

Resulting from the Cartagena protocol on Biosafety, a platform, the Biosafety Clearing-House, was established in which information from the CBD is registered and any law from any country can be more easily searched and retrieved (SCBD 2001–2011). The World Association of Zoos and Aquariums (WAZA 2005), the International Union for Conservation of Nature (IUCN), the Convention on

International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the World Wildlife Fund (WWF) are involved with the CBD. Because regulations from the CBD are aimed at the conservation of biodiversity the implementation of ex-situ measures is intended as a support to in situ practices. There are independent charitable organizations that also subscribe to AnGR ex-situ preservation and carry out valuable work in the field. The Frozen Ark project is a global consortium of research and conservation bodies that aims to strengthen the cryopreservation of material from endangered species (www.frozenark.org).

Besides the CBD, another important organism outlining international standard-setting in relation to AnGR conservation is the World Organization for Animal Health (Office International des Epizooties, OIE). Regarding measures relevant for germplasm banking, the OIE delegates agreed to a code for safeguarding the international trade of animals, which was published in two volumes under the name of "Terrestrial Animal Health Code" (Terrestrial Code). The official version of the document is revised annually and published in English, French, and Spanish. The 20th edition (OIE 2011) incorporates modifications agreed at at the 79th OIE General Session in May 2011, and aims to prevent sanitary conflicts during animal trade. Some chapters directly affect the import/export of germplasm banking in swine, and also indicate the appropriate preparation of frozen semen extenders (Table 11.1).

The committee for Companion Animals, Non-Domestic and Endangered Species of the International Embryo Transfer Society (IETS-CANDES) is an advisory board that provides guidance to international governmental regulatory agencies, recognized animal specialty groups and organized conservation programs about different issues related to reproductive biotechnologies, for what it also submits recommendations about the conservation and genetic management of species. A link to import/export regulations for biological materials in different countries can be found on its web (IETS-CANDES 2012).

Table 11.1 Chapters and articles from the 2011 © OIE Terrestrial Animal Health Code relevant for swine germplasm banking

Chapter	Title	Article	Content
4.6	Collection and processing of bovine, small ruminant and porcine semen	4.6.7	Conditions applicable to the handling of semen and preparation of semen samples in the laboratory
5.4	Animal health measures applicable before and at departure	5.4.2	Semen, embryo/ova, and hatching eggs
		5.4.4	Certificate
5.10	Model veterinary certificates for international trade in live animals, hatching eggs, and products of animal origin	5.10.1	Notes for guidance on the veterinary certificates for international trade in live animals, hatching eggs, and products of animal origin
		5.10.3	Model veterinary certificate for international trade in embryos, ova, and semen (Fig. 11.1)

Additional information available in the OIE website: http://www.oie.int/publications-and-documentation/general-information/

Each country should establish its own legislation on behalf of these international organisms, thus ensuring that the principal guidelines on gene banking are implemented and fair trade is applied in all territories. For consultation of country-based policies, the germplasm owner must apply to the competent organism in the country of origin and, in case of transboundary movement, to the one in the country of importation. As examples, the Department of Justice in Canada has established regulations to control germplasm importation (DJC 2011), and the New Zealand government demands an export certification (MAF 2011). In Europe, apart from national laws there is the European Regional Focal Point for AnGR (ERFP). This regional platform supports the in situ and ex situ conservation and sustainable use of AnGR and facilitates the implementation of FAO's Global Plan of Action for AnGR in Europe. It published the Guidelines for the Constitution of National Cryopreservation Programmes for Farm Animals (ERFP 2003). It is a good complement to the FAO's guidelines for developing a cryopreservation program for swine from the European perspective, but it is also a useful tool for other regions and it contains a full list of the national germplasm banks in Europe.

Because of gaps in germplasm regulation national governments are free to agree to policies that are not contemplated by international or regional organisms. On the opposite, germplasm stakeholders must refer to international or regional organisms (or even make their own decisions) when a case is not stated in national policies. The backflow of information that characterizes relations among the parts implicated in AnGR banking is shown in Fig. 11.1. In any case, at least four major issues should be addressed for correct practices (please consult the FAO and ERFP guidelines for extended information).

11.1.3.1 Germplasm Property

All nations consider livestock as private property, which includes any product derived from the animal. Setting up a germplasm bank requires strict control of the personal/institutional access to the material stored. Since private property is defined by contract, the owner stipulated in the contract must be consulted for permission to gain access to the collection. In the case of boar germplasm banking, the sperm stored are, in most cases, property of the breeder. Instead of transferring the entire property rights from the breeder to the responsible germplasm bank, a material transfer agreement (MTA) is desirable. Under this agreement, the responsible germplasm bank is granted the right to manipulate the collection, whereas the breeder maintains ownership. The MTA document should stipulate the ownership rights of the breeder and the responsible germplasm bank (including intellectual property rights or benefit sharing and access to confidential data), the economical terms of storage, the conditions before releasing material to a third party, a health veterinary certificate from the boar whose sperm have been collected, and a commitment to best storage practice. Equally, a material acquisition agreement (MAA) should be formulated as a standard contract to stipulate the conditions of use of the material after its transfer.

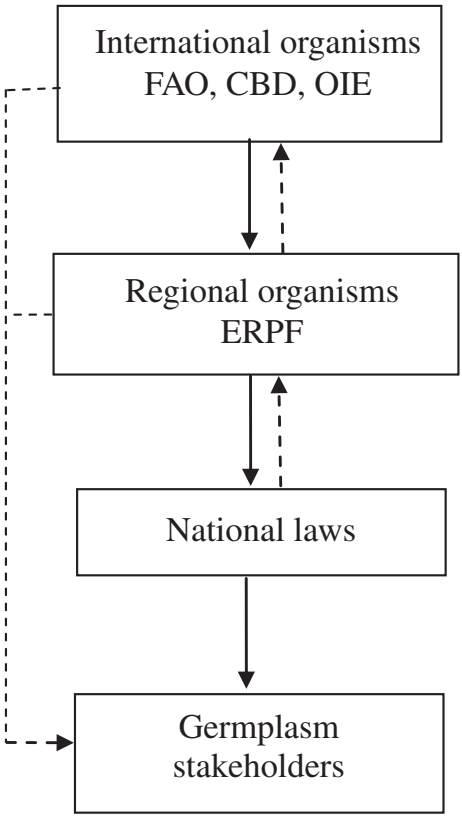

Fig. 11.1 Flow chart resuming information backflow among the parts implicated in animal germplasm banking management

It is important the germplasm bank does not become an isolated entity. The FAO advises inviting different stakeholders to participate in the project with the aim of maximizing management efficiency (the Ministry of Agriculture, breed associations, individual farmers, private companies, the FAO National Coordinator for AnGR). Making contact with the corresponding Ministry of Agriculture to obtain information about legal duties and public funding is recommended as a first step in the creation of a germplasm bank.

11.1.3.2 Biosecurity

The material stored, including the freezing diluents, must be free from pathogena (expedition of a health veterinary certificate is compulsory) and isolated from external hazards. There are OIE guidelines about this concern and according to

FAO recommendations (FAO 2011) the chamber where samples are stored should be closed to unauthorized personnel, maintain its structural integrity in case of major challenges, assure the availability of nitrogen and electricity, and be set up for biological level-2 protocols to maintain health standards. Nonetheless, personal security must be guaranteed when handling liquid nitrogen by monitoring oxygen levels and wearing protective gloves and glasses. Basic security tips can be found in a safety data sheet and in different supplier webpages. Certifications on biosecurity can be expedited by external certification bodies and permit international recognition of good practices of the germplasm bank.

11.1.3.3 Exportation/Importation

The germplasm bank must be protected from non-certified new entries and also must conform to health standards for semen shipment. In this respect, the OIE Terrestrial Animal Health Code and the national regulations of countries participating in the exchange must be consulted. The OIE permits public access to veterinary health certificate templates (Fig. 11.2) and customized models are also found online at a national level, like the Canadian (CFIA 2011). The general safety standards for transport of compressed nitrogen, classified as chemical hazard class 2 (identification number UN1066), must also be observed.

11.1.3.4 Databasing

Registry of data is mandatory in a germplasm bank. Such a need for tracking samples has led to the development of archives, containing all kinds of information from the material stored. As cryobanks have gradually increased, common databases have appeared to centralize the management of different repositories simultaneously. One of the first common databases was developed by the European Association for Animal Production (EAAP). Later, the FAO launched a different database for non-European countries called Domestic Animal Diversity Information System (DAD-IS). Both entities were incompatible until the development of the European Farm Animal Biodiversity Information System (EFABIS) project from the European Union. The two databases are currently linked under the name of FABISnet, creating a global system that permits the flow of information between both entities and others that request to join (Groeneveld et al. 2007).

The objective of FABISnet is to coordinate and synchronize the management of AnGR around the world through an integrated network involving regional and national germplasm programs. As commented above, major regional programs in FABISnet are EFABIS at the European level (http://efabis.tzv.fal.de/), and DAD-IS at the non-European level (http://dad.fao.org/). An example of a national database is the National Animal Germplasm Program (NAGP) in the

COUNTRY :

Part I: Details of dispatched consignment		
	I.1. Consignor: Name : Address:	I.2. Certificate reference number: I.3. Veterinary Authority:
	I.4. Consignee: Name: Address:	
	I.5. Country of origin: ISO code*	I.6. Zone or compartment of origin**:
	I.7. Country of destination: ISO code*	I.8. Zone or compartment of destination**:
	I.9. Place of origin: Name: Address:	
	I.10. Place of shipment:	I.11. Date of departure:
	I.12. Means of transport: Aeroplane ☐ Ship ☐ Railway wagon ☐ Road vehicle ☐ Other ☐ Identification :	I.13. Expected border post: I.14. CITES permit No(s).**:
	I.15. Description of commodity:	I.16. Commodity code (HS code): I.17. Total quantity:
	I.18.	I.19. Total number of packages:
	I.20. Identification of container/seal number:	I.21.
	I.22. Commodities intended for use as: Artificial reproduction ☐ Other ☐	
	I.23.	
	I.24. Identification of commodities: Species (Scientific name) Breed* Donor identity Date of collection Approval number of the centre/team Identification mark Quantity	

Fig. 11.2 Model veterinary certificate for international trade in embryos, ova, and semen (2011 © OIE Terrestrial Animal Health Code, Article 5.10.3). Reproduced with the kind authorization of the World Organisation of Animal Health (http://www.oie.int/eng/en_index.htm)

COUNTRY :

Part II. Zoosanitary Information

II.a. Certificate reference number

II. The undersigned Official Veterinarian certifies that the embryos, ova and semen described above satisfy(ies) the following requirements:

Official Veterinarian:

Name and address (in capital letters):

Official position:

Date:

Signature:

Stamp:

Fig. 11.2 (continued)

USA (http://www.ars-grin.gov/), which at the same time is part of the Germplasm Resources Information Network of the Department of Agriculture.

FABISnet links individual databases maintaining the same restricted access to certain contents. National databases can include the information they consider relevant and keep other information to be accessed only through their national web. Moreover, some data regarding European samples are much more complete

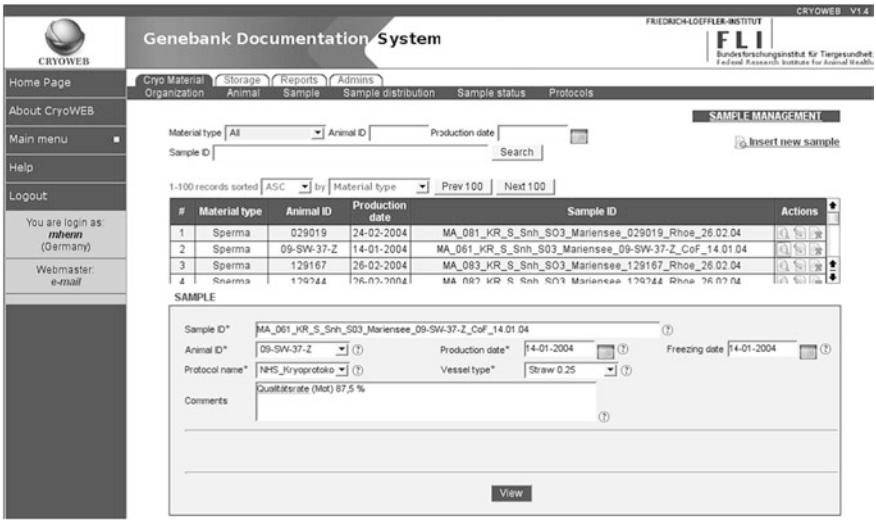

Fig. 11.3 Cryoweb open source software for Genebank documentation. Reproduced with the kind authorization of Dr. Zhivko Duchev, Institute of Farm Animal Genetics, Neustadt, Germany. The software can be downloaded at http://cryoweb.tzv.fal.de/

in EFABIS than in DAD-IS, and the opposite. Any country can create a national web-based information system integrated in FABISnet. The list of countries and AnGR regional and national coordinators enrolled in FABISnet can be consulted both in EFABIS and DAD-IS webs and private breeders should contact their national coordinator to join. FABISnet works with the free licensed CryoWEB software that facilitates introducing and managing cryopreserved sample data (Fig. 11.3). The software can be downloaded at http://cryoweb.tzv.fal.de/download.html.

A fundamental key to databasing is the identification of samples. Semen straw labeling according to ERFP guidelines should include species, breed, and identification of animal, country, collection center, and production date. These data are often requested in health certificates for trading and can be entered in the CryoWEB.

11.2 Preserving the Genetic Heritage in Pigs

11.2.1 Slow Versus Rapid Freezing: Which is the Best Method?

Sperm cells can currently be preserved for up to 15 days in refrigeration (liquid storage; see Chap. 10) or for longer by freezing water inside them. There are two methods for freezing sperm: slow freezing (ice-equilibrium freezing) and

rapid freezing (ice-free freezing). According to the two-factor hypothesis (see Sect. 11.1.1) both slow and rapid freezing induce damage to cells through different mechanisms. Then, how can we manage to freeze sperm successfully?

Since two major conflicts affect frozen sperm cells (dehydration and shrinkage), two major answers have been proposed depending on the characteristics of freezing. Both rely on the use of molecules called cryoprotectants, discovered in the fluids of ectothermic animals living in cold environments (Eastman and DeVries 1986; Storey and Storey 1990) and used for sperm since the 1930s (Milovanov and Selivanova 1932; Bernstein and Petropavlovsky 1937). These are added to the freezing extender and interfere with the expansion of ice by increasing the solute concentration (colligative or permeating cryoprotectants) or by stabilizing the biological membranes (non-permeating cryoprotectants).

If cooling is carried out at slow rates dehydration is minimized by using a mixture of colligative and non-permeating cryoprotectants at low concentrations. In contrast, if cooling is performed at fast rates nucleation is completely avoided by using high concentrations of colligative cryoprotectants. But since the viability after thawing depends not only on the freezing protocol but also on the sample only 50 % or more sperm in porcine ejaculates can usually survive under optimal freezing conditions (Green and Watson 2001). In the slow freezing of boar sperm the mixture of cryoprotectants together with progressive cooling permits the cell to become entrapped in non-frozen (glassy) areas surrounded by ice veins, the reason this process has been called "ice-equilibrium freezing" or "cryopreservation". In rapid freezing, the cell is entirely surrounded by a matrix of glassy material. In this case, the use of toxic percentages of colligative cryoprotectants is not a problem since the cell has no time to respond. The osmotic conditions are controlled by adding these substances in the freezing extender in a multiple step sequence consisting of increasing concentrations. The poor availability of water in this extender stops the expansion of ice and the medium turns into a glassy or vitrious phase instead of being frozen, which is why this process has been called "ice-free freezing", "non-equilibrium freezing" or simply "vitrification".

Scientists still do not agree on which method is the best for freezing biological samples. The literature is controversial on that point and many different outcomes on cell viability are retrieved even for the same method. The use of one or the other depends on personal preferences, on the background of the researcher, on the equipment available, and, mainly, on the suitability of the cell type (See Sect. 11.2.4). In fact, the selection of a method is not crucial for the survival of the sample. It all depends on the personal ability for fitting the procedure to the biological and physical characteristics of the sample in question and the application, in all cases, of a rapid thawing rate (>1,000 °C min^{-1}) to avoid the effects of ice crystals and hyperosmosis (Mazur 1963).

For both slow and rapid freezing some considerations about the storage of samples should be taken into account. In Fig. 11.4 the basic material required when owning a germplasm bank is shown. A monthly fee may be paid to the research center for storage of the straws in case the owner prefers not to deal with it. Frozen samples are stored in tanks of different capacities, the biggest usually containing

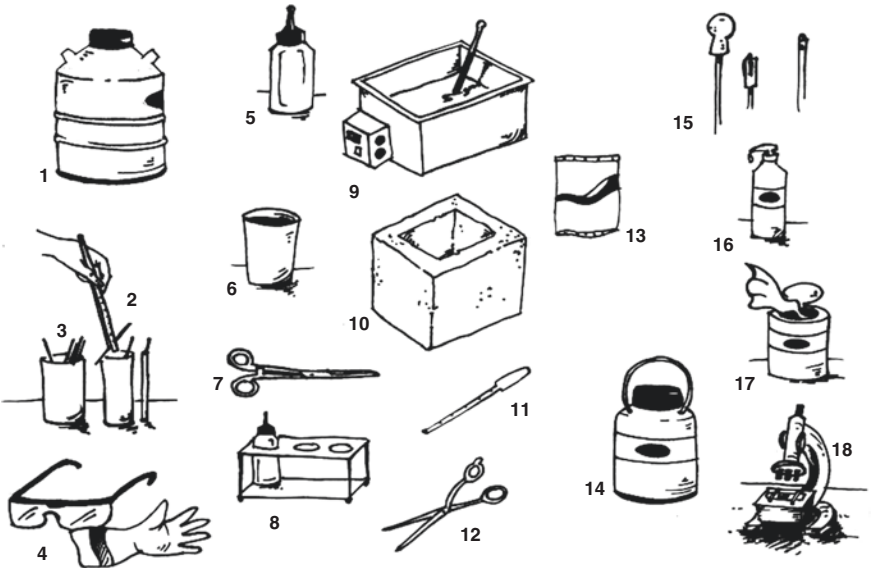

Fig. 11.4 Owning a germplasm bank: basic tools. *1* Dewars for refilling purposes and liquid nitrogen containers with canisters for storage of sperm samples; *2* long tweezers; *3* Goblets for the straws; *4* eye and hand protection for handling liquid nitrogen; *5* dose bottles for dropping thawed sperm; *6* rigid plastic cup for nitrogen transvasing; *7* long Halstead mosquito forceps for holding goblets and cups; *8* rack for dose bottles; *9* Water bath at 37 °C that fits the rack (*8*); *10* foam box for liquid nitrogen; *11* Disposable Pasteur pipettes; *12* Scissors; *13* Thawing extender; *14* transportable Dewar for straws; *15* Catheters for intrauterine insemination with dose bottles (*5*) adapters; *16* non-spermicidal lubricant for catheters; *17* cleaning and drying wipes; *18* microscope for verifying the motility of sperm before insemination

50 L of liquid nitrogen. A periodic maintenance consisting of frequent refillings with liquid nitrogen is important, since the maximal evaporation rate in a closed tank can be of three liters per week. The narrower the neck of the tank, the lower the evaporation rate, so the size of the tanks should fit the number of samples to be stored.

11.2.2 Characteristics of Boar Sperm Relevant to Freezing

The ability of boar sperm to survive freezing has been always questioned. Such bad reputation is mainly owed to the composition of the sperm membrane, with a low cholesterol to phospholipid molar ratio (0.26) (Parks and Lynch 1992; Cerolini et al. 2001), and a high content of unsaturated phospholipids (De Leeuw et al. 1990; Maxwell and Johnson 1997; Maldjian et al. 2004; Chen and Liu 2007). Unsaturated phospholipids give the cell membrane a high fluidity and enhance its permeability whereas cholesterol produces the opposite effect (Alberts et

al. 2008). Cooling under the 5 °C onsets a phase transition in plasma membrane lipids, from liquid-crystalline to a jellified state, at a given time point depending on the nature of the lipid so that some types of unsaturated phospholipids become jellified earlier than others. The result is a mixture of phases that compromise bonds among lipids so that ruptures appear (Drobnis et al. 1993). Such a phenomenon is not completely restored even in the presence of cryoprotectants (Holt and North 1984). Cholesterol is believed to interfere with the condensation into jellified phases and hence helps to maintain the integrity of the membrane (Quinn 1985; Blesbois et al. 2005). If the membrane is broken, sperm functions are compromised and obviously sperm cannot correctly respond to the challenges posed by freezing. In this respect, it has been demonstrated that boar sperm become more sensitive to freezing after their passage through the epididymis, where there is an important loss of cholesterol, (Nikolopoulou et al. 1985; Simpson et al. 1987).

Although the aforementioned features are true for the porcine species, certain genetic and ambiental factors also give particular traits to the sperm that help in overcoming these handicaps, thus producing notable differences among breeds and individuals for sperm resistance to freezing (Medrano and Holt 1998; Holt 2000; Hernández et al. 2006). For instance, slight differences in the shape and volume of the boar sperm head interfere with osmotic regulation during exposure to hypertonic conditions, as it occurs in ice formation (Thurston et al. 2001; Peña et al. 2005; Petrunkina et al. 2005a; Pesch and Bergmann 2006; García-Herreros et al. 2008). The amount of long-chain polyunsaturated fatty acids in the sperm plasma membrane (Waterhouse et al. 2006) and the presence of certain proteins in sperm before freezing, like the HSP90AA1 (Casas et al. 2009, 2010b), also participate in acquiring resistance. Genetics is thus believed to be crucial in the freezing success of boar sperm (Thurston et al. 2002a, b; Medrano et al. 2009) but it is not the only factor. Ejaculates from a same individual sometimes display different tolerance to freezing, which is why the presence of good freezability ejaculates (GFEs) and poor freezability ejaculates (PFEs), depending on their post-thaw viability, is generally accepted (Casas et al. 2009, 2010a, b). This means that ambiental factors must have a role in the matter together with genetics, both producing direct alterations to the whole ejaculate or to sperm populations. The combination of genetic and ambiental factors depicted by GFEs is still under research.

The characteristics of boar sperm that influence dehydration and ice-mediated injuries during freezing also give rise to other dramatic adjustments under the name of “cold-shock”. The sensitivity of the membrane to form jelly phases under 5 °C leads to its destabilization and the loss of its selective permeability. Together this triggers the influx of ions in the cytosol, which switches to a metabolic cascade whose effects resemble the events of true capacitation (Buhr et al. 1994; Bailey et al. 2000; Green and Watson 2001; Petrunkina et al. 2005b): increase in membrane permeability to ions, reorganization of the sperm plasma membrane, cholesterol release, signalling cascade for protein phosphorylation and sperm motility hyperactivation, release of reactive oxygen species

(ROS) (Guthrie et al. 2008; Awda et al. 2009) and, apoptotic-like features (Peña et al. 2009). Collateral damage includes decreased mitochondrial membrane potential (Flores et al. 2009, 2010), membrane lipid peroxidation (White 1993), chromatin unstability (Courtens et al. 1989; Flores et al. 2008a, 2011) and motility and viability impairment (Watson 2000; Cremades et al. 2005; Flores et al. 2008b, 2009). This capacitation-like status, called cryocapacitation, shortens the lifespan of sperm and interferes in their response to the signalling events required for fertilization and in their survival in the oviducts (Buhr et al. 1994; Green and Watson 2001; Petrunkina et al. 2005b). As stated before, frequently and even under the most optimal conditions half of the boar spermatozoa die after freezing, which results in 20 % less farrowings after AI (Johnson et al. 1981).

Although post-thaw sperm quality is thoroughly checked by the research center that processes the ejaculate for freezing, sperm motility should be rescreened on-farm at the moment of thawing, just before insemination. A basic microscope would suit this purpose. The minimal value for acceptance of a boar ejaculate in a freezing program is 80 % for parameters such as total sperm motility, morphology, osmotic tolerance (acrosome intactness) and membrane integrity, whereas for progressive sperm motility it is 60 % (Casas et al. 2009, 2010a, b) (see Chap. 9 for details on quality assessment). These values must be displayed by the ejaculate to account for the decrease in viability that usually occurs after freezing. It is reported that boar ejaculates with the best freezability (GFEs) are those that present a value of sperm membrane integrity and progressive motility that is at least 40 %; otherwise it is considered a PFE. The post-thawing parameters are usually assessed just after thawing and after 240 min to ensure sperm are viable within the insemination-to-ovulation interval (Casas et al. 2010a).

Developing new tests to detect ejaculates with poor freezability before the freezing process will be useful to increase the confidence of farmers. The motility values for linearity and straightness in boar sperm at 5 °C have been reported to be inversely related to levels of cold-shock, and the combination of both can predict around 70 % of sperm cryosurvival. Similarly, the abundance of HSP90AA1 in the sperm cytosol is a good predictor, since it distinguishes between good and poor freezability ejaculates before freezing (Casas et al. 2009, 2010b). However, when investing in the creation of a frozen sperm bank the reproductive performance of a boar and its ejaculate freezability could take second place to other traits that the germplasm owner wishes to preserve.

The improvement of the freezing protocol has been a major advancement in the last decades (Pursel et al. 1973; Hernández et al. 2007) to lessen the intrinsic weakness of frozen sperm, but it is also important to consider the optimization of AI by synchronizing the ovulation of sows to establish proper insemination timing (not earlier than four hours before ovulation) (Waberski et al. 1994) and by using intrauterine insemination techniques (see Sect. 12.2.2) (Gadea 2004; Vázquez et al. 2008). Polge and collaborators were the first to achieve farrowings with FT boar sperm (Polge et al. 1970).

11.2.3 Cryopreservation of Boar Sperm: Slow Freezing

Slow or ice-equilibrium freezing is characterized by the coexistence of two phases or fractions in the sample: an unfrozen fraction and a crystal fraction. When cooling, the temperature of the sperm solution decreases until nucleation occurs at freezing point. At this moment, the temperature stabilizes reaching a temperature plateau, as there is equilibrium between cooling and the formation of ice (latent heat of ice fusion). The more ice crystals, the more concentrated the solution, and the more difficult to attach water molecules to form ice. This causes the freezing point to progressively decrease and this is the reason the plateau is slightly inclined in solutions compared to pure water. When all water in the solution has solidified and the latent heat of ice fusion has dissipated, the equilibrium breaks and the temperature decreases again. At a given point, called the glass transition temperature, the remaining non-frozen solution turns into an amorphous metastable net around the ice crystals (Morris 2007; Taiz and Zeiger 2010). It is in this vitrified structure where the sperm are concentrated (Zavos and Graham 1983; Mazur and Koshimoto 2002; Morris 2007; Casas 2010).

Cryopreservation must ensure that the combination of the freezing extender, packaging properties, and cooling rates permits rapid nucleation, uniform expansion of the ice wave, and progressive dissipation of the latent heat of ice fusion (Zavos and Graham 1983; Berger and Fischerleitner 1992). If not, the process of supercooling may take place, which consists of a lack of nucleation at the freezing point of the sample solution (Debenedetti and Stillinger 2001; Giovambattista et al. 2004). This usually happens when applying high cooling rates in samples not prepared for vitrification, especially those packed in low surface-to-volume ratio containers. Consequently, the solution cools far below its freezing point with increasing possibilities for nucleation. When nucleation occurs, there is a sudden warming of the sample up to the freezing point to abruptly decrease again after the latent heat of ice fusion has dissipated (Morris 2007). Such an event is not desirable as ice formation is not equilibrated with the vitrified parts of the solution. For this reason, induced nucleation can eventually be performed at the start of cryopreservation.

Cryopreservation was the first method implemented for freezing boar sperm and is the most widespread nowadays. Yet in the eighteenth century, Spallanzani demonstrated that fertilization with frozen sperm was possible (Spallanzani 1776), but it was not until the 1970s that two successful models of cryopreservation were developed: the American or Beltsville method (Pursel and Johnson 1975), and the German or Hülsenberger method (Westendorf et al. 1975). Both are based on the use of freezing extenders containing the following substances as cryoprotectants: egg yolk, glycerol, and Orvus-ES® or Equex STM® Paste (commercial concentrated synthetic detergents, in paste form, to emulsify lipids in the egg yolk). The difference relies on the kind of sugar added and on the mode of cryopreservation. The Beltsville method adds glucose and freezes sperm in the shape of round pellets on carbonic ice, whereas the Hülsenberger method uses lactose and packs semen into straws that are frozen over vapors of liquid nitrogen. The latter

Table 11.2 Cryopreservation slope set for boar sperm in a programmable freezer

Step	Initial temperature (°C)	Final temperature (°C)	Cooling rate (°C/min)	Time
A	5	−5	−6	1 min 40 s
B	−5	−80	−39.82	1 min 53 s
C	−80	−80	0	30 s
D	−80	−150	−60	1 min 10 s

provides better sanitary conditions as sperm are enclosed in a package, for what it is possibly the most popular.

Cryopreservation requires removal of the seminal plasma to dilute sperm in a freezing extender, which mainly contains low density lipoproteins (LDL) from the egg yolk as non-permeating cryoprotectants. These egg yolk LDLs have been proved to be the best cryoprotectants, possibly because they compensate the lack of cholesterol in the boar sperm membrane and model the shape of ice crystals so they are less damaging (Andreeva et al. 2008). To allow the interaction of the egg yolk with the boar sperm membrane, it is preferable to follow a slow descent in a water bath from 17 °C, the temperature of commercial doses, to 5 °C. Although higher cooling rates are tolerated in most ejaculates (Juarez et al. 2011), some could not withstand the cold shock. Temperatures below 5 ºC would impair sperm if any colligative cryoprotectant was included in the freezing extender. Although lactose acts as a colligative cryoprotectant in the Hülsenberger method, it does not permit alone the formation of an unfrozen fraction large enough to host sperm. Glycerol is the colligative cryoprotectant of choice, although it is toxic at elevated concentrations and so no more than 6 % (v:v) is added. Together with glycerol, the addition of a synthetic detergent will facilitate the emulsion of the egg yolk lipids at low temperatures.

The addition of glycerol precedes a slope from 5 to −150 °C at different precise cooling rates, for which the use of a programmable freezer (Thurston et al. 2003) is required. Before its introduction, researchers used to suspend sperm on vapors of liquid nitrogen and cooling rates could not be controlled. One of the current freezing programs for boar sperm consists of 5 min and 13 s of cooling (Table 11.2). It first provides a slow cooling rate to permit uniform nucleation and the accommodation of sperm to the osmotic conditions. Thereafter, rapid cooling rates avoid dehydration of the cell, and a few seconds standing at −80 °C allows the correct vitrification of glycerol (Thurston et al. 2003; Zondervan et al. 2007). Once at −150 °C, the samples can be plunged into liquid nitrogen (−196 °C) and stored indefinitely.

Certain modifications from the original cryopreservation methods are adapted by different laboratories. A common one is the retention of boar sperm ejaculates at 17 °C from 3 h (Eriksson et al. 2001) to 24 h (Tamuli and Watson 1994) before cryopreservation, the so-called “holding time”. This period gives higher tolerance to low temperatures by making sperm membranes rather insensitive to cold shock, although the mechanism behind this is still not clear (Pursel et al. 1973; Tamuli and Watson 1994; Johnson et al. 2000). In this respect, it is thought that effects

of cold shock on the plasma membrane architecture are prevented or reversed by certain factors in seminal plasma (Muiño-Blanco et al. 2008; Okazaki et al. 2009). The benefits of seminal plasma on sperm fertility have also been reported when it is added to the thawing solution that recovers sperm after cryopreservation, as first reported by Larsson and Einarsson (Larsson and Einarsson 1975).

Another modification introduced respect the original methods is the use of different kind of packaging. Once sperm are at 5 °C, the sample must be packed inside a container resistant to low temperatures and with a high surface/volume ratio to ensure uniform freezing and rapid thawing. Suitable packages are the 0.25 or 0.5 mL plastic straws, engineered by the Danish Sørensen (Sørensen 1940), and the 5 mL flat plastic polyethylene terephthalate bags (FlatPack) (Ekwall 2009; Eriksson and Rodríguez-Martínez 2000). Adoption of one or other container requires different machinery for filling, sealing, and storing.

Some companies are developing media free from animal-origin compounds because of legal compliances on extenders (see Chap. 10). Among other options, the use of egg yolk phospholipid synthetic liposomes is a good alternative to egg yolk in sperm freezing procedures. Liposomes modify the composition of cellular membranes by saturated lipid and cholesterol transfer, which can moderate the response of cells to low temperatures (Parks et al. 1981; Watson 1981; Holt and North 1988; Wilhelm et al. 1996; He et al. 2001; Zeron et al. 2002; Pillet et al. 2011; Röpke et al. 2011).

Thawing cryopreserved boar sperm is a simpler procedure than freezing, but not less important, so its correct performance is essential recovering quality parameters and avoiding ice crystal enlargement. Sperm samples are directly transferred from liquid nitrogen to a 37 °C water bath where they are left for at least 20 s. Afterwards, the content of the package (i.e. straw or bag) is poured inside a recipient containing thawing extender at the same temperature and further processed for AI or for research purposes. In the former case, the sample is thawed on-farm at the very same moment of insemination.

11.2.4 Vitrification: Rapid Freezing

Vitrification is considered an alternative to standard cryopreservation and has been used so far in mammals for freezing embryos, oocytes, stem cells, and organs (Tucker and Liebermann 2007). Compared to the slow freezing method, vitrification has economic advantatges and is cost effective because there is no need for freezing instruments and because vitrification/warming requires only a few seconds (Palermo et al. 1992; Saki et al. 2009). Unfortunately, the different insemination techniques used in pigs require large sperm doses and effective vitrification demands very low sample volumes, so it is scarcely performed in the species. At the same time, pig oocyte and embryo rapid freezing are still in an experimental phase. As this freezing system has been relatively unexplored in boar sperm compared to cryopreservation we may have to wait some years before it develops its

full potential. In spite of, we have considered worth mentioning vitrification in this Chapter given that some trials have been carried out with sperm in other species.

Pioneer works that set the basis for the development of vitrification as a technique for freezing biological material involved embedding cells in supercooled water; that is, water that exists in liquid state below its freezing point. This happens when there is no element in contact with water that could trigger crystallization. Since it is a delicate equilibrium (metastable state) the probabilities of ice formation increase as temperature decreases (Debenedetti 1996), so researchers sought for methods to reduce its incidence.

Luyet (1937) first mentioned the possibility of using the vitrification technique and described it as a process by which a liquid turns into a solid in the absence of ice crystals. A good physical definition is the solidification of a solution at low temperatures not by ice crystallization but by extreme elevation of its viscosity during cooling (Isachenko et al. 2008). Vitrification is thus the supercooling of a high concentrated liquid so that the intercellular and intracellular fluids metamorphose into a glassy matrix that hinders the spontaneous triggering of nucleation. Phase transition from water into ice is replaced by glass transition from supercooled water into glass at a much lower temperature. Chemical reactions are slowed down in this matrix, stabilizing molecules inside and preserving their activity. One of the conditions for vitrification is the occurrence of agents in the solution that protect against the denaturation of proteins, which take in turn an active part in the formation of the matrix by increasing the viscosity of the solution (Debenedetti 1996). This role is played by cryoprotectants and these are naturally present in certain animals living in cold environments. These molecules prevent chilling injuries by depressing the freezing point of corporal fluids (Knight et al. 1984), which is the same working principle for vitrification protocols.

Luyet and Hodapp (1938) were the first to demonstrate, in frogs, that it was possible to freeze sperm by vitrification. A few years later, Schaffner (1942) vitrified fowl spermatozoa using a modification of Luyet's technique, while Hoagland and Pincus (1942) directly plunged raw human and rabbit sperm in liquid nitrogen. All subsequent attempts to vitrify mammalian spermatozoa did not result in satisfactory survival (Parkes 1945; Polge et al. 1949; Smith 1961). Despite obtaining results difficult to reproduce, these systems have established the bases of most current vitrification technologies. It was not until some decades later that Rall and Fahy (1985) successfully applied the vitrification technique to the preservation of mouse embryos. Since that first report this method has been investigated extensively and applied to female gametes and embryos of different mammalian species (Chen et al. 2001; Cervera and Garcia-Ximénez 2003; Isachenko et al. 2004a; Silva and Berland 2004). However, it has been challenging to standardize it because of the need for high concentrations of permeable cryoprotectants and the osmotic and cytotoxic effects they produce (Gilmore et al. 1997; Holt 1997; Katkov et al. 1998; Mazur et al. 2000).

Briefly, three factors affect the probability of vitrification: cooling rate, viscosity, and volume of the sample. Contrary to what occurs with cryopreservation, the

vitrification of cells demands elevated cooling rates and viscosity, and a high surface-to-volume ratio, which is achieved by different techniques.

The critical cooling speed for the vitrification of pure water varies dramatically depending on the method used (Karlsson and Cravalho 1994). The general technique is rapid non-equilibrium cooling (>10,000 °C/min; Leibo 1989; Leibo and Songsasen 2002; Shaw and Jones 2003; Nawroth et al. 2005), which differs from traditional cryopreservation protocols in that dehydration takes place before cooling begins. Such elevated cooling rates have risks of their own, nonetheless. In particular, glass fractures may form within the sample at temperatures below the glass transition temperature. Moreover, to prevent ice crystal formation vitrified samples must be "warmed" (a term preferred to "thawing" when talking about vitrification) as fast as they have been cooled, so thermal shock may cause fracture formation either during the cooling process or during the warming process (Arav 1999); after storage, warming is achieved by direct melting of the frozen suspension in a water bath, as in cryopreservation. Hence, achieving vitrification depends on a reciprocal relationship between cooling and warming rates, but the cryoprotectant concentration also matters: the lower the concentration the higher (by an exponential factor) the required cooling and warming rates (Fahy et al. 1987). Thus, partial or total intracellular vitrification can eventually be observed even during slow cooling (Vajta et al. 2009) as cells such as spermatozoa display a high content of soluble macromolecules (such as proteins and sugars) that make the intracellular matrix highly viscous (Isachenko et al. 2003, 2007).

Increasing viscosity is the role of cryoprotectants in order to compromise nucleation, as viscosity hinders accession to water molecules. The typical aim of a vitrification protocol is to increase the speed of temperature descent to keep the concentration of cryoprotectants, although high, as adjusted as possible (Nawroth et al. 2005). In this rapid non-equilibrium cooling, apart from high cooling rates, elevated concentrations of cryoprotectants are used (40–60 %; Leibo 1989; Leibo and Songsasen 2002; Shaw and Jones 2003). Cryoprotectant permeation takes place before cooling begins together with dehydration. Given that high concentrations of cryoprotectants have a marked toxic effect (Fahy 1986; Pegg and Diaper 1988; Shaw et al. 2000), it is possible to decrease toxicity by using a combination of two of them (e.g. ethylene glycol and DMSO), and/or to expose cells to precooled concentrated solutions in a stepwise manner (Fahy et al. 1984; Fahy 1986). Another strategy is to reduce the amount of cryoprotectants and simultaneously increase the cooling and warming rates (Liebermann et al. 2002). Some of the deleterious effects of cryoprotectants on mammalian sperm can be avoided by adopting optimal regimes of addition and removal (Sherman 1973; Watson 1979; Critser et al. 1988; Pérez-Sánchez et al. 1994; Gao et al. 1995; Leffler and Walters 1996; Katkov et al. 1998; Katkov 2002). These regimes are, however, ineffective for animal spermatozoa treated with high concentrations of cryoprotectants.

Reduction in the use of some permeable and osmotically active non-permeable cryoprotectants has been suggested as an alternative (Nawroth et al. 2002). More recently, the use of carbohydrate supplements (glucose, sucrose, and trehalose) before directly plunging samples into liquid nitrogen has also been studied

in human spermatozoa (Schulz et al. 2006; Isachenko et al. 2004a, b and 2008). The dogma has been established that vitrification of large cells, tissues, and even organs can only be effective by using high concentrations of permeable and non-permeable cryoprotectants (Fahy 1988). The total concentration of such substances in the sample must be at least 50 % to reach the threshold of stable vitrification. Concurrently, the speed of cooling and warming can be lowered although they are still relatively high. These conditions can be very damaging for cells and lead to subsequent biochemical alterations and lethal osmotic injury (Fahy et al. 1984).

Nonetheless, survival without cryoprotectants is also possible, at least in the case of vitrified sperm, and could obey to the occurrence of large amounts of osmotically inactive water bound to macromolecular structures such as DNA and protamines, or to the presence of high weight components in sperm that affect the viscosity and glass transition temperature of the cytosol (Isachenko et al. 2004a; Rama Raju et al. 2006). This kind of cryopreservation is useful on sperm due to their scant cytoplasm and low tolerance to cryoprotectants, unlike other larger cells such as oocytes and embryos or those of embryonic tissues (Nawroth et al. 2002; Isachenko et al. 2004b). Sánchez et al. (2011) confirm that vitrification without the use of cryoprotectants on dog sperm is able to preserve >95 % of DNA integrity.

The importance of the sample volume is stressed in freezing protocols as it accounts for homogeneous freezing of the entire specimen. For example, small flattened spermatozoa display more optimal surface-to-volume ratio compared to oocytes and embryos (Isachenko et al. 2003, 2007). However, the volume does not only refer to the cell itself but also to the amount of fluid in which it is immersed as it must provide rapid transfer of the external temperature to the cell. Nowadays, vitrification protocols only permit the use of very small specimen volumes to attain efficient glassification.

The sample size is minimized by using different carrier systems, as reviewed by Saragusty and Arav (2011): open-pulled straws (OPS; Vajta et al. 1997), Flexipet® denuding pipettes (FDP; Oberstein et al. 2001; Liebermann et al. 2002), micro drops (Papis et al. 2001), electron microscopy copper grids (Martino et al. 1996; Hong et al. 1999), hemi-straws (Vanderzwalmen et al. 2000), the Cryotop® (Kuwayama and Kato 2000), the CryoLoop™ (Lane et al. 1999; Mukaida et al. 2001), or, nylon meshes (Matsumoto et al. 2001), among others. In daily practice, OPS, carrying up to 10 μL spermatozoa suspension, is a common method of choice (Vajta et al. 1998; Isachenko et al. 2005). Depending on the biological material, however, other techniques should also be considered (Liu et al. 2008). In the case of OPS, current industrial technology does not yet enable the manufacture of a standard diameter of the pulled part of the straw. This shortcoming is reflected in a non-uniform vitrification regime. On the other hand, industrial suppliers offer plastic capillaries of regular cylinder shape with stable (fixed) diameter for medical applications. The vitrification process can be standardized using these capillaries, which have a great potential for assisted reproduction (Isachenko et al. 2011). Other of the aforementioned practices have been patented and are commercialized as straightforward methods with great success.

11.3 New Trends in Boar Sperm Preservation

Cryopreservation is up to now the only practical resource for long-term preservation of boar sperm. Some research groups have started seeking alternatives to conventional freezing protocols mainly because of the awkwardness and cost of handling liquid nitrogen, and of the space requirements of tanks, which would still be a matter in case vitrification was standardized in the species. Lyopreservation is currently a promising option and provides a new exciting field to explore.

Lyopreservation defines the art of preserving biomaterials at ambient temperature and it follows the principles of the well-known technique of lyophilization, otherwise called freeze-drying or cryodessication, with the purpose of becoming a long-term storage solution more affordable than freezing (Holt 1997; Lovell-Badge 1998; Meyers 2006; Kawase and Suzuki 2011). Most eukaryote cells (sperm included) do not withstand dessication (Day and Stacey 2007) and so their manipulation is sought to mimic the anhydrobiotic strategies of those organisms that tolerate extremely dry periods (Potts 2001; Alpert 2005).

Although as yet few publications exist on the topic, there is increasing interest in this technique and its related literature has grown in the last decade. The major concern in the lyopreservation of sperm is to find a process that optimizes the percentage of cell dehydration so that it does not affect its viability. To date, there are three major protocols tested: freeze-drying, convective drying (also named "passive", "air" or "evaporative drying"), and spin-drying. Although they all preserve the genetic integrity of the sample, thus being suitable for intracytoplasmatic sperm injection (ICSI); to date none of them is able to preserve the motility of the cell, which has become one of the biggest challenges to overcome.

Freeze-drying is a pioneering protocol as it comes directly from the original lyophilization procedure (Nail et al. 2002; Oetjen and Haseley 2004). Polge et al. (1949) laid the foundations of sperm lyopreservation by freezing fowl sperm in highly concentrated media, so that the sample vitrified and lost water. Sherman (1954, 1957), Saacke and Almquist (1961) and Meryman and Kafig (1963) did the same with human and bull sperm but the procedure was difficult to reproduce. The first protocol was based on cryopreservation of sperm followed by sublimation of ice into gas using a vacuum freeze-dryer. The current protocol is almost the same and consists of a freezing step followed by two holding times at different temperatures and pressures (primary and secondary drying) to sublimate the ice and to remove unfrozen water molecules (Kawase et al. 2007). However, almost 50 years elapsed before obtaining the first living foetuses from lyophilized sperm and that was done in mice (Wakayama and Yanagimachi 1998). Live offspring from freeze-dried spermatozoa were successfully obtained after ICSI from mice, rabbits, rats, hamsters, and horses (Hirabayashi et al. 2005; Kaneko et al. 2007; Hochi et al. 2008; Li et al. 2009; Choi et al. 2011; Muneto and Horiuchi 2011) but the procedure is still under optimization for other species, including humans and porcine livestock (Larson and Graham 1976; Jeyendran et al. 1981; Hoshi et al. 1994; Keskintepe et al. 2002; Kwon et al. 2004; Liu et al. 2004, 2005; Poleo et al. 2005; Lee and

Niwa 2006; Martins et al. 2007a, b; Nakai et al. 2007; Kusakabe et al. 2008; Loi et al. 2008; Sánchez-Partida et al. 2008; Abdalla et al. 2009; Czarny et al. 2009; Meyers et al. 2009; Watanabe et al. 2009). The problem of freeze-drying is that the porous spaces left by ice induce the collapse of the cell (Yang et al. 2010). Research related to this technique is focused on drying the sample below its collapse temperature and its critical moisture without altering its viability (Fonseca et al. 2004).

Convective drying was performed for the first time by Bhowmick et al. (2003). Sperm is air-desiccated inside a chamber either at room temperature or through injection of highly purified nitrogen gas. The major inconvenience is that a thin glassy skin forms on the surface of the sample, which hinders its homogeneous dehydration and subsequent rehydration (Biggers 2009). Compared to freeze-drying the simplification of dessication permits saving on specialized machinery. The optimal design of convective desiccation protocols requires accounting for the size of the cell, its membrane permeability (Lp), and the starting thickness of the solution (Chen et al. 2006).

Spin-drying is a variant of convective drying and was introduced by Chakraborty et al. (2011). It consists of spinning sperm until the water content is expelled by centrifugal forces, reducing the thickness of the glassy surface layer and permitting fast dehydration. It shares the advantage of the convective protocol plus an improvement in membrane integrity due to osmotic shock reduction, even though sperm motility is not yet recovered. Current studies aim to prevent alterations in the different organelles of sperm, which may be the key to motility maintenance.

Scientists introduce lyoprotectants in the lyopreservation medium in order to simulate the mechanisms of anhydrobiosis. Because most of the lyoprotective substances cannot be found inside the sperm and they are non-permeable, they require different techniques to be internalized, namely, induced poration, transgenesis and lipofection (Garrett et al. 1999; Shirakashi et al. 2002; Stoll and Wolkers 2011).

Lipofection is the fusion of phospholipid bilayer vesicles (liposomes) with the plasmatic membrane for drug and acid nucleic delivery inside cells or for modification of the lipid composition of the membrane (Felgner et al. 1987). This procedure was tested on boar, bull, and ram sperm in 1978 (Evans and Setchell 1978) and was subsequently applied in the eighties and nineties in different experiments involving spermatozoa (Davis and Byrne 1980; Graham et al. 1987; Baranov et al. 1990; Bachiller et al. 1991; Padilla et al. 1991; Wilhelm et al. 1996; Arts et al. 1997; Gamzu et al. 1997; Garrett et al. 1999). The use of egg yolk phospholipid liposomes as an alternative to egg yolk in sperm cryopreservation (see Sect. 11.2.3) is a preview of the potential benefits of lipofection both in freezing and in lyopreservation procedures. Lipofection can effectively reduce chilling sensitivity in boar sperm (He et al. 2001) and may also be used to mimetize the intracellular content of dehydration-resistant species. Indeed, liposomes are themselves resistant to freeze-drying (Shulkin et al. 1984), which suggests sperm could be also resistant to desiccation with the aid of lyoprotectant-loaded liposomes that would preserve both the membrane and the intracellular content.

Lyoprotectants can be non-reduced disaccharide sugars (Kawai et al. 1992; Oliver et al. 2004; Mc Ginnis et al. 2005), chelating agents (Kaneko and Nakagata 2006), antioxidants (Sitaula et al. 2009) or other proteins (Brockbank et al. 2011). Some dehydration responsive proteins have recently been discovered in desiccation-tolerant plants (Choudhary et al. 2009), insects (Cornette and Kikawada 2011) and prokaryotes (Potts et al. 2005), and a number of them encode for mitochondrial-related ones, which may account for protecting this organelle and, in turn, the sperm motility. Deferoxamine, a chelator that entraps ions to repress metabolic reactions, also aids in protecting cells during dehydration stress in certain species (Potts et al. 2005; Buitink and Leprince 2008; Choudhary et al. 2009; Farrant et al. 2009; Sitaula et al. 2009; Tejedor-Cano et al. 2010; Tolleter et al. 2010). On the other hand, the dissacharide trehalose is one of the main players in anhydrobiosis and is naturally present inside cells in desiccation-tolerant organisms (Erkut et al. 2011; Hengherr et al. 2011).

Disaccharides bind to residual water found in the inner and outer membrane layers, where water binds to the phospholipid head groups and is retained while moisture is above a critical point. Under this point disaccharides maintain membrane integrity and minimal moisture to overcome dehydration. Together with other lyoprotectants they also provide a vitrified state in which metabolic processes are limited and the cell is thus preserved in a latent state without energy consumption (Stoll and Wolkers 2011). In 2005, an optimized protocol for freeze-drying red blood cells was established by loading them with trehalose via lipofection (Kheirolomoom et al. 2005). Trehalose has been included in lyopreservation protocols for mice (Mc Ginnis et al. 2005; Li et al. 2009; Elmoazzen et al. 2009), boars (Meng et al. 2010), bovine livestock (Martins et al. 2007a, b; Sitaula et al. 2010) and *rhesus macaque* (Klooster et al. 2011), and it is also beneficial as a non-permeating cryoprotectant for it is thought to avoid protein denaturation (Jain and Roy 2009).

The combination of different strategies found in nature is pinpointed as key to the success of sperm lyopreservation (Crichton et al. 1994; Brockbank et al. 2011). The introduction of different components in the liposomes, not only trehalose but also stabilizers of mitochondrial membranes, could be the solution for preserving both membrane integrity and motility during sperm desiccation and rehydration.

11.4 Conclusion

Gene banking is the most effective strategy of the pig industry for preserving characters of interest from the genetic drift after intensive selection work has been carried out. The development and enhancement of breeds and pig lines is greatly facilitated by cryopreservation and, possibly in a near future, by lyopreservation of sperm cells, embryos and oocytes. At the same time, gene banking allows uniform availability of sperm samples throughout the year, regardless of poor quality semen production periods, and takes advantage of isolation in case of infectious diseases in livestock. However, the low endurance of certain boar ejaculates to

freezing protocols demands more refined handling techniques together with early detection of poor freezability. The definition of molecular markers of freezing resistance is stressed for this latter purpose and there are candidates under research and development. Last but not least, proper management of a gene bank requires the legal definition of property arrangements, biosecurity, sample shipment, and databasing to assist good practices and international trading.

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