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Production of mesenchymal stem cell derived-secretome as cell-free regenerative therapy and immunomodulation: A biomanufacturing perspective

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Abstract: The potential of mesenchymal stem cells (MSCs) in regenerative medicine has been largely known due to their capability to induce tissue regeneration *in vivo* with minimum inflammation during implantation. This adult stem cell type exhibit unique features of tissue repair mechanism and immune modulation mediated by their secreted factors, called secretome. Recently, the utilization of secretome as a therapeutic agent provided new insight into cell-free therapy. Nevertheless, a sufficient amount of secretome is necessary to realize their applications for translational medicine which required a proper biomanufacturing process. Several factors related to their production need to be considered to produce a clinical-grade secretome as a biological therapeutic agent. This viewpoint highlights the current challenges and considerations during the biomanufacturing process of MSCs secretome.

Abbreviation

MSCs:	Mesenchymal stem cells
CM:	Conditioned medium
FBS:	Fetal bovine serum
LPS:	Lipopolysaccharides
IFNs:	Interferons
IL-1β:	Interleukin 1 beta
IL-6:	Interleukin 6
IL-7:	Interleukin 7
IL-8:	Interleukin 8
IL-11:	Interleukin 11
IL-12:	Interleukin 12
HGF:	Hepatocyte growth factor
TNF-α:	Tumor necrosis factor α
TGFβ1:	Transforming growth factor-beta 1
VEGF:	Vascular endothelial growth factor
BDNF:	Brain-derived neurotrophic factor
IGF-1:	Insulin-like growth factor 1
bFGF:	Basic fibroblast growth factor
EGF:	Epidermal growth factor
GM-CSF:	granulocyte/monocyte growth factor
M-CSF:	Macrophage colony stimulating factor
PGE2:	Prostalglandin E2
miR-21:	microRNA-21
miR-210:	microRNA-210
IDO:	Indoleamine 2,3-dioxygenase
CXCL5:	C-X-C Motif Chemokine Ligand 5

Brief Introduction

The capability of mesenchymal stem cells (MSCs) to differentiate into multiple lineages with less immune rejection makes them become a good candidate for cell-based regenerative therapy. Aside from their applications in cell-based therapy, the study of MSCs secretome as a cell-free therapeutic agent was increased significantly. This secreted compound were natively produced by MSCs to maintain their homeostasis and homing mechanism *in vivo*. Based on their original function, several studies revealed that the secretome-contained-conditioned media shows therapeutic effects such as immunomodulation and improving tissue regeneration through a similar mechanism with their native function *in vivo* (Ahangar *et al.*, 2020). Compared to cell-based therapy, the secretome exhibits

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less immunogenicity because of the absence of immunogenic surface proteins that are expressed in living cells, which much advantageous for translational medicine (Vizoso *et al*., 2017).

Currently, the importance of regenerative medicine and immunomodulation has even become greater. Since a new variant of coronavirus, entitled severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified in late 2019, World Health Organization (WHO) denominated coronavirus disease 2019 (COVID-19) as a global pandemic infection. This disease makes the lower respiratory tract more prone to infection, which results in acute lung injury/acute respiratory distress syndrome (Anft *et al*., 2020). Hence, the MSCs secretome introduces a possible cell-free regenerative approach for tissue regeneration and immunomodulation which is urgently needed in this COVID-19 pandemic era.

Composition of MSCs secretome and its therapeutic potential

Secretome consisted of a complex mixture of bioactive compounds that were originally secreted by the cells into the extracellular environment, which are, composed of many factors such as peptides, proteins (including growth factors/cytokines or enzymes), nucleic acid, lipids, metabolites, or extracellular matrix. These biomolecules can be secreted as a free soluble form or contained inside the extracellular vesicle (Beer *et al*., 2017). The detailed composition of each component was previously described by Tsuji *et al*. (2018).

To the best of our knowledge, most of the current studies are concentrated on nucleic acid, peptide, growth factors/hormone/cytokine, enzymes, extracellular matrix, and Lipid components (Table 1). These compounds have been known to contribute to some therapeutic potential, which can be produced by *in vitro* MSCs culture for possible translational medicine. To date, some trials was performed to assess their effectiveness toward clinical treatment, for example, skin regeneration (Kim *et al*., 2020) or wound healing (Gugerell *et al*., 2021; Simader *et al*., 2017). Recently, a large number of clinical trials for secretome based-immunomodulation and regenerative therapy for COVID19 also has been reported (Chouw *et al*., 2021). Since the secretome consisted of multiple bioactive components, further *in vivo* comparison study between administration of manually formulated known bioactive components and MSCs derived-secretome complex. This information may provide additional insight into secretome efficacy in a disease-specific application.

The importance of optimum secretome biomanufacturing process

To realize their translational application, an adequate amount and a proper quality of secretome need to be produced for several therapeutic applications through biomanufacturing procedure which needs to be optimized for secretome production (Fig. 1). Furthermore, the MSCs secretome which was produced *in vitro* consisted of a similar cocktail that regulates various mechanisms as a response to a certain condition in the native tissue environment. Since this condition can be partially mimicked by the culture system, the culture conditions can be modified based on the desired therapeutic effect. A recent proteomic- and bioinformatic-study by Wangler *et al*. (2021) revealed that the exposure of healthy, traumatic, or degenerative-conditioned medium may stimulate the MSCs to secrete a similar protein complex which contributed to their response in each specific condition *in vivo*.

The secretome variability among different MSCs sources and individual

MSC can be isolated from a wide range of sources including bone marrow, adipose tissue, umbilical cord, liver, and dental tissues. Although there is a general similarity in composition among MSCs secretome from various resources, the secretome profile and quantity can be varied among the tissue-specific sources, depending on the donor`s physiological condition and their niche of surrounding tissue (Billing *et al*., 2016). This variability may contribute to their therapeutic effect in clinical applications. Therefore, secretome profiling of donor condition- and tissue-specific MSCs are essentially required to be further evaluated to obtain the general profile of secretome components that potentially play a specific role in tissue regeneration and immunomodulation. For example, a study revealed that there is a different concentration of proangiogenic factors among MSCs secretome derived from different sources, such as human adipose tissue, bone marrow, and umbilical cord Wharton's jelly (Kehl *et al*., 2019). Another study showed that the MSCs derived from various patient conditions secreted different amounts of anti-inflammatory factors (Gray *et al*., 2016).

Selection of culture system for secretome production

Another consideration related to large-scale biomanufacturing needs to be carefully considered in the selection of the culture system. For this purpose, the three-dimensional (3D) suspension culture system is much preferred over conventional monolayer (2D) culture. This system may eliminate the necessity of growth surface which may be limiting the scalability. Additionally, the 3D tissue-like formation can provide better cell-cell interaction which resulted in a significantly higher secretome production (Kim *et al*., 2020; Miranda *et al*., 2019).

Several culture systems was commonly applied to scale up the MSCs, such as Spinner flask, stirred tank bioreactor, vertical wheel bioreactor, or roller bottle. Each culture system exhibits different designs and operations that may impact the released secretome profile (Hassan *et al*., 2020). One of the potential biomanufacturing approaches to improving the quality and cost-effectiveness is by using dialysis culture technologies, such as hollow fiber (Barckhausen *et al*., 2016) or simple dialysis culture by modified culture insert with 4–12 kDa molecular weight cutoff dialysis membrane on the bottom side (Torizal *et al*., 2021). This culture system enables the localization and accumulation of high molecular weight secretome components, such as the one that enveloped by extracellular vesicle or as complex soluble proteins while continuously refining the small molecule nutrition in the basal media by continuous removal of waste metabolic byproducts, such as lactate and NH_3 (Fig. 2), and supplying the nutrition from the culture medium (Torizal *et al*., 2021). In addition, this system also enables a cost-effective high-density culture that not only saves the exogenous synthetic growth factors usage but also accumulated and concentrates the secretome in a certain compartment, making it easier to isolate and characterize (Barckhausen *et al*., 2016; Torizal *et al*., 2021).

TABLE 1

Several examples of MSCs secretome components and their possible therapeutic potential

MSCs secretome component		Source	Possible therapeutic potential	Reference
Nucleic acid	miR-21	Bone marrow-derived-MSCs	Improved intravertebral disc regeneration	(Cheng *et al.*, 2018)
	miR-210	Bonemarrow-derived-MSCs	Promotion of angiogenesis in myocardial infarction	(Wang *et al.*, 2017)
Peptide	Human β-defensins	Bonemarrow-derived-MSCs and Umbilical Cord-MSCs	Growth inhibition of Gram-negative (E. Coli)	(Sung *et al.*, 2016)
	Lipocalin-2	Bone marrow-derived-MSCs	Inhibition of bacterial growth	(Gupta *et al.*, 2012)
	Cathelicidin LL-37	Bone marrow-derived-MSCs	Growth inhibition of Gram-negative (P. Aeruginosa) and Gram-positive (S. Aureus; S. Pneumoniae)	(Sutton *et al.*, 2015)
	Hepcidin	Bone marrow-derived-MSCs and Menstrual-MSCs	Growth inhibition of bacteria	(Alcayaga-Miranda *et al.*, 2015)
Growth factors, hormones, and cytokines	CXCL5	Adipose-derived-MSCs	Regeneration during cavernous nerve injury	(Zhang *et al.*, 2013)
	bFGF, EGF, HGF	Adipose-derived-MSCs	Hematopoiesis, vasculogenesis, and mammary epithelial duct formation	(Kilroy *et al.*, 2007)
	GM-CSF, M-CSF	Adipose-derived-MSCs	Regulation of hematopoiesis	(Kilroy *et al.*, 2007)
	IL-7 IL-6, IL-8, IL-11, TNFα	Adipose derived-MSCs	Proinflammatory factors	(Kilroy *et al.*, 2007)
	PGE2	Bone marrow-derived-MSCs	Regulating the proinflammatory response	(Aggarwal and Pittenger, 2005)
Lipids	Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, lysoPC, and sphingomyelin	Bone marrow-derived-MSCs	Promoting inflammatory response against infection	(Campos *et al.*, 2016)
Enzyme	Indoleamine 2,3-dioxygenase (IDO)	Bone marrow-derived-MSCs	Growth inhibition of Gram-positive (S. Aureus; S. Epidermidis; E. Faecium; Group B. Streptococci) and parasite (Toxoplasma gondii), and reduction in virus replication (cytomegalovirus)	(Meisel *et al.*, 2011)
Extracellular matrix	Fibronectin, laminin, and Type I collagen	Adipose-derived-MSCs	Improved macrophage differentiation and secretion of their proinflammatory cytokines, such as IL-6, IL-8, IL-10, and MCP-1. Enhanced osteogenic differentiation	(Novoseletskaya *et al.*, 2020)

Effects of the hydrodynamic condition of culture systems on the release of regenerative- and immunomodulatory factors

The spheroid suspension culture enables a higher density culture which significantly enhanced cell production per volume unit than the monolayer culture. Their tissue-like-three-dimensional structure provides more physiologically relevant conditions which support a better production of their regenerative- and immunomodulation cytokine (Cesarz and Tamama, 2016).

To improve the oxygenation and proper mixing of the medium, a dynamic scalable culture system such as a spinner flask can be employed to proliferate the MSCs to produce a larger amount of secretome. Although the excess hydrodynamic stress can induce cell death, the proper amount of these hydrodynamic stimuli has been known to improve the release of specific cytokine composition related to their differentiation, tissue regeneration, and immunomodulation.

The previous study was revealed that the exposure of hydrodynamic force from MSCs spheroid expanded in suspension using bioreactor was significantly enhanced the production of BDNF, VEGF, and IGF-1, which is an important component to induce regeneration in various tissue (Marques *et al.*, 2018).

Generating expected secretome composition by biochemical stimulation

Naturally, MSCs produce cytokine factors that modulate inflammation and regeneration as the response to certain stimuli that occurred in the tissue. In *in vitro* conditions, the

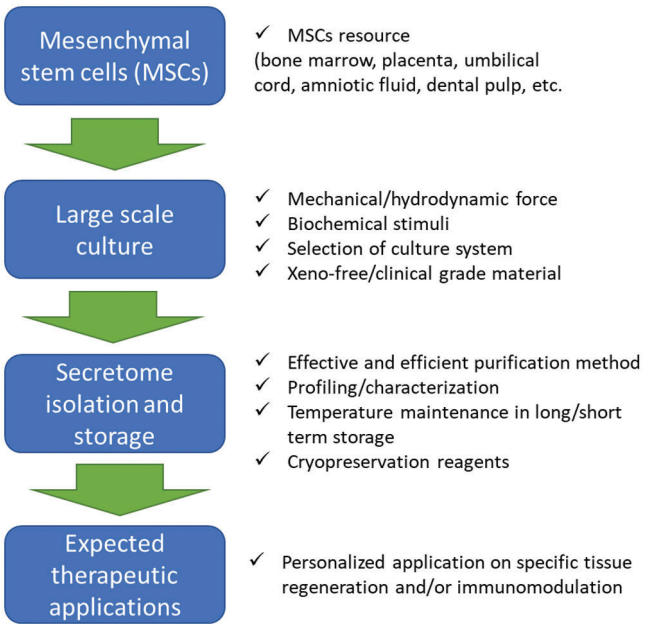

FIGURE 1. Several factors need to be considered to improve secretome production from MSCs.

profile of secretome components may be adjusted by mimicking this *in vivo* condition (Merino-González *et al.*, 2016). Several studies have shown that expanded MSCs in *in vivo* like-hypoxic conditions can significantly enhance the proliferation and production of autocrine factors (Paquet *et al.*, 2015; Teixeira *et al.*, 2015). The low oxygen concentration caused by ischemic-like conditions through the addition of H_2O_2 has been known as an induction method to increase the secretion of proangiogenic factors that can be useful during tissue regeneration, such as HGF and VEGF (Bai *et al.*, 2018). Another example is the addition of immunogenic substances such as lipopolysaccharides (LPS) that may induce the production of proinflammatory cytokines, such as IL-1, IL-6, IL-10, IFNs, IL-12, and TNF-α altogether with regeneration factor HGF and VEGF (Lee *et al.*, 2015; Ti *et al.*, 2015); while the inclusion of IFN-γ and TNF-α in culture media has shown to increase the release of cytokines complex that regulate anti-inflammatory factors and bone regeneration (Lu *et al.*, 2017; Sivanathan *et al.*, 2014).

The necessity of xeno-free materials for clinical-grade secretome production

Since the MSCs secretome would be applied for human therapy, the usage of animal-derived (xenogeneic) material needs to be avoided. For example, the fetal bovine serum (FBS) may contain non-human allergenic proteins or unknown transmittable pathogenic substances (Panchalingam *et al.*, 2015; Versteegen, 2016). In addition, this serum also consisted of variability in composition which may result in a heterogeneity of batch to batch secretome composition (Pachler *et al.*, 2017).

The development of serum-free media may provide a solution for this problem. Recently, some xeno-free and GMP (good manufacturing practice)-grade media such as human platelet lysates (HPL) and serum-free media/xeno-free FDA-approved culture medium (SFM/XF) was generally showed similar performance of secretome components compared to FBS-based medium (Guiotto *et al.*, 2020; Oikonomopoulos *et al.*, 2015). Nevertheless, the HPL based-medium formulation showed a lower production of immunosuppressive-related cytokine compared to the FBS-based medium which possibly correlated with a complex pathway inhibition induced by the HPL supplementation component that still needs to be investigated (Oikonomopoulos *et al.*, 2015).

MSCs secretome isolation method and storage

The usage of high-abundance serum protein such as Albumin from supplemented FBS was often mask the lower-abundance

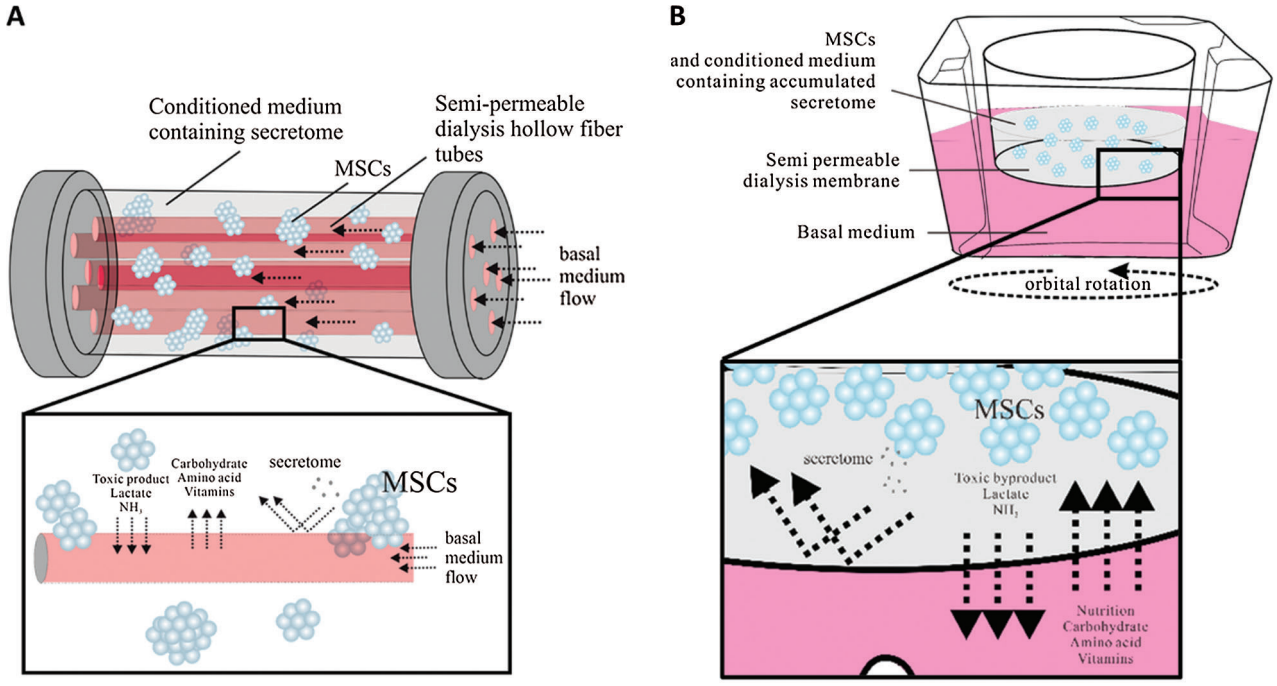

FIGURE 2. Suggested dialysis-based-culture system for MSCs secretome production: (A) Hollow fiber bioreactor and (B) simple dialysis culture system.

secretome, both in free form and in extracellular vesicles. Therefore, this component needs to be separated from the conditioned medium (CM) (Stastna and van Eyk, 2012). Centrifugation is the current common method to recover the secretome from the serum protein enriched-medium. However, the centrifugal force resulted from the centrifugation may disrupt the secretome, resulting in low recovery and decreased bioactivity (He *et al.*, 2014; Helwa *et al.*, 2017). Several technologies have been developed to improve the isolation with high purity, such as antibody- or chromatography-based isolation to separate the secretome from the serum protein used in MSCs culture, but these methods require a high-cost operation. High throughput and cost-effective isolation methods need to be developed in the future (Li *et al.*, 2017). Alternatively, the serum free-media as previously described can be used to address this problem.

In general, the isolated secretome can be stored at –20°C–80°C for up to 6 to 7 months (Zhou *et al.*, 2006). Increasing temperatures, such as more than 4°C or 37°C may slowly degrade cytokine or extracellular vesicle as well as its containing components (Yu *et al.*, 2014). The freeze-thaw cycle also needs to be minimized to preserve the structure and function of the secretome components. In order to reduce the degradation and preserve the proteins component, the aid by protease inhibitor is strongly suggested (Zhou *et al.*, 2006).

Conclusion and Future Perspectives

MSCs secretome holds a therapeutic potential to improve tissue regeneration and modulates the immune response *in vivo*. However, several challenges need to be addressed to ensure the efficacy of their translational applications. First, the accurate secretome characterization based on the MSCs' source and culture condition needs to be analyzed to obtain their general profile. Based on the profiling pattern, the specific resource can be selected to obtain the expected secretome composition. Secondly, further study needs to be performed to explore possible adjustment of the culture environment or induction that may direct the release of a specific composition of the secretome. The specific induction can be tailored to produce a secretome composition that can be specifically targeted for personalized regenerative- of disease-specific therapeutic effect; and finally, the improvement and standardized methods for secretome biomanufacturing, including large-scale culture systems, isolation, and storage is necessary to realize a safe, potent clinical grade cell-free therapy.

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