8 Biobanking of Human Mesenchymal Stem Cells: Future Strategy to Facilitate Clinical Applications

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Abstract

Human mesenchymal stem cells (hMSCs), a type of adult stem cells that hold great potential in clinical applications (e.g., regenerative medicine and cell-based therapy) due to their ability to differentiate into multiple types of specialized cells and secrete soluble factors which can initiate tissue repair and regulate immune response. hMSCs need to be expanded *in vitro* or cryopreserved to obtain sufficient cell numbers required for clinical applications. However, long-term *in vitro* culture-expanded hMSCs may raise some biosafety concerns (e.g., chromosomal abnormality and malignant transformation) and compromised functional properties, limiting their use in clinical applications. To avoid those adverse effects, it is essential to cryopreserve hMSCs at early passage and pool them for off-the-shelf use in clinical applications. However, the existing cryopreservation methods for hMSCs have some notable limitations. To address these limitations, several approaches have to be taken in order to produce healthy and efficacious cryopreserved hMSCs for clinical trials, which remains challenging to date. Therefore, a noteworthy amount of resources has been utilized in research in optimization of the cryopreservation methods, development of freezing devices, and formulation of cryopreservation media to ensure that hMSCs maintain their therapeutic characteristics without raising biosafety concerns following cryopreservation. Biobanking of hMSCs would be a crucial strategy to facilitate clinical applications in the future.

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Keywords

Human mesenchymal stem cells • Cryopreservation • Clinical trials • Clinical applications • Slow freezing • Vitrification • Limitations • Challenges

Abbreviations

hMSCs	Human mesenchymal stem cells
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GvHD	Graft-versus-host disease

8.1 Introduction

Regenerative medicine, a growing area of medicine, aims to treat diseases which are not easily treated, e.g., cardiovascular diseases and diabetes, by repairing the injured tissues and restoring their functions [1]. Human stem cells, particularly human mesenchymal stem cells (hMSCs), hold great promise in regenerative medicine due to their multilineage differentiation potential and paracrine function (ability to secrete soluble factors), which can regulate immune response and initiate tissue repair or regeneration [2–5]. Further, hMSCs are relatively easy to be isolated from various tissues in human body (e.g., adipose tissues, bone marrow and periosteum), and ethical concerns related to the use of embryonic stem cells can be avoided [6–8]. To date, hMSCs have been applied in clinical trials targeting clinical disorders such as graft-versus-host disease (GvHD), stroke and cardiovascular diseases [9]. Such clinical applications may require trillions of hMSCs, which can be produced through extensive *in vitro* cell expansion [10]. However, long-term *in vitro* cell expansion may raise biosafety concerns (e.g., chromosomal aberration and malignant transformation) and reduce therapeutic potency of hMSCs [11, 12]. It has been reported that patients treated with hMSCs at early passage have improved clinical outcome over those treated with late passage cells [13]. Therefore, an effective method of long-term preservation and storage of hMSCs at early passage is essential.

Cryopreservation is the only method used to preserve and store cells, including hMSCs, for a long-term period [14]. Cryopreservation adopts a principle which utilizes ultralow temperatures (approximately −196 °C, e.g., in liquid nitrogen) to halt the metabolic activity of cells while maintaining their life and cell functionality [15, 16]. Cryopreservation allows transportation and pooling of cells to reach cell numbers required for clinical applications while maintaining their functional properties [17]. It also allows the completion of quality control and safety testing of cells prior to clinical applications [18, 19]. A bank of efficacious and healthy cryopreserved cells would be available as a ready off-the-shelf supply, allowing better timing of therapy [15]. Further, cryopreservation produces a bank of cells at specific passages with intact functional properties and genetic characteristics. These validated cells can be used to initiate new experiments, maximize the long-term use of cells, and minimize experimental variation [20].

A successful cryopreservation requires the development of protocols that achieves high cell recovery and maintain cell functionality, including proliferation potential, multi-lineage differentiation potential, immunomodulatory property, migration ability and secretory profile, which are identical to that of the cells at pre-storage state [21]. As the utilization of cryopreserved cells continues to increase, researchers are demanding a standardized protocol which can achieve higher cell recovery and the cells recovered are biochemically and physiologically identical to that of pre-storage state at the structural, genomic, proteomic and functional levels [22]. Further, the

cells should be xeno-free, nontoxic and immune privileged in human [14]. However, many challenges remain thus far, including sub-optimal cell recovery, addition of toxic or animal components in the cryopreservation medium and potential contamination [23]. Therefore, a remarkable amount of resources has been utilized in research in optimization of the cryopreservation methods, development of freezing devices, and formulation of cryopreservation media, in order to ensure that hMSCs maintain their therapeutic characteristics without raising biosafety concerns following cryopreservation [15].

8.2 Clinical Applications of hMSCs

The first clinical trial of *in vitro* culture-expanded hMSCs isolated from bone marrow was conducted in patients with hematologic malignancies in year 1995 [24]. Since then, the clinical application of hMSCs has been further explored. From year 2010 to 2014, the clinical trials database (http://www.clinicaltrials.gov) demonstrated 685 registered clinical trials of hMSCs in different clinical phases to evaluate their potential therapeutic applications worldwide. The number of registered clinical trial of hMSCs has been rising since year 2010 (Fig. 8.1a). Most of these trials are aimed to treat cardiovascular diseases (e.g., myocardial ischemia and stroke), musculoskeletal diseases (e.g., osteoarthritis and rheumatic diseases), neurological diseases (e.g., spinal cord injury and amyotrophic lateral sclerosis) and immune system diseases (e.g., GvHD and multiple sclerosis) (Fig. 8.1b). Majority of these trials are in Phase I (safety evaluation), Phase II (establishment of the efficacy of hMSCs against a placebo), or a mixture of Phases I/II studies. Only an inconsiderable number of clinical trials are in Phase III (establishment of the efficacy of hMSCs against many standard or well-known treatments) or a mixture of Phases II/III or Phase IV (description of the additional information such as treatment's risk and optimal dosage) (Fig. 8.1c). In general, most trials have reported that no sign of significant adverse effects was seen in the patients receiving hMSCs-based therapies [25]. Further, many completed clinical trials have showed the efficacy of hMSCs in treating clinical disorders, including GvHD, amyotrophic sclerosis, liver cirrhosis, stroke and acute myocardial infarction [26].

As of 31st December 2014, there are 23 registered clinical trials of cryopreserved hMSCs (derived from bone marrow) in patients with clinical disorders such as myocardial infarction, GvHD and Crohn' disease. The first clinical trial using infusion of cryopreserved hMSCs (named as Prochymal® or remestemcel-L) in patients with GvHD was conducted in year 2005. Prochymal is an off-the-shelf stem cell product, which is frozen and stored in Plasma-Lyte®A (an isotonic solution for intravenous infusion) containing 5 % human serum albumin and 10 % DMSO. For administration, Prochymal was thawed and diluted with Plasma-Lyte®A to achieve the cell concentration needed for infusion and to reduce final concentration of DMSO in the infused product to 3.75 %. The viability of cells after thawing was at least 70 %, as determined by Trypan blue assay [27–30]. Prochymal has been approved by Canada as the first adult stem cell-based therapy to treat GvHD. No identifiable adverse effect or acute toxicity was seen in the patients receiving this therapy [27–29]. Prochymal is currently undergoing Phase III trials for refractory Crohn's disease, and Phase II trials for the treatment of myocardial infarction, diabetes mellitus type 1 and chronic obstructive pulmonary disease. On the other hand, MultiStem®, another product of cryopreserved hMSCs (derived from bone marrow), which is also stored frozen in a mixture of PlasmaLyte, human serum albumin and DMSO, undergoes Phase II trials for ischemic stroke, acute myocardial infarction, acute respiratory distress syndrome, and inflammatory bowel diseases (e.g., ulcerative colitis) [31].

Taken together, hMSCs have only been approved for the treatment of GvHD, whereas significant efforts are required in clinical trials in order to prompt the use of hMSCs in the treat-

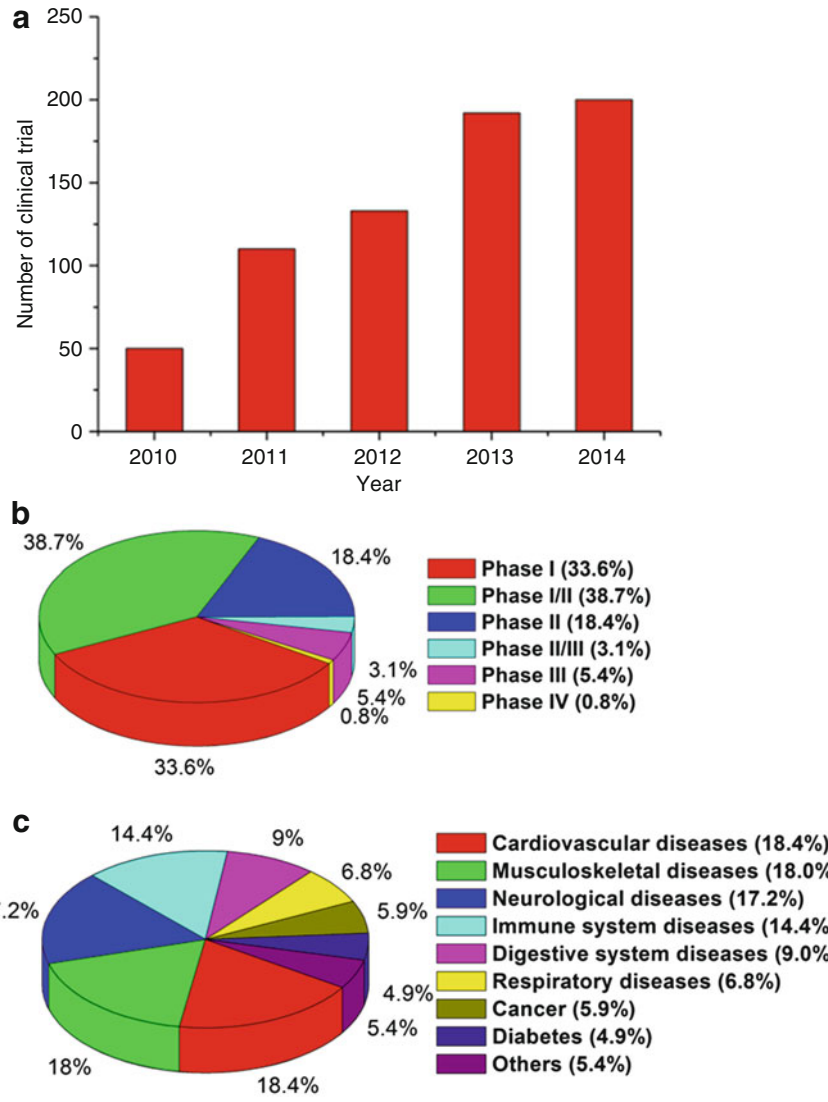

Fig. 8.1 (**a**) Number of registered clinical trials of hMSCs from year 2010 to 2014. (**b**) Clinical trials of hMSCs classified by phase. (**c**) Clinical trials of hMSCs classified by category of studied disease (Data from ClinicalTrials.gov)

ment of other clinical disorders in the future. Since cryopreserved hMSCs offer great potential for clinical application, most research has been focused on optimization of cryopreservation condition of hMSCs (including hMSCs isolated from umbilical cord, adipose tissues and amniotic fluid other than bone marrow) in order to produce more off-the-shelf hMSCs for clinical trial and future clinical applications.

8.3 Existing Methods of Cryopreservation of hMSCs

8.3.1 Slow Freezing

The slow freezing method is the preferred method of cryopreservation of high volume cells such as hMSCs and cell lines [32]. With a freezing rate of

1 °C/min (an optimal cooling rate for mammalian cells) [33], a large number of cells can be frozen in a cryovial at a low concentration (<1.5 M) of cryoproctective agents (CPAs) [34]. This freezing rate allows cellular dehydration and reduces the risk of intracellular ice formation. To achieve such a low freezing rate, direct contact of cells with non-sterile liquid nitrogen is not required during freezing, thus avoiding potential contamination with other microorganism or pathogens [14]. Therefore, a large amount of cryopreserved cells which are contamination-free can be pooled to provide sufficient cells for off-the-shelf clinical use. A uniform freezing rate can be generated in a passive or active manner. Passive freezing approach utilizes a commercially available freezing container filled with alcohol, e.g., "Mr. Frosty" (temperature is lowered at freezing rate of 1 °C/min due to the slow freezing property of isopropanol), whereas active freezing approach utilizes a programmable controlled rate freezer [22]. However, it has been reported that both freezing approaches, display similar potential to maintain phenotype, viability, and functional properties of hMSCs [35]. With the advance of technology a programmed freezer termed "Cell Alive System" (CAS) has been developed, which vibrates the water molecules and cells using alternating magnetic field and electric field during the process of freezing to prevent intra- and extra-cellular ice formation [36]. It has been reported that the risk of freeze injury to MSCs can be further reduced by using this system [37]. Among various types of slow freezing methods, freezing protocol which adopts passive freezing approach is favorable for cryopreservation of hMSCs due to low cost and high cryopreservation efficiency.

To preserve hMSCs efficiently using a slow freezing method, it is essential to optimize and determine the ideal CPAs used to preserve hMSCs. The roles of CPAs in cell cryopreservation are to stabilize the cell membrane, minimize osmotic stress to the cells, and protect cells against intracellular and extracellular ice crystal formation which are both harmful to cells [38, 39]. CPAs are divided into permeating CPAs (e.g., dimethyl sulfoxide (DMSO) and glycerol) and non-permeating CPAs (e.g., sucrose, trehalose and fetal bovine serum (FBS)). Permeating CPAs can penetrate the cell membrane due to their low molecular weight and remove the water from the cells to prevent the formation of intracellular ice [40–42]. Non-permeating CPAs is unable to pass through the cell membrane but it is able to protect the cell membrane by forming a viscous glassy shell around the outer surface of cells and regulate intracellular and extracellular osmotic pressure [40, 41] (Fig. 8.2).

Among the CPAs, DMSO with relatively high membrane permeability and FBS being rich in protein and growth factors is commonly utilized for cell cryopreservation [43]. DMSO with a concentration of 10 % (v/v) combined with FBS (20–90 %) (v/v) has been commonly used to preserve hMSCs [44–46], but both exhibit a considerable disadvantages. DMSO is cytotoxic at the temperatures beyond 4 °C [47]. The clinical uses of cells preserved in 10 % DMSO have caused many adverse effects (e.g., neurotoxicity and respiratory depression) in recipients [48, 49]. DMSO may induce undesired differentiation of stem cells to neuronal-like cells [50]. On the other hand, FBS contains a number of proteins and peptides which can initiate xenogeneic immune responses [51, 52]. In addition, FBS could possibly transfer pathogens [47]. Therefore, it is recommended to minimize or exclude the use of FBS as part of the cryopreservation medium [53]. Overall, an ideal CPA should maintain functional properties and high survival rate of cells after thawing, and allow for cell transplantation without raising biosafety issues (e.g., xenogeneic immune response, cytotoxicity and tumourigenesis).

8.3.2 Vitrification

Vitrification is a process which requires a very high freezing rate to convert a cell-laden CPA suspension directly from its aqueous phase to a glass state upon contact with liquid nitrogen [54]. Generally, two approaches have been used to preserve cells using vitrification – equilibrium vitrification and non-equilibrium vitrification. In

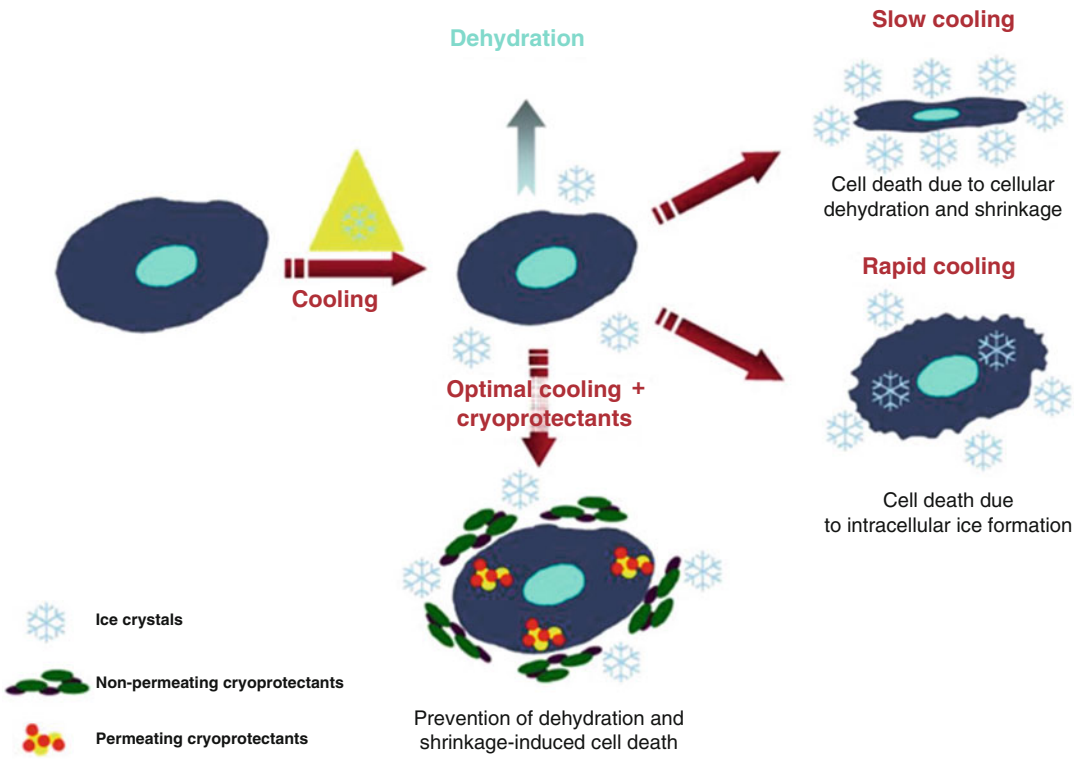

Fig. 8.2 The role of CPAs in cell cryopreservation. Cryopreservation requires CPAs (either permeating or non-permeating) to protect cells from freeze injury (e.g., cell death) by avoiding the formation of intra- and extracellular ice crystals which are both harmful to cells during freezing (Reproduced from Martin-Ibanez et al. [65])

equilibrium vitrification, cells are exposed to multimolar CPA mixtures in a stepwise manner, which helps to reduce chemical toxicity, followed by immersion into liquid nitrogen [55]. hMSCs have been vitrified using this approach, which could successfully maintain their functional properties and viability [56–58]. However, this approach has high potential to cause osmotic shock and chemical toxicity to cells [55].

In non-equilibrium vitrification, extremely high freezing rates has been performed with only one CPA at a low concentration for cell preservation [55]. In this approach, vitrification systems are divided into carrier-based systems and carrier-free systems. Cryoloops (700,000 °C/min), quartz microcapillaries (250,000 °C/min) and plastic straws (2500 °C/min) are among the carriers that have been developed for vitrification with each of them offering a different freezing rate [34]. The utilization of higher freezing rate allows vitrification using CPA at a lower concentration, thereby reducing the risk of osmotic damage and chemical toxicity to the cells. On the other hand, carrier-free systems which can generate micro-droplets of cell-laden CPA followed by ejection into liquid nitrogen, have been developed to further increase freezing rate of vitrification [34]. hMSCs have been vitrified using a carrier-free system without the need for directly contacting the cells with liquid nitrogen [59], and the results seem promising.

8.3.3 Slow Freezing Versus Vitrification

Post-thaw viability of cells was reported to be higher using vitrification compared to slow freezing method as the high freezing rate reduces the time of intra- and extracellular ice formation [60]. However, vitrification which adopts an approach of direct cell-to-liquid nitrogen contact, may lead to potential pathogenic contamination

[61]. The Hepatitis B virus and *Aspergillus sp.* are among the pathogens which have been reported to contaminate liquid nitrogen [61, 62]. The source of contamination may come from non-sterile liquid nitrogen itself or cross-contamination from infected samples in the liquid nitrogen storage tank [63]. Therefore, good operational skill is required for vitrification. Large scale cryopreservation of cells is impractical. Vitrification is usually applied to small volume cells such as embryonic stem cells, embryos and oocytes because it requires low volume of cell suspension to achieve high freezing rate [64]. Therefore, it is not recommended to cryopreservation of hMSCs in large volumes. Further, the recycling of cells is labor-intensive as it needs manual picking up of individual cell colonies [60], which may lead to the cell loss. The advantages and disadvantages of slow freezing and vitrification are described in brief in Table 8.1. Collectively, the slow freezing method is preferable because cryopreservation of hMSCs can be performed on a large scale, the post-thaw viability of cells is high, and it avoids the risk of pathogenic contamination, allowing cryopreserved hMSCs to be used for clinical applications with low risk of pathogen transmission.

Table 8.1 Comparison between slow freezing and vitrification method

No	Aspect	Slow freezing	Vitrification
1.	Concentration of CPAs required	Low	High
2.	Risk of freeze injury	High	Low
3.	Post-thaw viability	High	Higher
4.	Risk of toxicity of CPAs	Low	High
5.	Potential pathogenic contamination	Low	High
6.	Operational skill	Easy	Good operational skill is needed

Adapted from Yong et al. [14]
CPAs cryoprotective agents

8.4 Challenges in Cryopreservation of hMSCs for Clinical Applications

8.4.1 Slow Freezing

Today, the issue of adverse effects in recipients of cells cryopreserved in 10 % DMSO and FBS using a slow freezing method is challenging [60, 65]. Although the transplantation protocol has included the step of post-thaw washing of the cells to remove DMSO prior to transplantation [66, 67], but washing the cells by centrifugation prior to removal of DMSO can cause a significant cell loss. Further, total removal of DMSO from cryopreserved cells is time-consuming and complex [68]. Therefore, the development of an alternative cryopreservation medium or a cryopreservation medium consisting of a reduced concentration of DMSO is required [69]. To date, the introduction of non-toxic polymers (e.g., polyvinylpyrrolidone) and disaccharides (e.g., sucrose and trehalose) as a CPA [35, 70], have not achieved the aim of replacing DMSO for cryopreservation of hMSCs as they are less efficient than DMSO in maintaining the viability of hMSCs, possibly due to their inability to penetrate cell membrane. However, when trehalose encapsulated in genipin-cross-linked Pluronic F127-chitosan nanoparticles was delivered into hMSCs for cryopreservation, it was found that cells are capable to maintain their functional properties and high viability following cryopreservation [71]. This indicates that non-permeating CPAs, e.g., trehalose, may be more efficient when they are delivered intracellularly for cryopreservation of hMSCs. Further investigation is needed to assess the biodegradability and cytotoxicity of the genipin-cross-linked Pluronic F127-chitosan nanoparticles, and explore more efficient methods to deliver CPAs intracellularly. Further investigation is also required to explore alternative CPAs to replace DMSO completely.

Recently, it has been proposed that xeno-free cryopreservation medium can be used to preserve hMSCs [14]. For instance, 5 % human albumin solution has been proposed to replace FBS for cryopreservation of hMSCs [72]. Further, our recent study has demonstrated that hMSCs preserved in xeno-free cryopreservation medium consists of only 5 % DMSO were capable to maintain high viability and their functional properties following cryopreservation [73]. However, it has been reported that cells (e.g., ESCs and lymphocytes) cryopreserved in DMSO may display chromosomal abnormality and changes in telomere length, which might in turn lead to tumour formation [74, 75]. These concerns have established the need for assessment of tumourigenic potential and chromosomal abnormality in cryopreserved hMSCs prior to clinical applications. It has been demonstrated that cryopreservation does not alter chromosome structures and numbers in hMSCs, indicating no sign of chromosomal abnormality [45, 76–78]. Further, our recent study has demonstrated that cryopreserved hMSCs including those preserved in 5 % DMSO display low tumourigenic potential, indicating their low risk in tumourigenesis [79]. These results indicate that hMSCs preserved in 5 % DMSO which have low risk in inducing xenogeneic immune response and cytotoxicity, could be an ideal cell source for clinical applications in the future. Further investigation is needed to assess immunomodulatory property, migration ability and secretory profile of hMSCs preserved in 5 % DMSO following cryopreservation.

8.4.2 Vitrification

To vitrify cells for clinical applications, many issues need to be addressed, including small scale cryopreservation, high risk of contamination and cell loss due to the ineffective collection of frozen cells. With the advance of technology, the development of an ejection-based micro-droplet generation system permits efficient vitrification of micro-droplets in a continuous and high throughput manner, which has significantly improved the performance for cryopreservation of hMSCs. Further, a closed, sterile, and automated vitrification system (e.g., ejection-based micro-droplet generation system) which allows vitrification of cells via indirect contact with liquid nitrogen should be developed to avoid potential contamination and reduce cell loss [32, 59]. Xu's group has developed a device which can vitrify micro-droplets of cell-laden CPA printed on a silver film via boiling heat transfer without the need for direct contact with liquid nitrogen [59]. hMSCs subjected to vitrification using such approach were found to maintain cell phenotype, high viability and osteogenic differentiation potential. This device allows vitrification with low risk of contamination and cell loss, indicating its potential use in vitrification of hMSCs for clinical applications. Further investigation is needed to perform further assessment of cell functionality in hMSCs subjected to vitrification using this device. In addition, a sterile polyetrafluoroethylene cartridge with filteration and ultraviolet radiation has been suggested to sterilize liquid nitrogen prior to vitrification of cells since liquid nitrogen has been reported to be potentially contaminated with infectious agents [80].

8.4.3 Thawing

Besides freezing, it is essential to select the optimum method to thaw the frozen cells. The standard thawing method is to warm the cells at a rapid rate (>100 °C/min) at 37 °C in a water bath until all the ice melted [68], resulting in recovery of a high numbers of viable cells following thawing. Thawing frozen cells at such a rapid rate could minimize ice recrystallization and cellular exposure time to high concentrations of CPA [22]. A dry warming procedure has been proposed to thaw the frozen cells to avoid possible contamination of cells with microorganism presence in the water bath [81]. Therefore, a controlled-rate thawing chamber was developed to thaw frozen cells at a warming rate of 10 °C/min, resulting in a high viability of cells, which is comparable to those thawed with the standard method [82]. After thawing, the cells are washed

by centrifugation to remove CPAs, which are particularly toxic (e.g., DMSO) prior to clinical applications. However, the process of washing may result in a significant cell loss which may in turn affect the clinical outcome. In the future, it is essential to develop a controlled-rate dry warming device which is low cost, reliable and portable for thawing frozen cells, and a method which can remove CPAs while minimizing cell loss for effective clinical applications.

8.5 Conclusion and Future Perspectives

Nowadays, the demand for organ transplantation has been rising rapidly due to the increasing incidence of chronic diseases (e.g., liver cirrhosis and myocardial ischemia), which lead to the end-stage failure of many vital organs (e.g., liver and heart). The supply of organs from deceased donors has remained low and insufficient to meet the increasing demand. So, shortage of organs for transplantation has become a major crisis worldwide. To solve the organ shortage problem, regenerative medicine which emphasizes on the use of human stem cells in the treatment, has evolved rapidly. Among various types of stem cells, hMSCs possess paracrine function and immunosuppressive property, which support their use in various clinical settings. Implantation of such multi-functional hMSCs may treat the fatal diseases while addressing the problems of organ shortage crisis. However, long-term culture-expanded hMSCs may raise biosafety concerns and demonstrate decreased therapeutic potency, limiting their widespread use. Cryopreservation could retain the therapeutic characteristics of hMSCs with low risk of biosafety issues, indicating their potential use in clinical applications. Therefore, the development of biobank of hMSCs is essential as healthy and efficacious cryopreserved cells can be pooled for off-the-shelf use when needed, allowing better timing of therapy. To date, many challenges remains in addressing the limitations of the existing cryopreservation protocols (from freezing process to thawing process and therapeutic administration), including sub-optimal cell recovery, potential contamination and addition of toxic or animal-based agents into the cryopreservation medium. A standardized and safe cryopreservation protocol of hMSCs which can achieve high recovery of cells and maintain cell functionality should be established. In the future, biobanking of hMSCs would be a crucial strategy to facilitate clinical applications.

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