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REVIEW

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Effects, methods and limits of the cryopreservation on mesenchymal stem cells

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Abstract

Mesenchymal stem cells (MSCs) are a type of cell capable of regulating the immune system, as well as exhibiting self-renewal and multi-lineage differentiation potential. Mesenchymal stem cells have emerged as an essential source of seed cells for therapeutic cell therapy. It is crucial to cryopreserve MSCs in liquid nitrogen prior to clinical application while preserving their functionality. Furthermore, efficient cryopreservation greatly enhances MSCs' potential in a range of biological domains. Nevertheless, there are several limits on the MSC cryopreservation methods now in use, necessitating thorough biosafety assessments before utilizing cryopreserved MSCs. Therefore, in order to improve the effectiveness of cryopreserved MSCs in clinical stem cell treatment procedures, new technological techniques must be developed immediately. The study offers an exhaustive analysis of the state-of-the-art MSC cryopreservation techniques, their effects on MSCs, and the difficulties encountered when using cryopreserved MSCs in clinical applications.

Keywords Mesenchymal stem cells, Cryopreservation, Clinical application, Cryoprotective agents, Methods.

Background

Friedenstein's research team recovered mesenchymal stem cells (MSCs), a subset of non-hematopoietic stem cells, from mouse bone marrow [1]. The source of MSCs is no longer restricted to bone marrow with the advancement of technology. Nowadays, researchers can extract MSCs from a variety of tissues such as bone marrow [2], adipose tissue [3], amnion [4] and umbilical cord [5]. The requirements for MSC identification are becoming clearer with the expansion of their sources. In 2006, the International Society for Cellular Therapy's Mesenchymal and Tissue Stem Cell Committee proposed a set of minimal requirements to characterise human mesenchymal stem cells (hMSCs): firstly, MSCs must be plastic-adherent under standard culture conditions; secondly, MSCs must express CD105, CD73 and CD90, and not express CD45, CD34, CD14 or CD11b, CD79a or CD19 or HLA-DR surface molecules; and thirdly, MSCs must differentiate in vitro into osteoblasts, adipocytes and chondroblasts [6] (Fig. 1).

Therapeutic therapy extensively employs MSCs in the management of hematological diseases [7], plastic repair [8], and even COVID-19 [9] due to their potent immunosuppressive and immunomodulatory properties, as well as their capacity to differentiate into a variety of distinct cell types. To obtain the necessary quantity required for therapeutic applications, it is essential to either increase and cultivate MSCs or source them from a variety of donors. Future utilization of off-the-shelf cells necessitates long-term storage, with cryopreservation being the only technique that can successfully maintain cells over an extended period [10]. Cryopreservation can maintain the bulk of cells needed for therapeutic applications while preserving their properties [11]. Without cryopreservation, cells must undergo continuous passage, which could

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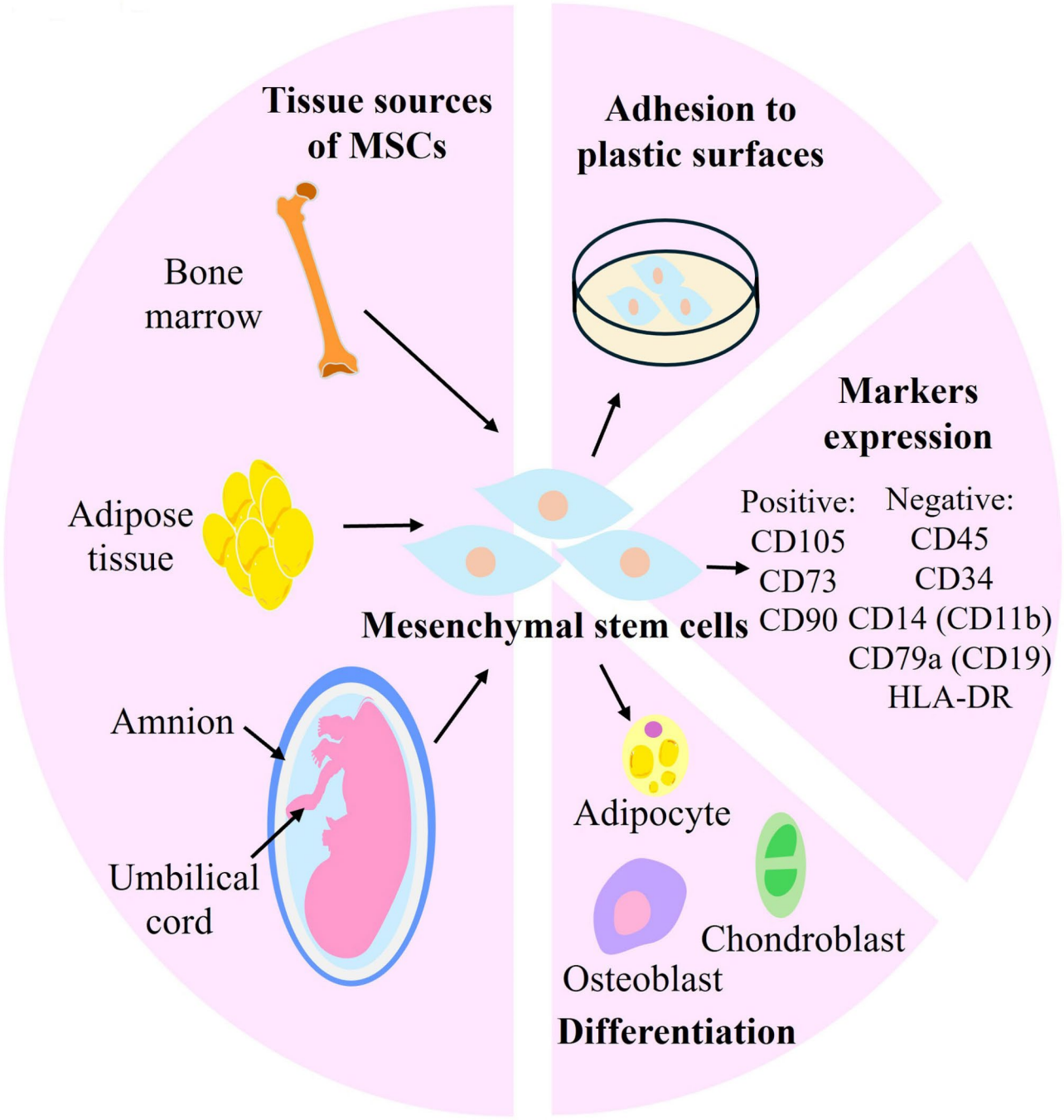

Fig. 1 Sources of MSCs and minimal criteria for defining human MSCs. The figure (left) shows the different sources of MSCs including bone marrow, adipose tissue, amnion and umbilical cord. The figure (right) depicts the minimal criteria to define human MSCs. First, MSCs are plastic-adherent and spindle-shaped morphology. Second, they must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Last, they must differentiate to osteoblasts, adipocytes and chondroblasts in vitro

potentially lower DNA methylation levels, alter epigenetic modifications such as telomere shortening, and result in random loss of genomic regions [12]. Therefore, in order to protect the cells from the trouble of continual passage, cryopreserving the cells in liquid nitrogen at a temperature of minus 196 degrees Celsius represents the optimal approach.

Liquid nitrogen preservation confers cells with resistance to infection and eliminates their need for metabolism [13]. Consequently, cells with certain genetic traits and full functionalities are made available for therapeutic

study through cryopreservation. The use of liquid nitrogen storage is simple and easy to operate, and has gained widespread adoption. Modern cryopreservation technology enables the long-term preservation of living cells and tissues, providing a cellular resource for therapeutic applications such bone marrow transplantation [14], blood transfusion [15] and in vitro fertilization [16, 17]. In addition, cryopreserved MSCs can be used to treat other diseases. While cryopreserved MSCs have been shown potential benefits in the treatment of liver [18] and diabetes [19], further research is required to confirm their true therapeutic usefulness efficacy.

MSCs can now be cryopreserved using a variety of techniques, including vitrification and slow freezing, although each technique has limitations [20]. In this paper, the advantages of these two approaches will be discussed. For example, cryoprotective agents (CPAs), such as dimethyl sulfoxide (DMSO), have the potential to pose hazards to cells when used to shield them against freezing damage [21]. Replacing dimethyl sulfoxide with dimethyl sulfoxide-free CPAs is a desirable option in order to minimize damage to the cells, but this necessitates the development of CPAs and cryopreservation procedures. In addition to a discussion of the effects of cryopreservation on MSCs and the challenges encountered in their clinical use, this study offers a comprehensive overview of the techniques for cryopreservation of MSCs.

Cryopreservation methods for MSCs

The two primary techniques, slow freezing and vitrification, are used to cryopreserve MSCs. Slow freezing is the cryopreservation of cells at a rate slow enough to dehydrate the cells sufficiently while minimizing the formation of ice crystals within the cells [22]. Vitrification allows cells and their extracellular environment to solidify into a glassy shape without forming ice [22]. Although there are many methods of MSCs cryopreservation, the method of thawing is the same. Cryopreserved MSCs are often thawed by quickly heating them in a water bath at 37 °C (at a rate of more than 100 °C/min) until all ice crystals are dissolved [23]. To enhance safety during cell thawing, it could be preferable to use drying heating equipment instead of a water bath because the water may be contaminated with microorganisms [24]. Centrifuging MSCs after thawing is necessary to eliminate CPAs, especially hazardous ones like DMSO. Developing new techniques for eliminating CPAs is crucial to reduce cell loss, as the current approach results in a considerable percentage of cell loss. Currently, there is room for improvement in the freezing and thawing processes used for MSCs.

Slow freezing

The mechanisms of slow freeing mainly include gradual dehydration, using CPAs and controlling the cooling rate. Gradual dehydration means that during the preservation process, the water inside the cell gradually exudes out through the cell membrane, reducing the formation of ice crystals inside the cell [17]. This is usually achieved by placing the cells in the temperature which is gradually reduced, such as at 4 ° C for a period of time, then gradually cooled to -80 ° C, and finally transferred to liquid nitrogen (-196 ° C) for long-term preservation. Adding low concentration penetrants such as DMSO or non-permeating agents (NPAs) like sucrose or trehalose, these small molecules can penetrate the cell membrane, reduce the freezing point of water, and improve the permeability of the cell membrane to water, thereby reducing the damage of ice crystals to cells [17]. The cooling rate needs to be strictly controlled, usually kept within −3 ° C /min, to ensure that the cells have enough time to dehydrate and reduce the formation of ice crystals.

The steps of slow freezing involve mixing the MSCs with the CPAs and placing them in the cryopreserved tubes. Then, store them in a freezer or other freezing apparatus at temperature of -20 °C. Subsequently, the cells are cooled down to -80 °C and stored in liquid nitrogen at -196 °C until their utilization is required [25] (Fig. 2). Approximately 70–80% of cells survive when employing this gradual freezing procedure [26]. Slow freezing is the recommended technique for clinical and laboratory MSC cryopreservation because of its ease of operation and minimal risk of contamination [27]. Because of its great effectiveness in cryopreservation, slow freezing remains the preferred method for MSC cryopreservation to this day.

However, optimizing the use of CPAs is required in order to make better use of the slow freezing approach for MSC cryopreservation. The reason why CPAs can protect cells in the cryopreservation is that CPAs can instantly pass through the plasma membrane, because of plasma membrane far lower permeability to CPAs than to water [28]. Thus, the difference between the osmotic pressure inside and outside the cell is reduced, which can reduce the cell damage caused by a large number of water molecules flowing out of the cell during the freezing process. Improper addition or removal of CPAs can potentially harm cells due to their intrinsic toxicity [29]. For example, the concentration of CPAs is too high or added or removed too quickly. Therefore, the conditions for the addition and removal of CPA should be strictly controlled to ensure the safe and effective preservation of MSCs.

The process of removing CPAs is reversed with the addition situation. Rinsing CPAs during thawing rapidly reduces their external concentration, leading to excessive cell expansion, cell damage, and cell lysis [30]. As a result, techniques for cleaning CPAs must guarantee that cells can withstand volume fluctuations and prevent harm

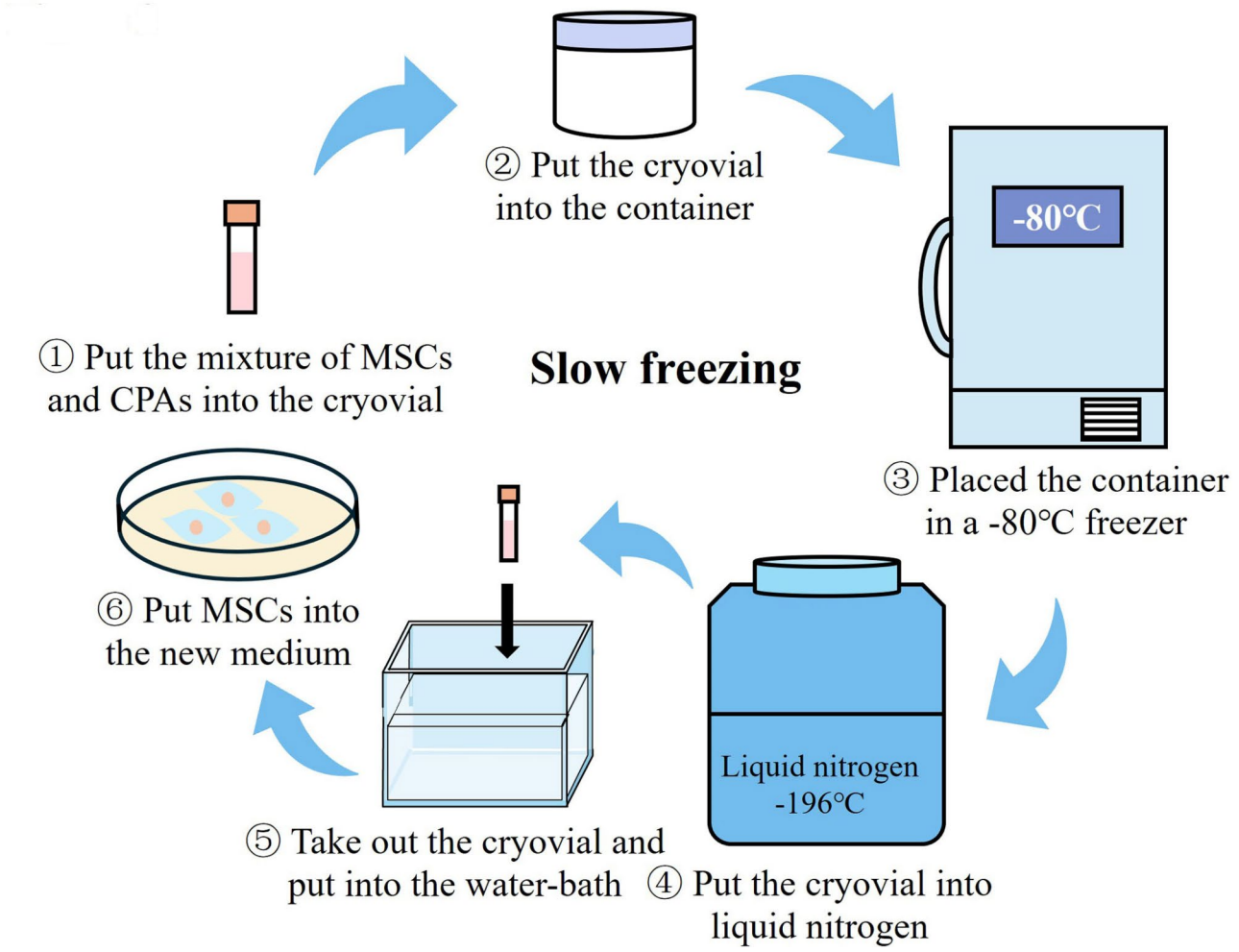

Fig. 2 The major steps of the slow freezing and thawing for MSCs. Step 1 to 4 is the part of cryopreservation, and step 5 to 6 is the part of thawing. Major steps of slow freezing procedure include CPAs addition, step freezing from room temperature to -196℃ with different freezing equipment, and storage in liquid nitrogen. Major steps of thawing procedure are using the water-bath to warm and adding new medium to recover cells

from osmotic pressure. Cleaning DMSO in particular is crucial due to its potential hazards, even at room temperature. The study has shown that transfusion of stem cells containing DMSO can trigger allergic responses in individuals requiring hematopoietic stem cells [31]. Additionally, Bombard's research concludes that among the four CPAs (glycerol, DMSO, ethylene glycol (EG), propylene glycol (PG)), PG has the worst cryopreservation effect, while EG and PG exhibit similar cell toxicity, which is lower than DMSO, and glycerol has the lowest cell toxicity but results in the worst cryopreservation effect [32]. Although there are many different CPAs, people need to consider whether to focus on safety or efficacy when cryopreserving MSCs.

Vitrification

The main mechanisms of vitrification include the use of high concentrations of cryoprotectants and high cooling rates. High concentration CPAs are combined to form the vitrification freezing solution. These protectants increase the viscosity of the solution by strongly hydrating with water molecules, thereby preventing the formation of ice crystals during rapid cooling [33, 34]. This method uses low-temperature liquid nitrogen to quickly freeze the viscous liquid resulting from a high concentration of CPAs into a glassy state [35]. Sufficient CPA concentration or rapid cooling can increase viscosity and prevent the formation of ice crystals [36]thereby protecting the structure and function of the cells.

There are two methods to accomplish vitrification. The first is the equilibrium vitrification approach, which involves a balance between the cells and the specific formulation of CPAs [33]. By controlling the concentration and penetration time of the CPAs, the cells are fully dehydrated before freezing and reach osmotic equilibrium with the cryoprotectant. Then cells are quickly put into liquid nitrogen for vitrification. This method aims to reduce the formation of ice crystals during

cryopreservation, thereby protecting cells from mechanical damage. The alternative technique is non-equilibrium vitrification, which pays more attention to cooling rate and high concentration of CPAs [33]. It uses a high concentration of CPAs to rapidly penetrate the cells and immediately put the cells into liquid nitrogen, so that the cells reach the vitrification state in a very short time. This method inhibits the formation of ice crystals by increasing the cooling rate and the concentration of cryoprotectants. These two methods can better protect cells from freezing damage, so they are suitable for situations that require high cell survival and functional recovery, such as the cryopreservation of embryonic stem cells and reproductive stem cells.

In the process of vitrification, it is crucial to select CPAs carefully due to their varying critical temperatures and cooling rates, which are crucial for preventing ice formation and considering their toxicity across different cell types [37]. In vitrification, EG, sucrose, DMSO, and fetal bovine serum (FBS) solutions are frequently utilized [38]. Researchers have effectively used different combinations of these CPAs, particularly EG and DMSO, on a variety of tissues and cells, including amnion-derived MSCs [39], cord blood [40], and embryos [41]. High concentrations of CPAs are necessary for the equilibrium vitrification process, which can potentially be detrimental to cells [42]. Consequently, the vitrification freezing method often requires both careful preparation of the CPA mixture and the gradual injection of the CPA at lower temperatures to ensure maximum safety. However, this approach often poses a risk of toxicity or osmotic damage to the cells, as lower temperature exacerbates osmotic injury [43]. By employing very quick cooling rates, non-equilibrium vitrification prevents the formation of ice crystals [17]. During the thawing process, when the system moves between the ice nucleation and crystal development zones, it changes into a metastable vitrified state. The rate of warming becomes very crucial in maintaining an ice-free state of the system [44]. If the warming rate is too slow, devitrification will occur at this point, causing the development of ice crystals [44]. Thus, when thawing cells, it is essential to operate quickly.

Apart from the issues related to osmotic shock and toxicity, vitrification offers several advantages. Firstly, it prevents the formation of intracellular ice crystals, protecting the cells from the potential harm [33]. Compared to conventional cryopreservation techniques, cells can be exposed to a solution containing fewer cryoprotectants for a shorter duration throughout the freezing process. Finally, vitrification can lead to cost saving by eliminating the need for sophisticated and expensive equipment such as programmed freezers. Human embryonic stem cells (hESCs) [45], human umbilical cord MSCs (hUCMSCs) [46], and human bone marrow MSCs (hBMSCs) [47] are increasingly using vitrification for cryopreservation in current research practices. The Table 1 provides a comprehensive comparison of the two methods - slow freezing and vitrification and shows that these two methods both have advantages and disadvantages. Vitrification can save time and cost, however, slow freezing is the best method for MSCs cryopreservation, because it is simple and easy to operate in different laboratories. When cryopreserving MSCs, people can choose the corresponding method according to their own needs.

Table 1 Comparison of slow freezing and vitrification methods

Characteristics	Slow freezing	Vitrification
Time	More than 3 h	Less than 10 min
Speed of freezing	Slow	Fast
Sample volume	100–250μL	1–2μL
Cost	Expensive, freezing machine needed	Inexpensive, no special machine needed
Ice crystal formation	Yes	No
Post thaw viability	High	High
Concentration of CPA	Low	High
Risk of CPA toxicity	Low	High
Potential pathogen contamination	Low	High
Procedure	Simple	Complex

This table compares some characteristics between slow freezing and vitrification, and shows the difference in the two methods

Effects for cryopreservation on MSCs

Compared to other cell types, such as tumor cells, MSCs require the detection of additional characteristics in order to assess the impact of cryopreservation. In addition to the standard evaluation of cell phenotypic and survival, it is imperative to analyze MSCs' capacity for multilineage differentiation and immunomodulation following cryopreservation [48]. If cryopreservation compromises their immunomodulatory and differentiation processes, MSCs may be unable to prevent transplant rejection or differentiate into various types of cells to aid in the regeneration and repair of damaged organs [49].

The biosafety of cryopreservation on MSCs requires thorough assessment. During cryopreservation, stem cells may be vulnerable to genomic instability, potentially affecting cell division and proliferation, as well as leading to epigenetic modifications. According to Kang's study, DMSO affects histone acetylation, which disrupts the transcriptional program for the mother-to-embryonic transition. Therefore, it is crucial to exercise caution when using DMSO as the CPA [50]. In addition, it is essential to assess the genetic stability of cryopreserved MSCs prior to therapeutic use. Testing the homing capacity of cryopreserved MSCs is also necessary since it is crucial to determine if MSCs can be homed to the intended locations when reinjected into the body [51].

Cell viability

Essentially, the assessment of cell viability is crucial during cryopreservation, as only live cells can be used for additional research or applications. Therefore, the identification of cell activity is critical. A variety of techniques, such as trypan blue exclusion, the MTT test, and annexin V-propidium iodide (annexin V-PI) [52], can be employed to evaluate the viability of MSCs following cryopreservation. When keeping cells in liquid nitrogen at a temperature of around −196 °C, CPAs are necessary to preserve cell viability [53]. During cryopreservation, the various kinds and concentrations of CPAs have a major impact on cell viability [54]. Cryopreserved hBMSCs exhibit greater cell survival rates when DMSO doses below 10% are used in these CPAs, according to Chen's research [55]. In comparison to the 10% DMSO+90% FBS treatment, Zhang's team discovered that the 1.0 M trehalose (Tre)+20% glycerol (Gly) treatment demonstrated a significantly higher efficiency in preserving human adipose-derived MSCs (hADSCs) activities after thawing, resulting in enhanced outcomes in both cell viability and proliferation capacity [56]. Therefore, it is necessary to select suitable CPAs and concentration for different MSCs to ensure that they still maintain cell viability after cryopreservation.

Cell phenotype and proliferation capacity

The International Society for Cellular Therapy's Mesenchymal and Tissue Stem Cell Committee has established specific minimal requirements for human MSCs, which include adherence to plastic under standard culture conditions and expression CD105, CD73, and CD90. They also need to lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules [6]. It is therefore necessary to identify the morphology and characteristics of MSCs during cryopreservation. Numerous investigations have demonstrated that MSC morphology and characteristics remain unchanged during cryopreservation, whether they are hBMSCs [57] or hADSCs [58]. The above information fully meets the minimum human requirements for MSCs.

The population doubling time (PDT) of cells, which refers to the time it takes for the number of cells to double, utilized to investigate cell growth dynamics, and serves as a measure for assessing MSCs' proliferative capacity [59]. The shorter the population doubling time, the stronger the proliferative capacity of the cells. Table 2 demonstrates that several studies have found that cryopreservation does not affect the PDT of various MSCs. These findings suggest that MSCs can maintain their ability to proliferate following cryopreservation.

Details on the population doubling time in different MSCs after cryopreservation by different research groups. This table aims to provide the PDT of different MSCs remains unchanged after cryopreservation, and specific cell types and time for cryopreservation.

Immunomodulatory ability

Multiple studies have shown that MSCs can attenuate immune responses and autoimmune activity by releasing chemicals like interleukin-10 (IL-10) and interleukin-4 (IL-4), which stop the activation of immune cells [69]. The implantation of MSCs benefits from the reduced immune response. And MSCs' ability to regulate the immune system is dependent on how well they inhibit lymphocytes or peripheral blood mononuclear cells (PBMCs) [70]. Inhibiting lymphocytes and PBMCs can significantly mitigate tissue damage caused by an exaggerated immune response. Alhamwe's research shows that exosomes derived from hBMSCs can boost the production of TGF-β1 and IL-10 from the peripheral blood monolayers of asthmatic patients thereby promoting the expansion of regulatory T cells grow and dampening the immune system [71]. In general, reducing the immune response in various ways can improve MSC viability within the implant recipient's body. Research has shown

Table 2 Studies reporting the population doubling time in different MSCs after cryopreservation

Study	Species	Source	The time of cryopreservation	The population doubling time after cryopreservation
Gamble et al. [60]	Human	Adipose tissue	-	No change
Ngeun et al. [61]	Rabbit	Adipose tissue, Bone marrow	4 Weeks	No change
Wiese et al. [62]	Human	Umbilical cord, Bone marrow	-	No change
Bella et al. [63]	Canine	Adipose tissue	7 Years	No change
Schmelzer et al. [64]	Human	Adipose tissue, Amniotic tissue, Bone marrow, Chorionic tissue, Liver, and Umbilical cord	-	No change
Yalvaç et al. [65]	Human	Tooth germs	6 months	No change
Nguyen et al. [66]	Human	Umbilical cord	-	No change
Navakauskienė et al. [67]	Human	Placenta	Long-term storage	No change
Fu et al. [51]	Human	Umbilical cord	24 h	No change
Hennes et al. [68]	Human	Amniotic fluid	1 week to 6 weeks	No change

that cryopreservation moderately reduces the immunomodulatory potential of MSCs compared to fresh MSCs, but does not significantly alter it [72, 73]. However, the immunosuppressive impact of cryopreserved MSCs on lymphocytes or PBMCs can recover to a level comparable to that of fresh MSCs within a short period following recovery, typically less than 7 days [74]. According to these studies, cryopreservation affects MSCs' ability to modulate immunity, but this effect is reversible. Prior to therapeutic use, cultivate cryopreserved MSCs for a brief duration may enhance their immunoregulatory capacity and restore their immunomodulatory potential.

Cell differentiation capacity

Based on the established minimum standards by the International Society for Cellular Therapy's Mesenchymal and Tissue Stem Cell Committee [6], MSCs must differentiate into osteoblasts, adipocytes, and chondroblasts under laboratory conditions. Therefore, it is crucial to assess the MSCs' differentiation capacity after cryopreservation using histochemical staining [75]. Von Kossa or Alizarin Red can stain MSCs red or black when they develop calcium deposits during osteogenic stimulation [75, 76], indicating the capacity to differentiate into osteocytes of the MSCs. Similarly, Oil Red O staining can be utilized to detect round lipid droplets characteristic of adipocytes following induction [75, 77], while Alcian blue staining of the proteoglycans in the induced cartilage blue reveals if MSCs have the capacity to develop into chondrocytes [75, 78]. According to recent research, cryopreservation has no appreciable impact on MSCs' capacity for differentiation, particularly in the areas of osteogenic, lipogenic, and chondrogenic differentiation [79, 80].

Biosafety

Conducting MSC characterization studies, animal tests, and clinical safety evaluations is critical to forecasting potential risks and ensuring the safety of MSCs in therapeutic applications [81]. Considering the significant correlation between gene mutations and cancer, MSCs used in therapeutic settings should not possess genomic alterations linked to cancer. This necessitates a careful examination of the MSCs' genetic makeup. In extreme circumstances, an inadequate safety evaluation may result in immune rejection during the treatment of allogeneic donor MSCs [82].

According to some research, cryopreservation with DMSO may change the stem cells' chromosomal stability and cell cycle [83, 84]. Prior to clinical application, it is critical to analyze the total chromosomal content of MSCs by karyotype, as chromosome alterations are one of cancer's features [85]. Kyrotype analysis can identify aneuploidy and chromosomal abnormalities, such as inversion, deletion, translocation, and duplication, commonly observed in genetic illnesses [86]. According to Zhang's research, MSC chromosomal makeup and number remain unchanged after cryopreservation [87]. On the other hand, DMSO may induce epigenetic modifications in MSCs, including telomere shortening and the random loss of genomic regions [12], mediated by pathways involving histone modification and DNA methylation. Pollock's research through the genomic analysis has confirmed that the different freezing media evaluated have different effects on the levels of DNA hydroxy methylation in MSCs [88]. To find the best CPAs for clinical use, more comparative research on the biological safety of other CPAs is required.

Homing potential

"Homing of MSCs" refers to the process by which foreign MSCs implant, move to a specific tissue or organ, and carry out their biological tasks [89]. For instance, MSCs migrate to the wounded location upon receiving an injury signal, where they impede the inflammatory process that facilitates healing [90]. Numerous variables, including chemokines, adhesion molecules, growth factors, and inflammatory factors, control the homing of MSCs [91]. Certain investigations have shown that cryopreservation has no influence on MSCs' homing potential (Paul Köhli [92] and Morgana T. L. Castelo-Branco [93]). However, other research has suggested that cryopreservation diminishes MSCs' homing potential [94]. Therefore, the effects of cryopreservation on the homing potential of MSCs still require further investigation.

Limits for cryopreservation on MSCs

Several challenges remain to be addressed before the clinical application of MSCs, including concerns related to immunogenicity, donor heterogeneity, in vitro expansion, and cryopreservation [95]. Cryopreservation remains a critical issue that requires resolution. For example, how to select suitable CPAs to avoid intracellular ice crystal formation and how to avoid epigenetic modification of MSCs after cryopreservation [96].

The choice of CPAs for MSCs is an important issue, because some CPAs may lead to cryopreserved MSCs encountering challenges related to genetic stability, including chromosome abnormalities, genetic mutations, and epigenetic alterations [12]. The traditional CPA is DMSO, but recently more clinical research has begun to use CPAs without DMSO to preserve the genetic integrity of MSCs, since some studies have revealed that DMSO may induce genetic alterations in MSCs [83, 84]. It is imperative to emphasize strict control the development and research of CPAs and the evaluation of the optimal dose of CPAs such as DMSO in order to mitigate genetic instability in cryopreserved MSCs and

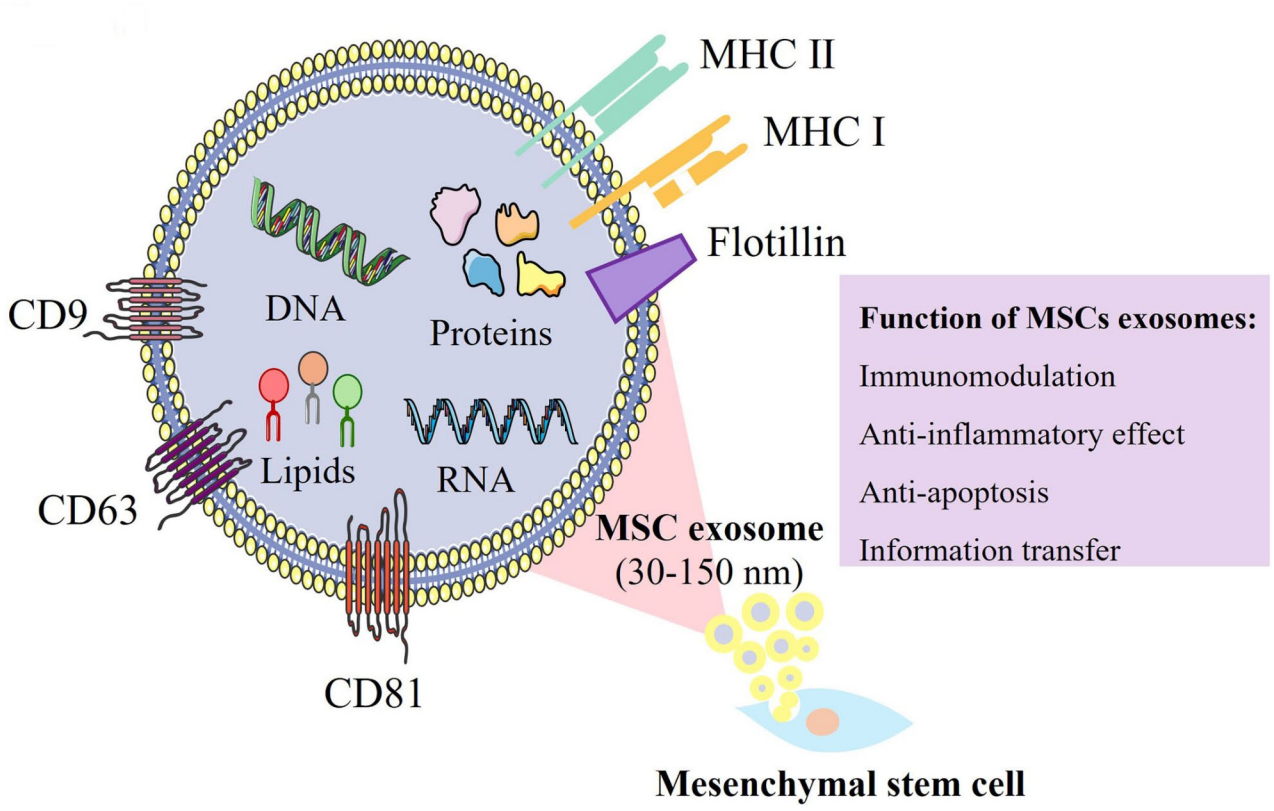

Fig. 3 Composition and function of mesenchymal stem cells (MSCs) derived exosomes. MSCs derived exosomes are small lipid bilayer vesicles with the diameter of 30–150 nm, which can contain proteins, lipids and nucleic acids and can be used as carriers for material exchange and information transfer between cells. They also have the same function as MSCs, including immunomodulation, anti-inflammatory and anti-apoptotic effects

retain their superior biological characteristics. In addition, it is also necessary to distinguish between CPAs used in experimental research and clinical application, because CPAs used in the former also need to be paired with serum, which is from animal and easy to produce non-human virus contamination risk, such as prions [97]. However, most clinical ready-to-use MSCs preparations contain DMSO, which has certain vascular and liver toxicity, so further optimization of CPAs is also required. Therefore, select appropriate CPAs to protect MSCs from these problems, and the safety and efficacy of MSCs in clinical applications can only be guaranteed in this way.

Furthermore, the identification of markers and biological traits of MSCs remains a challenging task [98]. Depending on the tissue source, MSCs have diverse phenotypes, differentiation potential, and expansion potential [99]. The International Society for Cellular Therapy's Mesenchymal and Tissue Stem Cell Committee proposed minimal criteria in 2006 to define human MSCs. These criteria included adherence to plastic under standard culture conditions; expression of CD105, CD73, and CD90 but not CD45, CD34, CD14 or CD11b, CD79a or CD19, or HLA-DR surface molecules; and the ability to differentiate into osteoblasts, adipocytes, or chondroblasts in vitro [6]. This offered an unambiguous standard for distinguishing hMSCs from various sources. Subsequently, in 2013, the committee revised these criteria to incorporate the identification of MSCs' immunological [100]. In 2015, the committee decided that three types of analysis—quantitative RNA analysis of specific gene products, flow cytometry analysis of functionally significant surface indicators, and protein-based assay of the secretome—would help them make the best matrix assay approach [101]. As these criteria continue to evolve and expand in number over time, the process of identifying MSCs is expected to become more straightforward and transparent.

However, there is no standard procedure for evaluating the effectiveness of MSCs upon thawing, leading to varying techniques being employed across different laboratories for this purpose [102]. Modifications to the whole cryopreservation procedure, including the CPAs, the length of storage, and the cell concentration, are under investigation [103]. While numerous research has shown that MSCs' cell shape [57, 58], marker expression [57, 58], proliferative potential [59], and ability to differentiate into three different types of cells [79, 80] remain stable following cryopreservation. However, variations have been observed in terms of immunomodulatory capacity [72, 73], cell viability [54, 55], and genetic stability

[12] of cryopreserved MSCs. And some researches have indicated that cryopreservation can lead to delay cell death, resulting in loss of viability and function, without immediate manifestation of cellular damage symptoms [104]. Further research is warranted to investigate the implications of injecting MSCs into patients immediately after thawing, as apoptotic cell injection may potentially impede the clinical therapeutic efficacy of MSCs [105]. Additionally, it is crucial for cryopreserved MSCs to endure and thrive in injured tissues, where they will be exposed to challenging conditions such as hypoxia, nutritional stress, and host immune responses [106]. These factors can significantly impact the survival and implantation performance of transplanted MSCs [107]. It is anticipated that advancements in technology will contribute to the development of improved strategies for preparing clinical-grade MSCs with enhanced biosafe, survival and regenerative potential.

What's more, MSCs help create an anti-inflammatory environment by suppressing pro-inflammatory cells and fostering anti-inflammatory cells at the site of injury [108]. Interactions between cells and intercellular communication via certain cellular secretions may enable the regulation of the immune system [109]. MSCs secrete growth factors and cytokines enclosed within extracellular vesicles, like exosomes [110], which are tiny lipid bilayer vesicles with a diameter of around 30–150 nm. These exosomes can encircle proteins, lipids, and nucleic acids, serving as carriers for material exchange and information transmission between cells [110]. MSCs release exosomes (MSCs-Exo) that exhibit immunomodulatory effects and influence immune cell activity in various ways [111, 112] (Fig. 3). For example, MSCs-Exo have been shown to attenuate immune and inflammatory responses while inhibiting the activation of different immune cells, including T, B, and NK cells [113]. Despite its diverse applications, few studies have explored the multiple roles of MSCS-Exo in MSCs during cryopreservation. Future research is expected to focus on further understanding the immunomodulatory properties of MSCS-EXO in MSCs cryopreservation along with developing analytical techniques for assessing these properties.

Conclusion

It is important to develop a well-thought-out cryopreservation plan that can protect MSCs' functional properties, like their ability to differentiate and survive, thereby enabling their effective utilization in clinical settings [114]. Since MSCs with complete biological functions are essential for effective clinical practice, particularly in the treatment of conditions like diabetes, it is critical to optimize cryopreservation procedures. This includes careful selection of CPA types and concentrations, to reduce the potential adverse effects on MSCs. Tailoring CPAs and concentrations to specific types of MSCs is also important. While some clinical studies have reported promising outcomes using freezing MSCs with DMSO-free cryopreservation solutions, further comprehensive exploration into their safety and efficacy will be necessary in future investigations. Additionally, prior to clinical application, a thorough assessment of the biological properties of cryopreserved MSCs is essential to ensure their safe and effective use. Safety remains the foremost priority in clinical application. If MSCs show rejection, toxicity, and other phenomena, they should be stopped immediately, or the most serious even lead to the death of the patient. Therefore, MSCs must be strictly tested and the implant must be carefully observed during the implantation process to ensure favorable responses. Apart from that, regular follow-ups and medical examinations are a necessity for MSC transplant recipients, with prompt hospital visits advised if any discomfort arises. In the future, it is possible to create a high-throughput, completely automated, and sterile system for MSC cryopreservation tailored specifically for clinical settings. It will significantly promote the utilization of cryopreserved MSCs in the clinical phase and enable clinicians to more efficiently prepare MSCs for their patients. We must overcome the challenges outlined in the review to establish a standardized process for the cryopreservation of human MSCs for clinical applications.

Abbreviations

MSCs	Mesenchymal stem cells
hMSCs	human mesenchymal stem cells
CPAs	Cryoprotective agents
DMSO	Dimethyl sulfoxide
NPAs	Non-permeating agents
EG	Ethylene glycol
PG	Propylene glycol
FBS	Fetal bovine serum
hESCs	human embryonic stem cells
hUCMSCs	human umbilical cord MSCs
hBMSCs	human bone marrow MSCs
annexin V-PI	annexin V-propidium iodide
Tre	Trehalose
Gly	Glycerol
hADSCs	human adipose-derived MSCs
PDT	the population doubling time
IL-10	Interleukin-10
IL-4	Interleukin-4
PBMCs	Peripheral blood mononuclear cells
MSCs-Exo	MSCs release exosomes

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