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In vitro expansion of mesenchymal stem cells for clinical use

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ABSTRACT

Mesenchymal stem cells (MSCs) now are popular stem cells for clinical applications. To date, MSCs were accepted in various disease treatments with several FDA-approved treatments in some countries. One important requirement for the clinical usage of stem cells is the production of stem cells. Actually, the treatment efficacy of MSC transplantation depends on the quality of transplanted MSCs. This review aimed to present some guidelines for MSC production according to good manufacturing practice that helps to maintain the quality of stem cells from batch to batch as well as the clinical satisfaction.

Keywords: Good manufacturing practice, Mesenchymal stem cells, Clinical application, Stem cell therapy.

Introduction

Stem cell transplantation is a novel treatment method for many diseases, especially degenerative diseases. There are reports of the clinical application of stem cells for more than 70 diseases. Mesenchymal stem cells (MSCs) have become popular for disease treatment in recent years via two approaches: personalized medicine (autologous transplantation) and as a stem cell drug (allogeneic transplantation) (Larsen and Lewis, 2011; Squillaro et al., 2015).

According to clinicaltrials.gov (2015), there are currently about 560 clinical trials using MSCs from several sources (**Fig. 1**). Many clinical trials are being performed in East Asia (176/560) and North America (111/560) (**Table 1**). Although there are more than 20 diseases that can be treated by MSC transplantation, researchers have focused on two groups of diseases: degenerative and

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immune system-related diseases. Unlike other kinds of stem cells, MSCs exhibit two therapeutic properties including a differentiation potential for specific cell types such as osteoblasts (Montespan et al., 2014; Shao et al., 2015), chondroblasts(Berninger et al., 2013; Perdisa et al., 2015), and adipocytes (Gruia et al., 2015; Lee et al., 2015), and immunomodulation of certain kinds of immune cells such as T cells, B cells, natural killer cells, dendritic cells, and T regulatory cells (Cardoso et al., 2012; Melief et al., 2013; Saeidi et al., 2013). Therefore, in earlier clinical studies, MSCs have been differentiated into specific cells to recover the degenerated cells in injured tissues, whereas recent clinical studies have used the immunomodulation of MSCs to treat immune dysfunction.

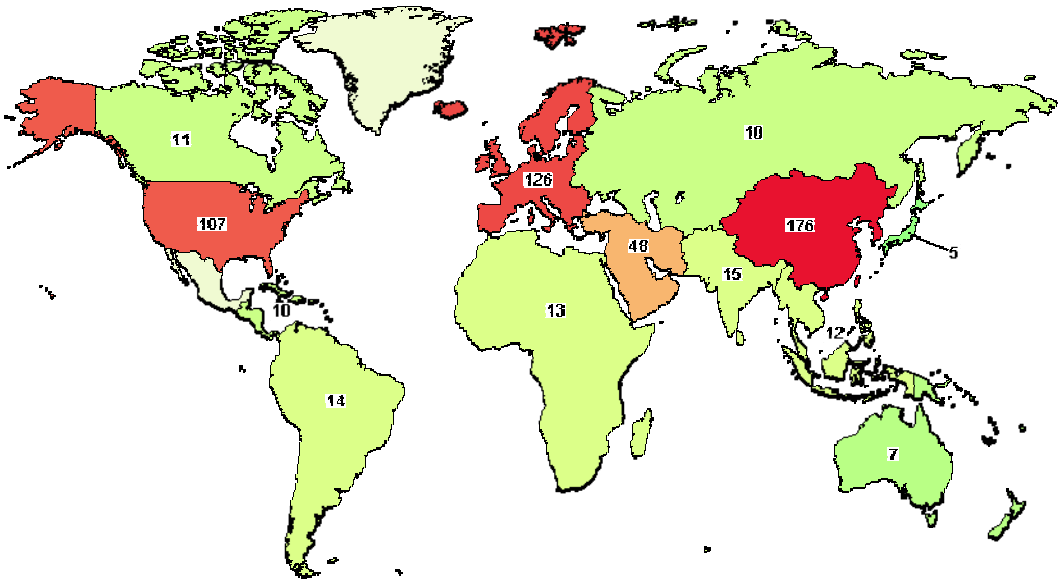

Figure 1. Clinical trials using MSCs according to clinicaltrials.gov. There are more than 500 clinical trials registered at clinicaltrials.gov. Many clinical trials are being performed in East Asia and North America.

Table 1. Distribution of clinical trials using MSCs worldwide (according to clinicaltrial.gov, November 20th, 2015)

Region Name		Number of Studies
Africa		13
Central America		10
East Asia		176
	Japan	5
Europe		126
Middle East		48
North America		111

	Canada	11
	United States	107
North Asia		10
Pacifica		7
South America		14
South Asia		15
Southeast Asia		12
World		560

Recent studies have shown that allogeneic MSCs can perform better immunomodulation than autologous MSCs. These results triggered the use of allogeneic MSCs in clinical applications. Commercialized MSC-based products have been developed and approved as stem cell drugs in some countries (**Table 2**). Osteocel (NuVasive), Trinity (Orthofix), and LiquidGen (Skye Orthobiologics) use allogeneic MSCs as the main component for bone regeneration and reduction of inflammation. MSC-based products have also been approved in Canada and Korea for certain diseases. Cartistem is stem cell drug containing umbilical cord blood-derived MSCs, which was approved in Korea as a drug for osteoarthritis. In 2012, Prochymal (Osiris Therapeutics), an allogeneic MSC-based product, was approved in Canada for graft-versus-host disease treatment. To date (2015), there are nine commercialized MSC-based products approved worldwide. Interestingly, most of them are allogeneic MSC-based products (**Table 2**).

Although MSCs are widely used in clinical treatments, there still are some issues related to the quality and safety of MSCs. In order to maintain MSC quality and reduce the risks after MSC transplantation, MSCs should be produced in accordance with good manufacturing practice (GMP) guidelines.

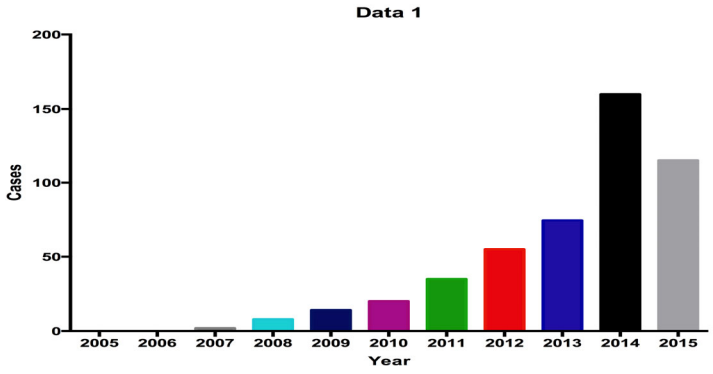

Figure 2. Clinical trials using MSCs (clinicaltrials.gov). The number of clinical trials using MSCs dramatically increase from 2007 to date. In 2015, the number of clinical trials was recorded to June, 2016.

Ex vivo-expanded MSCs

Media

Popular media for MSC expansion are α-minimal essential medium or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum FCS. In recent studies, DMEM/F12 (1:1) has been used as a basal medium for MSCs, except for MSCs from umbilical cord blood, which use Iscove's modified Dulbecco's medium. Using these media, MSCs can be grown but with a long doubling time (more than one month) to achieve useful quantities. To stimulate MSC growth and to reduce the doubling time, cytokines or growth factors (GFs) are added to the medium. Three GFs are commonly used: epidermal growth factor (EGF), basic fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (Tarte et al., 2010). Although these media are simple, inexpensive, and convenient for ex vivo MSC culture, they contain a xenogeneic source of GFs and proteins (from FCS) with a high risk of disease transmission. Potential risks are also related to allergies against xenoproteins and transmission of prions and viruses.

In the next generation of culture media for MSCs, FCS has been replaced by human blood-derived products. Both autologous and xenogenic blood-derived products have been successfully used for ex vivo culture of MSCs. Autologous blood-based products are optimal for ex vivo culture of MSCs for clinical application. However, it is sometimes detrimental for patients to undergo blood withdrawal. Therefore, allogeneic blood has been used as a replacement. Allogeneic samples must be subjected to serological and nucleic acid testing of blood-transmitted viruses, such as human immunodeficiency virus (HIV) and hepatitis C virus, with a supplemental step of virus inactivation before use as a supplement in culture medium.

There are two forms of blood-based products used in ex vivo culture: plasma platelet lysate (PL) and platelet rich plasma (PRP). Recent reports show that PRP is the most reliable and used product to grow MSCs from diverse sources, such as bone marrow, adipose tissue, umbilical cord, and dental pulp tissue. Only 2%–8% PRP stimulates MSC proliferation with a higher efficacy than FCS (Fekete et al., 2012). In fact, PRP is a pool of many GFs, including EGF, acidic FGF, PDGF, transforming growth factor, keratocyte growth factor, hepatocyte growth factor, and insulin-like growth factor. These are human GFs and efficiently stimulate MSCs compared with bovine GFs in FCS (Fekete et al., 2012). Studies have shown that PL- or PRP-based media efficiently maintains the phenotype and genotype of cells in long-term culture. Furthermore, the self-renewal, differentiation potential, and surface marker expression of MSCs are preserved during long-term culture in PL- or PRP-supplemented medium.

Although in vitro-cultured MSCs in media based on PL or PRP are clinically used to treat diseases via local injection or intravenous transfusion, several independent reports show that PRP or PL can drive spontaneous differentiation of MSCs in vitro. For example, Kasten et al. showed that bone marrow-derived MSCs grown in medium supplemented with PL commit to an osteoblastic lineage (Kasten et al., 2008), whereas Van Pham et al. (2013) showed that PRP drives ADSC differentiation into chondroblasts(Van Pham et al., 2013). For this reason, depending on the application, medium supplemented with PL or PRP should be carefully evaluated before use in ex vivo culture.

The third generation of media is completely defined and lacks any biological products from animal or human origins. At least five companies have successfully developed this type of medium. To replace non-defined components such as FCS, PL, and PRP, GF cocktails have been used to supplement culture media. Some of these media are produced under GMP guidelines and have obtained FDA approval as medical devices. These media also maintain the phenotypic and functional characteristics of cultured MSCs (Chase et al., 2010). The most significant problem of these media is the use of a specific protein to ensure primary cell attachment. In FCS, PL or PRP, there are proteins that facilitate MSC

attachment to the surface of flasks or dishes. Conversely, for defined media, substrates must be coated to the flask/dish surface before plating MSCs to assist MSC attachment. Although xenogeneic proteins have been removed in this culture system, some coating substrates originate from animal or non-defined components.

Culture platforms

To date, there are two platforms for ex vivo culture of MSCs: monolayer and suspension culture. In both platforms, MSCs must adhere to a surface. In fact, MSCs only grow in an adherent state. In monolayer culture, MSCs are plated in flasks or dishes with a treated surface. In a clinical study, T75 or T125 flasks showed more advantages than T25 flasks. In suspension culture, MSCs adhere to microbeads suspended in media. Suspension culture of MSCs on microbeads is a new technique and easy to scale up to obtain MSCs in short-term culture (Hervy et al., 2014; Wise et al., 2014). However, most clinical applications of MSCs use monolayer culture.

Monolayer culture is the traditional technique to culture MSCs. This technique allows MSCs to proliferate for a long time without changes in MSC properties or genetic stability. Studies show that MSCs maintain their karyotype until the 25th passage (Chen et al., 2014b). In another study, aneuploidy has been detected by some studies when MSCs are cultured (Tarte et al., 2010). However, they also confirmed that these modifications did not cause tumorigenesis of MSCs (Chen et al., 2014a; Tarte et al., 2010; Wang et al., 2013). MSCs also maintain stemness after long-term culture with a stable phenotype, self-renewal, and differentiation potential (Wang et al., 2013). Although monolayer culture has a high risk of contamination withbacteria or fungi because of many steps during culture depending on manipulators as well as the need for a class A cabinet, monolayer culture of MSCs is the standard for ex vivo expansion of MSCs. Most expanded MSCs used in clinical applications are cultured as a monolayer. Using this technique, the quality of the flask or dish is very important. In well-treated flask surfaces, MSCs growbetter. Ventilated flasks are recommended for MSC monoculture. Closed systems for MSC expansion have been developed in recent years. Closed culture systems are considered to be optimal for clinical applications of MSCs. They can significantly decrease the number of steps, exposure to the environment, and the risk of contamination. However, not all closed systems for ex vivo cell culture satisfy GMP requirements. The first generation of closed systems was multilayer, such as CellStacks (Corning, Corning, NY, USA) or Cellfactory (Nunc, part of Thermo Fisher Scientific Inc., Waltham, MA, USA), which could be stacked in incubators. These systems increase the surface area for culture to enable the cell expansion up to 1 billion pure MSCs in 2–3 weeks (Tarte et al., 2010). However, many limitations still exist because they were not completely closed systems and required a class A cabinet for each manipulation.

The second generation is a fully closed and automated bioreactor. The main advantages of bioreactors are a large surface area to volume ratio, a closed system, automated inoculation and harvesting, and automated control of culture parameters. Terumo (Somerset, NJ, USA) has developed a fully automated bioreactor based on hollow-fiber technology to allow large-scale expansion of MSCs in a GMP-compliant system (Rojewski et al., 2013). Although this system can provide optimal tools for delivering MSCs of clinical grade, which comply with GMP, the behavior or properties of MSCs can change in this platform (Guo et al., 2014). In a recent study, a low oxygen concentration was used to maintain the growth and genetic stability of MSCs cultured in suspension culture (Bigot et al., 2015; Estrada et al., 2012; Hung et al., 2012; Oliveira et al., 2012). In another report, three-dimensional culture increased the anti-inflammatory properties of MSCs (Bartosh et al., 2010; Hong et al., 2015).

Harvesting adherent cells

MSCs must be cultured as adherent cells in both monolayer and suspension culture. After expansion,

MSCs should be harvested by an enzyme. Trypsin/EDTA solution is popularly used to detach MSCs from the surfaces of culture dishes/flasks or microbeads. However, trypsin is usually derived from porcine, and not optimal for GMP production of MSCs. Some recombinant enzymes produced under GMP compliance can replace Trypsin/EDTA, such as TrypLE (Invitrogen, Thermo) and TrypZean (Sigma-Aldrich, St Louis, MO).

These second-generation enzymes are gradually being used to harvest MSCs for clinical use. Mechanical detachment using cell scrapers has also been suggested to harvest cells cultured in dishes or flasks. Although a cell scraper-based method is simple, the percentage of alive detached cells can be affected. Recently, a new de-attachment method with GMP compliance combining EDTA and chilling was patented.

Cryopreservation of cellular products

There are two forms of MSC cryopreservation. Commonly, 1.5 or 2.0 mL cryotubes are used to store MSCs in cryopreservation medium. However, a vial only holds about 1×10^7 cells which is insufficient for transplantation. In fact, for MSC transplantation, 1×10^6 cells per kg of weight is required. Therefore, similar to HSC cryopreservation, some studies have used bags for MSC cryopreservation. However, the protocols for MSC cryopreservation may be different to HSC cryopreservation. Prochymal is a commercial product containing MSCs cryopreserved in a bag, whereas Cartistem contains MSCs cryopreserved in penicillin vials.

Cryopreservation media significantly affects the quality of MSCs after thawing. They not only directly affect MSC viability but also factors affecting clinical usage. Traditionally, culture media with serum and 10% DMSO have been used in most studies. DMSO is a popular cryoprotectant. However, it also has some limitations, especially because it damages cells when presenting at high concentrations during the thawing procedure. Moreover, if DMSO is not completely removed from the cryopreserved cells, it can cause adverse reactions in patients, such as nausea, vomiting, tachycardia, bradycardia, and hypotension. Therefore, in recent years, a second generation of cryopreservation medium with other kinds of cryoprotectants has been developed, such as methylcellulose, sucrose, trehalose, glycerol, hydroxyethylstarch, polyvinylpyrrolidone, and various combinations of these cryoprotectants. However, reports show that none of these cryoprotectants are superior to DMSO. Hence, recent studies have tried to reduce the percentage of DMSO to 5% or 2%. In addition to DMSO, the serum in medium also affects MSC quality. MSCs can be well preserved in 10% DMSO and 90% FCS. However, the high ratio of animal serum can cause some adverse effects in patients. Therefore, in recent studies, FCS has been reduced to 10% or replaced with human serum. However, cryopreservation medium containing serum also has risks related to viral transmission or xenoprotein-related reactions. Auto-serum is suitable to replace animal serum or allogeneic serum. Currently, defined, serum-free and animal component-free freezing media have been developed and commercialized, such as Cryostor™ CS 10 (StemCell Technologies), Plasmalyte-A (Baxter), and Synth-a-Freeze (Gibco, Thermo).

There are two methods for freezing cryotubes for MSC cryopreservation controlled rate freezing and uncontrolled freezing (three step freezing). In the control rate freezing method, a rate of 10°C per minute has been applied with good results of viable thawed cells. The three-step freezing method involves the cells passing through three temperatures: (1) 4°C for 30–60 min, (2) −20°C for 60–120 min, and (3) −85°C overnight, and then storage in nitrogen liquid. Although control-rate freezing is clearly better than uncontrolled freezing, the most significant limitation of controlled rate freezing is the high cost of controlled rate freezing systems. At present, cryopreservation boxes have been developed. Using these boxes, the freezing rate is controlled but fixed at a specific rate. These boxes are inexpensive and can be used for MSC cryopreservation. After cryopreservation, the thawing method significantly contributes to MSC quality, especially cell viability. Commonly, MSCs are rapidly thawed by incubating the vials in a 37°C water bath

for 1–2 min. The cells are then centrifuged to remove DMSO/cryoprotectants and cryopreservation medium.

Control of MSC quality

The first issue relates to MSC characteristics. Expanded MSCs should maintain their phenotypes in long-term culture. Spontaneous differentiation of MSCs always occurs during in vitro or ex vivo culture because of a heterogeneous population of MSCs. This process will proceed quickly or slowly depending on the culture conditions, especially the culture medium. Some studies have added GFs to inhibit spontaneous differentiation of MSCs. However, before application to patients, MSC characteristics must be checked.

Similar to other types of stem cells, MSCs have two important properties, self-renewal and a differentiation potential. Self-renewal is evaluated by a clonogenicity assay. This test involves seeding cells at densities of 1.5, 3, 5, and 10 cells/cm^2 in a 100-mm Petri dish. It is simple, inexpensive and highly reproducible. However, the time needed for this assay is longer than the shelf-life of the final product. Therefore, this assay should be performed during evaluation of the production procedure. Although MSCs exhibit self-renewal, they also undergo senescence after long-term culture. MSCs typically proliferate for 20–50 doublings, depending on the cell source and culture conditions (Cholewa et al., 2011; Izadpanah et al., 2006; Suchanek et al., 2007). Senescent cells display aneuploidy without transformation and exhibit mutations in certain genes, such as the p53 gene (Tarte et al., 2010), upregulation of hyaluronan and proteoglycan link protein 1, keratin 18, brain-derived neurotrophic factor, or renal tumor antigen, and downregulation of pleiotrophin(Schallmoser et al., 2010). To date, senescence is easy to evaluate by a β-galactosidase staining assay.

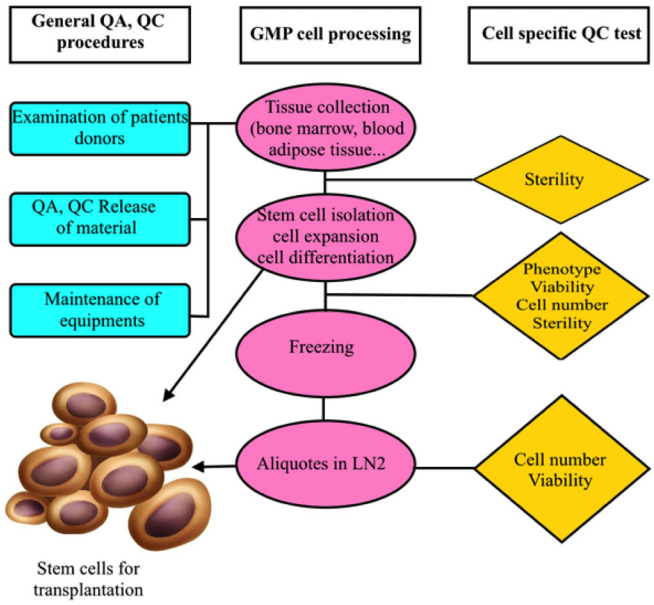

Figure 3. Flowchart of GMP-compliant production of MSCs for clinical application. All steps from donor selection to storage and delivery should be controlled and recorded.

Differentiation is also an important characteristic of MSCs. According to Dominici et al., MSCs must be able to differentiate into three kinds of mesodermal cells, namely, osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006). This suggestion has been used as a guideline to evaluate MSCs. Some reports show that

senescent MSCs have a reduced differentiation potential for only osteoblasts. Differentiation assays are easy to perform with commercial differentiation kits. When cultured in inducing medium for 14–21 days, MSCs differentiate into adipocytes, osteoblasts, or chondroblasts depending on the media. Similar to self-renewal testing, differentiation potential tests are also performed for 2–3 weeks. Therefore, this test is usually applied during evaluation of the production procedure.

To evaluate MSC quality before transplantation, there are two popular indicators, surface marker expression and cell viability. Assessment of both can be carried out by flow cytometry. For cell viability, collected MSCs are stained with 7-amino-actinomycin D (7-AAD), and dead cells are identified based on the signal of 7-AAD. Although there is no guideline or regulation concerning the percentage of live MSCs for clinical grafts, most studies only use MSC samples with more than 95% live cells. In terms of surface markers for MSCs, according to Dominici et al (2006), there are two groups of markers used to confirm MSCs: positive markers (CD13, CD44, CD73, CD90, and CD105) and negative markers (CD14, CD34, CD45, and HLA-DR). Profile marker kits for these have been commercialized to confirm MSC phenotypes (Dominici et al., 2006).

Conclusion

MSC production with GMP compliance (**Fig.3**) appears to be a compelling condition to use MSCs in clinical application. GMP will maintain the quality and safety of MSCs. Clinical grade MSCs are only produced by application of regulations as well as the requirements or elements of GMP. However, all procedures should originate from clinical demands. GMP is not a standard but a set of guidelines or rules for production procedures with the highest quality and safety.

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Competing Interests

The authors declare they have no competing interests.

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