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Chapter 3

Isolation and Cryopreservation of Animal Mesenchymal Stromal Cells

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Abstract

Scientific progress in cellular and molecular biotechnology has led to the development of advanced therapies, such as gene therapy, cell therapy, and tissue engineering. The application of stem cells as therapeutic agents has been investigated for several years in human medicine and, more recently, the same approach has been considered in the veterinary field as a novel opportunity for the treatment of animal diseases. Mesenchymal stem cell (MSC)-based therapies seem to contribute to the healing process by several mechanisms due to their peculiar biological features. It has been shown that MSCs could effectively differentiate into the required cell type to replace the damaged tissue. Furthermore, due to their autocrine and paracrine secretory activities, these cells are a powerful source of trophic mediators, growth factors, cytokines, and extracellular matrix components. The clinical application of MSCs needs great amounts of cells designed for *in vivo* implantation that can be obtained following their *in vitro* isolation, serial subcultivations, cryopreservation, and thawing. These procedures could determine their feature changes which could interfere with the therapeutic outcome. For these reasons, to preserve MSCs after *in vitro* manipulation for future applications, standardized quality controls and a reliable long-term cryopreservation method are required.

Keywords: mesenchymal stromal cells, cryopreservation, cryoprotectant, regenerative medicine

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1. Introduction

The aims of regenerative medicine are to renew cells, regenerate fully functional tissues, and organs or structures that are lost or damaged after disease, injury, or aging [1]. In recent years, the mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) have attracted much attention due to their potential use in regenerative medicine and tissue engineering as shown by the main applications described in the literature and the noteworthy progress that has been made toward their better understanding and characterization [2]. Those cells display a significant therapeutic plasticity as reflected by their advantageous characteristics: the ability to enhance tissue renovation, the immunomodulatory, and anti-inflammatory effects [3, 4] and the possibility to be used for both autologous and allogeneic therapies [5]. For these reasons, MSC-based cell therapies have been investigated for several years in human medicine and, more recently, the same approach has been considered in veterinary medicine as a novel potential therapy for animal diseases [6–9]. While most studies using animal models and even small clinical trials have utilized fresh MSC cultured on-site, cryopreservation of MSC is essential to the widespread application of MSC-based therapies. Cryopreservation allows for MSC to be prepared by specialized facilities, in large batches under the application of accepted quality control measures to ensure their safety. Currently, much information concerning the effects of cryopreservation on MSCs is difficult to interpret because MSCs are frequently isolated from different tissue sources and stored for variable periods of time. The capability of MSCs to survive to storage, maintain their phenotype, and differentiate along multiple lineage pathways upon thawing is of paramount importance if they are banked for future therapeutic purposes.

2. Mesenchymal stem cells

MSCs were first described as a specific cell population by Friedenstein's research group in the late 1960s [10]. Previously, stem cell populations were supposed to reside solely in adult tissues with a high turnover rate, such as blood, skin, hair, gastrointestinal epithelium, and bone. Indeed, these cells are present in variable amounts in specific stem cell "niches" (organs), in almost all the body tissues and even if the exact locations of these niches are poorly understood, there is growing evidence suggesting a close relationship with pericytes [11]. Generally, these cells remain in a quiescent state until activated by significant events, such as during tissue repair after injury or following transplantation, to regain tissues' homeostasis [12, 13]. MSCs are undifferentiated, self-renewable, multipotent adult stem cells originated from the mesoderm germ layer during the embryonic development, characterized by the ability to evolve both *in vitro* and *in vivo* along multiple lineage pathways [14]. Furthermore, MSCs have shown evidence of plasticity by trans-differentiating into a broad range of cell types of mesodermal origin (osteocytes, chondrocytes, adipocytes, and myocytes) [15, 16], but also deriving from other germ layers including ectodermal neurons [17] endodermal hepatocytes [18], endothelial cells [11], and cardiomyocytes [19].

3. Properties of MSCs for cellular therapy

Although MSCs first attracted attention due to their ability to differentiate into various cell types, current data suggest that MSCs, as a result of their peculiar biological features, may not only replace damaged tissues, but may be also capable of secreting several bioactive molecules with paracrine and autocrine properties. Such functional secretions of factors are responsible for trophic [20], antiapoptotic, angiogenic, and antiscar effects [21, 22]. MSCs have a further interesting characteristic, related to the capacity to exert immunoregulatory effects on cells of adaptive and innate immunity, such as T and B lymphocytes, dendritic cells, natural killer cells, and monocytes [23]. These immunomodulatory properties that have been extensively demonstrated by several *in vitro* and *in vivo* studies, seem to permit MSC-allogenic transplantation.

4. Sources of MSCs

In veterinary medicine, the first source reported to contain MSCs was the bone marrow (BM) that in the past was also the most widely used [15]. Nevertheless, more recent studies have identified MSCs with similar properties in almost all mammalian tissues such as skeletal muscle [24, 25], tendon [26], skin [27, 28], adipose tissue [29], periosteum [30], synovial membrane [31], dental pulp [32], peripheral blood [33], umbilical cord blood [34], amniotic fluids [35], and cornea [36].

5. Isolation of MSCs from bone marrow

The postnatal bone marrow (BM) has been the most studied tissue as a source of progenitor cells. It contains at least two cell populations: the hematopoietic stem cells (HSCs), located in proximity to the endosteum, and MSCs that surround the trabeculae and blood vessels [37]. HSCs are capable of regenerating the peripheral blood cell lines and the immune system, and the MSCs [38] are capable of giving rise to tissues of each of the three germ layers. Although MSCs derived from BM are easily separated from the nonadherent hematopoietic fraction of cells by culture and adherence to plastic dishes, BM harvest is an invasive and painful surgical procedure that requires the anesthesia and it could be associated with the risk of complications such as hemorrhage, infection, pneumothorax, or pneumopericardium in horses [39, 40]. Moreover, there is only a very low frequency (0.001–0.01%) of MSCs in bone marrow and these numbers decline with the age of the individual.

6. Isolation of MSCs from adipose tissue

The isolation of rat mature adipocytes and adipose tissue progenitor cells was described in literature for the first time by Rodbell [41]. The protocol was based on the fragmentation of

adipose tissue into small portions, followed by enzymatic digestion with collagenase type I at 37°C and the subsequent centrifugation to separate the different cell fractions. The obtained supernatant was composed of mature adipocytes and the pellet fraction consisted of the stromal vascular fraction (SVF) components, which comprise a heterogeneous cell population, including circulating blood cells, fibroblasts, pericytes, and endothelial cells, as well as "pre-adipocytes" or adipocyte progenitors. Stem cells and progenitor cells represent about the 3% of all cell populations [42]. Stem cells derived from the adipose tissue (ASCs) represent a purified population of the adherent stem cells present in the adipose tissue, since all other cell types are removed or die with time. Currently, ASC recovery is quick and easy to perform from the subcutaneous adipose tissue, as it could be successfully collected via lipectomy or from the tail base in horses and from the inguinal region [43] or during ovariohisterectomies in dogs and cats. Stem cells derived from the adipose tissue have been increasingly used for cell therapy both in humans and animals [44], either as freshly isolated, SVF cells, or as cultivated ASCs [43]. ASCs proliferate rapidly with a high cellular activity, making them an ideal source to obtain MSCs [45]. The most important advantage of adipose-derived stem cells is their abundance: from 1 g of adipose tissue an average of 0.5–2.0 × 10^6 SVF cells can be isolated, which gives 1–10% of stem cell yield [46]; in comparison, MSCs constitute only 0.001–0.01% of BM [15]. When autologous ASCs are used, the adipose tissue is collected 2 or 3 weeks before the treatment and the animal receives the cultivated cells, but long-term cultivation of ASCs before therapeutic use is not recommended, since the cells may lose their progenitor characteristics [47]. The use of allogeneic ASCs has been also performed; since these cells have immunoregulatory properties [48], this approach would allow the use of species-specific allogeneic cryopreserved cells, avoiding the need for collection of tissue from the patient [49].

7. Cryopreservation

Biopreservation has been characterized by a recent rapid growth since advances in cell therapy, stem-cell research, personalized medicine, cell banking, etc. drive the need for optimized storage protocols. Nevertheless, this field still experiences significant issues with the current techniques including suboptimal survival, loss of poststorage cell function, addition of animal components in storage solutions, and activation of cellular stress pathways which can lead to changes in gene expression and protein denaturation [50]. The clinical application of autologous or allogeneic MSCs requires on demand access to a ready off-the-shelf amount of viable therapeutic doses of MSC and therefore necessitates fast availability to cryopreserved MSC stocks. The aim of cryopreservation is to preserve the therapeutic properties of those cells that maintain unaltered the characteristics of the freshly isolated samples, but the freezing and thawing procedures could determine an alteration of the cellular osmosis which can cause cell injury.

7.1. Freezing

The freezing rate is a fundamental factor for all biological systems in the determination of viability following cryopreserved storage. Several studies have shown that successful cryopreservation of cells in suspension needs sufficiently high cooling rates to reach quickly low

temperatures and avoid slow-cooling injury, but low enough cooling rates to decrease the formation of intracellular ice and avoid rapid-cooling injury [51]. The responses to cooling rates are cell-type specific, as distinct cell types have different membrane permeability parameters. The intracellular dynamics during freezing or thawing could be influenced by many factors which influence cell viability after both of these procedures, affecting the therapeutic outcomes. Among these factors, the subtract desegregation stress before cryopreservation of the cells attached to the plastic, the intracellular ice formation during freezing which can compromise the integrity of the cell membranes and, after thawing, the risk of impairing the membrane and altering other cellular functional characteristics can be listed [52]. Currently, there are two procedures to achieve the efficient cryopreservation of MSCs: conventional slow-freezing and vitrification (rapid cooling). Both of these methods may lead to cell damage during loading/unloading of the cryoprotectant agents (CPAs), freezing, and thawing steps. The slow-freezing procedure is the most commonly used cryopreservation technique in clinics and research laboratories today, because it allows the preparation of large amounts of vials at one time. Cryopreservation by vitrification has shown higher cell survival and it has been recognized as a promising strategy for long-term cell banking. Nevertheless, the difficulty to generate a fast enough heating rate to minimize devitrification and recrystallization-induced intracellular ice formation during rewarming is one of the major problems to be overcome. However, the high CPA concentration that is required to achieve vitrification results in osmotic dehydration to cells. For these reasons, new vitrification methods have emerged as alternative techniques, which have shown the ability to significantly reduce cryoinjury. This approach has been improved for the cryopreservation of organized tissues where even extracellular freezing causes several damages. In fact, in a recent study reported by Wang et al. [53], magnetic induction heating of superparamagnetic nanoparticles was successfully applied to enhance rewarming, with promising results of the vitrified human umbilical cord matrix MSC survival.

7.2. Cryoprotectants

In addition to controlling the cooling rates, one of the major challenges to obtain an effective cryopreservation method is the selection of a suitable CPA, which minimizes the damaging effects of freezing. The most commonly employed CPA for cultured mammalian cells is dimethyl sulfoxide (DMSO) solution, because it is cheap and it has a relatively low cell toxicity. DMSO penetrates cell membrane, reduces intracellular ice formation, and prevents cell damage due to dehydration caused by extracellular ice formed during freezing; on the other hand, it can also decrease the survival rate [54, 55] or induce cell differentiation to neuronal-like cells when added to the cell culture medium [56]. The most common cryopreservation medium to store several types of stem cells has become a solution of 10% (v/v) DMSO and up to 90% (v/v) fetal bovine serum (FBS), despite showing disadvantages. To improve this procedure, MSCs have been cryopreserved using both DMSO and FBS free systems, comprising different polymers either alone or in combination with ethylene glycol, 1,2-propylene glycol, trehalose, sucrose, and/or glucose. In contrast to DMSO that penetrates quickly into the cell, the high molecular weight polymers such as polyvinylpyrrolidone, polyethylene glycol, polyethylene oxide, or polyvinyl alcohol are nonpenetrating and seems to act extracellularly

(at 10–40% concentrations), with the increasingly high viscosities at low temperature and avoiding that water molecules form ice crystals [52]. In a study reported by Renzi et al. [57], several cryopreservation solutions for MSCs isolated from equine, ovine, rodent bone marrow, and equine adipose tissue were compared: the best results regarding cell viability were obtained using a solution of fetal bovine serum added with 10% of DMSO. Conversely, in a previous study, Ock and Rho [58] reported that the survival and number of colonies formed by porcine MSCs were significantly decreased following short-term storage (less than a month) into liquid nitrogen (−196°C) and the amount of this decrease was inversely proportional to the DMSO concentration. Those data strongly suggest the use of 5% DMSO instead of conventional 10% DMSO for the cryopreservation of porcine MSCs, for minimizing the CPA toxicity on cells. However, slow freezing with reduced concentration of CPAs has gained much interest in order to decrease the effect of the osmotic shock and chemical toxicity. Nevertheless, the commonly used CPAs are highly toxic at 37°C (body temperature) and could not be applied to patients. For this reason, multistep washing is required to completely remove the highly toxic, cell membrane-permeable cryoprotectants from cryopreserved cells for clinical use, though this procedure is often associated with significant loss of precious cells (~10% during each washing step). Therefore, it is important to achieve cell cryopreservation with nontoxic CPAs. Recently, Rao et al. [59] demonstrate that nanoparticle-mediated delivery of trehalose into mammalian cells has great potential for cryopreserving the human primary adipose derived stem cells (hADSCs) and possibly other types of stem cells to facilitate their ready availability for clinical use. In fact, successful results on cryopreservation of hADSCs using only trehalose as cryoprotectant has been achieved with high survival and undamaged function post cryopreservation.

7.3. Thawing and viability assessment

As well as cooling, optimizing the thawing method of frozen MSCs is also important. Furthermore, in clinical transplantation applications the post-thaw viability assessment has shown to be of paramount importance. Several techniques have already been suggested for thawing frozen sample. A procedure of thaw and wash allows to remove DMSO and cell fragments, but may cause cell loss or cellular aggregation during centrifugation. Thaw, dilution, and wash procedure avoids the problem due to the centrifugation, allowing an osmolar equilibration, but the untoward effects of DMSO and cell debris infusion are not prevented. Currently, the standard method for thaw frozen MSCs, either from slow freezing or vitrification, is to warm them rapidly (>100°C/min) in a water bath at 37°C, until all ice crystals disappear. This method generally results in high post-thaw recovery of viable cells without using high-cost equipment, but it is safer to thaw cells using a dry warming procedure, due to the potential microbiological contaminations of the water bath [60]. Literature suggests that rapid thawing rates (>100°C/min) that can prevent damaging ice crystals during recrystallization are optimal choice and generally results in the best post-thaw recovery and viability of cells [61]. High post-thaw viability of MSCs, comparable to those thawed with the standard method, were obtained by Thirumala et al. [62] with a thawing procedure in a controlled-rate freezing/thawing chamber at 10°C/min. For evaluating the cryopreservation outcomes in terms of post-thaw cell quality and quantity,

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the selection of the correct viability measurement is essential. The most commonly utilized test, owing to its easiness and quickness, is the Trypan blue dye exclusion assay; however, this method has the disadvantage that it generally overestimates the viable population. Several reports suggested that fluorescence dyes are more accurate and reliable indicators of cell viability [63].

8. Microbiological controls

Biosafety assessment of cryopreserved MSCs is necessary to ensure the safe use of the cells prior to clinical applications. Specific tests for the detection of bacteria, yeast, fungi, mycoplasmas, and viruses should be used as a part of routine and regular quality control screening procedures. To detect low levels of contamination, samples from the cell cultures and their products may be inoculated in either liquid tryptic soy broth (TSB) for the detection of aerobes, facultative anaerobes, and fungi, fluid thioglycollate medium (FTM) for the detection of aerobic and anaerobic bacteria, or onto solid (trypticase soy agar, blood agar, Sabouraud's dextrose agar, and malt extract agar) growth media. These inoculated media may be incubated at different temperatures, reflecting conditions for pathogen culture (37°C) and environmental organisms with lower growth temperature optimal (25°C) in microbiological culture incubators, depending on the specific testing standards used. Mycoplasmas competes with the cells for the nutrients in the culture medium, typical signs of contamination consist in a reduction of the rate of cell proliferation, and changes in cellular physiology including gene expression, metabolism, and phenotype. Among the wide variety of techniques that have been developed to detect mycoplasma contamination of cell cultures, Uphoff and Drexler [64] recommended the PCR analysis for the screening, as it considered the most reliable and useful detection method. The presence of viral agents could be evaluated by a panel of tests to detect pathogens and adventitious viruses. Usually, this panel of tests includes: electronic microscopy, reverse transcriptase detection (as a general test for retroviruses), and other tests to find specific agents, depending on the animal species of the sample.

9. Storage of MSCs

MSCs should be preserved without direct exposure to liquid nitrogen, to reduce the risk of pathogenic cross-contamination. This issue enforces the stem cells banks to store materials at vapor phase of liquid nitrogen. However, recent evidence suggests that storage in vapor phase above liquid nitrogen still carries the risk of cross-contamination [65]. Potentially, infective agents may also enter storage directly from the facility atmosphere, contaminated surfaces, or leaking samples, and they can be accumulated in viable condition. Stem cell banks should also maintain secure liquid nitrogen storage equipment in cryogenic tanks monitored by a specific control and alarm system (−196°C), in order to avoid catastrophic loss of cryopreserved samples. Furthermore, proper storage requires the use of cryovials and labeling systems that

will withstand the intended storage conditions: labels and bar codes or other printing systems are chosen for extended storage periods.

10. Future perspectives

MSC-based therapy is a promising treatment in repair and regeneration of injured and pathological tissues. Nowadays, even if this innovative therapy in veterinary medicine is still limited, stem cell technology has attracted attention and is a quickly evolving field, among either competitive horses or companion animals, due to the limitations of pharmacological and other current therapeutic strategies. The clinical application of autologous or allogeneic MSCs requires a ready off-the-shelf amount of viable cells that maintain unaltered the characteristics of the freshly isolated samples. Although the long experience of cells' processing facilities, consensus is lacking on a universally accepted method for the effective cryopreservation protocol of MSCs and on the maximum time of cryopreserved storage. For these reasons, even if several successful clinical results have been reported by several groups, the methods of stem cells administration need to be improved and the protocols standardized, before a broad spectrum of clinical applications can be successfully achieved. Currently, the Italian Ministry of Health funded a research project to evaluate the safety and efficacy of animal cryopreserved MSCs for allogenic use. These cells are stored and available at the Italian Biobank of Veterinary Resources of IZSLER (http://www.ibvr.org) and the activity is in progress (data not published).

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