ABSTRACT

Method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells

The invention relates to a method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells (MSCs). The invention also relates to a method for treating and a therapeutic product for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising MSCs.

WE CLAIM:

1. A method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of atleast 1,25,000 cells/cm^2 pure clinical grade MSCs comprising over 95% cells which express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR for allogenic use the method comprising the steps of:
    a) separately collecting 10ml to 120ml human adipose tissue from multiple donors;
    b) digesting the adipose tissue of each donor separately with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
    c) seeding the cells contained in the separate SVFs into a culture medium;
    d) trypsinising and washing the cells once they reach confluence at passage 0;
    e) pooling the cells obtained from the adipose tissue of multiple donors;
    f) seeding the pooled cells of passage 0 into a culture medium;
    g) trypsinising and washing the cells once they reach confluence at passage 1;
    h) seeding the cells of passage 1 into a culture medium; and
    i) trypsinising and washing the cells once they reach confluence at passage 2,

wherein,

- step (a) is performed by ultrasound-assisted liposuction;
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion;
- cells are seeded in step (c), only if at least 60% cells are positive for CD90;
- the cells are seeded in to the culture medium in step (c) at a seeding density of at least 10000-50000 cells per sq cm;
- prior to seeding in step (f), the cells are characterized based on the percentage of cells which express positive markers CD73, CD90, CD105 and CD166, and negative markers CD45, CD34 and HLA-DR;

- the cells are seeded in to the culture medium in step (f) at a seeding density of 1000 to 5000 MSCs per sq cm and comprise at least 95% of the positive markers and at most 2% of the negative markers;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO), 75% to 25% alpha-Minimum Essential Medium (α-MEM), upto 100% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) or upto 100% alpha-Minimum Essential Medium (α-MEM); and
- prior to the trypsinising at steps (d), (g) and (i), 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

2. A method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 90,000 cells per cm^2 pure clinical grade MSCs comprising over 95% cells which express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR for autologous use, the method comprising the steps of:
    a) separately collecting 10ml to 120ml human adipose tissue from a human donor;
    b) digesting the adipose tissue with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
    c) seeding the cells contained in the SVF into a culture medium; and
    d) trypsinising and washing the cells once they reach confluence at passage 0;
    e) seeding the washed cells of passage 0 into a culture medium;
    f) trypsinising and washing the cells once they reach confluence at passage 1 as final product;

wherein,

- step (a) is performed by ultrasound-assisted liposuction;
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion;
- cells are seeded in step (c), only if at least 60% cells are positive for CD90

- the cells are seeded in to the culture medium in step (c) at a seeding density of at least 10000-50000 cells per sq cm
- prior to seeding in step (e), the cells are characterized based on the percentage of cells which express positive markers CD73, CD90, CD105 and CD166, and negative markers CD45, CD34 and HLA-DR;
- the cells are seeded in to the culture medium in step (e) at a seeding density of 1000 to 5000 cells per sq cm and comprise at least 95% of the positive markers and at most 2% of the negative markers;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO), 25% to 75% alpha-Minimum Essential Medium (α-MEM), upto 100% DMEM-KO or upto 100% α-MEM; and
- prior to the trypsinising at step (d) and (f) 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

3. A method for isolation of human adipose tissue derived stromal vascular fraction (SVF) cells to obtain a yield of at least 80-100 million cells comprising cells of which over 60% cells express CD90 marker for autologous, the method comprising the steps of:
    a) separately collecting 10ml to 120ml human adipose tissue from at least one human donor; and
    b) digesting the adipose tissue separately with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);

wherin,

- step (a) is performed by ultrasound-assisted liposuction; and
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion.

4. The method as claimed in any one of claims 1 to 3, wherein the washed cells after trypsinisation are frozen in a freezing mixture comprising multiple electrolyte solution supplemented with 5% human serum albumin and 10% dimethyl sulfoxide (DMSO).

5. The method as claimed in claim 1 or 2, wherein the culture medium comprises 25% DMEM-KO + 75% α-MEM.

6. A therapeutic product prepared according to the method of claim 1 for allogenic use.

7. A therapeutic product prepared according to the method of claim 2 or 3 for autologous use.

8. A therapeutic product for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising MSCs suspended in multiple electrolyte solution supplemented with human serum albumin and dimethyl sulfoxide (DMSO) wherein, over 95% MSCs express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells express negative markers CD45, CD34 and HLA-DR, and wherein the MSCs have undergone not more than 16 population doublings in vitro and are capable of at least 50 population doublings, the MSCs are capable of differentiating in adipocytes, osteocytes and chondrocytes, the MSCs express pluripotent markers REX1, ABCG2, NANOG, OCT4 and SOX2, and secrete growth factors IL-1RA, IL-2R, IL-6, IL-7, IL-8 (CXCL 12), IFN-α, MCP-1 (CCL2), MIP-1a, Eotaxin (CCL11), hGF, VEGF, and TGF-ß and show immunomodulatory activity.

9. An autologous therapeutic product for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising SVF cells wherein, over 60% SVF cells are positive for CD90, expressing pluripotent markers like REX1, ABCG2, NANOG, OCT4 and SOX2, and secrete growth factors like IL-1RA, IL-2R, IL-6, IL-7, IL-8 (CXCL 12), IFN-α, MCP-1 (CCL2), MIP-1a, Eotaxin (CCL11), hGF, VEGF, and TGF-ß.

10. A method of treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising administering one to three doses of the therapeutic product as claimed in any one of claims 6 to 8, each dose comprising 1 to 4 million MSCs per kg body weight.

11. The method as claimed in claim 10, wherein each dose comprises 2 million MSCs per kg body weight.

12. Use of the therapeutic product as claimed in any one of claims 6 to 8, for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy and osteoarthritis.

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FIELD OF THE INVENTION

This invention relates to a method for isolation, purification and industrial scale expansion of clinical grade human adipose tissue derived mesenchymal stem cells and to characterization of and uses for such cells.

BACKGROUND OF THE INVENTION

Adult stem cells are known to be useful in regenerative medicine. Bone marrow, adipose tissue, Wharton's jelly, placenta, etc are good sources of stem cells. Mesenchymal stem cells (MSCs), which are of mesodermal germ-origin, are able to differentiate into specialized cells of mesodermal origin such as adipocytes, myocytes, osteocytes and chondrocytes along with angiogenesis stimulating potential and immune-modulatory effects. MSCs also have immunosuppressive properties which stem from their ability to inhibit or halt maturation of dendritic cells and proliferation of T cells, B cells and NK cells. Their immunomodulatory action is mediated by the cytokines and chemokines they secrete. Several groups have demonstrated that mesenchymal cells within the stromal-vascular fraction (SVF) of subcutaneous adipose tissue display multi-lineage developmental plasticity in vitro and in vivo. Given the right conditions of growth factors, these adipose tissue-derived adult stem cells, which are also MSCs, are able to trans-differentiate into cells of germ-origin other than their own. Animal model and human studies have shown that these MSCs undergo cardiomyogenic, endothelial (vascular), pancreatic (endocrine), neurogenic, and hepatic trans-differentiation, while also supporting haematopoiesis.

Type-1 Diabetes Mellitus (T1D) previously known as Insulin Dependent Diabetes Mellitus (IDDM) or Juvenile-onset Diabetes is an autoimmune disease. The pathophysiology in this disease is the destruction of pancreatic β cells by infiltrating T cells leading to lack of insulin and subsequent hyperglycemia. It mostly affects the people below 20 years of age. The symptoms include excessive thirst, hunger, frequent urination, body weight loss, fatigue etc. High blood glucose levels can cause severe damage to organs in the long term leading to further complications. Insulin treatment, immunosuppressive drugs or pancreatic transplants are the currently existing treatment modalities for T1D. Pancreatic islet transplantation, inspite of being a viable option, is limited by the availability, immunocompatibility of the donor organ and the relapse of autoimmune destruction. Immunomodulatory drugs that target the

T-cell receptors or enhance T-regulatory cell function are also under investigation. The currently existing cell based therapies for T1D which are under clinical trials are T-regulatory cell therapy, adult bone marrow derived human MSCs, autologus umbilical cord blood infusion, hematopoeitic stem cell therapy and autologus dendritic cell therapy.

Stromal vascular fraction from adipose tissue is usually isolated by using standard technique of lipoaspiration. The technique of tumescent liposuction requires infusing a large quantity of lidocaine and epinephrine containing saline that causes target tissue to become swollen and firm. A liposuction cannula is a stainless steel tube which is inserted into subcutaneous fat through a small opening or incision in the skin. A micro-cannula has an outside diameter of less than 3 millimeters (mm). With special designs, micro-cannulas can remove fat very efficiently. The use of larger cannulas, for example those having an outside diameter ranging from 3 mm to 6 mm require larger incisions which usually leave visible scars.

A disadvantage of manual aspiration or tumescent liposuction is that it a traumatic method and causes heavy bleeding that yields blood mixed lipoaspirate. Moreover, epinephrine and lidocaine mixed lipoaspirate may affect the stromal vascular cell population and hence result in variable yields. Due to this variability, use of manual lipoaspiration for producing a standard dose of cells for therapeutic use has not been feasible. Low yields of cells may be insufficient for therapeutic use and administering multiple doses may be neither practical nor economically viable. In order to obtain a sufficiently large number of cells, the MSCs may be grown to around 10-15 passages and hence the final product for therapeutic use contains relatively aged MSCs.

Several nutrient media have been tried for culturing stromal vascular fraction for obtaining a homogeneous population of mesenchymal stem cells. Media like DMEM-Low Glucose, DMEM/F12, DMEM – high glucose, F12, Alpha-MEM, LP02, supplemented with fetal bovine serum, knockout serum replacement, Serum replacement 1 and Serum replacement 3 result in variable and often low yields of MSCs which display variable morphology. These media have not successfully been used in industrial scale expansion of human adipose tissue derived MSCs and are also not cost effective for producing therapeutic doses of MSCs.

In many clinical trials, efficacy has been shown to be related to the dose of MSCs administered, highlighting the need for an industrial scale process giving a high yield of MSCs. There is a high demand for human MSCs for numerous therapeutic applications but insufficient availability in the market. There is also a need for an efficient culturing system that gives an optimum yield at an affordable cost thereby reducing the demand-supply gap.

There is also a need for an optimal method of isolating, purifying and ultimately expanding human adipose tissue derived MSCs in the least possible passages and minimum population doublings in order to obtain highly potent and young clinical grade MSCs having multi lineage differentiation capacity, showing consistency in cell numbers and amenable to off-the-shelf clinical use.

SUMMARY OF THE INVENTION

According to an embodiment of the invention there is provided a method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 1,25,000 cells/cm^2 pure clinical grade MSCs for allogenic use comprising over 95% cells which express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR, the method comprising the steps of:

a) separately collecting 10ml to 120ml human adipose tissue from multiple donors;
b) digesting the adipose tissue of each donor separately with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
c) seeding the cells contained in the separate SVFs into a culture medium;
d) trypsinising and washing the cells once they reach confluence at passage 0;
e) pooling the cells obtained from the adipose tissue of multiple donors;
f) seeding the pooled cells of passage 0 into a culture medium;
g) trypsinising and washing the cells once they reach confluence at passage 1;

h) seeding the cells of passage 1 into a culture medium; and

i) trypsinising and washing the cells once they reach confluence at passage 2,

wherein,

- step (a) is performed by ultrasound-assisted liposuction;
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion;
- cells are seeded in step (c), only if at least 60% cells are positive for CD90;
- the cells are seeded in to the culture medium in step (c) at a seeding density of at least 10000-50000 cells per sq cm;
- prior to seeding in step (f), the cells are characterized based on the percentage of cells which express positive markers CD73, CD90, CD105 and CD166, and negative markers CD45, CD34 and HLA-DR;
- the cells are seeded in to the culture medium in step (f) at a seeding density of 1000 to 5000 MSCs per sq cm and comprise at least 95% of the positive markers and at most 2% of the negative markers;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO), 75% to 25% alpha-Minimum Essential Medium (α-MEM), upto 100% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) or upto 100% alpha-Minimum Essential Medium (α-MEM); and
- prior to the trypsinising at steps (d), (g) and (i), 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

According to another embodiment of the invention there is provided a method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 90,000 cells per cm^2 pure clinical grade MSCs comprising over 95% cells which express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR for autologous use, the method comprising the steps of:

a) separately collecting 10ml to 120ml human adipose tissue from a human donor;
b) digesting the adipose tissue with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
c) seeding the cells contained in the SVF into a culture medium; and
d) trypsinising and washing the cells once they reach confluence at passage 0;
e) seeding the washed cells of passage 0 into a culture medium;
f) trypsinising and washing the cells once they reach confluence at passage 1;

wherein,

- step (a) is performed by ultrasound-assisted liposuction;
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion;
- cells are seeded in step (c), only if at least 60% cells are positive for CD90
- the cells are seeded in to the culture medium in step (c) at a seeding density of at least 10000-50000 cells per sq cm
- prior to seeding in step (e), the cells are characterized based on the percentage of cells which express positive markers CD73, CD90, CD105 and CD166, and negative markers CD45, CD34 and HLA-DR;
- the cells are seeded in to the culture medium in step (e) at a seeding density of 1000 to 5000 cells per sq cm and comprise at least 95% of the positive markers and at most 2% of the negative markers;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO), 25% to 75% alpha-Minimum Essential Medium (α-MEM), upto 100% DMEM-KO or upto 100% α-MEM; and
- prior to the trypsinising at step (d) and (f) 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

According to yet another embodiment of the invention there is provided a therapeutic product for treating type-I diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes,

idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising MSCs are suspended in multiple electrolyte solution supplemented with human serum albumin and dimethyl sulfoxide (DMSO) wherein, over 95% MSCs express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells express negative markers CD45, CD34 and HLA-DR, and wherein the MSCs have undergone not more than 16 population doublings in vitro and are capable of at least 50 population doublings, the MSCs are capable of differentiating in adipocytes, osteocytes and chondrocytes, the MSCs express pluripotent markers REX1, ABCG2, NANOG, OCT4 and SOX2, and secrete growth factors IL-1RA, IL-2R, IL-6, IL-7, IL-8 (CXCL 12), IFN-α, MCP-1 (CCL2), MIP-1a, Eotaxin (CCL11), hGF, VEGF, and TGF-ß and show immunomodulatory activity.

It is to be understood that both the foregoing general description and the following detailed description of the present embodiments of the invention are intended to provide an overview or framework for understanding the nature and character of the invention as it is claimed. The accompanying graphical representations are included to substantiate the invention and are incorporated into and constitute a part of this specification.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the disclosure may be readily understood and put into practical effect, reference will now be made to exemplary embodiments as illustrated with reference to *the accompanying figures. The figures together with a detailed description below, are* incorporated in and form part of the specification, and serve to further illustrate the embodiments and explain various principles and advantages, in accordance with the present disclosure where:

FIG. 1 shows the morphology of the MSCs attached to culture flasks in Example 1.

FIG. 2 shows the expression of CD34, HLA-DR CD45 CD90, CD73, CD166 and CD105 on the MSCs obtained in Example 1, 2, 3 and 4.

FIG 3 shows the levels of growth factors secreted in the spent media.

FIG.4A and 4B show the SVF cell count yield when the adipose tissue was aspirated using ultrasound-assisted liposuction as compared to manual liposuction as done in Example 6.

FIG. 5 show the cell count results comparing the yields of SVF cells obtained after aspirating adipose tissue incubated at room temperature and analysed at different time-points as done in Example 7.

FIG. 6A and shows the relation between the yield of MSCs and seeding density of adipose derived MSCs in passage-1 and FIG. 6B shows the relation between yield of MSCs and seeding density of adipose derived MSCs in passage-2.

FIG. 7A, 7B and 7C shows the effect of MSC treatment on blood glucose level body weight loss and serum c-peptide level in diabetic animals.

FIG. 8A, 8B, 8C and 8D show the serum biochemistry results indicating therapeutic efficacy after administering MSCs obtained according to an embodiment of the invention.

FIG 9 demonstrates the differentiation capacity of human adipose tissue derived MSCs obtained according to an embodiment of the invention to differentiate into osteocytes, adipocytes and chondrocytes.

FIG. 10A and FIG. 10B respectively demonstrate expression of pluripotent markers on MSCs and SVF cells obtained according to an embodiment of the invention.

FIG. 11A shows thesuppression of Phytoheamagglutinin (PHA) induced T-cell proliferation by MSCs obtained according to an embodiment of this invention.

FIG. 11B shows the suppression of a one way Mixed Lymphocyte Reaction by MSCs obtained according to an embodiment of this invention.

FIG. 12A shows the effect of treatment with MSCs obtained according to an embodiment of the invention on lung weight while FIG. 12B shows the effect of

treatment with MSCs obtained according to an embodiment of the invention on hydroxyproline levels.

FIG. 13 A, 13B, and 13C show the results of histological analysis while assessing efficacy of adipose derived MSCs in bleomycin induced idiopathic pulmonary fibrosis in Swiss albino mice by autologous treatment

FIG. 14A, 14B and 14C show the homing and engraftment potential of AD-MSCs at the site of injury

FIG. 15A, 15B, 15C, and 15D show that AD-MSCs downregulate bleomycin-induced expression of pro-inflammatory and pro-fibrotic transcripts in damaged lungs.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

For simplicity and illustrative purposes, the present invention is described by referring mainly to exemplary embodiments thereof. In the following description, numerous specific details are set forth in order to provide a thorough understanding of the present invention. It will be apparent, however, to one of ordinary skill in the art that the present invention may be practiced without limitation to these specific details. In other instances, well known methods have not been described in detail so as not to unnecessarily obscure the present invention.

In the context of the invention, the term "combination media" as used in the specification refers to a culture media comprising 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) and 75% to 25% alpha-Minimum Essential Medium (α-MEM).

In the context of the invention, the term "clinical grade" as used in the specification refers to MSCs obtained according to an embodiment of the invention and having the same efficacy and safety after isolation, purification and expansion as their parent MSCs.

In the context of the invention, the term "confluence" as used in the specification means approximately 80 to 90% confluence of cells attained during cell culture.

In the context of the invention, the term "multiple electrolyte solution" as used in the specification includes normal saline, Plasmalyte-A and/or Ringer lactate.

According to another embodiment of the invention there is provided a method for isolation of human adipose tissue derived stromal vascular fraction (SVF) cells to obtain a yield of at least 80-100 million cells per 10- 120ml of fat comprising cells of which over 60% cells express CD90 marker for autologous, the method comprising the steps of:

a) separately collecting 10ml to 120 ml human adipose tissue from at least one human donor; and
b) digesting the adipose tissue separately with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);

wherin,

- step (a) is performed by ultrasound-assisted liposuction; and
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion.

According to yet another embodiment of the invention there is provided a therapeutic product prepared according to any of the above methods.

According to another embodiment of the invention there is provided an autologous therapeutic product for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising SVF cells wherein, over 60% SVF cells are positive for CD90, expressing pluripotent markers like REX1, ABCG2, NANOG, OCT4 and SOX2, and secrete growth factors like IL-1RA, IL-2R, IL-6, IL-7, IL-8 (CXCL 12), IFN-α, MCP-1 (CCL2), MIP-1a, Eotaxin (CCL11), hGF, VEGF, and TGF-ß.

Another embodiment of the invention provides a method of treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy,

rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising administering one to three doses of the therapeutic product comprising 1 to 4 million MSCs per kg body weight, preferably 2 million MSCs per kg body weight.

Another embodiment of the invention provides use of the therapeutic product for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis.

The media changes were seen to help in knocking out undesired cells and toxic wastes. Only upto 90% of the media was changed and at least 10% of the spent media was left behind for the purpose of conditioning. The media change also helped to eliminate cells which were not MSCs as cells which are not MSCs do not adhere to the culture flasks/chambers. Preferably, the culture medium comprises 50% DMEM-KO + 50% α-MEM; 75% DMEM-KO + 25% α-MEM or 25% DMEM-KO + 75% α-MEM. More preferably, the culture medium comprises 25% DMEM-KO + 75% α-MEM.

Optionally, the washed cells after trypsinisation can be frozen in a freezing mixture comprising multiple electrolyte solution supplemented with 5% human serum albumin and 10% dimethyl sulfoxide (DMSO) and stored under liquid nitrogen for subsequent use. The MSCs obtained in passage 0 can constitute a master cell bank (MCB), the MSCs obtained in passage 1 can constitute a working cell bank (WCB) and the MSCs obtained in passage 2 can constitute the clinical grade product i.e. investigational product (IP). The cells obtained at the end of passage 0 can be used for autologous clinical purposes. Alternatively, the cells at the end of passage 0 can be expanded to passage 1, without mixing the donor cells, and can still be used for autologous clinical purposes.. The MSCs from multiple donors obtained at the end of passage 0 can be mixed, expanded to passage 1, and further optionally, expanded to passage 2, and used for allogenic clinical use.

The maximum population doublings of adipose tissue derived MSCs is approximately 50. The IP in the present invention is administered at a total population doubling of 16 i.e at a stage when the MSCs are highly potent.

Clinical grade ready to use product is frozen in cryo bags containing 100 million cells, 10% injectable dimethyl sulfoxide (DMSO), 5% injectable Human serum albumin and suspended in multiple electrolyte solution, which is preferably normal saline.

The IP should be transported in liquid nitrogen charged dry shipper for administration. Before transferring the cells to the dry shippers, the dry-shipper chamber should be saturated using liquid nitrogen. After saturation of the dry shipper with liquid nitrogen, excess liquid nitrogen is removed by decanting it from the dry shipper. The IP sample which is to be transported is placed in a canister, which is provided in the dry shipper. The lid of the dry shipper is closed, the dry shipper is locked and sealed and transported to the site of administration within 7 to 9 days of charging. The dry shipper should not be exposed to direct sunlight, rain, or X-rays. After reaching the site of administration, the dry shipper should be placed at room temperature till the day of administration to the patient.

At the time of administration, the lid of the dry shipper is slowly opened. The cryo bags containing the IP are removed from the canister and thawed in a 37°C water bath. The cryo bags should be held upright and swirled continuously till the last crystals melt. Immediately 35ml of multiple electrolyte solution should be added to the cryo bag using a sterile syringe. The contents of the cryobag should then be mixed thoroughly by swaying the bag. The cell suspension is then ready to be administered at a dose of 1 to 4 million cells per kg body weight of the patient, preferably at a dose of 2 million cells per kg body weight.

In order that those skilled in the art will be better able to practice the present disclosure, the following examples are given by way of illustration and not by way of limitation.

Example 1: Preparation of MCB

Prior to collecting the adipose tissue from human donors, the donors were screened by performing regular blood tests and infectious diseases screening tests for HIV, HCV, CMV, VDRL etc. Once the donor passed the screening tests, adipose tissue was aspirated from the donor by VASER ultrasound assisted liposuction. The lipoaspirate from donors was collected and dispensed into separate T175 flasks. Adipose fraction was allowed to settle above the blood fraction. Once settling was complete, the blood fraction and oil droplets were removed completely. Equal volume of washing solution i.e. Dulbecco's Phosphate Buffered Saline (DPBS) with 2% Antibiotic/Antimycotic was added to the adipose fraction and shaken vigorously. Adipose fraction was allowed to settle above the washing solution, the infranatant was discarded and the procedure was repeated till the lipoaspirate became clear in appearance. After the final wash, an equal amount of 0.2% type I Collagenase A solution was added to the T175 flask containing adipose fraction so that the final concentration of Collagenase was 0.1%. The suspension was then mixed well and incubated at 37°C on a shaking incubator at 100 to 150 rpm till the adipose fraction got completely digested. The flask was taken out at 5-10 minute-intervals and observed for percentage of digestion. After complete digestion of adipose fraction, equal volume of neutralization media was added and mixed well. The mixture was centrifuged at 1800-2500rpm for 5-10minutes. SVF fraction was obtained as a pellet after centrifugation.

After dispensing the supernatant, the pellet was resuspended in complete media, strained through a 40-100 micron cell strainer, and a cell count for SVF was taken. The cell suspension was then centrifuged at 1000-1800rpm for 5-10 minutes. The supernatant was then collected and sent for sterility testing. The pellet was then resuspended in complete media. One aliquot of SVF fraction was sent for Flow marker analysis and viability testing.

SVF cells were seeded into tissue culture flasks/ chambers containing combination media on the basis of the flow marker analysis depending on the percentage of CD-90 in SVF population. CD-90 is a positive characterization marker for MSCs. Hence, the SVF cells to be seeded per sq. cm were decided by the percentage of CD-90 obtained by doing fluorescence-activated cell sorting (FACS). If the percentage of CD-90 cells is at least 60%, then 10,000-50,000 SVF cells/sq cm can be seeded, as was done in the present case.

The flasks/chambers containing SVF cells i.e. stem cells were incubated in pre-humidified 37 degree Celsius incubator. The cell culture flask or chamber was observed after 48 to 72hrs of incubation for attachment and initiation of cell growth. The SVF cells showed long, spindle shaped appearance similar to that of fibroblasts after attaching to the cell culture flask as is evident from FIG. 1.

The first media change was done after about 3 days from seeding. 70 to 90% of the spent media was aspirated and freshly prepared combination media was then added to cell culture flask or chamber. The second and third media change were similarly done after 5 to 6 days of the first media change respectively. Once the culture attained 80%-90% confluence, the flasks or chambers were harvested.

The spent media was removed from the flask or chamber and two aliquots were given for checking of sterility, endotoxin, mycoplasma and pH and these were found to be within acceptable ranges. The flasks or chambers were given two washes with DPBS. The wash was removed and 0.25% Trypsin EDTA was added and kept in 5% CO_2 incubator at 37^0C for 2 to 3 minutes and then the flask is observed for detachment of cells. Trypsin activity was stopped by addition of Neutralization media and the neutralized cells were collected in centrifuge tubes. The tissue culture flask or chamber was given one more wash with neutralization media and the same was collected. The neutralized cells were then centrifuged at 1400-1800rpm for 6-10 minutes. The supernatant was then discarded, and the pellet was resuspended in complete media. The cell count was taken and 1 aliquot of cells was given for FACS Flow analysis, and Differentiation. On analysis, it was observed that the expression of haematopoetic markers CD34, HLA-DR and CD45 was < 2 % as seen in FIG. 2. It was further observed that > 95 % of cells expressed a homogenous cell population of cell surface markers such as extracellular matrix protein CD90 (99.52 %), the surface enzyme ecto-5'-nucleotidase CD73 (99.93 %), the activated leukocyte cell adhesion molecule CD166 (99.32 %) the endoglin receptor CD105 (99.85 %). Cell viability was performed using by 7-AAD staining for exclusion of nonviable cells, followed by flow cytometric analysis and was found to be > 95% as shown in FIG. 2.

Table 1 gives the SVF cell counts obtained per donor, percentage of specific cell surface markers expressed, the number population doublings at passage 0 (from SVF to MSC), the number of MSCs obtained per sq cm which was calculated considering the percentage of CD 90 positive cells out of 10,000 SVF cells seeded per sq cm and the total MSC cell count.

Table 1

Donor Serial No	**SVF cell count in millions**	**CD90 (%)**		**Population doubling at passage 0 (from SVF to MSC)**	**Number of MSCs growing per sq. cm**	**Total MSC cell count (in millions)**
1	87	76.19		2.73	46,514	355
2	65	62.19		3.124	56,650	318
3	48.6	75.6		2.438	41,100	224
4	48	67.84		3.117	60,720	225

The remaining cell suspension was centrifuged at 1400rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in the desired volume of freezing mixture such that the concentration of cells in the freezing mixture was 1 million cells per ml. The cells in the freezing mixture were dispensed into prelabelled cryovials at the rate of 1ml per cryovial. These vials constituted the master cell bank (MCB). The cryovials were then frozen in a controlled rate freezer to attain -80°C. The cryovials were then transferred to a vapour phase liquid nitrogen tank for further storage. The above procedure was repeated for each of the adipose tissue donors so as to obtain a bank of cells from multiple donors.

Example 2: Expansion of adipose derived MSCs to Passage 1 for autologous use:
For seeding of passage 1 cells, passage 0 cells (MCB) from Example 1 were thawed, and separately seeded at the rate of 1000 cells/cm^2 to 5000 cells/cm^2, into 10-cell chamber stack having area of 6360 cm^2. The flasks / stacks were then transferred to humidified 5% CO_2 incubator at 37 °C. The cultures were maintained in 5% CO_2 incubator at 37°C in growth media comprising of combination media containing10% FBS, 200 Mm L-Glutamine and Antibiotic-Antimycotic w/v 10,000 U Penicillin,

10mg Streptomycin and 25 µg Amphotericin B per ml in 0.9% normal saline and bFGF 2ng/ml. Media changes were done once in 3 to 6 days till the culture attained 70-80% confluence. The cells were then trypsinized using 0.25 % Trypsin EDTA. These cells constituted Passage 1 cells. The trypsinized cells were cryopreserved in cryopreservation media comprising of100 million cells in multiple electrolyte solution containing 10% DMSO and 5% HSA, frozen to -80°C in programmable controlled rate freezer (PLANAR) and then stored in liquid nitrogen storage tanks at -196°C.

The spent media were checked for sterility, endotoxin and pH and these were found to be within acceptable ranges.

Example 3: Preparation of WCB from MCB

The cryovials of Example 1 were taken from the vapour phase of the liquid nitrogen storage tank and immediately placed in a water bath at 37°C.The vials were held straight and swirled in water bath till the last crystal dissolved out. The contents of the cryovials were then aspirated and resuspended in pre-thawed neutralization media. The tube containing cell suspension was centrifuged at 1400 to 1800rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in a desired volume of complete media, mixed well and the viable cell count was taken.

Since the cells were to be seeded at the rate of 1000 cells/cm^2 to 5000 cell/cm^2 preferably at 3000 cells/ cm^2, the cells of the four donors from the MCBs obtained in Example 1 were pooled in appropriate equal proportions to make the required quantity of cells and then the cells were seeded into culture flasks/ chambers containing combination media.

Here, the first media change was done after 3 to 6 days from seeding. 70 to 90% of the spent media was aspirated and freshly prepared combination media was then added to cell culture flask or chamber. The second media change was similarly done after 3 to 6 days of the first media change. Once the culture attained 80%-90% confluence, the flasks or chambers were harvested.

The spent media was then removed from the flask or chamber and two aliquots were given for checking of sterility, endotoxin, mycoplasma and pH and these were found

to be within acceptable ranges. The flasks or chambers were given two washes with DPBS. The wash was removed and 0.25% Trypsin EDTA was added and kept in 5% CO_2 incubator at 37°C for 2 to 3 minutes and then the flask was observed for detachment of cells. Trypsin activity was stopped by addition of Neutralization media and the neutralized cells were collected in centrifuge tubes. The tissue culture flask or chamber was given one more wash with neutralization media and the same was collected. The neutralized cells were then centrifuged at 1400-1800rpm for 5-10 minutes. The supernatant was then discarded, and the pellet was resuspended in complete media. The cell count was taken and 1 aliquot of cells was given for FACS Flow analysis, and Differentiation. The remaining cell suspension was centrifuged at 1000-1400rpm for 5-10 minutes. The supernatant was discarded and the pellet was resuspended in the desired volume of freezing mixture such that the concentration of cells in the freezing mixture was three million cells per ml. The cells in the freezing mixture were dispensed in to prelabelled cryovials at the rate of 1ml per cryovial. These vials constituted the working cell bank (WCB). The cryovials were then frozen in a controlled rate freezer to attain -80°C. The cryovials were then transferred to a vapour phase liquid nitrogen tank for further storage.

Example 4: Preparation of Clinical grade Product (IP) from WCB

The cryovials of Example 3 were taken from the vapour phase of the liquid nitrogen storage tank and immediately placed in a water bath 37°C. The vials were held straight and swirled in water bath till the last crystal dissolved out. The contents of the cryovials were then aspirated and resuspended in pre-thawed neutralization media. The tube containing cell suspension was centrifuged at 1400 to 1800rpm for 5 to 10 minutes. The supernatant was discarded and the pellet was re-suspended in a desired volume of complete media, mixed well and the viable cell count was taken. The cells were seeded at the rate of 1000 cells/cm^2 to 5000 cells/cm^2, preferably 3000 cells/cm^2, into 10-cell chamber stack having area of 6360 cm^2.

Here, the first media change was done after 3 to 6 days from seeding. 70 to 90% of the spent media was aspirated and freshly prepared combination media was then added to cell culture flask or chamber. The second media change was similarly done after 3 to 6 days of the first media change. Once the culture attained 80%-90% confluence, the flasks or chambers were harvested.

The spent media was then removed from the flask or chamber and two aliquots were given for checking of sterility, endotoxin, mycoplasma and pH and these were found to be within acceptable ranges. The flasks or chambers were given two washes with DPBS. The wash was removed and 0.25% Trypsin EDTA was added and kept in 5% CO_2 incubator at 37°C for 2 to 3 minutes and then the flask is observed for detachment of cells. Trypsin activity was stopped by addition of Neutralization media and the neutralized cells were collected in centrifuge tubes. The tissue culture flask or chamber was given one more wash with neutralization media and the same was collected. The neutralized cells were then centrifuged at 1400-1800rpm for 5-10 minutes. The supernatant was then discarded, and the pellet was resuspended in multiple electrolyte solution. The cell count was taken and 1 aliquot of cells was given for FACS Flow analysis, and Differentiation. The cell count was found to be ≥ 1,25,000 cells per cm^2 i.e. almost 600-800 million cells in 6360 sq cm cells chamber.

The remaining cell suspension was centrifuged at 1000-1400rpm for 5-10 minutes. The supernatant was discarded and the pellet was resuspended in multiple electrolyte solution. The cell suspension was then filtered through a 20-40micron strainer and then centrifuged at 1000-1400rpm for 5-10minutes. The washing with multiple electrolyte solution was repeated twice and the supernantant was discarded and the pellet was resuspended in the desired volume of multiple electrolyte solution. The cell suspension was then filtered through a 40micron strainer and then centrifuged at 1400rpm for 10minutes. The washing with multiple electrolyte solution was repeated twice and the supernantant was discarded and the pellet was resuspended in the desired volume of freezing mixture comprising multiple electrolyte solution containing 5% human serum albumin (HSA) and 10%dimethyl sulfoxide (DMSO) which serves as a cryoprotectant such that the concentration of cells in the freezing mixture was 100 million cells per 15 ml. The cells in the freezing mixture were dispensed in to prelabelled cryobags at the rate of 15ml per cryobag. These bags constituted the Investigational Product (IP). The cryobags were then frozen in a controlled rate freezer to attain -80°C. The cryobags were then transferred to a vapour phase liquid nitrogen tank for further storage.

Example 5: Secretome analysis of human adipose tissue derived SVF cells and MSCs

Spent media was collected from SVF cells and confluent human adipose tissue derived MSC cultures at the end of Examples 1, 2, 3 and 4. To analyse the spent media for estimation of growth factors, chemokines and cytokines secreted by human adipose tissue derived MSCs into the spent media, multiplex Enzyme linked immunosorbent assay (ELISA) or multiplex bead immunoassay kits were used, as per the kit instructions.

FIG. 3 shows the levels of growth factors secreted in the spent media. Levels of growth factors in SVF, MCB, WCB and IP were comparable.

Example 6: Comparison between SVF isolated from adipose tissue extracted by manual liposuction and ultrasound-assisted liposuction

This was done to determine the optimal technique to aspirate adipose tissue which gave a good yield of SVF cells when the adipose tissue was further processed. Five adipose tissue samples were obtained by manual liposuction and ultrasound-assisted liposuction and these samples were further processed as per Example 1 upto the point at which SVF cells were obtained. The cells were counted and the yield was noted for each liposuction technique. The cells were also found to be viable by the trypan blue assay and 7AAD marker by flow cytometry. The SVF cell count yield was found to be higher when the adipose tissue was aspirated using ultrasound-assisted liposuction as compared to manual liposuction as shown in FIG.4A and 4B.

Example 7: Report for stability studies of adipose tissue stored at different time intervals before processing to obtain MSCs

This was done to determine the optimal time at which the adipose tissue should be processed to get maximum yield. Adipose tissue was aspirated by ultrasound-assisted liposuction. The samples were kept standing at room temperature for upto 24 hours during which time some amount of adipose tissue at 0 hours, 3 hours, 6 hours, 12 hours and 24 hours from liposuction respectively was sampled and processed in accordance with Example 2 upto obtaining SVF. The SVF was isolated and a cell count was taken. The cell count yield was noted at each of the time-points. Flow cytometry analysis was done for the same and the results obtained are shown in FIG

5. After comparing the yields obtained at different time-points, it is clear that adipose tissue when processed between 6 to 24 hours gave the optimal yield of SVF cells.

Example 8: Standardization for seeding density of MSCs

The seeding density standardization was done to determine the optimum number of MSCs to be seeded to get maximum cell yield and a population doubling of approximately 5 to 7 per passage. The cells which were stored in liquid nitrogen at the end of Example 1 were revived and were seeded in T-175 flasks with different seeding densities i.e from 1000 to 5000 cells/ sq. cm. The cells counts and population doublings were checked for 2 passages. At the end of passage 1, the cells were re-seeded with the same seeding densities in a T-175 flask. At the end of 2 passages, an average of cell counts and population doubling was taken and seeding densities between 1000 cells/ sq. cm to 3000 cells/ sq. cm were found to give optimum results as shown in FIG 6A (seeding density at passage-1) and 6B (seeding density at passage-2).

Example 9: Plasticity studies for MSCs

This was done to determine the plasticity of MSCs derived from adipose tissue. The cells from two donors were cultured in T-175 tissue culture flasks till 10 passages and the cell counts obtained at each passage was noted. The population doublings and population doubling time were calculated and noted. The number of population doublings was seen to decrease after passage 9. At the end of 10 passages the number of total population doubling was found to be approximately 50. Hence, it was concluded that MSCs can grow upto approximately 50 population doublings after which they start losing their potency/stemness.

Example 10: Standardization of media combination for optimum cell count yields

The cells frozen at passage 1 as per Example 2 and the MCB cells frozen at passage 0 as per example 1 from four donors were used for determining the media combination to be used for obtaining the optimum cell count yields. The media used were DMEM-KO, α-MEM, and combinations thereof.

The cryovials of the four donors were revived by thawing at 37°C. The vials were thawed till the last crystal in the vial was dissolved. The cells were re-suspended in a 15ml tube containing complete media. The tubes were centrifuged between 1000 to 1800rpm for 5 to 10 minutes at 24°C. The supernatant was discarded leaving behind a cell pellet. The pellet was resuspended in complete media and a cell viability count was taken by the trypan blue assay. The cell count for the WCB, which consisted of the cells of four pooled donors, and the cell count for MCB, which consisted of the cells of each of the individual donors, were recorded. These MCB and WCB cells were seeded at density of 1000 cells/cm^2 in five T-75 flasks with the respective media combinations as stated in Table 2. The cells were observed till they became 90% confluent. Once the cells reached 90% confluence they were harvested (using Trypsin-EDTA) and a cell count was taken for the cells grown in each of the five media combinations. The number of population doublings and the population doubling time (in hours) were calculated for the same. The WCB cells were grown till passage 2 only, since the cells used for therapy are administered at passage 2 in the IP. After recording the cell count, the MCB individual donor cells were re-seeded at the rate of 1000 cells/cm^2 in T-75 tissue culture flasks in the above five media combinations for each donor. The cells were observed till they reached 90 % confluence and were harvested at passage 2. The cell counts for all individual donors in each combination were recorded.

Table 2 indicates the MSC counts obtained from the WCB pooled donors and MCB individual donors when 75000 WCB cells and 75000 MCB cells were seeded in different combinations of media.

Table 2

Media	Seeding Density (per cm^2)	Cells/cm^2 Individual donor at passage 2 (in millions)	Cells/ cm2 of Pooled Donor at passage 2 (in millions)
100% DMEM-KO	1000	57625	55833
100% α-MEM	1000	58208	69167
50% DMEM-KO +	1000	62500	79000

50% α-MEM			
75% DMEM-KO + 25% α-MEM	1000	62750	91500
25% DMEM-KO + 75% α-MEM	1000	62417	91833

From Table 2 it is clear that the media combinations comprising 50% DMEM-KO + 50% α-MEM; 75% DMEM-KO + 25% α-MEM and 25% DMEM-KO + 75% α-MEM gave a higher yield of cells as compared to the individual media ie 100% DMEM-KO or 100% α-MEM. Hence, there appears to be a synergy in the above combinations of media for obtaining optimum yield of MSCs

Example-11: Preparation of Off-The-Shelf-Product for clinical use

As mentioned in Example 4, industrial scale production of adipose derived MSCs was done in a 10-cell stack so as to get multiple dosages. MSCs were washed and trypsinized. Cells were washed minimum 3 to 4 times to remove all the trypsin. Cells were re-suspended in multiple electrolyte solution supplemented with 10% DMSO, 5% human serum albumin. 100 million cells were packed per cryobag in 15 ml of freezing mixture media and gradually frozen in a control rate freezer at the rate of 1°C per minute until -80°C. The cryobag was then stored in vapour phase liquid nitrogen container.

For clinical use, the cryobag was thawed at 37°C in a water bath for 2 minutes. 35 ml of multiple electrolyte solution was added to make volume 50 ml. This dilution made DMSO 3% and human serum albumin 0.75 to 1.50% which was within the allowable range for human use without causing any toxicity, side effects or shock. The diluted solution could then be safely and efficaciously injected intravenously at the rate of 1 ml per minute.

Table: 3: Final product in suspension and its stability showed that injectable normal saline gives better stability of the cells during transport and after final dilution

Stability (Hours)	Normal Saline (%)	Plasmalyte-A (%)	Ringer Lactate (%)
FRESH CELLS			

0	93.70	94.26	93.30
24	92.86	80.30	75.26
48	90.30	75.43	29.36
FROZEN-THAWED			
0	95.76	95.76	95.07
24	91.75	90.76	89.90
48	90.38	85.55	80.35

Example 12: Efficacy of human adipose derived MSCs in Streptozotocin induced diabetes in Sprague Dawley rats by treatment with a therapeutic product prepared according to the method of preparing the product for allogenic use according to an embodiment of the invention

Streptozotocin is an antibiotic which destroys pancreatic β cells mimicking the symptoms of diabetes. The pre-clinical efficacy of human adipose tissue derived MSCs were studied in Streptozotocin induced diabetes in Sprague Dawley rats.

24 healthy male Sprague Dawley rats, weighing around 150-180g were housed in separate cages under standard conditions. They were given food and water *ad libitum*. The duration of this study was 28 days. Diabetes was induced by administrating intraperitoneally a dose of 55mg/kg body weight Streptozotocin dissovled in citrate buffer. Animals having a blood glucose level greater than 300mg/dl were considered diabetic and recruited for the study.

The treatment regimen used is illustrated in Table 4 below:

Table 4

Gr. No.	**Group name**	**No. of animals/ group**	**Induction (administered intraperitoneally)**	**Test substance**	**Dosage (cells/kg body weight)**
1	Naïve control	8	-	None	-
2	Diabetic control	8	Streptozotocin	Vehicle	-

	(Vehicle)		(55mg/kg)		
3	Test group treated with MSCs	8	Streptozotocin (55mg/kg)	MSC (administered intravenously)	$8x10^6$

The assessment parameters were blood glucose after 6 hours fasting, body weight, serum c-peptide and serum biochemistry. No pre-terminal deaths were observed.

The Naïve control and MSC treated groups were compared to the Diabetic control group. Statistical analysis was performed using One Way ANOVA followed by Dunnett's test. The results are shown in FIG 7Ashows a significant decrease in the blood glucose level in the MSC treated group as compared to the Diabetic control group. FIG 7B shows that MSC treatment prevented body weight loss in the diabetic animals. FIG 7C shows a significant increase in the serum c-peptide level in the MSC treated group as compared to the Diabetic control group.

The serum biochemistry results shown in FIGs 8A, 8B, 8C and 8D clearly indicate that MSC treatment lowered the elevated serum levels of biochemical markers for liver and kidney function in diabetes.

As is evident from the above results, a dose of $8x10^6$ human adipose tissue derived MSCs was efficacious in improving the diabetes parameters i.e. the MSC treated group showed a significantly lower blood glucose level and higher serum c-peptide level as compared to the control group. On MSC treatment the animals gained weight as compared to the Diabetic control group which had a constant body weight throughout the study period. The biochemical parameters also indicated an improvement in the disease condition on MSC treatment.

Example 13: Multilineage capacity and pluripotency of MSCs

The ability of human adipose tissue derived MSCs isolated, purified and culturally expanded according to an embodiment of the present invention, to differentiate into the various lineages was investigated. Cells obtained as per Example 1, 2, 3 and 4 were plated and cultured in the specific differentiation media for adipogenic,

chondrogenic and osteogenic differentiation, an undifferentiated unstained control of human adipose tissue derived MSCs was also maintained. Differentiation into adipocytes was confirmed by observing the lipid droplets after Oil red O staining as seen in FIG. 9, mineralization of the matrix / calcium deposition as assessed by Alizarin red S staining demonstrated the osteogenic differentiation potential of AD-MSCs as seen in FIG. 9 and a micromass culture, characteristic to chondrocytes was observed on staining with Alacian blue as seen in FIG. 9. In order to determine the pluripotent capacity of the SVF cells and MSCs, the expression levels of pluripotent markers REX1, ABCG2, NANOG, OCT4 and SOX2 were compared to human dermal fibroblasts. The expression levels of these pluripotent markers were found to be significantly higher in the SVF cells and MSCs isolated, purified and culturally expanded according to an embodiment of the present invention as compared to normal human dermal fibroblasts as seen in FIG. 10A and 10B.

Example 14: Immunoassay

To assess the immunomodulatory potential of the MSCs, two immunological reactions were set up: A one way mixed lymphocyte reaction (MLR) and a Phytoheamagglutinin (PHA) induced T-cell proliferation assay.

MSCs obtained at the end of Example 2 (Autologous MSCs) and Example 4 (Allogenic MSCs) were harvested and seeded into 96 well plates at 1000 cells/well density and allowed to adhere for 24 hours. The next day, they were inactivated by incubation with Actinomycin-D (5μg/ml) for 15 minutes at 37°C.

To set up the one way MLR, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples of 4 donors by density gradient centrifugation with Histopaque (Sigma Aldrich, USA). The PBMC stimulator population was generated by mixing equal populations of 3 donors, followed by Actinomycin-D inactivation. The fourth PBMC donor served as the responder population. 10^5 stimulators and $2x10^5$ responder PBMCs were added to each well with MSCs. The positive control wells contained no MSCs. The negative controls included inactivated MSCs, inactivated stimulator PBMCs and responder PBMCs plated individually in the same concentrations, as in the test wells. The MLR was allowed to proceed for 5 days before estimation of cell proliferation using the BrdU cell proliferation kit. The

endpoint was calculated in terms of percentage T-cell proliferation relative to the positive control, which is assumed to show 100% proliferation.

To set up the PHA stimulation assay, individual populations of 3 native PBMCs were added to wells containing inactivated MSCs. The positive control wells contained no MSCs. PHA was added to each test and positive control well at a concentration of 10 µg/ml. The negative controls included inactivated MSCs and native PBMCs plated individually in the same concentrations, as in the test wells. No PHA was added to the negative control wells. The assay was allowed to proceed for 3 days before estimation of cell proliferation using the BrdU cell proliferation kit. The endpoint was calculated in terms of percentage T-cell proliferation relative to the positive control, which is assumed to show 100% proliferation.

FIG. 11A shows the dose dependent suppression of Phytoheamagglutinin (PHA) induced T-cell proliferation by Autologous and Allogenic human adipose derived mesenchymal stem cells (AD-MSCs). T-cell proliferation induced by PHA, in the absence of AD-MSCs, serves as the positive control.

FIG. 11B shows the dose dependent suppression of a one way Mixed Lymphocyte reaction by Autologous and Allogenic human adipose derived mesenchymal stem cells (AD-MSCs). A Mixed lymphocyte reaction, in the absence of AD-MSCs, serves as the positive control.

Example 15: Safety Profile of Human AD-SVF and AD-MSC's

The *in vivo* acute toxicity study for ex vivo expanded Allogenic Human Adipose derived Mesenchymal Stem cells (MSCs) was conducted using Sprague Dawley rats and Swiss Albino mice as the test species. The animals from each species were divided into two groups, i.e. the test group and the vehicle control group with 10 animals per group (5 males and 5 females). The investigational product, MSCs, were administered by a slow single intravenous injection, at the dose of 40 x 10^6 cells/kg body weight to the test group animals. The vehicle control group was similarly administered with the vehicle i.e. Ringers lactate solution containing 3% DMSO and 1.5% human serum albumin. The animals were observed for mortality and other clinical signs for a period of 14 days post-dosing and their body weights were

recorded prior to dosing (day 1) and twice weekly thereafter. All animals were sacrificed at termination of the study on day 15 and subjected to a complete necropsy. The body weight gain by treated/control group animals was not adversely affected during the 14 day observation period post-dosing. No gross pathological alterations were encountered at terminal necropsy in tissues/organs of any of the animals in this study.

The *in vivo* acute toxicity study for Ex vivo expanded autologous Human Adipose derived Mesenchymal Stem cells (MSCs) was conducted using Swiss Albino mice and Wistar rats as the test species. For each species, the animals were divided into 9 groups with 10 animals per group (5 males and 5 females). There were 3 control groups and 6 test groups for both mice and rats. The investigational product, MSCs, were administered by a slow single intravenous injection, at the dose of 30, 40, 50, 100, 150, 200 x 10^6 cells/kg body weight to test groups IV, V, VI, VII, VIII, IX respectively. Control group I was intravenously administered with Plasmalyte A alone, Control group II was administered with Plasmalyte A + 1.5% HSA and Control group III (Vehicle control) was administered with the vehicle Plasmalyte A + 1.5% HSA + 3% DMSO. The animals were observed for mortality and other clinical signs for a period of 21 days post-dosing and their body weights were recorded prior to dosing (day 0) and once weekly thereafter and also at termination (day 22). All animals were sacrificed humanely at the termination of the study and subjected to a complete necropsy.

The *in vivo* acute toxicity study for Human Adipose derived Stromal vascular fraction cells (SVF) was conducted using Sprague Dawley rats and Swiss Albino mice as the test species. The animals from each species were divided into two groups, i.e. the test group and the vehicle control group with 10 animals per group (5 males and 5 females). The investigational product, SVF, were administered by a slow single intravenous injection, at the dose of 30, 40 and 50 x 10^6 cells/kg body weight to the test group animals. The vehicle control group was similarly administered with the vehicle i.e. Ringers lactate solution containing 3% DMSO and 1.5% human serum albumin. The animals were observed for mortality and other clinical signs for a period of 14 days post-dosing and their body weights were recorded prior to dosing (day 1)

and twice weekly thereafter. All animals were sacrificed at termination of the study on day 15 and subjected to a complete necropsy.

The body weight gain by treated/control group animals was not adversely affected during the 14 day observation period post-dosing. No gross pathological alterations were encountered at terminal necropsy in tissues/organs of any of the animals in this study.

Based on the findings of this acute toxicity study, in absence of any incidence of deaths and adverse effects among treated animals, the minimum lethal dose (MLD), the median lethal dose (LD_{50}) and the maximum tolerated dose (MTD) of Allogenic Human Adipose Derived Mesenchymal Stem Cells (MSCs) in mice and rats were estimated to be greater than 40 x 10^6 cells/kg body weight a dose which is greater than ten times (10X) the maximum therapeutic dose anticipated for use with human subjects. The maximum tolerated dose (MTD) of Autologous Human Adipose Derived Mesenchymal Stem Cells (MSCs) in Swiss albino mice was estimated to be 150 x 10^6 cells/kg body weight and that in Wistar rats was estimated to be 50 x 10^6 cells/kg body weight, a dose which is greater than ten times (10X) the maximum therapeutic dose anticipated for use with human subjects..

The maximum tolerated dose (MTD) of Autologous Human Adipose Derived Stromal vascular fraction cells (SVF) in mice and rats were estimated to be greater than 50 x 10^6 cells/kg body weight a dose which is greater than ten times (10X) the maximum therapeutic dose anticipated for use with human subjects.

Example 16: Efficacy of human adipose derived MSCs in bleomycin induced idiopathic pulmonary fibrosis in Swiss albino mice by treatment with a therapeutic product prepared according to the method of preparing the product for autologous use according to an embodiment of the invention

Male Swiss albino mice (10-12 weeks of age, weighing 18-22 g) were obtained and housed in individually ventilated cages in a temperature-controlled room, with access to food and water *ad libitum*. All animal experiments were double blinded. Pulmonary Fibrosis was induced in Swiss albino mice by intratracheal administration of 1 unit/kg

body weight of Bleomycin sulfate reconstituted in normal saline. The animals were randomly divided into 3 groups (n=6): Sham control group, Vehicle control group and AD-MSC treated group. Pulmonary fibrosis was assessed by macroscopic and histological analyses of the lungs (described below). To probe the efficacy of AD-MSCs obtained according to an embodiment of the invention, a group of mice (n=6) were given intravenous administration of 40×10^6 cells/kg AD-MSCs suspended in normal saline via the tail vein on day 3, 6 and 9. On day 24, animals were euthanized and the lung tissues were collected for the assessment of multiple parameters.

Hydroxyproline estimation

The hydroxyproline content was assessed to indicate the extent of collagen deposition in the lungs. To estimate the hydroxyproline content, lung tissue homogenates were hydrolyzed in 6N HCl (Sigma Aldrich) for 16 h at 120°C. An aliquot of these hydrolysates was then assayed using a hydroxyproline estimation kit (BioVision Research Products; CA, USA). Briefly, hydrolysates were incubated with Chloramine-T, followed by color development with Erlich's reagent at 65°C for 90 min. The absorbance at 550 nm was measured by iMark Microplate Reader (Bio-Rad; CA, USA). Hydroxyproline levels were determined by plotting standard curve using serial dilutions of hydroxyproline standard. Results were quantified as µg/ lung.

Bleomycin treatment significantly increased the collagen deposition in the lungs as evidenced by increased lung weights and a corresponding increase in levels of hydroxyproline as shown in FIG. 12A and 12B. More importantly, intravenous administration of AD-MSCs, showed significant inhibition of bleomycin-induced lung fibrosis and reduction in collagen deposition as evidenced by a reduction of lung weights and decreased levels of hydroxyproline as shown in FIG. 12A and 12B.

FIG. 12A shows a reduction in % lung weight is observed in the AD-MSC treated group when compared to the vehicle control group. All values are averages ± S.E.M. of 6 mice. Levels of collagen deposition in the lung is determined by hydroxyproline estimation in FIG. 12B where vehicle control mice show increased levels of hydroxyproline due to bleomycin-induced lung damage. On treatment with AD-MSCs a reduction in levels of hydroxyproline was observed. All values are averages ± S.E.M. of 6 mice. Results presented are representative of 3 separate experiments.

Histological evaluations

Lung biopsies were fixed in 10 % neutral buffered formalin. Paraffin embedded sections (5 μm thickness) of the lung specimens were stained with Hematoxylin (Sigma Aldrich) and Eosin (Loba Chemie; Mumbai, India) and Masson's Trichrome (Sigma Aldrich) stain for collagen deposition. Sections were graded by an investigator blinded to the treatment groups. Modified Ashcroft's scoring was performed on a scale of 0 to 7 to determine the extent of fibrosis as described elsewhere.

FIG. 13 A, 13B, and 13C show the results of histological analysis. The analysis confirmed the bleomycin-induction of fibrosis. Lung tissue sections from diseased mice, but not from normal mice, revealed severe fibrosis, characterized by the presence of fibrotic masses, collapsed alveoli with severely thickened alveolar septa along with extensive tissue scarring. In contrast, tissue sections from AD-MSC-treated bleomycin mice revealed attenuation in fibrosis with mild thickening of alveolar septa, protection against bleomycin-induced lung fibrosis and maintenance of alveolar architecture.

In FIG 13A, representative images of H&E stained sections of lungs are presented. Compared to normal saline (vehicle control) treated bleomycin-induced mice, tissue sections from AD-MSC-treated bleomycin mice revealed attenuation in fibrosis with mild thickening of alveolar septa, protection against bleomycin-induced lung fibrosis and maintenance of alveolar architecture. In FIG. 13B epresentative images of Masson's trichrome stained sections of the lungs showing collagen staining are presented. In FIG. 13C, histological appearances of the Masson's trichrome stained sections were scored for fibrosis using the Ashcroft's modified scoring criteria. All values are averages ± S.E.M. of 6 mice.

AD-MSCs homing and localization studies

To qualitatively assess the homing potential of systemically administered AD-MSCs, cells were stained with PKH-67 fluorescent cytosolic dye (Sigma Aldrich) prior to injection into mice. Post sacrifice, fluorescence imaging of cryotome sections of the lung tissues was carried out using the Nikon Eclipse Ti inverted fluorescence microscope (Nikon Instruments Inc.; NY, USA). PKH-67 tagged cells were visualized

using the green fluorescent filter (excitation - 490 nm, emission - 502 nm). To observe their distribution in the lung tissues, the sections were then stained with 10 μM Hoechst 33342 (AnaSpec Inc.; Fermont, CA) and visualized under the blue fluorescent filter (excitation - 358 nm, emission - 461 nm). The images were then superimposed using the NIS elements imaging software to create a composite image.

In order to investigate the homing and engraftment potential of AD-MSCs, cells were tagged with PKH-67 cytosolic stain, immediately before intravenous administration to mice. Fluorescence photomicrographs of lung sections of AD-MSC treated animals revealed that PKH-67 labeled cells demonstrated homing and engraftment potential towards the damaged lung tissue and were detected even at 21 days after administration as shown in FIG. 14A, 14B and 14C.

FIG. 14A shows fluorescence photomicrographs of cryosections showing nuclear staining of mice lungs with Hoechst 33342. Nucleus is stained blue. FIG. 14B shows fluorescence photomicrographs of cryosections showing engraftment of PKH-67 tagged AD-MSCs at the site of injury in lungs. AD-MSCs are indicated by green fluorescence. FIG. 14C is merged image showing localization of AD-MSCs in the lung parenchyma.

Pro-inflammatory and pro-fibrotic marker analysis in lung

Total cellular RNA was extracted from lung tissues of mice and purified using the Pure Link RNA Mini kit (Ambion) . RTQ-PCR analysis was carried out using the Express SYBR GreenER qPCR Supermix kit (Invitrogen). The primers used were designed using Primer 3 software and custom synthesized at Bioserve. Melting curve analysis was performed at the end of PCR and changes in gene expression were analyzed using the 'StepOne Plus' software (version 1.1). The relative expression of each gene was calculated using the comparative Ct method. Relative quantification of target mRNA expression was calculated and normalized to Gapdh expression. The results are presented as the $\log_2$ fold change of mRNA expression as compared to the amount present in vehicle control samples.

To demonstrate the reduction in inflammation and fibrosis of bleomycin-induced lung injury by AD-MSCs, mechanisms that mediate this effect were studied. RNA isolated

from tissue sections of lungs of mice from various groups was subjected to RTQ-PCR analyses using appropriate primers for:

- Pro-inflammatory genes-Tgfb, Tnf, Il1b, Il2 as shown in FIG. 15A.
- Pro-fibrotic genes –Col1a1, Col3a1, Ctgf, Bfgf as shown in FIG. 15B.
- Matrix metalloproteinases- Mmp2, Mmp3, Mmp13 as shown in FIG. 15C
- Tissue inhibitor of metalloproteinases- Timp1, Timp2, Timp3 as shown in FIG. 15D.

Gapdh was used as the loading control. Results presented are normalized to loading control. All values are averages ± S.E.M. of 6 mice.

AD-MSCs downregulated the expression of proinflammatory cytokines Il2, Il1b, Tnf and Tgfb which led to a reduction in inflammation as shown in FIG. 15A. AD-MSCs also downregulated the expression of pro-fibrotic mediators Bfgf, Ctgf, Col3a1 and Col1a1 as shown in FIG. 15B. Furthermore, AD-MSCs downregulated the elevated expression of matrix metalloproteinases (MMPs) as shown in FIG. 15C which in turn downregulated the expression of tissue inhibitor of metalloproteinases (TIMPs) as shown in FIG. 15D, thus maintaining the MMP-TIMP balance and preventing the restructuring of the matrix caused due to bleomycin-induced lung injury. This reduction in cellular restructuring led to a reduction of collagen deposition and subsequent attenuation of fibrosis.

It is evident from the above results that AD-MSCs show promise in attenuating symptoms of pulmonary fibrosis due to their immunomodulatory potential. MSCs with their immunomodulatory, hypo-immunogenic and multipotent characteristics, help to manage inflammation and begin the reversal of fibrosis in pulmonary tissues. Administration of AD-MSCs at different stages during the disease phase had a multi-factorial effect of the progression of pulmonary fibrosis in Swiss albino mice. Administration on day 3, when the alveolar microenvironment was severely inflamed induced the paracrine mechanism of AD-MSCs which then initiated the mitigation of inflammation. The day 6 dose served as a booster dose to maintain this effect and further act against infiltrating T-cells. The day 9 dose was administered to target the fibrotic stages of the disease. Of note, AD-MSCs after engraftment at the site of injury

down-regulated pro-inflammatory cytokines like TGF-β, TNF-α, IL-1 and IL-2 in lungs of diseased mice.

Histopathology of lung tissue showed severe fibrosis in the untreated animals, while the AD-MSC treated group showed mild alveolar thickening and significant clearing of fibrotic lesions. This was corroborated by the decreasing trend observed in the hydroxyproline content of the lungs and the reduced % organ weight of the AD-MSC treated group. Further, gene expression analysis of lung tissues from animals sacrificed on Day 24 showed a reduction in mRNA transcripts of several pro-fibrotic markers, MMPs and TIMPs implicated in the progression of pulmonary fibrosis. Homing and engraftment studies showed that AD-MSCs homed into the lung and were retained in the alveolar spaces even after 21 days of administration. A significant portion of systemically administered MSCs tend to get trapped in the microenvironment of the lung. The homing and engraftment of AD-MSCs in lung may have been augmented by engagement of their CD44 receptor by hyaluronic acid and osteopontin since bleomycin exposure is known to increase the expression of hyaluronic acid in lung. Alternatively, release of cytokines by immune-infiltrating cells may also affect the homing and engraftment status of AD-MSCs in lung. These factors make AD-MSCs an ideal candidate for targeting pulmonary diseases.

It is clear that AD-MSCs obtained according to an embodiment of this invention, after systemic autologous administration, home in to the site of injury due to paracrine mechanisms, engraft in the lung parenchyma wherein due to their immunomodulatory potential, inhibit the production of pro-inflammatory cytokines and pro-fibrotic mediators, and are efficacious in ameliorating the symptoms of pulmonary fibrosis.

What has been described and illustrated herein are preferred embodiments of the invention along with some of their variations. The terms, descriptions and figures used herein are set forth by way of illustration only and are not meant as limitations. Those skilled in the art will recognize that many variations are possible within the spirit and scope of the invention, which is intended to be defined by the claims in the

complete specification—and their equivalents—in which all terms are meant in their broadest reasonable sense unless otherwise indicated.

WE CLAIM:

1. A method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of atleast 1,25,000 cells/cm^2 pure clinical grade MSCs comprising over 95% cells which express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR for allogenic use the method comprising the steps of:
    a) separately collecting 10ml to 120ml human adipose tissue from multiple donors;
    b) digesting the adipose tissue of each donor separately with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
    c) seeding the cells contained in the separate SVFs into a culture medium;
    d) trypsinising and washing the cells once they reach confluence at passage 0;
    e) pooling the cells obtained from the adipose tissue of multiple donors;
    f) seeding the pooled cells of passage 0 into a culture medium;
    g) trypsinising and washing the cells once they reach confluence at passage 1;
    h) seeding the cells of passage 1 into a culture medium; and
    i) trypsinising and washing the cells once they reach confluence at passage 2,

wherein,

- step (a) is performed by ultrasound-assisted liposuction;
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion;
- cells are seeded in step (c), only if at least 60% cells are positive for CD90;
- the cells are seeded in to the culture medium in step (c) at a seeding density of at least 10000-50000 cells per sq cm;
- prior to seeding in step (f), the cells are characterized based on the percentage of cells which express positive markers CD73, CD90, CD105 and CD166, and negative markers CD45, CD34 and HLA-DR;

- the cells are seeded in to the culture medium in step (f) at a seeding density of 1000 to 5000 MSCs per sq cm and comprise at least 95% of the positive markers and at most 2% of the negative markers;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO), 75% to 25% alpha-Minimum Essential Medium (α-MEM), upto 100% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO) or upto 100% alpha-Minimum Essential Medium (α-MEM); and
- prior to the trypsinising at steps (d), (g) and (i), 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

2. A method for isolation, purification and industrial scale expansion of human adipose tissue derived mesenchymal stem cells (MSCs) to obtain a yield of at least 90,000 cells per cm^2 pure clinical grade MSCs comprising over 95% cells which express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells which express negative markers CD45, CD34 and HLA-DR for autologous use, the method comprising the steps of:
    a) separately collecting 10ml to 120ml human adipose tissue from a human donor;
    b) digesting the adipose tissue with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);
    c) seeding the cells contained in the SVF into a culture medium; and
    d) trypsinising and washing the cells once they reach confluence at passage 0;
    e) seeding the washed cells of passage 0 into a culture medium;
    f) trypsinising and washing the cells once they reach confluence at passage 1 as final product;

wherein,

- step (a) is performed by ultrasound-assisted liposuction;
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion;
- cells are seeded in step (c), only if at least 60% cells are positive for CD90

- the cells are seeded in to the culture medium in step (c) at a seeding density of at least 10000-50000 cells per sq cm
- prior to seeding in step (e), the cells are characterized based on the percentage of cells which express positive markers CD73, CD90, CD105 and CD166, and negative markers CD45, CD34 and HLA-DR;
- the cells are seeded in to the culture medium in step (e) at a seeding density of 1000 to 5000 cells per sq cm and comprise at least 95% of the positive markers and at most 2% of the negative markers;
- the culture medium comprises 25% to 75% Dulbecco's Modified Eagle's Medium-Knockout (DMEM-KO), 25% to 75% alpha-Minimum Essential Medium (α-MEM), upto 100% DMEM-KO or upto 100% α-MEM; and
- prior to the trypsinising at step (d) and (f) 70 to 90% of the culture medium is changed at 3 to 6 day intervals after seeding until confluence is reached.

3. A method for isolation of human adipose tissue derived stromal vascular fraction (SVF) cells to obtain a yield of at least 80-100 million cells comprising cells of which over 60% cells express CD90 marker for autologous, the method comprising the steps of:
    a) separately collecting 10ml to 120ml human adipose tissue from at least one human donor; and
    b) digesting the adipose tissue separately with collagenase, centrifuging the digested tissue and collecting the stromal vascular fraction (SVF);

wherin,

- step (a) is performed by ultrasound-assisted liposuction; and
- after the collection at step (a), the tissue is incubated at room temperature for 6 hours to 24 hours prior to digestion.

4. The method as claimed in any one of claims 1 to 3, wherein the washed cells after trypsinisation are frozen in a freezing mixture comprising multiple electrolyte solution supplemented with 5% human serum albumin and 10% dimethyl sulfoxide (DMSO).

5. The method as claimed in claim 1 or 2, wherein the culture medium comprises 25% DMEM-KO + 75% α-MEM.

6. A therapeutic product prepared according to the method of claim 1 for allogenic use.

7. A therapeutic product prepared according to the method of claim 2 or 3 for autologous use.

8. A therapeutic product for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising MSCs suspended in multiple electrolyte solution supplemented with human serum albumin and dimethyl sulfoxide (DMSO) wherein, over 95% MSCs express positive markers CD73, CD90, CD105 and CD166, and less than 2% cells express negative markers CD45, CD34 and HLA-DR, and wherein the MSCs have undergone not more than 16 population doublings in vitro and are capable of at least 50 population doublings, the MSCs are capable of differentiating in adipocytes, osteocytes and chondrocytes, the MSCs express pluripotent markers REX1, ABCG2, NANOG, OCT4 and SOX2, and secrete growth factors IL-1RA, IL-2R, IL-6, IL-7, IL-8 (CXCL 12), IFN-α, MCP-1 (CCL2), MIP-1a, Eotaxin (CCL11), hGF, VEGF, and TGF-ß and show immunomodulatory activity.

9. An autologous therapeutic product for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising SVF cells wherein, over 60% SVF cells are positive for CD90, expressing pluripotent markers like REX1, ABCG2, NANOG, OCT4 and SOX2, and secrete growth factors like IL-1RA, IL-2R, IL-6, IL-7, IL-8 (CXCL 12), IFN-α, MCP-1 (CCL2), MIP-1a, Eotaxin (CCL11), hGF, VEGF, and TGF-ß.

10. A method of treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy, and osteoarthritis comprising administering one to three doses of the therapeutic product as claimed in any one of claims 6 to 8, each dose comprising 1 to 4 million MSCs per kg body weight.

11. The method as claimed in claim 10, wherein each dose comprises 2 million MSCs per kg body weight.

12. Use of the therapeutic product as claimed in any one of claims 6 to 8, for treating type-1 diabetes mellitus, critical limb ischemia, multiple sclerosis, Duchenne muscular dystrophy, rheumatoid arthritis, cerebral stroke, type-II diabetes, idiopathic pulmonary fibrosis, dilated cardiomyopathy and osteoarthritis.

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