Chapter 6

Freezing Technology: Control of Freezing, Thawing, and Ice Nucleation

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Abstract

From early dry-ice-based freezers and passive coolers, cryopreservation devices have come a long way. With increasing interest in the field of cryobiology from new scientific applications, the importance of reliable, traceable, and reproducible cold chain devices is sure to increase, ensuring more precise cryopreservation and enabling better post-thaw outcomes, both for the user and for biological samples. As with any cryopreservation process, it is important to optimize each part of the cold chain for each lab's biological samples, cryocontainers used, and logistical restraints. In this chapter we describe how freezing technology can be used for cryopreservation of cells.

Key words Controlled freezing rate, Ice nucleation, Thawing, Freezing technology

1 Introduction

For successful cryopreservation of most eukaryotic cells, control of the freezing, cryogenic storage, and thawing procedures is essential for optimal cell recovery. Recent advances in cellular therapies employing cryopreservation, often administered directly and immediately post-thawing, require stringent controls to ensure that the required level of cell function is achieved without the need for a "recovery" period of in vitro culture. Such therapies often require one or several cooling, storage, and warming stages, with a typical process shown in Fig. 1.

Historically, cryopreservation of cell lines was carried out using a passive cooling system with samples enclosed in, for example, a polystyrene box that was then cooled by placing in a suitable refrigerated environment. More recently low-cost systems such as a "Mr. Frosty" or "CellCool" designed for use with −80 °C refrigerators have become commercially available. These are widely used

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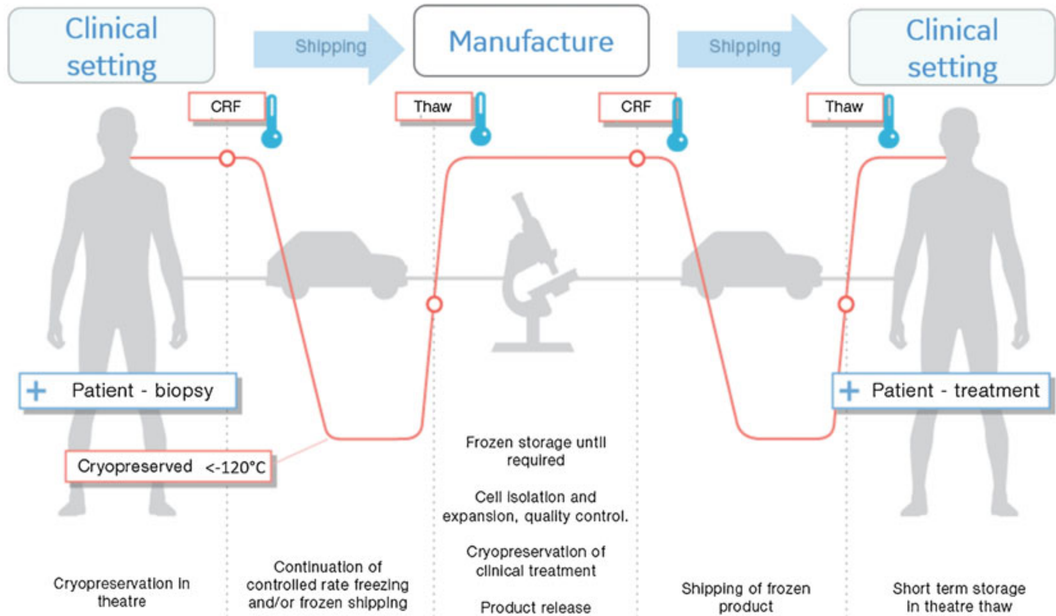

Fig. 1 A typical workflow for a cell therapy, which may involve cooling, thawing, storage, and shipment at several parts of the process. Each of these steps requires precise control and technologies to ensure that the final therapy has optimal post-thaw viability and function

for the cryopreservation of generic immortalized and cryo-resistant cell lines. Starting in the 1970s, the need for accurately controlled and variable cooling rates led to the development of controlled-rate freezers (CRF) [1–3]. The first of these systems used heaters and liquid nitrogen (LN_2) to control cooling rates that could be adjusted for different cell lines. More recently, systems that avoid the need for LN_2 have become more common [4].

A suitable storage temperature for cryopreserved (but not dried), biological samples must be below the glass transition temperature of the extracellular solution (~−120 °C for DMSO-based cryoprotectants), to prevent time-sensitive cellular degradation [5–9]. When cryopreservation emerged as a discipline in its own right (in the early 1950s), eukaryotic samples were routinely stored in dry ice containers (~−78 °C), significantly limiting viable storage time. In modern times secure long-term storage is in either liquid nitrogen or the vapor phase immediately above it. Mechanical, ultra-low freezers (~−150 °C) are also widely used.

The final step in a cold chain before therapeutic or research use is thawing which is, typically, the least-controlled part of the process. Viability and performance can be lost here as easily as in any other phase of the cryopreservation process. The most commonly used thawing procedure is direct immersion of the frozen sample container in a warmed water bath (37 °C), which produces rapid thawing. Dry thawing systems have also been developed in response to constraints imposed by current cleanroom procedures and clinical practice [10–12].

2 Materials

2.1 Cooling

1. Biological sample—For optimal cryopreservation of a cell suspension, it is important to optimize the cryoprotectant(s) and cooling rate for the specific cellular system [13–15]. These have been discussed in detail elsewhere, and we point the reader to the works referenced and theme 4 of this text.
2. Cryocontainer—Cryovials (typically <5 mL volume) are constructed of plastic with a flexible neck ring to relieve pressures during cooling and warming. They are widely used, particularly in research, but for the storage of medical-grade therapies, hermetically sealed cryocontainers are required [16]. These fall into three broad categories—cryobags, sealed vials, and straws [16, 17]. These latter containers are also common for the cryopreservation of small (<0.3 mL) biological samples such as sperm, eggs, and embryos [16]. It is important to use cryocontainers designed with specific structural properties for safe use at ultra-low temperatures, as inappropriate plastics, for example, can become brittle and shatter on warming.
3. Freezing devices—The three main choices for cryopreservation are a passive cooler, a controlled-rate freezer (CRF) using LN_2, or a CRF that is LN_2-free [1–4]. When small sample numbers are involved and viability can be recovered from rapid post-thaw cell growth, passive coolers can be a practical option. They provide an opportunity for low-cost, low-footprint cryopreservation and can be particularly appropriate in a research environment where cryopreservation is an enabling technology.
4. Both LN_2 and LN_2-free CRFs provide controlled, accurate, and reproducible cooling. A choice between them may rest, for example, on cooling rates required (LN_2 devices have higher maximum rate), available infrastructure and resources, and the work location (LN_2 freezers cannot be operated in cleanrooms) [4, 16, 18].

2.2 Ice Nucleation During Cooling

1. Samples cooled to just below their equilibrium freezing point.
2. Cryopen (*see* **Note 1**) or pre-chilled forceps (*see* **Note 2**).

2.3 Storage and Transfer

1. Liquid nitrogen vapor-phase storage tank.
2. Ultra-cold mechanical freezer.
3. Dry ice (*see* **Note 3**) or insulated chilled container.

2.4 Thawing

1. Thawing system.
2. Cryopreserved samples.
3. Prepared culture medium or washing solution.
4. Dry ice (*see* **Note 3**) or insulated chilled container.

3 Methods

3.1 Cooling

3.1.1 Passive Coolers

1. Prior to cryopreservation the passive cooler should be stored in a 4 °C refrigerator. To prepare, fill the system with coolant (usually propanol) if required and place the device in a 4 °C fridge for at least 1 h before use, to allow for temperature equilibration.
2. Prepare biological samples in the chosen cryoprotectant medium and load them into a suitable cryocontainer. Transfer promptly to the passive cooler to minimize any toxicity (*see* **Notes 4** and **5**). Fill unused spaces in the cooler with vials containing cryoprotectant medium only (*see* **Note 6**) to ensure more consistent cooling within the device.
3. Place the passive cooler into a −80 °C mechanical freezer (*see* **Notes 6–8**). It is recommended that the cooler is placed toward the back or bottom of any freezer, where thermal variations are minimal, and that a chest (top opening) freezer is used, as opening the door will cause less thermal variation here than in a side-opening freezer.
4. During the cooling process, it is important that the mechanical freezer remains closed as even brief opening will disrupt the cooling cycle.
5. When the cooling run is complete, transfer the biological samples to long-term storage below −120 °C. The samples should be thermally protected during this transfer as they can warm rapidly, risking a negative impact on post-thaw outcome (*see* **Note 3**). For a transport time of only a few minutes, leaving the cryocontainers in the passive cooler will protect them from excessive warming. For longer times, or delicate samples, transfer on dry ice or insulated chilled container for transport is required (*see* **Note 3**).

3.1.2 Controlled-Rate Freezers

1. Preparation of the freezer. As with passive coolers, it is recommended to prepare the freezing device prior to preparing the cells. The chamber/sample holder of the CRF should be pre-cooled to desired start temperature (*see* **Note 9**).
2. Selection of starting temperature. The starting temperature of the cryopreservation protocol should be as low as possible to reduce cryoprotectant toxicity and limit the overall cooling time. It must, however, be above the freezing point of the cryoprotective medium to avoid the risk of ice formation in the sample before the commencement of the intended cooling protocol, which could have damaging effects. Starting temperatures of +4 °C to −2 °C are typically used.

3. Selection of cooling rate. The importance of cooling rates in cryopreservation is well-known and should be optimized for each cell type (*see* **Note 10**) [5, 6, 13, 19]. These cooling rates may be linear—i.e., one cooling rate throughout the cooling process, or multistep, i.e., different cooling rates at different steps in the protocol.
4. Loading of biological samples. Prepare biological samples in chosen cryoprotectant medium at an appropriate temperature (e.g., on ice) and load into suitable cryocontainers (*see* **Note 4**). Transfer quickly to the CRF to minimize any toxicity (*see* **Notes 4**, **5**, and **11**). Start the cooling procedure.
5. Ice nucleation if required (*see* Subheading 3.2).
6. End temperature. An appropriate temperature must be chosen where controlled cooling is no longer required, and samples can be transferred to long-term storage (*see* **Notes 3**, **12**, and **13**). CRFs should stop cooling and maintain this final temperature until all biological samples have been removed. Programming a temperature a few degrees below the minimum endpoint temperature will help protect the biologics from any unintended warming during transfer to long-term storage (*see* **Notes 12** and **13**).
7. When the cooling run is complete, transfer the biological samples to long-term storage below −120 °C. The samples should be protected from the environment during this transfer, as they can warm rapidly which will negatively impact post-thaw outcomes. Transfer on dry ice or similar coolant may be required (*see* **Note 3**).

3.2 Ice Nucleation During Cooling

Manual nucleation can be used to nucleate a biological sample at a defined temperature, which is beneficial for some cell types [20]. A small section of the cryocontainer surface is cooled to significantly below the equilibrium freezing point causing ice nucleation in the limited, excessively supercooled region. Ice can then propagate through the remainder of the sample at a relatively high temperature (*see* **Note 14**). Manual nucleation can be achieved using a dedicated device such as a cryopen (a small device which, by expelling liquid nitrous oxide, can cool a small area to below −50 °C [21], *see* **Notes 1** and **2**) or the tips of pre-cooled forceps. Alternatively, an appropriate ice-nucleating agent can be included in the cryocontainer (*see* **Note 15**). For manual nucleation:

1. Ensure that cryopreservation protocol includes a constant temperature holding step approximately 5 °C below the equilibrium freezing point of the cryopreservation medium (*see* **Note 6**).
2. Maintain the holding temperature for at least 5 min to allow biological sample temperature to equilibrate.

3. Access the cryocontainers as rapidly as possible, and, holding them so as not to further warm their content, use the cryopen to cool the surface of the cryocontainer. This cooling should be focused until ice is seen to nucleate. Be careful to cool only one point of the vial (*see* **Notes 2** and **16**).
4. Ice nucleation will cause the cryocontainer contents to turn from clear liquid to opaque ice.
5. Return the cryocontainer to the controlled-rate freezer as rapidly as possible and resume the cooling profile when all samples have been nucleated.
6. If samples take longer than ~ 30 s to nucleate, return the samples and reduce the device's hold temperature a further 2 °C.

3.3 Storage and Transfer

Storage of biological samples containing live cells for any more than a few days must be below the glass transition temperature of the cryoprotectant medium [5, 8]. In DMSO-based cryoprotectants, this is typically ~−120 °C. Storage is therefore in LN_2 vapor or in an ultra-low mechanical freezer. Appropriate safety considerations should always be in place where this hardware is present. Inadequate handling during transfer between the cooling device and long-term storage location can lead to damaging sample warming.

1. Ensure that the controlled rate or passive cooler has reached the final temperature and that the biological samples have equilibrated below at least −50 °C [6, 22].
2. Prepare a polystyrene box containing dry ice or other appropriate cold-transfer system and ensure that space is available in the long-term storage (*see* **Note 3**).
3. Remove cryocontainers by their lids, or area containing the least biological material, and place quickly into the dry ice or transfer vessel. Always use insulated gloves and other appropriate personal protective equipment.
4. Add biological samples to appropriate location in long-term storage. It is essential to ensure that samples are positioned in the storage container to minimize warming of surrounding, already stored samples during retrieval of the required material (*see* **Note 17**). Dispersal of duplicate samples to different locations in a storage tank is recommended, as is the use of duplicate tanks.
5. Maintain the freezer at the end temperature until transfer to storage is complete. If there is a problem during transfer, then samples can be temporarily returned to the cooling device.

3.4 Thawing

It is important to note that, for optimal results, correct thawing and post-thaw treatment of a cryopreserved biological sample are as

important as the earlier cooling process. Immersion of cryocontainers in a warmed water bath is the established, and probably still most common, method of thawing cryopreserved biological samples. In the past decade, dry thawing systems have become more common due to lower contamination risks and greater consistency.

3.4.1 Water Bath

1. Warm water bath to 37 °C (*see* **Note 18**).
2. Remove the biological sample from storage device, being careful not to warm other samples in the same storage location.
3. Place sample in water bath, and where possible avoid wetting the lid or inlet/outlet ports of the cryocontainer. Use of a submerged rack or floating vial holder is often useful here.
4. Visually inspect the sample, and when only a small ice crystal remains visible, remove the sample and quickly begin any washing or post-thaw steps [23]. As a rule of the thumb, 2 mL cryovials containing 1 mL of liquid take ~ 2 min to thaw from approximately −120 °C in a 37 °C water bath (*see* **Note 19**).

3.4.2 Dry Thawing Systems

1. Ensure that the correct thawing system is used—some are optimized for vials, others cryobags, and some both.
2. Remove the biological sample from storage, being careful not to warm other samples within the same storage location.
3. Place sample in the equipment for the required time.
4. Visually inspect biological sample when thawing is believed to be complete, to ensure no more than a small ice crystal remains.
5. Promptly start any washing or post-thaw steps [23].

4 Notes

1. A cryopen is a small device (in shape and size like a pen), containing nitrous oxide under pressure. When a small volume of the gas is released onto the outer surface of the sample container, a transient and localized region of deep cooling is generated that cools the inner container surface sufficiently to initiate ice nucleation.
2. In the absence of a cryopen, it is possible to use some pre-chilled forceps or other metal surface to nucleate ice. Pre-chilling can be done by dipping the tip of the forceps in LN_2 and then used in the same way as a cryopen by touching the side of the cryocontainer with them. The forceps or metal implement should be re-cooled between samples, as these will warm rapidly in the air.

3. Any suitably cooled and insulated container is effective for transfer. If no dry ice is available, cold-box gel kits pre-cooled to −80 °C or below can be used, as well as any specific cold-transfer device which can maintain the endpoint temperature or lower. Using a small Dewar containing liquid nitrogen is not advisable due to the possibility of LN_2 ingress and the risks of spills and splashes of LN_2 from carrying open containers (*see* **Note 17**).
4. When transferring procedure-ready samples on ice, it is important to ensure that the cryocontainer is dried immediately before loading into the cooling device. Any water remaining on the outside of the sample will freeze on cooling and may result in the sample being stuck to the cooler when it reaches its target temperature.
5. When labelling cryocontainers, it is critically important to use ink and materials which are resistant to both low temperatures and water. Labels designed for only ambient temperatures can become brittle and lose their adhesive properties during ultra-low temperature storage.
6. In this chapter we have at several points recommended that a "dummy" vial or cryocontainer, containing cryoprotectant medium but no cells, is used. This can be set up to record the actual temperature within the cryocontainer that, due to the thermal mass of the sample and the physical properties of the cryocontainer, will lag behind the device programmed temperature. Once the actual sample temperatures have been established, the programmed cryopreservation protocol can be modified as required. The process can be carried out without using dummy cryocontainers in every run as the cooling/thawing process should be consistent when carefully repeated, but for maximum security, a dummy cryocontainer could be included in each run. For dummy cryocontainer thermocouple recommendations (*see* **Note 8**).
7. To alter cooling rates in a passive cooler, a range of mechanical freezers can be used. For example, using a −150 °C freezer will increase the cooling rate by approximately 70% compared to −80 °C system. When changing cooling environment, using a thermocouple in a vial containing cryoprotectant medium to test actual cooling rates is useful (*see* **Notes 6** and **8**). While many passive coolers claim a cooling rate of 1 °C/min when placed into a −80 °C environment, this is a calculated, mean value and will only be achieved for a small part of the cooling process. It will be impacted by sample load and freezer temperature fluctuations.
8. When setting up a dummy cryocontainer, it is recommended that a thermocouple attached to a data logger is used. The

thermocouple lead should be small and flexible enough to be inserted into a vial or other cryocontainer easily. The entry point must be securely sealed. The thermocouple junction (at the end of the lead) should be in the center of the liquid of small vials (<2 mL), and several spatially separated thermocouples may be used in larger containers. Many types of thermocouple are available, and type T can be recommended due to their better accuracy at low temperatures when compared to other common types.

9. When loading and cooling cryocontainers such as vials, it is important that they are upright for consistency. Cryobags should be cryopreserved with as few air bubbles as possible, as these insulate the biological sample on both cooling and thawing, leading to suboptimal outcome.
10. For most somatic mammalian cells in suspension, cooling rates of 1 °C/min are usually a good starting point for optimization studies [24]. For larger cell structures, such as spheroids and organoids, the longer time required for dehydration suggests that a slower rate such as 0.3 °C/min is a better starting point. Some specific cell types, such as sperm cells and erythrocytes, require relatively fast cooling rates (10 °C/min or greater).
11. When cooling multiple samples in one cooling run in an LN_2-based CRF, it is advisable to leave a gap between individual cryocontainers. These freezers cool by blowing cold nitrogen into the freezing chamber at a specific rate. This can result in differing cooling rates between those samples directly exposed to this cold nitrogen and those that may be partially insulated by other samples surrounding it. For sensitive cells this can lead to variable and/or suboptimal results on thaw.
12. This endpoint temperature should always be below −50 °C, and −80 °C is a commonly adopted final temperature. The −80 °C achieved when a passive cooler is placed into mechanical freezer is a convenient end temperature for such devices.
13. As the temperature of a biological sample temperature typically lags behind freezer temperature, albeit slightly, it is preferable to wait a few minutes after the freezer reaches its endpoint temperature before transfer of the sample to long-term storage.
14. Ice nucleation is volume dependent, and larger samples (>2 mL) usually nucleate with minimal supercooling, whereas smaller volumes are more susceptible to excessive supercooling. Where possible, biological sample volumes should be as large as possible when dealing with known, nucleation-sensitive material.
15. Some freezing protocols include an ice-nucleating agent in the cryoprotective medium. These are reagents which are included

in small quantities to initiate nucleation at a higher temperature than would otherwise occur, without the need for manual handling of the sample [20, 25].

16. It is usually better to aim to cool the upper section of the liquid in a cryovial. This is because on cooling, most cells will sink slowly under gravity. Cooling the upper section of the liquid will ensure that the minimum number of cells will be present in the extremely supercooled area. This is only possible, however, in vertical setups such as cryovials. In cryobags cryopreserved vertically, this becomes impractical. For cryostraws, the best area to nucleate is some of the supercooled liquid far away from the biological sample.
17. It is recommended that samples are stored in the vapor phase immediately above LN_2 and not submerged in the liquid itself. Submerged cryocontainers, if damaged, may allow LN_2 ingress which can contaminate the biological sample and other content in the storage vessel. It can also lead to the sample container exploding on thawing if a damaged area becomes sealed with ice, as the liquid nitrogen remaining in the container will rapidly expand as converting to gas.
18. Replacing water regularly and including a disinfectant reagent in the water bath are recommended to reduce any contamination risk to the sample and the lab in general.
19. It is important on thaw to remove the biological sample and start any wash steps quickly once thawing is complete. Samples left in a warm environment may start to experience cryoprotectant toxicity.

References

1. Thurston LM, Holt WV, Watson PF (2003) Post-thaw functional status of boar spermatozoa cryopreserved using three controlled rate freezers: a comparison. Theriogenology 60:101–113
2. Perez-Oteyza J, Bornstein R, Corral M, Hermosa V, Alegre A, Torrabadella M, Ramos P, Garcia J, Odriozola J, Navarro JL (1998) Controlled-rate versus uncontrolled-rate cryopreservation of peripheral blood progenitor cells: a prospective multicenter study. Group for Cryobiology and Biology of Bone Marrow Transplantation (CBTMO), Spain. Haematologica 83:1001–1005
3. Meryman HT (2007) Cryopreservation of living cells: principles and practice. Transfusion 47:935–945
4. Creemers E, Nijs M, Vanheusden E, Ombelet W (2011) Cryopreservation of human sperm: efficacy and use of a new nitrogen-free controlled rate freezer versus liquid nitrogen vapour freezing. Andrologia 43:392–397
5. Baboo J, Kilbride P, Delahaye M, Milne S, Fonseca F, Blanco M, Meneghel J, Nancekievill A, Gaddum N, Morris JG (2019) The impact of varying cooling and thawing rates on the quality of cryopreserved human peripheral blood T cells. Sci Rep 9:3417
6. Meneghel J, Kilbride P, Morris JG, Fonseca F (2019) Physical events occurring during the cryopreservation of immortalized human T cells. PLoS One 14:e0217304
7. Massie I, Selden C, Hodgson H, Fuller B (2013) Storage temperatures for cold-chain delivery in cell therapy: a study of alginate-encapsulated liver cell spheroids stored at −80 °C or −170 °C for up to 1 year. Tissue Eng Part C Methods 19:189–195
8. Hubel A, Spindler R, Skubitz AP (2014) Storage of human biospecimens: selection of the

optimal storage temperature. Biopreserv Biobank 12:165–175
9. Attarian H, Feng Z, Buckner C, MacLeod B, Rowley S (1996) Long-term cryopreservation of bone marrow for autologous transplantation. Bone Marrow Transplant 17:425–430
10. Röllig C, Babatz J, Wagner I, Maiwald A, Schwarze V, Ehninger G, Bornhäuser M (2002) Thawing of cryopreserved mobilized peripheral blood—comparison between waterbath and dry warming device. Cytotherapy 4:551–555
11. Triana E, Ortega S, Azqueta C, Pomares H, Valdivia E, Duarte R, Massuet L, Martín-Henao GA (2013) Thawing of cryopreserved hematopoietic progenitor cells from apheresis with a new dry-warming device. Transfusion 53:85–90
12. Pinki SS, Mohan G, Rafi AM, Innah SJ, Thomas T (2017) Rapid dry plasma thawing system: an alternative to conventional thawing baths. Asian J Transfus Sci 11:147–150
13. Mazur P, Leibo S, Chu E (1972) A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells. Exp Cell Res 71:345–355
14. Elliott GD, Wang S, Fuller BJ (2017) Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology 76:74–91
15. Baust JM, Campbell LH, Harbell JW (2017) Best practices for cryopreserving, thawing, recovering, and assessing cells. In Vitro Cell Dev Biol Anim 53:855–871
16. Bielanski A (2012) A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices. Theriogenology 77:467–482
17. Haack-Sørensen M, Ekblond A, Kastrup J (2016) Cryopreservation and revival of human mesenchymal stromal cells. In: Mesenchymal stem cells. Springer, New York, pp 357–374
18. Grout BWW, Morris G (2009) Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer. Theriogenology 71:1079–1082
19. Morris GJ, Acton E, Murray BJ, Fonseca F (2012) Freezing injury: the special case of the sperm cell. Cryobiology 64:71–80
20. Morris GJ, Acton E (2013) Controlled ice nucleation in cryopreservation—a review. Cryobiology 66:85–92
21. Winkler JL, Jeronimo J, Singleton J, Janmohamed A, Santos C (2010) Performance of cryotherapy devices using nitrous oxide and carbon dioxide. Int J Gynaecol Obstet 111:73–77
22. Eskandari N, Marquez-Curtis LA, McGann LE, Elliott JA (2018) Cryopreservation of human umbilical vein and porcine corneal endothelial cell monolayers. Cryobiology 85:63–72
23. Shu Z, Heimfeld S, Huang Z, Liu C, Gao D (2015) Progress in cryopreservation of stem cells and immune cells for cytotherapy. Progr Stem Cell Transplant. https://doi.org/10.5772/60620
24. Stacey GN, Healy L, Man J, Hunt CJ, Morris J (2017) Fundamental points to consider in the cryopreservation and shipment of cells for human application. Bioprocessing for cell based therapies. John Wiley & Sons Ltd, Hoboken, NJ, pp 167–185
25. Massie I, Selden C, Hodgson H, Fuller B (2011) Cryopreservation of encapsulated liver spheroids for a bioartificial liver: reducing latent cryoinjury using an ice nucleating agent. Tissue Eng Part C Methods 17:765–774

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