

A METHOD OF LYOPHILIZATION OF MESENCHYMAL STROMAL CELL (MSC)SECRETOME FOR BIOPROTEINS AND BIOACTIVES PRESERVATION**Field of Invention**

5 The present invention relates to a method for lyophilizing secretome. More particularly, the present invention relates to a method of lyophilization of mesenchymal stromal cell (MSC) secretome for bioproteins and bioactives preservation.

10 Background of the invention

Lyophilization is a water removal process typically used to preserve perishable materials, to extend shelf life, or make the material qualitatively more stable and convenient for transportation. Lyophilization works by freezing the material, then reducing the pressure and adding heat to allow the frozen water in the materials to sublime. Mesenchymal stromal cell (MSC) secretome is typically subjected to lyophilization for stability, long-term storage, transportation, and usage from the needs of super-freezing condition at about -20°C, and to be transported in a freeze box without degrading all of the important bioproteins and bioactives in it. The cell-free secretome derived from MSCs has the capability to recapitulate many of the properties and effects that have been described from its parent cell, and even though it is regarded as the by-

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product of the MSCs, it consists with abundance of bioactive compounds that has regenerative, anti-apoptosis and immunomodulator properties. In order to preserve these properties and to be able to be used as a raw material, it
5 needs to exist in a more stable form. Its current form that can exert and retain such properties is when the MSC secretome liquid is stored at -20°C. The method of freeze drying or lyophilization is opted as it is the most efficient procedure to change the state of the MSC secretome
10 into a dry powder form and still retain some of the highly valuable bioproteins and bioactives, and later be used as a regenerative product. Currently, the lyophilization process includes only three steps/stages, that includes pre-freezing, primary drying (sublimation), and secondary drying
15 (desorption) stage. The currently known technique of lyophilization has several disadvantages including:

- a) The MSC secretome is unable to uniformly and consistently be dried off to the desired moisture content, hence impacting the efficacy and stability of
20 the product.
- b) The bioprotein is unable to be stable in the final end product after lyophilization has completed.
- c) The product yield with the current technique introduces significant problems including slanted cake, lifted
25 cake, dusting, cracked cake, and worst is melt-back.

- d) The duration of the MSC secretome to exist in its unstable liquid form is longer during the pre-freezing stage after being freeze-thawed to be prepared for the lyophilization process.
- 5 e) Degradation of the bioprotein is highly possible by applying the current techniques, and hence this will affect the end concentration and quality of the intended final product.
- 10 Therefore, in light of the foregoing discussion, there exists a need to overcome the aforementioned drawbacks associated with the conventional lyophilization technique.

These and other objects and advantages of the present
15 invention will become apparent to those skilled in the art from a consideration of the following specification and claims.

Summary of the Invention

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According to an aspect of the present invention, there is a provided method of lyophilization of Mesenchymal Stromal Cell (MSC) secretome for bioproteins and bioactives preservation. The method includes obtaining a frozen MSC
25 secretome. The method also includes preparing a vial using

a natural liquid form of MSC secretome and mixing each of the vial with a cryoprotectant of selection and stored at -20°C. The method also includes loading the vials containing the mixture into a freeze dry machine shelf pre-cooled to
5 about -20°C.

In an embodiment, prior to loading the vial into the freeze dry machine shelf, the frozen MSC secretome is thawed in a biosafety cabinet at 16°C to 20°C for a duration of about 15 to 20 minutes. The MSC secretome is pipetted into the vial
10 after being completely thawed around 4°C to 7°C and the cryoprotectant of selection is added into each of the vial. The mixture of the MSC secretome and the cryoprotectant of selection is mechanically mixed until homogenous. A rubber stopper is placed on the vial and the vial is stored in the
15 freezer at -20°C.

In an embodiment, the freeze dry machine shelf temperature is pre-cooled to about -20°C for about 1-2 hours to reach a set temperature, during preparing the vial.

In an embodiment, the vial is loaded into the freeze dry
20 machine shelf that is pre-cooled, upon the freeze dry machine shelf reaching about -20°C and setting the temperature to -40°C for pre-freezing and -20°C during the drying process. The pre-freezing and drying process in the freeze dry machine shelf is performed for about 12 to 48
25 hours.

The vacuum chamber is released and the door is opened for unloading the vials for crimping process, testing and storage. In an embodiment the cryoprotectant of selection to be mixed with the MSC secretome is chosen from at least one
5 of: mannitol, sucrose and trehalose, depending on a final product appearance intended. In an embodiment, the freeze dry machine shelf is pre-cooled for a duration of 1 to 2 hours.

10 The lyophilization of Mesenchymal Stromal Cell (MSC) secretome for bioproteins and bioactives preservation of the present technology produces a product that is not only highly stable and suitable for long-term preservation and easy to be transported, but also soluble in certain diluents
15 to be reconstituted as a raw starting material for product development and can be mainly used aesthetically for skin rejuvenation, regeneration, anti-apoptosis and immunomodulation agents.

20 These and other aspects of the embodiments herein will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following descriptions, while indicating preferred
25 embodiments and numerous specific details thereof, are given

by way of illustration and not of limitation. Many changes and modifications may be made within the scope of the embodiments herein without departing from the spirit thereof, and the embodiments herein include all such
5 modifications.

Brief Description of the Drawings

Other objects, features, and advantages of the invention will be apparent from the following description when read
10 with reference to the accompanying drawings. In the drawings, wherein like reference numerals denote corresponding parts throughout the several views:

Figure 1 depicts a freezer from which a frozen mesenchymal
15 stromal cell (MSC) secretome is initially obtained for lyophilization on the MSC secretome and later for storage of vial mixed with MSC secretome and cryoprotectant, in accordance with an exemplary scenario;

Figure 2 depicts a biosafety cabinet for thawing, mixing and
20 preparing the MSC secretome, in accordance with an exemplary scenario;

Figure 3 depicts a freeze dry machine shelf, in accordance with an exemplary scenario;

Figure 4 depicts the freeze dry machine shelf during the pre-freezing and drying process, in accordance with an exemplary scenario; and

Figure 5 illustrates a flow diagram depicting the steps
5 involved in a process of lyophilization of Mesenchymal Stromal Cell (MSC) secretome for bioproteins and bioactives preservation, in accordance with an embodiment.

10 Detailed Description of the Preferred Embodiments

In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of the invention. However, it will be
15 understood by those of ordinary skill in the art that the invention may be practiced without these specific details. In other instances, well-known methods, procedures and/or components have not been described in detail so as not to obscure the invention. Reference will now be made in detail
20 to the preferred embodiments of the present invention, examples of which are illustrated in the accompanying drawings.

Various embodiments of the present invention provide a
25 method of lyophilization of Mesenchymal Stromal Cell (MSC)

secretome for bioproteins and bioactives preservation. In an embodiment, an original raw material of MSC secretome is derived from umbilical cord mesenchymal stem cells (UC-MSCs) and stabilized with 0.9% sodium chloride. The MSCs derived

5 cell-free secretome is discovered to be able to recapitulate many of the properties and effects that have been described from a parent cell, even though it is regarded as the by-product of the MSCs. It consists with abundance of bioproteins and bioactives that has regenerative, anti-

10 apoptosis and immunomodulator properties. As used herein the term "bioproteins and bioactives" includes, but is not limited to any bioactive materials found in the MSC supernatant such as chemokines, cytokines, interleukins, growth factors, proteases, exosomes, micro-vesicles,

15 extracellular-vesicles and micro ribonucleic acids (RNAs) that is secreted by the MSCs. In order to preserve these properties and to be able to be used as a raw material, it needs to exist in a more stable form. Its current form that can exert and retain such properties is when the MSC

20 secretome liquid is stored at -20°C. The method of freeze drying or lyophilization is opted as it is the most efficient procedure to change the state of the MSC secretome into a dry powder form, while retaining most of the highly valuable bioproteins and bioactives that can be used as a

25 regenerative product and immunomodulator agents. Various

embodiments of the present technology provide a method of lyophilization of Mesenchymal Stromal Cell (MSC) secretome for bioproteins and bioactives preservation. The main objective of the present technology is:

5 a) To ensure transformation of MSC secretome that is convenient and more stable for long term storage, transportation, and usage from the needs of super-freezing condition at least -20°C, and the needs to be transported in a freeze box without degrading all of the important
10 bioproteins and bioactives in it.

b) To be able to maintain certain concentration of identified bioproteins and bioactives after physical transformation occur and found it to be stable in dried powder form.

15 The lyophilization technique disclosed in the present invention includes a pre-treatment stage, a pre-cool stage, a pre-freezing stage, a primary drying stage and a secondary drying stage. With these two additional new steps namely pre-treatment and pre-cool stage, the product is more stable
20 and kept in a condition that is not favorable for the degradation of the bioproteins and other bioactives. The present technology ensures that the yield of the final freeze-dried MSC secretome is more consistent, qualitatively more stable and all of the listed problems such as meltdown,
25 lifted cake, dusting are significantly reduced while

improving the production efficiency, product yield and its clinical application. The introduction of the pre-treatment and pre-cool stage during the lyophilization process, where the product and machine shelf is cooled at -20°C before the

5 vial is to be loaded into the machine, in order to maintain the integrity and suitable density of the frozen MSC secretome, leads to a more uniform and stable end product, minimal to zero cake lifting and melt-back with the ability to retain and preserve up to approximately 10% of the

10 bioproteins concentration in it with minimal moisture content of 1.0%. Additionally, the pre-treatment and pre-cool stages of the product and machine shelves provide a more stable environment for the MSC secretome in avoiding second thawing from occurring to preserve the integrity of

15 the active bioproteins, bioactives and ensure that the machine is ready and prepared to perform the lyophilization process. Moreover, the newly adopted procedure is able to shorten the time of the production as the product is able to reach its eutectic point faster and resulted in a shorter

20 time taken for the primary drying to about 14 hours as compared to the conventional procedures at 48 hours longest. This increases the productivity of the product and more cost-effective using this new procedure of lyophilization on the MSC secretome.

With reference to Figures 1-5, Figure 1 depicts a freezer 100 from which a frozen MSC secretome is initially obtained for lyophilization on the MSC secretome, in accordance with an exemplary scenario. In an embodiment, the MSC secretome
5 is initially stored in the freezer 100 maintained at -20°C in order to preserve the bioproteins and bioactives. Once the lyophilization procedure is likely to commence, the MSC secretome is transferred out from the freezer 100 for initial pre-treatment process. After obtaining the natural
10 liquid form of the MSC secretome by thawing, a vial is prepared using the natural liquid form of the MSC secretome by mixing each of the vial with a cryoprotectant of selection.

The vials containing the mixtures are stored into the
15 freezer 100 at -20°C .

In an embodiment, the frozen MSC secretome is thawed in a biosafety cabinet at 16°C to 20°C for a duration of about 15 to 20 minutes. Figure 2 depicts a biosafety cabinet 200 for
20 thawing the MSC secretome, in accordance with an exemplary scenario. The MSC secretome is subsequently pipetted into the vial upon complete thawing and the cryoprotectant of selection is added into each of the vial. The cryoprotectant of selection to be mixed with the MSC secretome is chosen
25 from at least one of: mannitol, sucrose and trehalose,

depending on the final product appearance intended. In several embodiments, the composition of cryoprotectant of selection includes, but not limited to 1-10% w/v. The selection of cryoprotectant and its concentration to be used
5 also plays an important role in getting a high-quality result from the whole lyophilization process. The mixture of the MSC secretome and the cryoprotectant of selection is mechanically mixed until homogenous. A rubber stopper is placed on the vial and the vial is stored in the freezer at
10 -20°C, this procedure constitutes a pre-cool stage and the vacuum setting is turned off at this point.

A freeze dry machine shelf temperature is pre-cooled to about -20°C for about 1-2 hours to reach a set temperature, during preparing the vial. Figure 3 depicts a freeze dry
15 machine shelf 300, in accordance with an exemplary scenario. In several embodiments, the temperature used for pre-treatment and pre-cooling step includes, but is not limited to -20°C, -40°C and -60°C. Once the freeze dry machine shelf 300 reaches the set -20°C, the vial is loaded
20 into the freeze dry machine shelf 300 and the temperature is set to -40°C for pre-freezing and -20°C for drying. Pre-freezing and drying processes are performed in the freeze dry machine shelf 300 for about 12 to 48 hours. Figure 4 depicts the freeze dry machine shelf 300 during the
25 pre-freezing and drying processes, in accordance with an

exemplary scenario. When the pre-freezing process begins, the temperature of the freeze dry machine shelf 300 is set at -40°C with vacuum condition to be turned off as well. The duration for this stage is between 4 to 6 hours. Once the
5 desired temperature (eutectic point) has been achieved, the primary drying (sublimation) stage will take place for the duration of 8 to 14 hours, at 0.0 millibar (mbar) to 1.5 mbar vacuum condition. A secondary drying (desorption) stage starts once the primary drying ends after a duration of
10 about 5 to 8 hours with the same vacuum condition setting. Once the secondary drying procedure ends, the chamber temperature of the freeze dry machine shelf 300 cascades from a temperature range 8°C to 10°C to a temperature range of 25°C to 30°C. Subsequently, the vacuum chamber of the
15 freeze dry machine shelf 300 is released and the door of the freeze dry machine shelf 300 is opened for unloading. The post lyophilization stoppering procedure take place at 0.2 mbar for a duration of 5 to 15 minutes to complete the whole process. The vials are transferred for crimping process,
20 testing, and followed by storage. The freeze-dried powder of MSC secretome in a sealed glass vial is physically stable for long term storage of temperature from 5°C to 20°C up to 12 months. In several embodiments, the protein content of the freeze-dried powder of the MSC secretome includes but is
25 not limited to 6.0-10%. In an embodiment, the moisture

content of freeze-dried powder of the MSC secretome includes, but is not limited to 1.0-2.0%.

Lifting cake generally takes place during the primary drying
5 stage. It is reasonably known and understood that the cake
separates slightly from the inner wall of the vial that open
up a low-resistance path for the escaping water vapour to
follow instead of through the partially dried solids. This
unintended water vapor flow causing a drag force being
10 exerted between the cake and the vial walls, results in the
lifting off from the bottom of the vial. This will make
reconstitution difficult and cannot be used and be rejected
from the completed batch. The cake lifting can also lead to
incomplete drying and causing the uncertainty in the final
15 moisture content. It can lead to a longer primary drying
time as well and introduce non-uniform end product, while
also affecting the stability of the end product. The
pre-treatment and pre-cooling of the present method enables
the yield of the final freeze-dried powder of MSC secretome
20 to be more consistent and the problems such as meltdown,
lifted cake, dusting are significantly reduced, thereby
increasing the production efficiency and its clinical
application.

Figure 5 illustrates a flow diagram depicting the steps involved in a process of method of lyophilization of Mesenchymal Stromal Cell (MSC) secretome for bioproteins and bioactives preservation, in accordance with an embodiment.

5 In an embodiment at step 502, a frozen MSC secretome is obtained. At step 504, a vial is prepared using a natural liquid form of MSC secretome, mixing each of the vial with a cryoprotectant of selection, and stored at -20°C . In an embodiment, the frozen MSC secretome is thawed in a

10 biosafety cabinet at 16°C to 20°C for a duration of about 15 to 20 minutes. The MSC secretome is pipetted into the vial upon complete thawing and cryoprotectant of selection is added into each of the vials. The mixture of the MSC secretome and the cryoprotectant of selection is

15 mechanically mixed until homogenous. A rubber stopper is placed on the vials and the vials are stored in the freezer at -20°C . At step 506, the vials containing the mixture are loaded into a freeze dry machine shelf pre-cooled to about -20°C .

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The method of lyophilization of MSC secretome for bioproteins and bioactives preservation of the present technology produces a product that is not only suitable for long-term preservation and easy to be transported, but also

25 highly soluble in certain diluents to be reconstituted as a

raw starting material for product development and can be mainly used aesthetically for skin rejuvenation, regeneration, anti-apoptosis and immunomodulation agents.

- 5 Various embodiments of the present technology are applicable for various clinical applications, such as determining pathogenesis of several diseases, anti-ageing therapy, and the like. The material produces of the present technology contains high level of bioproteins and bioactives and it
- 10 will be stable in this new 'dried powder form' following lyophilization process. The bioproteins and bioactives contain various type such as fibroblast, growth factors, cytokines, and other known bioactives. An example of the main bioproteins is Fibroblast growth factor 2 (FGF2).
- 15 Fibroblast growth factor 2 (FGF2), also known as basic fibroblast growth factor (BFGF) prevents endothelial cells from undergoing cell death and promotes endothelial cell proliferation and angiogenesis. Also, fibroblast growth factor-2 (FGF-2) is a member of a large family of proteins
- 20 that bind heparin and heparan sulphate and modulate the function of a wide range of cell types. Another example is Vascular endothelial growth factor (VEGF), which is considered the master regulator of angiogenesis during growth and development, as well as in disease states such as
- 25 cancer, diabetes, and macular degeneration. In healthy humans, VEGF promotes angiogenesis in embryonic development

and is important in wound healing in adults. Transforming growth factor (sometimes referred to as tumor growth factor, or TGF) is used to describe two classes of polypeptide growth factors, TGF α and TGF β . These
5 bioactives are known to induce cellular transformation, and are not the only growth factors that induce cellular transformation.

The roles of bioproteins and bioactives in the dried form
10 are: 1) Bioproteins stimulate the growth and development of new blood vessels (angiogenesis) that contribute: a) The pathogenesis of several diseases (i.e. cancer, atherosclerosis), b) Normal wound healing and c) Tissue development; 2) The basic bioprotein namely fibroblast
15 growth factor (FGF-2) reduces and prevents expression lines and wrinkles of the human skin through the activation of new skin cells and stimulates the proliferation of cells of mesodermal, ectodermal, and endodermal origin, mainly fibroblasts and keratinocytes; 3) Bioprotein and FGF2 plays
20 a relevant role in antiaging therapy because it is related to the induction of collagen and elastin synthesis responsible for skin elasticity and resistance, characteristics that are diminished with skin aging, both intrinsic and extrinsic; and 4) Administration of FGF2
25 protects against radiation-induced cell death in the

endothelium in the mouse small intestine and reduces lethality from intestinal radiation toxicity. Endothelial targeted FGF2 protects against long-term radiation injury in the lung; 5) Other bioactives namely VEGF and TGF are known
5 to be involved in angiogenesis during growth and development, as well as induce cellular transformation.

As will be readily apparent to those skilled in the art, the present invention may easily be produced in other specific
10 forms without departing from its essential characteristics. The present embodiments are, therefore, to be considered as merely illustrative and not restrictive, the scope of the invention being indicated by the claims rather than the foregoing description, and all changes which come within
15 therefore intended to be embraced therein.

Claims

1. A method of Lyophilization of Mesenchymal Stromal Cell
(MSC) secretome for bioproteins and bioactives
5 preservation, the method comprising:

obtaining (502) a frozen MSC secretome;

preparing (504) a vial using a natural liquid form
of the MSC secretome, mixing each of the vial with a
cryoprotectant of selection and stored at -20°C (pre-
10 treatment stage); and

loading (506) the vials containing the mixture of
the vial with a cryoprotectant of selection into a
freeze dry machine shelf pre-cooled to about -20°C
(pre-cool stage).

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2. The method as claimed in claim 1, wherein loading the
vial into the freeze dry machine shelf comprises:

thawing the frozen MSC secretome in a biosafety
cabinet at 16°C to 20°C for a duration of about 15 to
20 minutes;

pipetting the MSC secretome into the vial upon
complete thawing and adding the cryoprotectant of
selection into each of the vial;

mechanically mixing the mixture of the MSC secretome and the cryoprotectant of selection until homogenous; and

5 placing a rubber stopper on the vial and storing the vial in the freezer at -20°C.

3. The method as claimed in claim 1, wherein the freeze dry machine shelf temperature is pre-cooled to about -20°C for about 1-2 hours to reach a set temperature, during preparing the vial.

4. The method as claimed in claim 1, further comprising:
loading the vial into the freeze dry machine shelf that is pre-cooled, upon the freeze dry machine shelf reaching about -20°C and setting the temperature to -40°C for pre-freezing and -20°C for drying; and
15 performing pre-freezing and drying processes in the freeze dry machine shelf for about 12 to 48 hours.

20 5. The method as claimed in claim 1, further comprising:
releasing the vacuum chamber and opening the door for unloading the vials for crimping process, testing and storage.

25 6. The method as claimed in claim 1, comprising:

choosing the cryoprotectant of selection to be mixed with the MSC secretome, from at least one of: mannitol, sucrose and trehalose, depending on a final product appearance intended.

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7. The method as claimed in claim 1, wherein the freeze dry machine shelf is pre-cooled for a duration of 1 to 2 hours.

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