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Review

Mesenchymal stromal cells derived from various tissues: Biological, clinical and cryopreservation aspects

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ABSTRACT

Originally isolated from bone marrow, mesenchymal stromal cells (MSCs) have since been obtained from various fetal and post-natal tissues and are the focus of an increasing number of clinical trials. Because of their tremendous potential for cellular therapy, regenerative medicine and tissue engineering, it is desirable to cryopreserve and bank MSCs to increase their access and availability. A remarkable amount of research and resources have been expended towards optimizing the protocols, freezing media composition, cooling devices and storage containers, as well as developing good manufacturing practices in order to ensure that MSCs retain their therapeutic characteristics following cryopreservation and that they are safe for clinical use. Here, we first present an overview of the identification of MSCs, their tissue sources and the properties that render them suitable as a cellular therapeutic. Next, we discuss the responses of cells during freezing and focus on the traditional and novel approaches used to cryopreserve MSCs. We conclude that viable MSCs from diverse tissues can be recovered after cryopreservation using a variety of freezing protocols, cryoprotectants, storage periods and temperatures. However, alterations in certain functions of MSCs following cryopreservation warrant future investigations on the recovery of cells post-thaw followed by expansion of functional cells in order to achieve their full therapeutic potential.

1. Introduction

Mesenchymal stromal cells (MSCs) have emerged as a prominent candidate for cell-based therapies, tissue repair and immune modulation. Intensive research has been directed at elucidating their characteristics and the mechanisms by which they elicit their therapeutic effects. Translating basic research to clinical applications for a wide range of degenerative diseases and autoimmune disorders requires a steady supply of viable and functional cells. Following the example of hematopoietic stem/progenitor cells, which have been previously cryopreserved and successfully transplanted at a later time, MSCs can likewise be stored and thawed as needed. The challenge becomes the optimization of freezing protocols to ensure that MSCs retain the characteristics of their freshly isolated counterparts.

In this review we begin with a brief historical background on the identification and characterization of MSCs and the tissues, apart from bone marrow, from which they have been derived. The rationale for cryopreservation of MSCs for cellular therapy and tissue engineering applications is then discussed. We describe the cryobiological responses of cells and tissues, including the two-factor hypothesis of cryoinjury and the roles of cooling rates and cryoprotective agents in mitigating freezing-induced damage. We focus on the traditional and novel approaches that have been employed to cryopreserve MSCs and the current status of MSC manufacturing, banking and clinical transplantation. We recognize that variability in outcome of MSC-based clinical trials is likely not due to differences in the manner by which MSCs from various sources are cryopreserved, but rather to alterations in some of the crucial functional characteristics of MSCs following their cryopreservation.

2. Mesenchymal stromal cells (MSCs)

2.1. Identification and characterization

Nearly half a century ago, Friedenstein and colleagues observed spindle-shaped cells with dense cytoplasm and large nuclei in

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cultures of murine bone marrow, which, after a few days enlarged into polygonal cells with characteristic features of osteoblasts or bone-forming cells [69]. Thus, it became apparent that aside from the blood-forming (hematopoietic) stem cells, non-hematopoietic cells exist in the bone marrow, which are capable of generating colony-forming unit-fibroblasts having osteogenic potential. In 1991, Caplan introduced the term "mesenchymal stem cells" (MSCs) to designate cells in the "mesoderm" (middle germ layer) from which bone, cartilage, tendon, ligament, fat, skin, muscle, and marrow stroma are derived [30]. This terminology has been criticized and deemed technically inappropriate because the original concept of MSCs specifically referred to cells in the bone marrow having limited self-renewal and differentiation capacity, whereas the current notion has been extended to include cells from almost every post-natal tissue to which stem cell characteristics have been ascribed [22]. However, in consideration of the widespread usage of the acronym MSCs, throughout this review we will use the same abbreviation but refer to these cells as "mesenchymal stromal cells." Moreover, in recognition of the fact that the therapeutic potential of MSCs rely on tissue-specific characteristics of these cells we will always indicate the tissue from which they were derived.

From their initial isolation until now, it has been widely accepted that primary MSC cultures are a heterogeneous population of cells with varying capacities of self-renewal and differentiation [91,131,162]. Due to a lack of unique markers, MSCs have been minimally defined using criteria based on their propensity to adhere to the plastic surface of culture vessels, expression of CD73, CD90 and CD105, absence of CD34, CD45, CD14 and HLA-DR, and by their tri-lineage differentiation into adipocytes, chondrocytes and osteocytes under inductive culture conditions [51]. These properties persist during the cultivation of MSCs independent of the degree of cell confluence during expansion [83]. In view of the functional potency of MSCs as immune modulators, a standardized assessment of their immunological properties has also been proposed [115]. Despite these attempts to establish the identity of MSCs, confounding issues remain such as the similarities that MSCs share with stromal fibroblasts [87], and with pericytes that closely enfold endothelial cells in capillaries and microvessels [131]. Controversies aside, the versatility of MSCs has generated remarkable interest for applications in cellular therapy, regenerative medicine and tissue engineering. In fact, to date over 500 clinical trials are testing MSCs as therapy for a diverse range of diseases (Fig. 1), although less than 2% of these studies have published results (http://www.clinicaltrials.gov). Interestingly, the most promising applications are those of bone marrow-derived MSCs for disorders of the bone and cartilage [156]. It is now increasingly recognized that tissue regeneration induced by MSCs may be contingent upon the tissue from which they were derived.

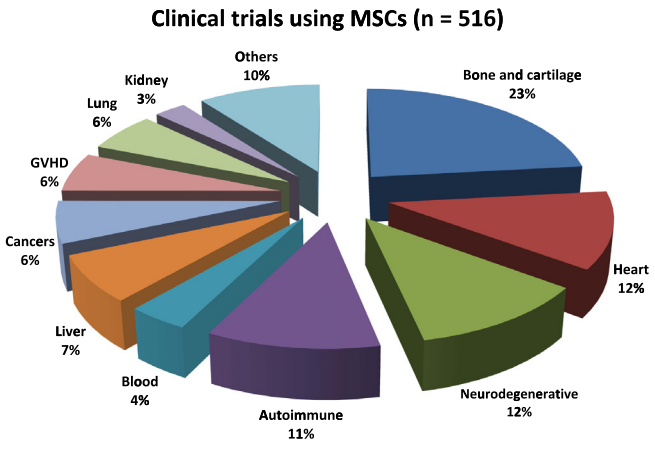

Fig. 1. MSCs are increasingly being tested as therapy for a diverse range of diseases. This graph summarizes the number of clinical trials as reported on the website http://www.clinicaltrials.gov (Accessed 2015 Jan 28). GVHD (graft-versus-host disease).

2.2. Tissue sources

Bone marrow has been for many years the main source of MSCs, but its collection from patients and donors is an invasive and painful procedure. Moreover, there is only a very low frequency (0.001–0.01%) of MSCs in bone marrow and these numbers decline with age [31,123]. MSCs were shown to be present in essentially all adult murine organs and tissues [189]. A rational explanation proposed for this observation is that any tissue that is able to repair itself must harbor a reserve of progenitor cells that can contribute to the renewal and replenishment of cells in that tissue [151]. There is also emerging evidence for the perivascular origin of MSCs, as they have been shown to assemble around blood vessels [36,40,42]; thus, any tissue or organ where blood flows will naturally have MSCs closely associated with or adhering to vascular walls. MSCs have been isolated from multiple adult human tissues such as adipose tissue [81,220], articular cartilage [4,90], brain [8,157], dental tissues [47,80,154,158,207], endometrium and menstrual blood [192,199] and skin [172]. In addition, perinatal organs and tissues that are generally discarded after delivery, namely amniotic fluid [6,197], amniotic membrane [111], placenta [5,97], Wharton's jelly [15,110], umbilical cord tissue [180,215] and cord blood [23,59,132] have been shown to be rich sources of proliferative MSCs. Among these, cord blood has been cryopreserved and banked as a source of transplantable hematopoietic stem/progenitor cells for patients with hematological disorders [13]; however, the existence of non-hematopoietic MSCs in cord blood could shift the paradigm and expand its utilization in cellular therapy.

2.3. Properties of MSCs useful for cellular therapy

2.3.1. Proliferation capacity

The precursor cells for osteogenic tissue that Friedenstein's group described in their seminal papers [69,70] were characterized by high mitotic activity. The ability of MSCs to proliferate *in vitro* is traditionally evaluated using a colony forming unit-fibroblast assay or by measuring the population doubling time. MSCs exhibit a typical growth curve consisting of an initial lag phase followed by a logarithmic phase and lastly a senescent phase. Under optimal conditions, during the log phase of growth (around passages 2–4), MSCs can easily be expanded upwards of 50 population doublings making it possible to attain therapeutic doses. For example, for clinical application in graft-versus-host disease at least 1–2 $\times$ 10^6 MSCs per kg of patient body weight is generally administered by systemic infusion twice per week over the course of 2 weeks [107,171].

2.3.2. Differentiation potential

Aside from bone, MSCs derived from bone marrow can differentiate into other mesodermal lineages such as cartilage, adipocytes and connective stromal cells when cultured under appropriate induction media formulations [163]. Similar to the hierarchical differentiation of hematopoietic cells, bone marrow-derived MSCs undergo a mesengenic process as they differentiate to these lineages and to tendon, ligament and muscle [31]. For example, bone marrow and adipose tissue-derived MSCs have been primed to differentiate to cardiomyocytes, and this finding has been translated into clinical trials for cardiovascular repair [160,167]. However, the underlying mechanism appears to be the secretion of biomolecules that can stimulate tissue regeneration by resident

cardiogenic cells (see next section). It has also been suggested that MSCs might be capable of transcending germ layer boundaries and differentiating into ectodermal lineage cells such as neurons [217], and into endodermal lineage cells such as hepatocytes [100]; however, there is accumulating evidence against this idea of trans-differentiation [21]. In fact, recent evidence suggests that MSCs are unlikely to induce tissue regenerative processes by *in situ* differentiation or by directly replacing damaged cells [16]. Although not endowed with their previously touted pluripotency, MSCs are nevertheless recognized to exert therapeutic effects through other mechanisms.

2.3.3. Release of bioactive factors

MSCs secrete a wide array of growth factors and cytokines which can exert autocrine effects (in which the secreted molecules interact with receptors on the same cell) or paracrine effects (in which the secreted molecules interact with other cells). These interactions can promote the formation of new blood vessels and re-establish blood supply (angiogenic), prevent programmed cell death (anti-apoptotic), stimulate progenitors to divide (mitotic), prevent scarring (anti-fibrotic), recruit resident progenitor cells (chemotactic) or provide the microenvironment favoring the differentiation of resident stem cells (cooperative) [32,190]. In addition, MSCs possess broad immunomodulatory properties, by which they can sense and control inflammation [20].

2.3.4. Immunoregulatory properties

At the onset of inflammation, endogenous MSCs are activated to secrete soluble factors that inhibit the proliferation of T cells and mount an anti-inflammatory response [58,166]. In contrast to this immunosuppressive response, allogeneic MSCs may elicit an innate immune response by releasing chemokines that recruit macrophages, neutrophils and lymphocytes to sites of inflammation [7,121,188]. MSCs have been shown to affect other cells of the immune system including B cells, NK cells, dendritic cells and macrophages [190]. In order to exert their effects, MSCs need to migrate to inflammatory sites.

2.3.5. Migratory properties

MSCs communicate with other cells in the human body and appear to "home" to areas of injury in response to signals of cellular or tissue damage [48,105]. Whereas tracking studies have shown that a majority of MSCs get trapped in the lungs following intravenous infusion, they tend to disappear from the lungs within hours and migrate to other tissues such as the liver, spleen and kidney, and preferentially to sites of injury [9,37,120]. Despite their limited lifespan, it is believed that MSCs can exert their therapeutic effects via other cell types [55]. The mode of recruitment of MSCs is by chemotaxis, a directional migratory response to a gradient of soluble chemoattractants. We and others have shown that stromal cell-derived factor (SDF)-1, a chemokine that is up-regulated in sites of injury, is critical for the chemotaxis of MSCs through its interaction with its receptor CXCR4 [126,191]. Moreover, we also found that the expression of CXCR4 in human cord-blood derived MSCs can be enhanced in order to improve their efficacy for tissue repair [133–135,168].

2.4. Clinical and tissue engineering applications of MSCs

Applications of MSCs for cellular therapy and regenerative medicine have been the subject of numerous reviews [46,65,139,171,186,196,198]. Their availability, ease of expansion, and amenability to genetic or tissue engineering manipulations make MSCs particularly attractive for cellular therapy applications. As discussed above, the therapeutic efficacy of MSCs appears to be derived from their ability to secrete a broad range of bioactive molecules that are immune-modulatory, anti-apoptotic, anti-fibrotic, anti-inflammatory, pro-angiogenic, chemotactic or stimulatory of tissue regeneration in response to injury [26,52,118,170,190]. It appears that MSCs are able to detect injury and inflammation and concomitantly activate multiple restorative pathways in response to local stimuli from the injured tissue by releasing growth factors, chemokines, enzymes, *etc.* that induce the resident progenitor cells in the tissue to repair itself.

The secretory nature of MSCs can further be exploited by genetically manipulating MSCs to express missing or defective proteins in patients with congenital or acquired deficiency disorders. For example, we have modified cord blood-derived MSCs using lentiviral transduction to express coagulation Factor IX for potential transplants in hemophilia B patients [49]. Furthermore we have encapsulated Factor IX-engineered MSCs in fibrinogen–alginate microcapsules and shown them to have enhanced cell viability and Factor IX secretion [182]. The use of MSCs as vehicles for delivering proteins via exosomes for therapeutic purposes such as glial cell-derived neurotrophic factor for Parkinson's disease, nerve growth factor for Alzheimer's disease and brain-derived neurotrophic factor for Huntington's disease have also been explored [211]. In the setting of allogeneic hematopoietic stem cell transplantation, MSCs have been used to promote engraftment and for immunosuppression in graft-versus-host disease (GVHD) [25,94,196,214]. In fact, the first commercially available MSC therapeutic approved by Health Canada, Prochymal (Osiris Therapeutics, Inc., Columbia, MD, recently acquired by Mesoblast, Melbourne, Australia), is indicated for the management of GVHD.

Prochymal has demonstrated immunomodulatory properties to regulate T-cell-mediated inflammatory responses by inhibiting T-cell proliferation and down-regulating the production of the pro-inflammatory cytokines such as tumor necrosis factor-alpha and interferon gamma. It is marketed as a cell suspension of *ex vivo* expanded adult human bone marrow-derived MSCs, supplied as a 15-mL formulation with approximately 100×10^6 viable cells in Plasma-Lyte A containing 5% human serum albumin and 10% dimethyl sulfoxide (Me_2SO). The doses are packaged in Cryocyte freezing containers and frozen for storage (24-month shelf-life) at liquid nitrogen (LN_2) vapor phase temperature ($\leqslant -135$ °C) until thawed and reconstituted before administration. The reconstituted product can be kept at room temperature and used within 5 h post-thaw. Prochymal is an example of how MSCs may be supplied as an off-the-shelf product by employing cryopreservation techniques. In a recent review of 49 clinical trials testing MSCs for various disorders, 35% employed MSCs that have been cryopreserved [98]. However, as we will discuss later, mixed outcomes, negative findings, or results showing only minimal or transient improvements in patients indicate that the post-thaw functionality of cryopreserved MSCs may be a factor contributing to their efficacy.

2.5. Rationale for cryopreservation of MSCs

The cryopreservation of primary cells has been extensively used in hematopoietic stem cell transplantation. In autologous patients, the cells are collected and cryopreserved for later clinical use. In allogeneic patients, cryopreservation permits the banking of cells for human leukocyte antigen typing and matching, facilitates the logistical transport of cellular products to transplant centers, and allows sufficient time for the screening of transmissible diseases in the donated cells before transplantation. Cryopreservation of MSCs can likewise reduce the constant need for fresh tissues, enable quality control and standardization of the same cell preparation at different times when the cellular product is needed, and provide a source of reference MSCs that can be used to validate

assays [202]. A bank of regulatory-tested cells would make available a ready off-the-shelf supply of cells for transplant, as well as allow better timing of therapy. It is essential to cryopreserve MSCs at an early passage in order to maintain a reservoir of healthy and efficacious cells for transplant, as the number of MSCs and their differentiation potential decrease with passage [203]. In fact, patients treated with MSCs at early passage had improved clinical outcome over those given late-passage cells [147]. Moreover, some MSCs, such as those derived from human amniotic membrane, cannot survive for long periods of culture [148]. In addition, MSCs under long-term *ex vivo* culture conditions could be prone to genotypic drift, chromosomal aberrations, phenotypic instability and contamination [18,200]. Cryopreservation could circumvent these problems and also save time and culture media. There have also been studies demonstrating that fetal MSCs harvested in the prenatal period can be cryopreserved during the remainder of the gestation period to be used for the repair of congenital disorders soon after birth or in utero [116,119]. Clearly, the fields of tissue engineering, gene therapy, regenerative medicine and cell transplantation rely to a great degree on the ability to preserve, store and transport these cells. In addition, a master cell bank of reference MSCs from various tissue sources could be important to advance research and clinical translation [202]. Ironically, the same cryopreservation process aimed at preserving living cells could also cause damage and compromise their survival. An understanding of the responses of cells and tissues to the physical changes that occur during the freezing process is essential in the design of protocols that will minimize cryoinjury and ensure maximum recovery of viable and functional cells.

3. Cryobiological responses of cells

3.1. Role of cooling rates

The purpose of cryopreservation is to maintain life by slowing down metabolic activity of cells at temperatures as low as that of liquid nitrogen (−196 °C). Several mechanisms have been proposed to explain cell behavior during cryopreservation. In the course of freezing cells in suspension, water crystallizes in pure form in the extracellular space, resulting in the concentration of dissolved solutes in the remaining liquid. A concentration gradient is created across the cell membrane and, if cooling takes place slowly enough, water moves out of the cells in response to the resultant osmotic stress. As cooling continues and more ice is formed, salts concentrate to high levels as the cells become dehydrated and shrink. Damage caused by extended exposure to increased solute concentration at intermediate temperatures is referred to as slow-cooling injury. On the other hand, if the cooling rate is too fast for the cells to maintain equilibrium by loss of intracellular water, the unfrozen solution within the cells becomes increasingly super-cooled resulting in intracellular ice formation. Damage caused by intracellular ice is referred to as rapid-cooling injury. This two-factor hypothesis of freezing injury (*i.e.*, solute effects and intracellular ice formation) as proposed by Mazur [140] has been elaborated upon by others [66,75,144,150,175,176]. Successful cryopreservation of cells in suspension requires sufficiently high cooling rates to reach low temperatures quickly, minimize solution effects and avoid slow-cooling injury, but low enough cooling rates to decrease the formation of intracellular ice and avoid rapid-cooling injury. A cell will proceed towards osmotic equilibrium by allowing water to get in or out until there is no osmotic gradient across the plasma membrane. However, these osmotic volume excursions can exceed the tolerance limits of cells, as shown in cord blood-derived $CD34^+$ cells [96], adding a third prominent injury mechanism to the slow-cooling and rapid-cooling injuries described above.

It is important to consider that different cell types have different membrane permeability parameters and therefore, it is logical that responses of cells to cooling rates are also cell-type specific. For example, optimal cooling rates range from 1 °C/min for mouse marrow stem cells to over 1000 °C/min for human red blood cells [141]. Also, confluent monolayers that have intercellular connections called gap junctions form more intracellular ice at a given temperature compared to cells that do not have gap junctions [1], and it has recently been recognized that the influence of cell junctions on intracellular ice is extremely complex [56,89]. Moreover, it was shown for dental pulp-derived MSCs that intracellular ice formation does not cause cell membrane rupture but rather protects the cells from dehydration during freezing [219]. In another study comparing bone marrow-derived MSCs from various species, the highest post-thaw cell viability was obtained in mouse MSCs (91.5 ± 5.6%) while the lowest was in human MSCs (82.9 ± 4.3%) [128]. These results highlight the importance of cellular characteristics in determining response to freezing. Because mouse MSCs are smaller and have higher surface-to-volume ratio than human MSCs (0.65 μm^{-1} *vs.* 0.34 μm^{-1}) mass transfer occurs faster in mouse MSCs which influences their survival rate [128].

In addition to controlling the cooling rates, cryoprotectants have been added to cell suspensions to minimize the damaging effects of freezing.

3.2. Role of cryoprotectants

Cryoprotectants are classified as either permeating or non-permeating depending on their ability to traverse the cell membrane [143]. Permeating cryoprotectants, such as Me_2SO, glycerol, ethylene glycol, propylene glycol (1,2-propanediol), methanol, ethanol, propanol and formamide, protect against slow cooling injury by reducing extracellular ice formation, preventing excessive concentration of solutes and minimizing cell dehydration to a tolerable degree [129]. Their protective property is shared by many low molecular weight compounds having a high solubility in aqueous solutions and which, at high multi-molar concentrations, are able to depress the freezing point of water, thereby reducing the amount of ice formed at any temperature during cooling [142,145]. The rate at which a given one of these compounds penetrates the cells varies for different cell types and cell sources [143]. Non-permeating cryoprotectants can protect cells at lower molar concentrations, but they generally require more rapid rates of freezing to confer protection [143]. Examples include polyvinylpyrrolidone, sugars such as trehalose, sucrose, lactose and glucose, sugar alcohols such as mannitol and sorbitol, and the polymer hydroxyethyl starch (HES). Many mechanisms of action of non-permeating cryoprotectants have been proposed [29,142,143]. For example, because HES does not penetrate the cells, its extracellular concentration increases, creating an osmotic stress on cells which results in a loss of intracellular water at high subzero temperatures. In other words, non-permeating cryoprotectants remove water from the cells primarily during the initial phases of freezing when they are concentrated in the extracellular regions [142]. The cryoprotective effects of trehalose have been attributed to its interactions with lipid membranes, stabilization of proteins during freezing–thawing processes, and its ability to form a glassy matrix that can contribute to the inhibition of potentially lethal intracellular ice [29].

Although intended to confer protection as their name implies, cryoprotectants can also cause injury to cells and several investigations have focused on determining, minimizing or eliminating their toxicity [3,57,60,62–64,103].

4. Approaches to cryopreservation of MSCs

4.1. Conventional methods of MSC cryopreservation

MSCs for clinical use are most commonly frozen in 5% or 10% Me_2SO in an electrolyte solution (*e.g.* Plasmalyte) with an added protein (*e.g.* human serum albumin). The freezing rate is typically 1 °C/min to 5 °C/min until roughly −100 °C at which point the cells are placed in liquid nitrogen or its vapor-phase [84]. However, this procedure is based on the cryopreservation protocols developed for hematopoietic stem cells and lymphoid cells and is not optimized for MSCs [186]. It is important to consider that the parameters that are crucial in mitigating cell damage during cryopreservation are not only dependent on whether the cells are in suspension or in an adherent monolayer, but also on the cell source. For example, it was shown that when suspended and adherent human bone marrow-derived MSCs were cooled at 1°/min to −80 °C and stored in liquid nitrogen, the post-thaw viability was 30–35% lower in the adherent cells [212,213]. In addition, post-thaw viabilities of cells isolated from different tissue sources differed significantly, with adipose tissue being a more robust stem cell source than dental pulp and bone marrow [44]. Therefore, an understanding of the physical and biological processes that take place when cells are frozen and thawed is essential in developing protocols to ensure that MSCs maintain their structural integrity and retain their functional properties.

4.2. Effects of cooling rates, cryoprotectants, storage period and temperature on the characteristics and function of cryopreserved MSCs

Several studies have shown that MSCs from diverse sources can be cryopreserved in different ways using different cryoprotectants and employing various cooling rates, storage periods, and temperatures without compromising their proliferation potential, phenotypic characteristics and capacity for differentiation (Table 1). These investigations were focused on optimizing cell survival and function and ensuring that cryopreserved MSCs display the same characteristics as freshly isolated cells.

As early as 1997 it was shown that human bone marrow-derived MSCs that were cryopreserved in 10% Me_2SO (with an unspecified cooling profile) and stored in liquid nitrogen for 24 h can be sub-cultured for up to 15 passages and still retain their osteogenic potential [28]. Later, it was likewise demonstrated that osteoblast progenitor cells derived from human bone marrow aspirates – cryopreserved by cooling 1 ml cell suspension in 10% Me_2SO at 1 °C/min to −70 °C and after 24 h, storing in liquid nitrogen for 7 days – exhibit no significant difference in their proliferation rate and osteogenic potential compared to cells from fresh bone marrow [179].

Because of loss in replicative ability with extensive passaging, many subsequent investigations focused on cryopreservation of MSCs at an early passage. For instance, human bone marrow mononuclear cells were either plated immediately (MSC-Passage 1) or frozen in 1 mL cryovials in the presence of 5% Me_2SO and 10% fetal bovine serum (FBS) using an unspecified cooling protocol and stored for a week in liquid nitrogen and then thawed, cultured (F-MSC-P1) and analyzed [82]. The MSC-P1 was either analyzed directly or frozen for a week, then thawed, cultured (F-MSC-Passage 2) and analyzed. All batches of cells were shown to have similar morphology, proliferation potential and surface marker expression [82]. In another study, rabbit synovium-derived MSCs were expanded up to passage 5 and cryopreserved in 1 mL of commercial freezing media (CellBanker, Fukushima, Japan). Cryovials were placed in a cryofreezing container and then stored at −80 °C for 1 week. The cryopreserved MSCs showed no significant difference in viability (as measured by the spectrophotometric MTT assay), proliferation rate and expression of chondrogenic genes compared to fresh MSCs [153].

To investigate the effect of cryoprotectants on the post-thaw properties of MSCs different combinations of Me_2SO (1%, 4%, 8% and 10%) and trehalose (9%, 6% and 2%) in 90% FBS were tested on human adipose tissue-derived MSCs [173]. After adding 1 mL of freezing solution in 1.8 mL cryovials, the cells underwent uncontrolled cooling by placing directly at −20 °C for 30 min, then placing at −80 °C for 1 h, and were finally transferred into liquid nitrogen (−196 °C) for long-term storage (1, 6 and 12 months). The best freezing solution, composed of 4% Me_2SO and 6% trehalose, resulted in cells with more than 80% post-thaw viability as measured by flow cytometry using an annexin/propidium iodide assay kit. Furthermore, proliferation of cryopreserved MSCs was comparable to that of fresh cells; no apoptotic death was observed, and cells retained their differentiation multipotency. Finally, in the same study, the adherent cell population after long-term cryopreservation gave rise to MSCs with morphology, immunophenotype, proliferation, and differentiation potential similar to those of fresh cells [173].

Similarly, in an attempt to optimize the cryopreservation of dental pulp-derived MSCs, the cells were frozen in various concentrations (0.5, 1.0 and 1.5 M) of ethylene glycol, propylene glycol or Me_2SO using a cooling rate of 1 °C/min to −85 °C followed after 24 h by immersion in liquid nitrogen [207]. The viability (as measured by a trypan blue exclusion assay) of cells frozen in 1.0 M and 1.5 M Me_2SO were significantly better (about 91 ± 9%) than the viability of cells frozen in the other cryoprotectants. The use of 1.3 M Me_2SO (about 10% w/v) worked as well as the commercially available cryoprotectant containing 10% Me_2SO in a serum-free defined medium (Cryostor CS-10, BioLife Solutions Inc., Bothell, WA). Furthermore, MSCs did not show statistically significant differences in their doubling times during their log-phase of growth, or in their expression of surface markers whether they were stored at −85 °C (mechanical freezer) or at −196 °C (liquid nitrogen Dewar) for up to 6 months [207].

To further compare the effect of cryoprotectant, either 10% Me_2SO, 10% glycerol or 10% ethylene glycol was added to 90% FBS to cryopreserve human dental root-derived MSCs [47]. Cells at a concentration of 1×10^6/mL were slowly cooled by placing cryovials directly at 4 °C for 1 h, followed by uncontrolled cooling at −20 °C for 2 h, then at −80 °C overnight and finally at −196 °C for 6 months. There were no significant differences observed between fresh and cryopreserved MSCs in terms of cell viability (by trypan blue exclusion), colony-forming efficiency, proliferation rate, differentiation, phenotypic and karyotypic profiles and immunological responses [47].

In addition to cryoprotectant effects, various cooling rates and storage periods in liquid nitrogen have also been investigated. Amniotic fluid-derived MSCs were subjected to time-programmed slow cooling (consisting of 1 °C/min to −60 °C, then 3 °C/min to −100 °C followed by liquid nitrogen storage for 3 and 6 months) or a non-programmed protocol (consisting of cooling for 20 min in a −20 °C freezer, followed by 12–16 h in a −80 °C freezer, and storage in liquid nitrogen for 3 or 6 months) [99]. The cryoprotectants used were Me_2SO (5% or 10%), glycerol (5% or 10%), sucrose (30 or 60 mM), or trehalose (60 and 100 mM). Interestingly, the freezing protocols did not cause any significant differences in post-thaw cell viability as measured by trypan blue exclusion. Moreover, all cryoprotectants tested were able to maintain the surface marker expression, gene expression and differentiation of MSCs, although more viable cells were recovered using Me_2SO or glycerol rather than sucrose or trehalose [99].

Using rat bone marrow-derived MSCs, it was shown that the rate of freezing (slow cooling at 0.3 °C/min or fast cooling at 99 °C/min

Table 1
Studies on cryopreservation of MSCs from various tissues using different cryoprotectants, cooling protocols, storage temperatures and periods.

Tissue source	Cryoprotectant used	Cooling protocol	Storage temperature	Storage period	Refs.	Significant findings
Human bone marrow	5% Me_2SO	~1 °C/min in freezing container at −80 °C (24 h) OR controlled freezing (1 °C/min from 4 °C to 0 °C; 2 °C/min to −45 °C; 5 °C/min to −100 °C)	−196 °C (liquid nitrogen)	Indefinite	[84]	MSCs can be successfully cryopreserved in vials or freezing bags using uncontrolled or controlled rate freezing, respectively
	10% Me_2SO	1 °C/min in controlled rate freezer from 4 °C to at −80 °C	−196 °C (liquid nitrogen)	Not specified	[213]	There is a 35% decrease in viability of adherent cells in comparison to cells in suspension after cryopreservation
	10% Me_2SO	Controlled rate freezing from 4 °C to −80 °C at cooling rates of 1 °C/min OR 5 °C/min OR 10 °C/min	−196 °C (liquid nitrogen) OR −80 °C	Not specified	[212]	Adherent cells more susceptible to injury than cells in suspension; 1 °C/min better in preserving cell actin and mitochondria
	10% Me_2SO	Unspecified	−196 °C (liquid nitrogen)	24 h	[28]	Cryopreservation and thawing did not affect cell growth and osteogenic potential
	10% Me_2SO	~1 °C/min in −70 °C freezer (24 h)	−196 °C (liquid nitrogen)	7 days	[179]	Cryopreservation does not affect osteoblastic potential or cell viability
	CellBanker (commercial Me_2SO-based)	4 °C for 10 min; −30 °C for 1 h; −80 °C for 2–3 h	−152 °C	0.3–37 months	[114]	No difference in viability (~90%) with storage time
Monkey marrow	CellBanker (commercial Me_2SO-based)	Directly at −80 °C for 24 h	−150 °C	10 days	[195]	Bone formation potential retained after cryopreservation
Dog bone marrow	10% Me_2SO	4 °C for 1 h; −20 °C for 2 h; −80 °C for 10.5 h	−196 °C (liquid nitrogen)	3 years	[218]	Proliferation and *in vivo* regenerative ability are maintained after cryopreservation
Rat bone marrow	0–10% Me_2SO + 10–0% HES	One (0.3 °C/min to −100 °C) to 4-step slow cooling OR straight-freeze	−134 °C (vapor phase of liquid nitrogen)	>24 h	[152]	No difference in survival rates of MSCs among 7 cooling protocols tested; 5% Me_2SO/5% HES is comparable to 10% Me_2SO
Human amniotic fluid	Me_2SO or glycerol (5% or 10%); sucrose (30 or 60 mM); trehalose (60 or 100 mM)	Uncontrolled (−20 °C for 20 min; −80 °C for 12–16 h) OR controlled (1 °C/min to −60 °C; 3 °C/min to −100 °C)	−196 °C (liquid nitrogen)	3 OR 6 months	[99]	Me_2SO and glycerol are better than sugars as cryoprotectants. There is no significant difference in cell viability using either freezing protocol or storage period
Human teeth	10% Me_2SO OR 10% glycerol OR 10% ethylene glycol	4 °C for 1 h; −20 °C for 2 h; −80 °C overnight	−196 °C (liquid nitrogen)	6 months	[47]	No difference in post-thaw cell viability and proliferation using various CPAs
Human dental pulp	0.5, 1, OR 1.5 M ethylene glycol OR propylene glycol OR Me_2SO	~1 °C/min in freezing container at −85 °C for 24 h	−196 °C (liquid nitrogen) OR −85 °C freezer	1 week OR 1 month OR 6 months	[207]	1 M and 1.5 M Me_2SO is slightly better; 1 week storage gave highest recovery; no difference between LN_2 or −85 °C storage
Human adipose tissue	Different combinations of Me_2SO and trehalose OR 10% Me_2SO	−20 °C for 30 min; −80 °C for 1 h	−196 °C (liquid nitrogen)	1 month OR 6 months OR 12 months	[173]	Optimal medium is 4% Me_2SO and 6% trehalose, but viable cells obtained with all combinations. Best recovery after 1–6 months storage but still 70% after 12 months
Pig adipose tissue	10% Me_2SO	Not specified	−196 °C (liquid nitrogen)	3–12 months	[43]	Growth characteristics and karyotype were not affected by cryopreservation
Horse blood	10% Me_2SO	~1 °C/min in freezing container at −80 °C for a week	−196 °C (liquid nitrogen)	10–12 months	[137]	Stem cell characteristics were not affected by cryopreservation
Rabbit synovium	CellBanker (commercial Me_2SO-based)	~1 °C/min in cryofreezing container at −80 °C	−80 °C	1 week	[153]	Cryopreservation did not affect morphology, viability and differentiation

to −100 °C before storage at −134 °C) affected neither phenotypic markers in cryopreserved *vs.* fresh cells nor their ability to proliferate or differentiate into osteocytes [152]. The direct post-thaw viability as assessed by the spectrophotometric metabolic activity tetrazolium MTT assay was about 85% using other protocols which included one to four-step cooling stages or a "straight freeze" approach which involved direct plunge in liquid nitrogen vapor phase (−134 °C) after 10 min equilibration with cryoprotectant at 4 °C. The effect of different cooling rates on viability became evident only after 3 days of culture [152]. This was to be expected as it has been shown that cryopreservation-associated apoptosis and necrosis occur hours to days after thawing as various pathways are activated [17]. It is also important to note that MTT may not give an accurate numerical estimate of cell viability due to the increased metabolic activity per cell after cryopreservation as reported for WST-1 (a related tetrazolium assay) [102].

To test the effect of storage period, horse blood-derived MSCs were cryopreserved in 10% Me_2SO and 90% FBS (cooled at 1 °C/min to −80 °C and after 1 week transferred to liquid nitrogen) and stored for 10–12 months [137]. Post-thaw analyses revealed that MSCs retained their morphology, alkaline phosphatase and telomerase activities, karyotype profile, proliferation rate, expression of surface markers, and adipogenic, osteogenic and myogenic potential. The latter is particularly important in veterinary medicine as horses are susceptible to muscle injuries [137]. Likewise, MSCs from porcine adipose tissue were cryopreserved in 10% Me_2SO using an unspecified cooling protocol and stored in liquid nitrogen for up to 12 months [43]. The morphology, viability (as measured by flow cytometry using annexin/propidium iodide staining), expression of surface markers, cumulative population doublings and senescence rates were maintained after long-term storage. Cryopreservation did not produce any chromosomal aberrations nor did it affect adipogenesis or osteogenesis [43]. The effect of extended storage period was examined in another study where human bone-marrow derived MSCs were frozen in a commercially available Me_2SO-containing freezing solution (CellBanker, Tokyo, Japan) for up to 3 years [114]. First, the cells were allowed to equilibrate at 4 °C for 10 min, then cooled by uncontrolled cooling at −30 °C for 1 h, further cooled by placing at −80 °C for 2–3 days, then stored at −152 °C. The viability of cryopreserved cells determined using an automated nuclei dye exclusion cell counter was approximately 90% regardless of the length of storage (0.3–37 months). Cryopreservation did not affect the expression of surface markers and had no influence on the ability of MSCs to undergo osteoblastic differentiation [114].

Aside from *in vitro* properties after cryopreservation, the retention of *in vivo* function of cryopreserved MSCs has also been investigated. Canine bone marrow-derived MSCs were subjected to a 3-step slow freezing procedure (uncontrolled cooling by placing at 4 °C for 1 h, followed by 2 h at −20 °C, then 10.5 h at −80 °C in the presence of 10% Me_2SO) before plunging into liquid nitrogen [218]. After 3 years, the cells were thawed at 37 °C, assessed for viability by trypan blue staining and alkaline phosphatase activity, and then seeded onto collagen scaffolds. The MSC-scaffold complex was implanted into nude mice and was shown to retain osteogenic differentiation ability [218]. In addition, MSCs from monkey bone marrow that were cryopreserved (in CellBanker freezing medium by placing directly at −80 °C for 24 h and then kept in a −150 °C freezer for 10 days) and used in a quantitative implantation model showed extra-skeletal bone formation similar to fresh MSCs [195].

Human bone marrow-derived MSCs that have been cryopreserved in suspension have been thawed and cultured in 3D scaffolds prior to their use in tissue repair. In one study, the cells were cryopreserved in medium containing 10% Me_2SO and 20% FBS by exposing to 4 °C for 1 h, then placing at −20 °C for 2 h, followed by rapid freezing in liquid nitrogen. After 24 h the cells were quickly thawed at 37 °C and seeded in partially demineralized bone matrix scaffolds. The cryopreserved MSCs exhibited the same proliferation potential, alkaline phosphatase activity, osteocalcin secretion and osteogenic differentiation *in vitro* and *in vivo* as fresh cells [125]. Although the cells were frozen for only a day, the authors expect similar results for cells that are stored for a longer period.

In our lab we have been able to isolate MSCs from umbilical cord blood and have sub-cultured and frozen them at different passages using uncontrolled cooling at −70 °C in the presence of 10% Me_2SO and 10% FBS [168,191]. Cord blood was collected with the mother's informed consent in accordance with the guidelines of the University of Alberta Health Research Ethics Board, and experiments were conducted with their approval. For the purpose of this review and reported in a poster [136], we evaluated MSCs derived from one cord blood sample; from approximately 2×10^6 cells from passage 1 cultures we were able to achieve close to 50 population doublings and to generate several million cells in frozen stocks from passages 2–6. We obtained post-thaw viability by trypan blue exclusion of 80 ± 10% from stocks frozen for 5 years and 89 ± 5% from stocks frozen for 2 years [136]. On sub-culture we found that thawed cells retained their fibroblastic morphology and expression of CD73, CD90 and CD105 as determined by flow cytometry. Moreover, the cells exhibited an ability to differentiate to osteoblasts and chondrocytes *in vitro*, as detected by Alizarin red staining for calcium deposits and Alcian blue staining for proteoglycans, respectively. The expression of markers of pluripotency (Nanog, Oct-4, Rex-1 and Sox-2) and differentiation (nestin, osteocalcin and collagen X) were confirmed by RT-PCR [136]. We have then proceeded to use these cells in our investigations aimed at improving the *in vitro* migratory potential of MSCs [133–135,168] and transducing these cells to produce Factor IX [49,181,182]. Our studies demonstrated the high-efficiency recovery of viable, proliferative and functional cells after cryopreservation which can be employed for research, gene therapy or clinical transplantation.

Taken together, the above studies demonstrate that MSCs from diverse sources, cryopreserved using different cooling rates, in the presence of different cryoprotectants and stored for various lengths of time and at various subzero temperatures retain their biological properties post-thaw (Table 1). Indeed, MSCs have shown robustness and resiliency during cryopreservation unlike other cells which required optimal cooling rates [141] or defined protocols. For example, to achieve maximal recovery of functional mouse oocytes, they had to be cryopreserved in 1.5 M Me_2SO at a cooling rate of 0.5 °C/min and a plunge temperature of −80 °C [106]. In addition, whereas rabbit synovium-derived MSCs maintained their morphology and differentiation potential after storage at −80 °C in a commercial preservation solution, chondrocytes did not [153]. We note that the hematopoietic cell line, TF-1, has also shown robustness to cryopreservation protocols; it has even been demonstrated using interrupted rapid cooling to −40 °C that there is a narrow range of hold temperatures (−5 °C to −12 °C) and times (1–6 min) that confers cryoprotection in the absence of added permeating cryoprotectant [174]. Notwithstanding this robustness of MSCs to cryopreservation protocols, many groups have explored other strategies to improve the cryopreservation of MSCs for the purpose of reducing the toxicity of cryoprotectant, eliminating animal-derived serum in the freezing solution, minimizing freezing-induced cell damage or simulating the 3D configuration *in vivo* by using scaffolds.

4.3. Other strategies for MSC cryopreservation

4.3.1. Reducing Me_2SO

Me_2SO is the most widely used cryoprotectant for MSCs, but it is potentially toxic at molar concentrations and has been shown to

have unfavorable effects in animal models [74]. Adverse reactions including nausea, headache, hypotension, hypertension, diarrhea and abdominal cramps have been reported in patients infused with Me_2SO-containing hematopoietic stem cells [2,177]. Thus, it is desirable to reduce Me_2SO in cellular products for clinical use. It has been shown that a human hematopoietic stem cell line can be cryopreserved in the absence of Me_2SO or any other permeating cryoprotectant [174] with careful choice of cooling profile [175]. To this end, human adipose-derived MSCs were cryopreserved in standard medium (10% Me_2SO + 90% FBS) or in serum-free medium (IBMTSTEM, Fischer Procryotect, Zurich, Switzerland) supplemented with 2%, 5% or 10% Me_2SO [216]. The MSCs were cooled to −80 °C at 1 °C/min using a standard freezing container and after ⩾2 h were plunged into liquid nitrogen where they were kept for at least 24 h. No cells survived following cryopreservation in 0% or 2% Me_2SO. Membrane damage from extra- and intracellular ice formation and the appearance of small holes on the cell surface were detected by multiphoton laser-scanning cryomicroscopy and scanning electron microscopy, respectively. However, a reduction of Me_2SO from 10% to 5% resulted in no significant difference in post-thaw cell count and viability by flow cytometric propidium iodide exclusion, membrane integrity by scanning electron microscopy, and tri-lineage differentiation [216]. In a similar study, the effect of 5% Me_2SO was found to be comparable to 10% Me_2SO after cryopreserving porcine bone marrow-derived MSCs (by cooling at a rate of 1 °C/min from 25 °C to −80 °C and immediately plunging into liquid nitrogen where they were stored for less than a month) in terms of survival as measured by trypan blue exclusion, and expression of apoptosis-related genes and stem cell markers [155].

Furthermore, no significant differences in survival rates of human bone marrow-derived MSCs were detected when cells were cryopreserved (by placing the cell suspension in a Mr. Frosty freezing container at −80 °C for 24 h then transferring to liquid nitrogen vapor) in the presence of 10% Me_2SO or 5% Me_2SO when trehalose is added; however, using only 2.5% Me_2SO resulted in reduced viability and proliferation after 7 days of culture as measured by trypan blue exclusion [27]. Consistent with this result is the finding that 3% Me_2SO has no protective effect on human tooth germ stem cells that were cryopreserved by placing in a 1 °C/min freezing container at −80 °C then transferring to −196 °C the next day [45]. Moreover, Me_2SO concentrations under 4% are associated with reduced viability (as measured using MTT) in rat bone marrow-derived MSCs cryopreserved using various controlled rate freezing protocols [152]. Addition of hydroxyethyl starch (HES), which by itself does not confer protection to MSCs, can help reduce Me_2SO levels as a solution of 8% Me_2SO and 2% HES showed the highest post-thaw viability compared to other combinations tested [152].

Recently, it was shown that the addition of sugars such as lactose, sucrose, trehalose or raffinose (at 300 mM concentration) during the sub-culture and cryopreservation of human dermal MSCs (by cooling at 1 °C/min to −80 °C and plunging in liquid nitrogen) resulted in up to 60% survival even in the complete absence of Me_2SO and FBS [159]. Moreover, no significant differences in morphology or ability to differentiate to osteogenic and adipogenic cells were observed between unfrozen cells and cells cryopreserved in the presence of sugars or 10% Me_2SO and 20% FBS [159].

4.3.2. Animal serum-free formulations

Most cryopreservation protocols for stem cells employ FBS at concentrations ranging from 5% to 90%. Apart from providing nutrients to cells while in culture, serum stabilizes cell membranes, adjusts osmotic pressures and protects cells from free oxygen radicals formed during cryopreservation and long-term storage. However, animal-derived serum is not a desirable component of cellular products for clinical applications because it carries the risks of transmitting viruses, prions or proteins that may elicit immunological responses. Moreover, serum composition varies with source and batch. Thus, for ethical and practical reasons, there have been active investigations into finding well-defined serum alternatives. For example, Lonza (Basel, Switzerland) has developed Chemically Defined Mesenchymal Stem Cell Growth Medium (MSCGM-CD) which was employed recently to culture umbilical cord-derived MSCs [208]. The cells exhibited the same phenotype and differentiation capacity as those cultured in serum-containing media and produced more immunomodulatory factors.

The feasibility of using serum-free freezing solution with a reduced amount of Me_2SO (7.5%, 5% and 2.5%) in combination with polyethylene glycol and trehalose in cryopreserving human bone marrow-derived MSCs was also investigated by allowing cells to equilibrate with freezing solution for 10 min at 4 °C, cooling in a freezing container at 1 °C/min to −80 °C, remaining at −80 °C overnight, and then storing in liquid nitrogen for at least 1 week [127]. Although non-cryopreserved MSCs have faster proliferation rates (7 days to reach stationary growth phase *vs.* 9 days) compared to their cryopreserved counterparts, both show similar patterns of cellular morphology, intracellular pH and mitochondrial distribution. There was significant apoptosis 2 h after thawing, but after 24 h, metabolic rates were comparable in all cryopreserved cells. This study also indicated that in order to protect MSCs during cryopreservation there should be at least 5% Me_2SO if it is the only permeating cryoprotectant used. However, Me_2SO may be replaced with another permeating cryoprotectant, such as 1,2-propanediol, and human albumin (2%) may also be added to enhance cell survival [127].

The effects of these different freezing solutions were further evaluated on MSCs derived from the bone marrow of mice, rats and calves [128] using exactly the same freezing protocol as above [127]. It was demonstrated that a serum-free medium with 5% Me_2SO, 2% polyethylene glycol, 3% trehalose and 2% albumin is better at maintaining the cell viability (as determined by trypan blue exclusion), metabolic activity and differentiation potential of cryopreserved MSCs from these species than 10% Me_2SO with 10% or 90% bovine serum [128].

Cryoprotective properties of non-toxic alternatives to Me_2SO, such as glycerol and the biocompatible solutes ectoin and proline in the absence of serum but in the presence of methylcellulose, were also compared [149]. Glycerol protects from osmotic damage by reducing intra- and extracellular ice formation; ectoin protects against high salt concentration by acting as an osmolyte; and proline is an amino acid that can diffuse through cells and act as a cryoprotectant. Different concentrations of glycerol (5%, 10% and 20%), ectoin (1%, 5% and 10%) and proline (1%, 5% and 10%) were used to freeze (by cooling at 1 °C/min to a final temperature of −80 °C) a human MSC cell line and found to be non-toxic for up to 60 min incubation and at all concentrations tested. However, only ectoin conferred protection resulting in a high post-thaw survival of up to 72% according to trypan blue exclusion, suggesting that it is possible to cryopreserve MSCs in the absence of Me_2SO and serum [79]. Because a combination of two compatible solutes is more effective than each solute by itself, a human MSC cell line was cryopreserved in a basal freezing medium consisting of PBS and 0.1% methylcellulose and varying concentrations of ectoin and proline or using the commercial Me_2SO-free freezing solution Biofreeze (Biochrom, Berlin, Germany) [68]. The cells were pre-equilibrated with the freezing medium for 10 or 60 min on ice and cooled at 1 °C/min or 5 °C/min or 10 °C/min to −150 °C at which they were stored for at least 3 days. A pre-freezing incubation time of 60 min in 1% proline/10% ectoin and a cooling rate of 10 °C/min gave a high survival 48 h post-thaw (about 90%) but the highest survival (nearly 99%) was attained using Biofreeze [68].

In an attempt to eliminate FBS in the cryopreservation medium, neuropeptides (vasoactive intestinal peptide, glucose-dependent insulinotropic peptide, and pituitary adenylate cyclase activating polypeptide) have been used as alternatives [27]. High post-thaw viability (about 80% by trypan blue exclusion) was attained when human bone marrow-derived MSCs were cryopreserved (by placing directly in a freezing container at −80 °C and transferring to liquid nitrogen vapor after 24 h) in the presence of 5% Me_2SO, 30 mM trehalose and 8% human albumin. However, the enhancing effects of neuropeptides on cell survival and proliferation rates after cryopreservation still required the presence of FBS [27].

Instead of animal serum, human albumin (5%) and Me_2SO (5%) were used to cryopreserve the MSC-containing stromal vascular fraction from adipose tissues under controlled rate conditions (comprising of cooling from 4 °C to 0 °C in 6 min, then holding for 15 min at 0 °C; cooling from 0 °C to − °C in 9 min, holding for 2 min at −2 °C; cooling from −2 °C to −35 °C in 25.5 min, and finally cooling from −35 °C to −100 °C in 13 min) [146]. The cells were kept frozen for 14–193 days. The post-thaw viability as measured by 7-amino-actinomycin D (7AAD) flow cytometric analysis ranged from 71.7% to 98.3% and cells maintained their growth and differentiation to mesenchymal-specific lineages [146].

4.3.3. Sugars, anti-oxidants, apoptotic inhibitors and other additives

Some of the damage induced in cells during freezing can be attributed to dehydration, the production of oxygen radicals and the activation of apoptosis enzymes such as caspases. Therefore, the effect on cell survival of addition of trehalose (a sugar that can stabilize cell membranes by interacting with lipoproteins), catalase (an antioxidant) and zVAD-fmk (a general caspase inhibitor) in the cryopreservation solution has been investigated [185]. Amniotic fluid-derived MSCs were added to a serum-free freezing medium containing varying concentrations of Me_2SO (2.5%, 5% and 10%) and 60 mM trehalose, 100 μg/mL catalase and 30 μM zVAD-fmk, then cooled using a controlled rate freezer (unspecified cooling rate) and stored in liquid nitrogen for a minimum of 3 weeks. The population doubling, cell surface antigen expression and myogenic differentiation potential were similar in MSCs cryopreserved in 2.5% Me_2SO in the presence of the three additives compared to MSCs frozen in 10% Me_2SO and 30% FBS [185]. These results suggest that it is possible to reduce the amount of Me_2SO and eliminate serum in the cell freezing solutions and still maintain the viability and function of cryopreserved MSCs.

Surface-active compounds play important roles *in vivo* in the assembly and function of many biological structures such as the cell membrane where they have a tendency to be adsorbed at the surface. Pluronic F68 is a non-ionic low-foaming surfactant that has been shown to have protective effects on eukaryotic cell lines during their cryopreservation by stabilizing the cell membrane [78]. One group tested the effect of Pluronic 188 (F68) on MSCs during cryopreservation [50]. Human tooth germ stem cells were cryopreserved in freezing medium containing 10% Me_2SO, 20% FBS and 0.05% F68 by uncontrolled cooling at −20 °C for 15 min, then placing at −80 °C overnight before plunging into liquid nitrogen. Cells were thawed 1 day or 6 months later and assessed for viability by trypan blue exclusion, expression of apoptotic genes and differentiation-specific genes and subjected to fatty acid profiling [50]. F68 was found to increase cell viability and survival by inhibiting the expression of apoptotic genes Bax, caspase-3 and p53; however, F68 did not alter the alkaline phosphatase activity, the expression of collagen and osteocalcin and the differentiation potential after cryopreservation. It was suggested that a possible cryoprotective role of F68 on the survival of MSCs may be attributed to an alteration of lipid composition that stabilized the cell membrane [50].

Boron deficiency in plant cells is characterized by disruptions in the cell wall and membrane integrity; in animals, boron is a micronutrient required for bone maintenance. The same group referenced above [50] studied the effect of boron (in the form of sodium pentaborate pentahydrate) on the survival of dental-derived MSCs after long-term cryopreservation (storage in liquid nitrogen for 6 months) [45]. The MSCs were cooled at a rate of 1 °C/min to −80 °C before plunging into liquid nitrogen. They found that addition of borate at a concentration of 20 μg/mL to the cryopreservation solution containing 5% Me_2SO did not alter the surface antigen expression of MSCs, but enhanced their viability (as measured using the spectrophotometric MTS CellTitre96 assay kit) and osteogenic and chondrogenic differentiation [45]. The positive effect of boron on cell survival was attributed to an increase in the osmolality of the freezing medium causing cells to shrink and dehydrate rapidly thus preventing the deleterious effects of intracellular ice formation [45].

4.3.4. Programmed freezing in a magnetic field

The formation of intracellular ice when cells are cooled can have extremely damaging effects as described above. Based on the premise that magnetically-induced mechanical oscillations can prevent ice crystal formation by non-thermal vibration [112], a protocol was developed whereby murine bone marrow-derived MSCs were frozen in the presence of 10% Me_2SO at different plunge temperatures (−20 °C, −30 °C or −40 °C), using various hold times (0, 5, 15 or 25 min) at −5 °C, and increasing intensity of magnetic field (0.005, 0.1 and 0.2 mT) at a plunging temperature of −30 °C and a 15-min hold time at −5 °C [113]. The cells were then kept at −150 °C for 7 days, thawed and cultured for 48 h. Post-thaw survival was highest (60.8%) when the plunge temperature was −30 °C [113]. If the plunge temperature is higher than the optimal temperature, cells are damaged by intracellular ice formation but if the plunge temperature is too low, the cells are damaged by excessive dehydration [162]. The highest survival rate (75.8%) was also attained when the cells were held for 15 min. If the hold time is too short, the Me_2SO does not have enough time to permeate the cell, but if the hold time is too long, the cells are damaged by cytotoxic effects of Me_2SO. Overall, high survival and proliferation rates and maintenance of both adipogenic and osteogenic differentiation abilities were attained at a hold time of 15 min at −5 °C, a plunge temperature of −30 °C and a magnetic field intensity of 0.1 mT [113].

4.3.5. Vitrification

Vitrification, a process whereby a viscous liquid solution is transformed into a glass-like state (an amorphous solid without any ice formed), has been employed as an alternative to freezing for cryopreservation. Vitrification can be achieved by exposure to high concentrations (>6 M or 50% by weight) of cryoprotectants and rapid cooling [61,101]. Compared to the slow freezing method of cryopreservation, vitrification is a necessity for delicate systems that cannot tolerate the presence of ice. Biologics that are typically cryopreserved using vitrification include embryos [169], oocytes [201], articular cartilage [101], and potentially organs such as kidney [61]. Table 2 shows studies employing vitrification as a means of cryopreserving MSCs from various tissue sources. Vitrification was applied to human amnion-derived MSCs because these cells were unable to survive the damage of ice formation that accompanies slow freezing [148]. The cells were vitrified using a 2-step loading method consisting of an equilibration step in 20% ethylene glycol for 5 min at room temperature followed by vitrification in 40% ethylene glycol, 18% Ficoll, 0.3 M sucrose and 20% FBS. The cell suspension was cooled at a rate of 2500–5000 °C/min attained by plunging directly into liquid nitrogen. The vitrified MSCs showed post-thaw viability of 84.3 ± 3.2% as determined by trypan blue

Table 2
Studies on vitrification of MSCs.

Tissue source	Equilibration step	Vitrification solution	Cooling rate	Refs.	Significant findings
Human amnion	20% ethylene glycol at room temperature for 5 min	40% ethylene glycol, 18% ficoll, 0.3 M sucrose and 20% FBS	2500–5000 °C/min	[148]	Vitrified and non-vitrified MSCs were no different in terms of viability, morphology, differentiation potential and expression of stem cell and lineage markers
Human fetal liver	10% Me_2SO, 10% ethylene glycol, 0.1 M sucrose at room temperature for 2 min	20% Me_2SO, 20% ethylene glycol, 0.5 M sucrose at room temperature	Direct immersion into liquid nitrogen in 0.25 mL straws	[194]	Morphology, expression of surface markers and differentiation potential were not affected by vitrification
Sheep umbilical cord blood	Same as vitrification solutions for 2–20 min	*VS1:* 5% ethylene glycol, 35% 1,2-propylene glycol and 6% sucrose *VS2:* 5% ethylene glycol, 35% 1,2-propylene glycol and 5% sucrose, 1% polyvinyl alcohol	Direct immersion into liquid nitrogen in 0.25 mL straws	[205]	Higher viability of thawed MSCs was attained after vitrification in the presence of polyvinyl alcohol compared to programmed freezing
Human Wharton's jelly	20% ethylene glycol and 20% FBS for 5 min	40% ethylene glycol, 18% ficoll, 0.3 M sucrose and 20% FBS	Direct immersion of 0.5 mL cell suspension in 1.5 mL cryovial in liquid nitrogen	[109,138]	Morphology, cell surface markers and differentiation potential of MSCs were not changed after vitrification, and 95% post-thaw viability achieved

exclusion, and were not different from non-vitrified fresh MSCs in terms of morphological characteristics, expression of surface antigens and embryonic stem cell markers and tri-lineage differentiation under appropriate culture conditions [148].

Instead of the cryoprotectants used in the vitrification method described above, an equilibration solution containing 10% Me_2SO, 10% ethylene glycol and 0.1 M sucrose and vitrification solution with 20% Me_2SO, 20% ethylene glycol and 0.5 M sucrose, was used to vitrify human fetal liver-derived MSCs [194]. The equilibration and addition steps were both carried out at room temperature. The mean recovery of viable cells (86 ± 4.7% as assessed by trypan blue exclusion) was comparable to that obtained using the original vitrification procedure (84.2 ± 4.8%) although it was lower than that of non-vitrified control (93.5 ± 1.7%). The morphological appearance, expression of surface markers and differentiation to adipocytes and osteocytes were unaffected by the vitrification procedure [194].

Because existing protocols for vitrification use high concentrations of cryoprotectants which can be cytotoxic, additives have been used to mitigate damaging effects. One such compound is polyvinyl alcohol (PVA), a non-permeating cryoprotectant which has been previously proven to inhibit ice crystal formation and growth [204]. MSCs derived from sheep umbilical cord blood were vitrified in a solution containing 5% ethylene glycol, 35% propylene glycol, 5% sucrose and 1% PVA using the Opened Pulled Straw technique [201]. Cell viability as high as 95.4% was obtained using trypan blue exclusion, which was comparable to that obtained with non-vitrified control [205].

The same two-step vitrification method described above [148] was used to cryopreserve MSCs from human umbilical cord Wharton's jelly. The expression of surface antigens and tri-lineage differentiation were retained in the vitrified cells compared to their non-vitrified counterparts and a post-thaw viability of 95.54 ± 2.30% was attained by trypan blue staining [138]. More recently, this group demonstrated that vitrification does not alter the differentiation of these cells to male germ-like cells based on their protein expression of germ cell-specific markers [109].

4.3.6. Use of 3D scaffolds

Cryopreservation of MSCs adhering to fabricated scaffolds of biocompatible materials is a strategy that has been explored to prepare ready-to-use transplantation units for the repair of damage in bone, cartilage or skin. In order to overcome the difficulty of freezing adherent monolayers and to simulate the 3D conformation that exists *in vivo*, MSCs have been cryopreserved in encapsulated form in alginate microspheres. Alginate is a natural polysaccharide that offers the advantage of biocompatibility and biodegradability. Moreover, alginate is hygroscopic and by absorbing water can prevent the formation of large ice crystals during the freezing process. Suspended or alginate-encapsulated bone marrow-derived MSCs were cryopreserved in 5% or 10% Me_2SO by linear cooling at 1 °C/min to −80 °C before plunging into liquid nitrogen [165]. The percent viability was assessed by confocal microscopy after staining cells with a combination of fluorescein diacetate (FDA) and ethidium bromide (EB). The viability and metabolic activity of MSCs in suspension were found to be higher than their encapsulated counterparts in the presence of 5% Me_2SO, but were the same in 10% Me_2SO, which may be due to the slower suboptimal distribution of the Me_2SO in the hydrogel. By using a 3-step freezing procedure (comprising of a 1 °C/min cooling from 0 °C to −7 °C at which point ice nucleation was induced, followed by cooling at 1 °C/min to −40 °C, and finally a 10 °C/min cooling to −80 °C before plunging into liquid nitrogen) post-thaw viabilities of 90% were obtained for alginate-encapsulated MSCs cryopreserved in the presence of 10% Me_2SO. These encapsulated cells exhibited tri-lineage differentiation [165].

In order to further optimize this methodology, the attachment and spreading of MSCs within an alginate–gelatin scaffold prior to cryopreservation was investigated [108]. Umbilical cord-derived MSCs were seeded for 2 h in the scaffold, and the cell–scaffold complex was held in 10% Me_2SO for 5 min at 4 °C followed by cooling at a rate of 1 °C/min to −80 °C where it was kept overnight before plunging into liquid nitrogen. Viability was assessed by confocal laser scanning microscopy of FDA/EB labeled cells, and the attachment of cells to the scaffold as well as cell membrane disruptions were visualized using scanning electron microscopy. The cell viability, cell contacts, membrane integrity, motility and spreading were shown to be comparable to the non-frozen control [108]. Furthermore, tissue-engineered constructs developed from umbilical cord-derived MSCs in electrospun silk fibroin scaffold were successfully cryopreserved using a freezing medium consisting of trehalose (40 mM), ectoin (40 mM), catalase (100 μg) and Me_2SO (2.5%). The viability (based on propidium iodide staining), proliferation and differentiation of MSCs, and the mechanical integrity of the scaffold were similar to those of their non-cryopreserved counterpart [24]. Table 3

Table 3
Studies on cryopreservation of MSCs in scaffolds.

Tissue source	Scaffolding material	Cryopreservation protocol	Refs.	Significant findings
Human bone marrow	Alginate	Cryoprotectant: 5–10% Me_2SO *Protocol 1:* Cooling at 1 °C/min to −80 °C followed by immersion in liquid nitrogen *Protocol 2:* Three step cooling with ice nucleation at −7 °C and immersion in liquid nitrogen *Protocol 3:* Direct immersion in liquid nitrogen with approximate cooling rate of 200 °C/min	[165]	The highest post-thaw viability and metabolic rate were obtained using protocol 2 in the presence of 10% Me_2SO, and cells retained their ability to differentiate into adipogenic, osteogenic and chondrogenic lineages
Human umbilical cord	Alginate–gelatin	Cryoprotectant: 10% Me_2SO Cooling rate of 1 °C/min to −80 °C followed by immersion in vapor phase of liquid nitrogen	[108]	The cell viability, cell–cell contacts, membrane integrity, motility and spreading were comparable between cryopreserved and non-frozen control
Human umbilical cord	Nanofibrous silk fibroin	Different combinations of 2.5–10% Me_2SO, 40 mM trehalose, 40 mM ectoin and 100 μg catalase were used as cryoprotectant solutions and the cells equilibrated at 4 °C for 10 min before cooling at 1 °C/min to −150 °C	[24]	Freezing medium consisting of 2.5% Me_2SO, 40 mM trehalose, 40 mM ectoin and 100 μg catalase was most effective in maintaining viability, proliferation rate and differentiation of MSCs

summarizes the above studies on cryopreservation of MSCs in scaffolds, which indicate that the use of 3D scaffolds is feasible and presents potential applications for tissue-engineered cryopreserved cells in regenerative therapies.

5. Cryopreservation of tissues from which MSCs are derived

The desired scenario for regenerative medicine and tissue engineering includes a ready availability of clinically acceptable MSCs. As the tissue sources of MSC may not always be accessible on short notice, it may become necessary to cryopreserve tissues as they become available. In addition, some facilities lack the personnel and equipment that would otherwise be required to start the cultures at the very moment that the tissues are received. Moreover, because changes in gene expression occur immediately after isolation and persist during their monolayer expansion [14], it would be ideal to establish MSC cultures from the same donor tissue at passage 0 instead of using cells that have undergone long-term culture. Cryopreservation of the tissues from which the MSCs are derived, and initiating their isolation and culture at a later time, is a practical approach to circumvent these issues. The question that arises is whether the MSCs that are recovered from the frozen tissues will have the same functional properties as those isolated from fresh non-cryopreserved tissues. Several groups have compared MSCs isolated from fresh *vs.* cryopreserved tissues including bone marrow, umbilical cord, adipose tissue, dental pulp and intact teeth [10,34,39,122,124,158,178,187] (Table 4).

Bone marrow has been the original and traditional source of MSCs, although its cryopreservation has been performed primarily for the purpose of preserving the hematopoietic stem/progenitor cell populations for transplantation. Previously it has been shown that MSCs obtained from bone marrow mononuclear cells that have been cryopreserved for 4–8 weeks exhibit similar surface markers, proliferation potential and differentiation capacity to their counterparts from fresh bone marrow [34]. More recently, bone marrow cells (from autologous transplant patients) that have been cryopreserved for over 20 years were thawed and used to establish MSC cultures [187]. The cryopreservation protocol involved pre-incubation in freezing solution containing 10% Me_2SO and 10% autologous plasma in media for 30 min at 4 °C, followed by controlled rate freezing at 1 °C/min from 4 °C to −50 °C and at 10 °C/min from −50 °C to −80 °C before immersion in liquid nitrogen. Although the number of adherent of cells in the initial cultures after thawing was only 50% of the seeded cells compared to those from fresh bone marrow, the cells were able to recover under appropriate culture conditions and displayed similar morphology, cell growth patterns, cell surface marker expression and differentiation into osteogenic and adipogenic cells as MSCs established from fresh bone marrow samples [187].

MSCs harvested from the Wharton's jelly of umbilical cord exhibit greater proliferative potential than bone marrow-derived MSCs [122] which makes Wharton's jelly an ideal MSC source. In one study, segments of umbilical cord (2–3 cm) were immersed in medium containing 10% Me_2SO and 0.2 M sucrose and cooled at 1°/min to −80 °C [178]. After overnight incubation, the frozen tissue was transferred to liquid nitrogen and kept for 5–29 days. After quick thawing at 37 °C, the cord tissue was cultured as explants until cells started to emerge and form a confluent layer. The growth kinetics, surface antigen expression and differentiation of MSCs to adipocytes and chondrocytes were compared to those for MSCs obtained from non-cryopreserved explants from the same donor. Although MSCs took longer to grow out of the cryopreserved cord explants as compared to corresponding fresh explants, no significant change in population doublings were observed between them. The MSCs derived from cryopreserved tissue were likewise capable of *in vitro* differentiation and expressed the same surface markers, although their cumulative cell yield at the end of the third passage was lower than that from fresh umbilical cord samples [178]. Similarly, cord tissue was suspended in 10% Me_2SO and 20% cord plasma for 30 min at 4 °C and cooled using a controlled rate freezer to −180 °C (unspecified cooling rate) where it was kept for 5 years. After thawing, the tissues yielded MSCs with poor plating efficiency albeit with good functional recovery [10]. In contrast, one group reported failure to isolate MSCs from umbilical cord fragments cryopreserved in medium containing 10% Me_2SO, 5% glycerol and 20% FBS [35]. The tissues were allowed to equilibrate in the freezing medium for 30 min at 4 °C before placing in a freezing container designed for cooling at 1 °C/min to −80 °C, and then stored in liquid nitrogen for 1 week, 1 month or 6 months [35].

In another study, adipose tissue was harvested from patients undergoing liposuction procedures and samples from each patient were either processed within 24 h or frozen for at least a week [39]. The cryopreservation protocol involved putting the adipose tissue in 4 mL media containing 10% Me_2SO and 10% FBS at 4 °C, cooling in a controlled rate freezer (unspecified cooling rate), and submerging in liquid nitrogen. MSCs were successfully isolated from all of the fresh and frozen adipose tissues. The morphology, doubling time, plating efficiency, expression of phenotypic markers, and adipogenic, osteogenic, and chondrogenic differentiation of the MSCs were not compromised by cryopreservation nor was there any significant effect on the senescence of MSCs [39].

Dental pulp is another promising source of MSCs for cellular therapy and tissue engineering applications. It has been shown

Table 4
Studies on cryopreservation of source tissues of MSCs.

Tissue	Cryopreservation protocol	Storage period	Refs.	Significant findings
Bone marrow	Mononuclear cells were cryopreserved in 10% Me_2SO and stored in liquid nitrogen	4–8 weeks	[34]	MSCs derived from cryopreserved bone marrow mononuclear cells show high proliferation rate and retain their phenotype and differentiation potential as MSCs from fresh bone marrow cells
Bone marrow	Cells were equilibrated in 10% Me_2SO for 30 min at 4 °C, cooled at 1 °C/min to −50 °C, and at 10 °C/min to −80 °C before storage in liquid nitrogen	23–25 years	[187]	Although the initial recovery of MSCs from cryopreserved bone marrow was poor, subculture of surviving cells yielded MSCs with characteristic phenotype, good proliferation rates and capacities to differentiate to osteoblasts, adipocytes and neuronal cells
Human umbilical cord	Cord segments were cooled at 1 °C/min to −80 °C in the presence of 10% Me_2SO or 5% glycerol and 0, 0.2 M or 0.5 M sucrose, kept at −80 °C overnight before transfer to liquid nitrogen	5–29 days	[178]	MSCs from cryopreserved cord tissue express characteristic surface markers and were able to undergo differentiation. However, they took longer to grow and the cumulative cell yield was lower than MSCs from fresh cord tissue samples
Human umbilical cord	Cord segments were equilibrated for 30 min at 4 °C in 10% Me_2SO, frozen using a controlled rate freezer to −180 °C and stored in the vapor phase of liquid nitrogen	5 years	[10]	Cryopreserved cord tissue had poor plating efficiency and increased doubling times. However, once established, the cells exhibited MSC surface markers and adipogenic and osteogenic differentiation
Human umbilical cord	Cord segments were equilibrated in medium containing 10% Me_2SO and 5% glycerol for 30 min at 4 °C, cooled at 1 °C/min to −80 °C before storage in liquid nitrogen	1 week, 1 month, 6 months	[35]	Viable MSCs cannot be isolated from the Wharton's jelly of cryopreserved cord tissue
Human adipose tissue	Lipoaspirates were equilibrated in 10% Me_2SO at 4 °C for 30 min, cooled using a controlled rate freezer to −180 °C before immersion in liquid nitrogen	At least 1 week	[39]	MSCs obtained from cryopreserved adipose tissues exhibited similar morphology, surface markers, proliferative and differentiation potential as MSCs from fresh adipose tissues
Human dental pulp	Enzymatically digested or unprocessed dental pulp tissue was placed in 10% Me_2SO and cooled at 1 °C/min before storage in liquid nitrogen	At least 1 month	[207]	Optimal functional recovery was achieved using cryopreserved dental pulp tissue with enzymatic digestion and culture performed post-thaw
Intact adult teeth	Intact teeth were equilibrated in 10% Me_2SO for 2 h at 4 °C, cooled at 1 °C/min to −85 °C before immersion in liquid nitrogen	At least 1 month	[207]	Only two out of ten cryopreserved teeth yielded viable and functional MSCs
Intact baby teeth	Teeth were equilibrated in 10% Me_2SO at 4 °C for an hour, cooled at 1 °C/min to −80 °C, and stored in liquid nitrogen	7 days	[124]	A 30% success rate was achieved in recovering MSCs from thawed teeth, and the cells displayed lower proliferation rate and altered morphology compared to MSCs from fresh teeth

previously that cryopreservation (in 2 mL cryovials containing 10% Me_2SO, cooled at 1 °C/min to −85 °C before plunging into liquid nitrogen) of early passage MSCs from dental pulp leads to a high post-thaw recovery of viable cells as assessed by trypan blue exclusion [158]. The same group then attempted to cryopreserve dental pulp by placing enzyme-digested tissue in 10% Me_2SO and cooling at 1 °C/min before storing for at least a month in liquid nitrogen [207]. Intact teeth or undigested dental pulp tissue were also frozen by direct immersion into 10% Me_2SO for 2 h at 4 °C, followed by cooling at 1 °C/min in an isopropanol bath within a −85 °C mechanical freezer for 24 h before plunging into liquid nitrogen where they were stored for at least a month. The best recovery of MSCs was achieved by cryopreserving the dental pulp tissue with enzymatic digestion and cell isolation carried out post-thaw. Growth of MSCs from pre-digested tissue was delayed and only 2 out of 10 frozen intact teeth yielded MSCs [207]. Similarly, only a 30% success rate was achieved when establishing MSC cultures from cryopreserved deciduous teeth [124]. The teeth were immersed in 1.5 mL of 10% Me_2SO/90% FBS, equilibrated at 4 °C for 1 h, cooled at 1 °C/min to −80 °C where they were kept for 24 h before storing in liquid nitrogen for 7 days. The MSCs recovered from thawed teeth displayed lower proliferation rate and altered morphology [124].

6. Current status of cryopreserved MSCs for the clinic

Cryopreservation and banking under safe and quality-controlled conditions are crucial to the implementation and success of cell-based therapies [54]. Tremendous research, regulatory and industrial resources have been expended towards optimizing the protocols, freezing media composition, cooling devices and storage containers in order to ensure that MSCs retain their therapeutic characteristics after freeze/thaw [95,193]. The market approval of the cryopreserved MSC drug Prochymal by Health Canada in 2012, based on compelling phase II clinical trial results showing statistically significant improvement in survival among patients with severe refractory GVHD [25], may be considered the culmination of these efforts. Lately however, doubt has been cast upon the efficacy of this cellular product based primarily on the erroneous assumption that immediate post-thaw MSCs possess the same features as their pre-freeze counterparts [72]. A phase III industry-sponsored clinical trial (NCT00366145) on Prochymal conducted in the USA and licensed by the Food and Drug Administration failed to meet its primary clinical endpoint of achieving an increased GVHD overall complete response compared with placebo control [71]. This trial outcome does not support the clinical use of MSCs and is not consistent with published European clinical experience which claimed that multiple MSC infusions are effective in children with GVHD [12]. These conflicting trial outcomes raised the question of the appropriateness of MSCs for clinical application and also brought to light putative problems associated with MSC-based therapy, including immediate cell death upon infusion, poor homing capacity, increased risk of infection and leukemic relapse, among others [104]. Notwithstanding, it is claimed that MSC therapy benefited some patients to a certain degree; and although a myriad of factors could be put forth to explain why it failed in others, here we will consider only those related to the culture and cryopreservation of MSCs.

The company website of Prochymal (http://www.osiris.com) describes this product as being harvested from a single healthy bone marrow donor from which 10,000 doses can be derived. This would entail massive expansion and most likely increased number of passaging. In contrast, the European-based clinical trials typically harvest a number of cells equivalent to 5–10 doses per donor [71]. It has been previously shown that late passage MSCs are not as effective as their early passage counterparts in treating GVHD [11]. Recently, it was demonstrated that freeze–thawed

MSCs, in contrast to freshly harvested MSCs, have impaired immunomodulatory properties and that patients infused with freshly harvested cells from early passage cultures had better response rates [147]. Moreover, it is noteworthy that the retrospective study reporting favorable outcomes of MSC infusions in children with GVHD [12] involved transfusions of MSCs in their log phase of growth. In contrast, Prochymal was administered either immediately or up to 5 h after thaw by reconstituting the frozen product. Taken together, these factors may explain why the US-based phase III clinical trial, which used Prochymal, failed to reach its desired endpoint.

It is empirically known that a significant loss (about 20%) in the recovery of viable cells occurs after cryopreservation. Apoptotic and necrotic pathways are activated in cells 6–48 h post-thaw in response to low temperature exposure [17], and cryopreserved MSCs have a higher percentage of apoptotic cells than MSCs from fresh live cultures [38]. It has also been shown that thawed MSCs launch a "heat shock" response to the stress associated with cryopreservation which results in a dampening of their immune-suppressive properties [67,72,147]. Moreover, although cryopreservation does not alter the expression of adhesion molecules and homing receptors such as CXCR4 in MSCs, it disrupts F-actin polymerization [212,213] and reduces their homing to lungs [38]; however, a 48-h culture of post-thaw MSCs restores their homing ability [38,72]. It may therefore be necessary to allow the cells to recover first before they are infused. In support of this notion, human bone marrow-derived MSCs which were sub-cultured up to passage 3 were cryopreserved in 10% Me_2SO by cooling at 1 °C/min to −80 °C and plunging immediately into liquid nitrogen [93]. The cells thawed after 1, 3, 6, 7, and 8 months, not only attained 20-fold expansion after 5–8 days, but most importantly, their immunosuppressive properties were retained [93]. It has also been shown in adipose-derived MSCs that, instead of the harsh enzymatic or chemical treatment used to dislodge the cells which can strip them of important surface receptors, the cells can be cultured in synthetic hydrogels which permit reagent-free passaging while preserving their phenotype and potency [53]. Thus, while viable MSCs can be obtained from certain cryopreserved tissues, the isolation and cryopreservation of MSCs from fresh tissues present some benefit.

7. Conclusions

MSCs are favored above embryonic or induced pluripotent stem cells as a candidate cellular therapy product because of their easy availability and minimum manipulation. In addition, a comprehensive and systematic review of reported adverse events in clinical trials using MSCs show that their systemic infusion is safe and carries no risk of malignant transformation [117]. Cytogenetic aberrations were not observed in fresh and cryopreserved human bone-marrow derived MSCs cultured under xenogen-free GMP conditions [130]. Presuming positive outcome of clinical trials, MSC-based therapy could become the standard of care in patients with various injuries and disorders, but rigorous evidence of their efficacy remains to be proved.

It is apparent from the studies reported so far that viable and functional MSCs can be recovered following cryopreservation regardless of the tissue from which the MSCs are derived, using a wide variety of cooling protocols, added cryoprotectants, storage times and temperatures. Some cells inevitably die as a result of the freeze–thaw process while others have alterations in their biological properties which affect their therapeutic potential [164]. However, those cells that survive retain their proliferative potential and are able to generate large numbers of fully functional cells. In fact, it has been demonstrated that MSCs express proteins involved in replication and cellular defense mechanisms in response to the stress of cryopreservation that may contribute to their survival and recovery during freeze–thaw [77].

In light of the negative outcome of clinical trials that employed cryopreserved MSCs for infusion, we and others recommend that cryopreserved MSCs should be allowed to recover longer after thaw [38,67,93]. Also, because washing of cells after thawing can lead to cell loss, a better approach may be to plate the cells immediately after thaw (which involves some dilution of the cryoprotectant), and then to allow them to expand to confluency. Passaging will remove the residual cryoprotectant and animal-derived serum, and the cells can then be further cultured in animal serum-free medium or supplemented with human platelet lysate [19] or platelet-rich plasma in a Good Manufacturing Practice (GMP)-compliant lab [161,184]. The final cellular product could be delivered to the patient's bedside for infusion. Alternatively, it has been shown that sub-confluent cultures of MSCs that have been stored hypothermically at 4 °C for 2–4 days and then allowed to recover at 37 °C for 3 h yielded cells with normal proliferation, surface marker expression and osteogenic potential [76]. Moreover, the addition of chemical modulators such as resveratrol and salubrinal decreased apoptosis, necrosis and the levels of cell stress signaling proteins, thereby improving the viability of human MSCs following hypothermic storage [41].

Translating research-based procedures into large-scale manufacturing requires strict compliance with regulations to ensure the identity, safety, genetic stability, potency and efficacy of MSCs for use in the clinic [54,183,184,206,209]. Because massive expansion of MSCs would be needed to attain therapeutic doses, it is not practical to use conventional culture methods which will require hundreds of culture flasks and many open procedures. In response to this need, closed system large-scale bioreactors are becoming available [54,85,86]. As 2D expansion negatively affects MSC proliferation potential, successful MSC expansion has been achieved using growth factor stimulation and reduced oxygen tension in 3D bioreactors [92]. Also, spinner flask cultures using synthetic microcarriers under xeno-free conditions have allowed for a 3D environment in the scalable and cost-effective expansion of MSCs [33,88].

Finally, the success of MSC-based therapies would rely on cells whose proliferative capacity, differentiation potential, secretory profile, migration ability and immunomodulatory properties remain intact following cryopreservation. The efficacy of MSCs should be based on cell functionality, and assays predictive of potency for the appropriate clinical application must be employed and used as release criteria [73,210]. Future investigations should focus on the recovery and rescue of cells from freeze/thaw-associated injury followed by the expansion of fully functional cells and correlation of post-thaw cell handling with outcome studies. Moreover, the robustness displayed by MSCs in response to cryopreservation makes them an ideal cell source for tissue-engineered constructs for the repair or regeneration of diseased or damaged tissues. The cryopreservation of these constructs could be another focus of future research.

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