ABSTRACT

The present invention discloses a novel method of expanding mesenchymal stem cells derived from Wharton jelly part of the umbilical cord, dental Pulp, adipose tissue and any such mesenchymal tissue with minimum use of tissue and time, for the purposes of clinical research. The method comprises of transferring the explants from one culture medium to another thereby prolonging the Passage O (Po) phase of the expansion in order to obtain maximum yield of the cells without manipulation of the cells in homogenous passage state of cell division.

Figure 2

WE CLAIM

1. A novel method of expanding stromal mesenchymal stem cells wherein less time and tissues are required and wherein no manipulation of cells in homogenous passage state of the cell division is required and wherein the yield in the Po phase of the cell expansion is maximized, comprising the steps of:

   (a) Culturing the mesenchymal tissue fragments in a growth medium under suitable conditions and sufficient time in a mother plate;
   (b) Transferring the cultured adherent explants from mother plate to a fresh coated tissue culture plate ensuring that the stromal side is in contact with the culture dish;
   (c) Culturing the transferred explants in a fresh culture medium under suitable conditions and sufficient time; and
   (d) Repeating the steps (b) and (c) atleast thrice.

2. The method as claimed in Claim 1, wherein the mesenchymal tissue fragment is minced to atleast 1 – 2 mm^3 fragments prior to culturing.

3. The method as claimed in Claim 1, wherein the fragments are aligned and attached at regular intervals in the plate with culture medium.

4. The method as claimed in Claim 1, wherein the culture medium is a basal medium comprising DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; MSC-qualified Invitrogen) and penicillin-streptomycin-amphotericin B.

5. The method as claimed in Claim 1, wherein the culture is maintained in a humidified atmosphere with 5% CO_2 at 37° C.

6. The method as claimed in Claim 1, wherein the fibroblast like adherent cells migrates from the tissue fragments at least after 4 weeks.

7. The method as claimed in Claim 1, wherein the adherent cells are removed by trypsinization method.

8. The method as claimed in Claim 1 wherein the culturing of the transferred explants is repeated atleast thrice further once 60 – 70% of the confluency is reached by the adherent cells in each subsequent plate after culturing.

9. The method as claimed in Claim 1 wherein the explants are transferred from one plate to the subsequent plate manually using sterile forceps in a Biosafety cabinet.

10. A method and use of the method substantially as herein described with reference to the claims and attached figures.

Dated this 26th day of February 2013.

Signature: _____
Name: Ranjith Kumar
(Applicant)

Signature: _____
Name: Subhadra Dravida
(Applicant)

NEW COMPLETE PATENT APPLICATION

NO. OF PAGES: 3
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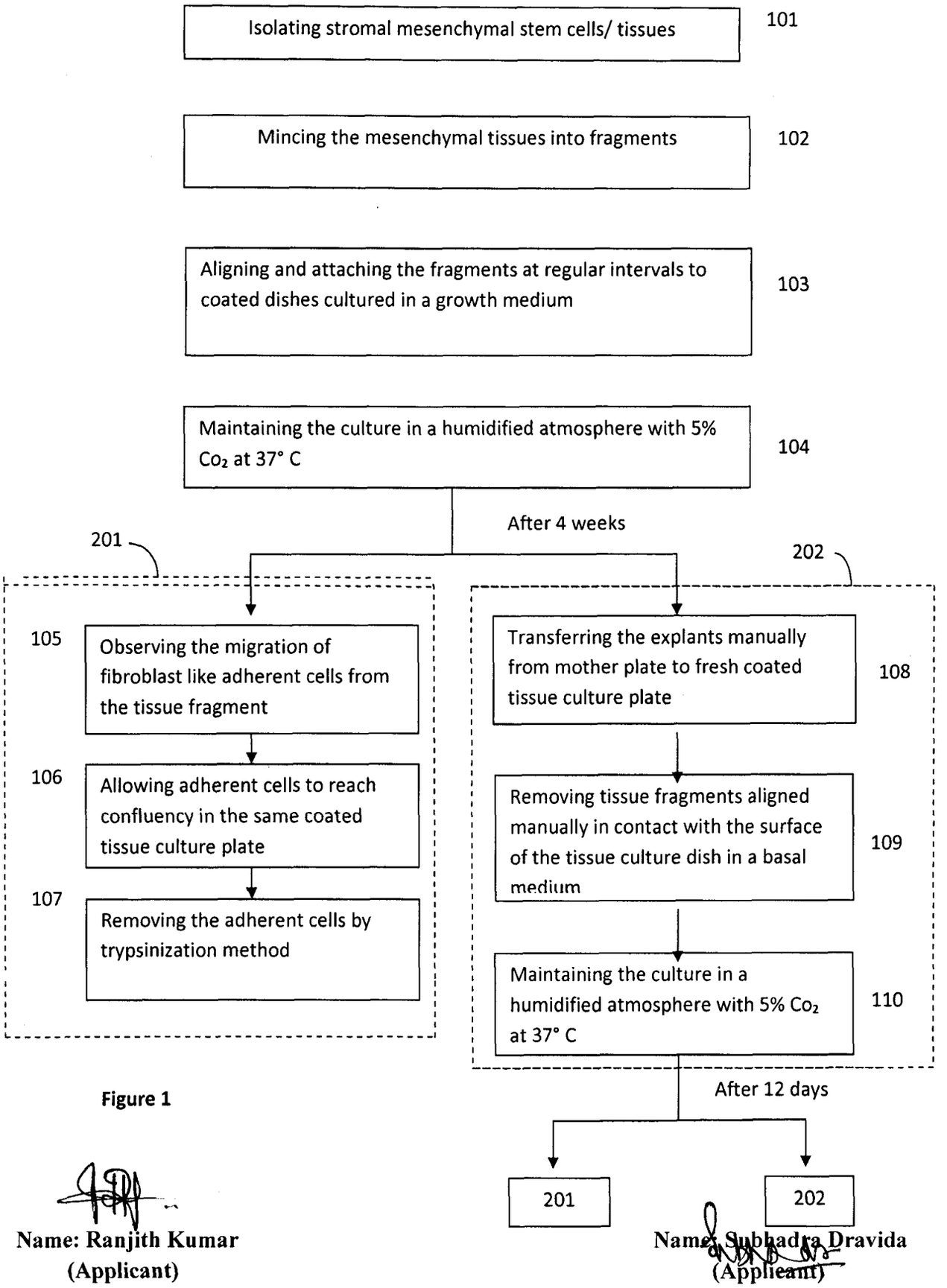

Figure 1

Name: Ranjith Kumar
(Applicant)

Name: Subhadra Dravida
(Applicant)

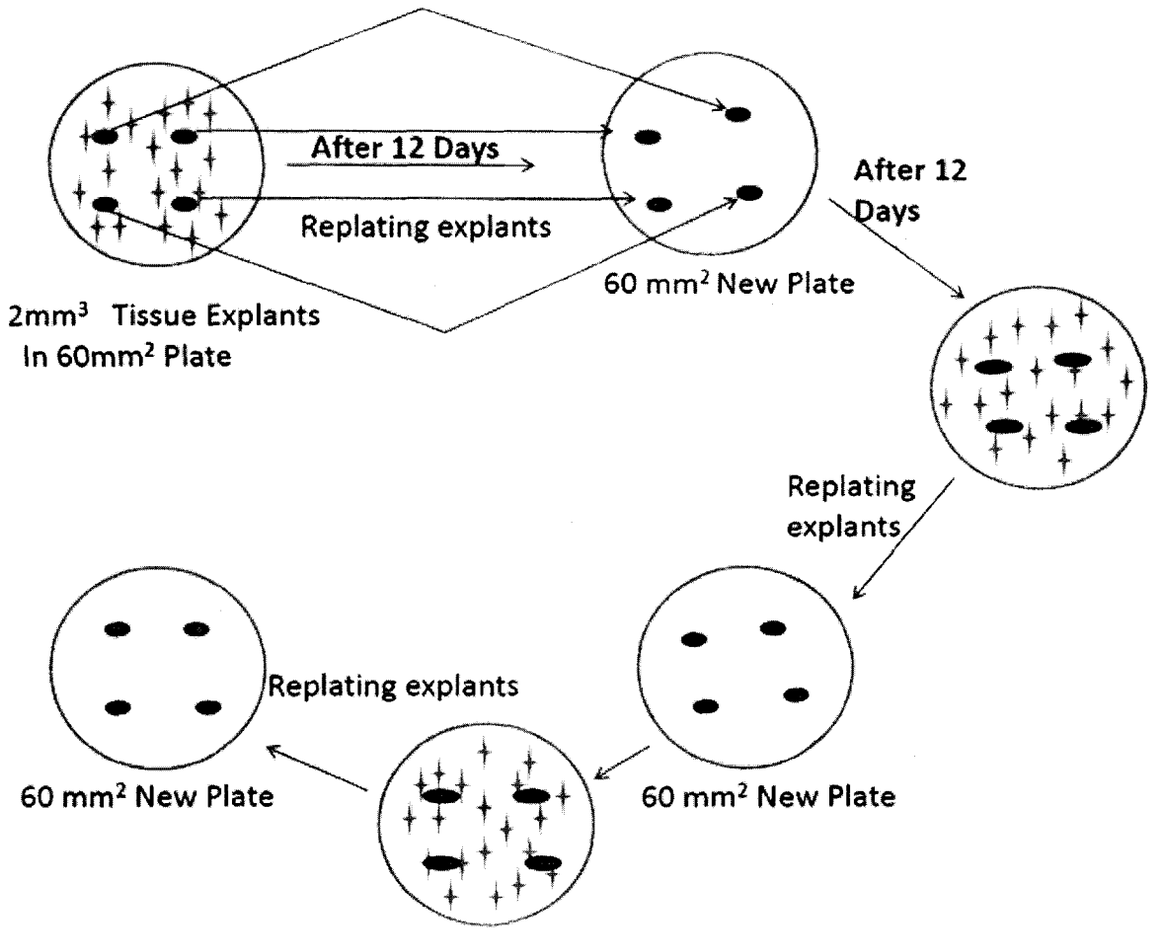

Figure 2

Name: Ranjith Kumar
(Applicant)

Name: Subhadra Dravida
(Applicant)

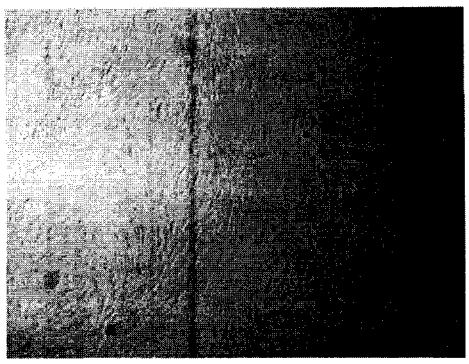

Figure 3

Name: Ranjith Kumar
(Applicant)

Name: Subhadra Dravida
(Applicant)

FIELD OF THE INVENTION

The present invention relates to the field of stem cell culture technology, particularly relates to a novel method of culturing and expansion of mesenchymal stem cells by maintaining the same passaging phase (Po) throughout the expansion without manipulation of the cells in homogenous passage state of cell division. The invention enables expansion to therapeutic dosages of stem cells and used mainly for the purposes of clinical research.

BACKGROUND AND PRIOR ART DISCUSSION

Mesenchymal Stem Cells (MSCs) are self-renewing, multipotent adult stem cells. MSCs are traditionally found in the bone marrow, but can also be isolated from other tissues including adipose, dental pulp, human umbilical cord or cord blood, and peripheral blood. Adipose-derived MSCs are isolated from human adipose (fat) tissues by lipoaspiration or biopsy. MSCs derived from the umbilical cord are isolated from Wharton's Jelly, the gelatinous substance within the human umbilical cord. MSCs are isolated from connective tissue precursor cells and can differentiate into bone, fat, and cartilage upon treatment with Osteocyte, Chondrocyte, or Adipocyte Differentiation Tool kits. Therefore, they are very useful in understanding cell differentiation as well as tissue engineering, orthopaedic and obesity research.

There are three human stromal mesenchymal stem cell population types namely Umbilical Cord-Derived Mesechymal Stem Cells,Adipose Derived Mesenchymal Stem Cells and Dental Pulp Derived Mesenchymal Stem Cells which are isolated from single-donor tissue. Although MSCs from primary adipose tissue and Wharton's Jelly are considered to be relatively easy to obtain, consistent isolation of stem cell populations from such material is costly and time consuming. Lipoaspirates and umbilical cord tissue represent a heterogeneous mixture of cell types, including adipocytes, endothelial cells, smooth muscle cells, pericytes and progenitor cells. The heterogeneity of the source material increases the potential so that the MSC culture will be contaminated and potentially over-run by another cell type.

MSCs are cryo preserved at second passage and tested for growth, morphology, marker expression3 and differentiation potential. MSCs are useful tools for stem cell differentiation research and for the creation of iPSC lines. MSCs have also been useful in tissue engineering cell therapy, and regenerative medicine applications.

Cell culture and expansion is a very important step in cell therapy and mesenchymal stem cells (MSCs) represent the second most selected stem cell group (after hematopoietic stem cells) in the adult stem cell market today. A recent upsurge in the use of primary and stem cells (e.g. MSCs) for cell based therapy has created a growing need for cell culture systems that can be used to expand these cell types.

Typically, MSCs are grown in monolayer T-flask cultures, which is labor intensive and space requiring if large amount of cells are to be produced. There is one article describing successful culture of porcine bone-marrow derived MSCs in spinner flasks. However, this is not a disposable system and the shear forces from the mechanical mixing may damage the cells.

Previous published results have shown that it is possible to expand various types of cells (e.g. Vero cells and MDCK cells) on micro carriers in the Wave Bioreactor. However, the applications for these cultures lie in the vaccine production area. Similar protocols cannot be applied to primary cell cultivation since they differ from cancer cell lines in several ways. For instance, MSCs have more sensitive and proliferative properties as they lose their multiline age capacity after certain number of passages. The method is said to provide large yields of stem cells. However, there is still a need of a new method suitable for expanding adherent cells, such as primary cells and stem cells, in bioreactors, for clinical scale production. This method relates to cell expansion by a method capable of starting from a small volume with a low number of cells and ending with high numbers of cells suitable for, for example celltherapy. This relates to a method for cell expansion, comprising the following steps. The active detachment step may, for example by allowing the cells to detach by using enzymes, thermo responsive agents and/or pH responsive agents. Alternatively, the cells are expanded by allowing passive migration from confluent cells. Step may be performed with intermittent or occasional rocking under very low speed, more gentle than in step preferably, the step is repeated at least once. In a preferred

embodiment, the core of microcarriers is provided with magnetic particles to facilitate sedimentation and/or decantation of culture media etc. It is critical that the volume in step is low to increase cell-to-micro carrier contact and preferably, the volume of the added cells and micro carriers in step is between 150-300 ml. Seeding density is 1-10 cells per micro carrier and the start amount of micro carrier should be at least 0.2 g (dry weight) per litre. The cells are primary cells or stem cells. The stem cells may be adult or embryonic or induced pluripotent stem cells, iPS. The cells may be nucleated cells from adipose tissue, bone marrow or cord blood. In one embodiment, the cells are pre-cultivated before step .This is preferably done in a separate container, such as a culture flask. In a preferred embodiment, the cells are adult mesenchymal stem cells. In a preferred embodiment, final 3-5 g (dry weight) microcarriers per litre are present, which leads to a final cell number of $300\text{-}500\times10^6$ MSCs in a 1 litre bioreactor. Preferably steps and are repeated until 3-8 g microcarriers per litre medium are reached. Up to 5 g without perfusion and up to 8 g with perfusion of the bioreactor bag with fresh medium etc. In another embodiment, the MSCs may be obtained from a purified blood (mononuclear cell fraction) or tissue sample, without any pre-cultivation. Thus, these cells may be provided directly into the above workflow. Preferably, the container is an inflated bioreactor bag. The cultivation may be performed under hypoxic conditions. The cells may be detached in last step inside the container or outside the container. In the latter case the cells and micro carriers are re-introduced into the container.

The concept that adult haematopoiesis' occurs in a stromal microenvironment within the bone marrow was first proposed by Dexter et al., leading to the establishment of in vitro culture conditions for long-term bone marrow culture (LTBMC). These studies demonstrated that an adherent stromal-like culture could support maintenance of hematopoietic stem cells (HSC). Similar adherent cell cultures supporting early B lymphopoiesis have been described and demonstrating full recapitulation of B-cell ontogeny from purified CD34 hematopoietic precursors. This demonstration of the essential role of stromal support in hematopoiesis shaped the view that cell–cell interactions in the marrow microenvironment were necessary for normal hematopoietic function and differentiation. Stromal cell cultures often are defined as the non hematopoietic adherent cell population obtained by direct plating of bone marrow. Marrow stromal may have relatively simpler or complex cellular compositions depending on the growth

media or plating substrate used. The majority of reported conditions used relatively undefined media compositions containing fetal bovine serum (FBS) or other animal sera, thus limiting the study of physiologic signals required for efficient attachment and culture expansion. However, ex vivo cultures results in consistently reproducible stromal cell cultures and have been evaluated in human clinical studies for support of autologous hematopoietic engraftment.

A standard conditions for generation of marrow derived mesenchymal stromal cultures and similar gradient centrifugation is reported, from which light-density cells are taken and plated at a standard plating density in a DMEM media base containing FBS. After allowing 2 days for adherence to non coated polystyrene, nonadherent cells are removed, and a feeding schedule established for a 14-day primary expansion period of adherent colonies. At this time, near-confluent cultures can be processed further by trypsinization and expansion through sequential passages to confluency. Cells may be expanded as many as 40 generations while still retaining their multipotent mesenchymal lineage capability, although growth rates are reduced. The expanded mesenchymal stem cells do exhibit a finite lifetime and do not display properties of immortalized cells. Variation between laboratories in the use of specific growth factors or inducers in propagating these stromal cultures has been reported and likely results in selective enrichment of progenitor differentiation. For example, maintenance of stromal cultures in the presence of dexamethasone is known to enhance lineage progression along the osteogenic or adipogenic lineages, which may explain the relatively complex patterns of cell morphology and differentiation seen in Dexter or Whitlock-Witte cultures.

Quito et al. reported the striking differences between stromal cultures supporting hematopoietic activity when k-FGF (fibroblast growth factor 4) was used to propagate the stromal culture. Delayed senescence and extended proliferation, in addition to improved hematopoietic support, were seen in cultures expanded with k-FGF. Majumdar et al directly compared an expanded mesenchymal stromal culture to Dexter culture with respect to hematopoietic support capability. Continued investigation into subpopulations of mesenchymally derived cells and their support of in vitro hematopoiesis may help define key regulatory stages of hematopoiesis in vivo.

The mesenchymal stem cell culture techniques involve harvesting, expanding by passaging several times to reach the therapeutic dose yield. This is time and resources consuming and labour intensive with manipulated cells in the yield for therapeutics.

US Patent 6649812 entitled "***Method of production of transgenic plants, wholly performed in the To generation, from meristems***" discloses a method of production of transgenic plants from meristems wherein the plants being wholly transformed in the To generation. It comprises the steps of genetically transforming a meristematic explants said sunflower; culturing the transformed meristematic explants on a selection medium; detecting among axillary buds and/or newly formed leaf buds, the buds which are transformed; removing said transformed buds detected in step c) from the cultured transformed meristematic explant; e) culturing said transformed buds removed in step d) on a selection medium; repeating steps c), d) and e) at least three times; and regenerating said cultured transformed buds to obtain a transgenic sunflower. At least 92% of transgenic sunflowers are wholly transformed in the T0 generation by this method. But this expansion is not applicable for the mammalian mesenchymal tissue explants as this is mainly for the production of transgenic plants.

Here a new approach of tissue handling to culture harvest and expand mesenchymal stem cells is recommended, with minimum use of tissue, resources and time and with un-manipulated cells in homogenous passage state of cell division

SUMMARY

The present invention discloses a novel method of expanding mesenchymal stem cells derived from a umbilical cord, dental pulp and other stromal mesenchymal tissues with minimum use of the tissue and time without any manipulation of cells in homogenous passage state of cell division characterised with maximization of the yield in the P0 phase of the cell expansion for the purpose of clinical research, the said method comprising the steps of:

(a) Mincing the mesenchymal tissue fragment in to atleast 1 – 2 mm^3 fragments;

(b) Aligning and attaching the fragments at regular intervals to coated dishes cultured in a basal medium;

(c) Maintaining the culture in a humidified atmosphere with 5% CO_2 at 37° C;

(d) Observing the migration of fibroblast like adherent cells from the tissue fragment after 4 weeks;

(e) Removing the adherent cells by trypsinization method;

(f) Transferring the explants manually from mother plate to fresh coated tissue culture plate using sterile forceps in a Biosafety cabinet;

(g) Removing tissue fragments aligned manually in contact with the surface of the tissue culture dish in a basal medium; and

(h) Repeating the above steps (c) to (g).

The above process is repeated with the removed explants to a maximum of four times once 60 – 70% of the confluency is reached by the adherent cells in each subsequent plate; hence the P0 phase of expansion is maintained throughout the expansion. Moreover, in a conventional cell culture, harvesting of the mesenchymal stem cell is a time consuming process in the Passage 0 (P0). The present invention can reduce the time period and get more number of mesenchymal stem cells in passage 0 (P0).

BRIEF DESCRIPTION OF THE DRAWINGS

For a more complete understanding of the embodiments of the present invention, reference is now made to the following description taken in connection with the accompanying in which:

Figure 1 illustrates a flowchart describing the steps involved in the expansion of the mesenchymal stem cells; and

Figure 2 illustrates the inventive steps of the present invention wherein the explants are re-plated in o the subsequent plate for further expansion maintaining the P0 phase throughout the expansion process.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Mesenchymal stem cells (MSC) possess the capacity for self-renewal and differentiation into several types of cells, including osteocytes, adiposities, chondrocytes, myocytes, cardiomyocytes and neurons. This culturing and expansion is suitable for all types of cells derived from stromal mesenchymal tissues like umbilical cord, dental pulp, adipose etc. The mesenchymal stem cells (MSC) from any of the above tissues are isolated and determined their relative capacities for sustained proliferation and multilineage differentiation [101]. Individual tissue components were dissected, diced into atleast 1–2 mm3 fragments, and aligned in explants cultures from which migrating cells were isolated using trypsinization.

The mesenchymal tissues isolated were then minced into fragments [102]. The fragments were aligned and attached at regular intervals to coated dishes cultured in basal medium [103]. Basal medium comprises of DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; MSC-qualified Invitrogen) and penicillin–streptomycin–amphotericin B.

Cultures were maintained in a humidified atmosphere with 5% CO2 at 37^0C [104]. Approximately 4 weeks after expantion, fibroblast-like adherent cells migrated from the tissue fragments [105]. The adherent cells were allowed to reach confluency in the same coated tissue culture plate [106] while the explants were transferred manually from mother plate to a fresh coated tissue culture plate using sterile forceps in the biosafety cabinet [108]. The cells in the mother plate were trypsinized [107]. The removed tissue fragments, which are otherwise called as explants here were again aligned manually with the blunt sterile forceps so as to make sure that the stromal side is in contact with the surface of the tissue culture dish and cultured in the growth medium [109]. Cultures were maintained in a humidified atmosphere with 5% CO2 at 37^0C [110].

Within the period of ten days, the adherent cells migrated from the tissue fragments. The cells were allowed to reach confluency in the same dish by culturing the cells with alternate day change of medium for one more week while the tissue fragments were manually transferred

maximum thrice after, into fresh coated tissue culture dishes after the 12 day growth period in the mother plates.

Example 1

The UCWJ were minced in to 1 – 2 mm^3 fragments which are aligned and attached to a 60 mm^2 coated petri plates at regular intervals wherein the plates are cultured with basal medium. The explants cultured in the coated plates are maintained in a humidified atmosphere with 5% CO2 at 37^0C. Approximately after 4 weeks after expansion, fibroblast-like adherent cells migrated from the tissue fragments. The adherent cells were allowed to reach atleast 60 – 70% confluency in the same coated tissue culture plate while the explants were transferred manually from mother plate to a fresh coated tissue culture plate using sterile forceps in the biosafety cabinet. The cells in the mother plate were trypsinized and enumerated as 3.2 million in number before freezing. The removed explants were again aligned manually with the blunt sterile forceps so as to make sure that the stromal side is in contact with the surface of the tissue culture dish and cultured in the growth medium. Cultures were maintained in a humidified atmosphere with 5% CO2 at 37^0C. It is observed that approximately about 12. 8 million cells were harvested from the same passage zero from a total $6mm^3$ explants size of the UC, used for culturing cells.

In other words, the explants plated in the first plate were dislodged and replated in to second fresh plate for cell initiation at 60-70% confluency. After the second plate reaches 60-70% confluency, the same explants were manually dislodged from the second plate to the third one and so on to a maximum of four times.

The cells from 3 UCs were successfully isolated and compared which is tabulated as below in Table – I. An average of 12.13 X 10^6 cells was the yield from a total of $6mm^3$ explants seeded in passage 0 itself. Further, figure 3 shows the micrograph of the mesenchymal stem cells that were harvested from UC explants (1^{st} plate at 70% confluency) at 20X magnification using the above method.

TABLE – 1

Sample	Total number of harvested cells from a total $6mm^3$ explants (UCWJ)
1	12.8×10^6
2	12×10^6
3	11.6×10^6

Example 2

The Dental Pulp was minced in to 1 – 2 mm^3 fragments which are aligned and attached to a 60 mm^2 coated petri plates, wherein the plates are cultured with basal medium and growth factors. The explants cultured in the coated plates are maintained in a humidified atmosphere with 5% CO2 at 37^0C. Approximately after 4 weeks after expansion, fibroblast-like adherent cells migrated from the tissue fragments. The adherent cells were allowed to reach atleast 60 – 70% confluency in the same coated tissue culture plate while the explants were transferred manually from mother plate to a fresh coated tissue culture plate using sterile forceps in the biosafety cabinet. The cells in the mother plate were trypsinized and enumerated as 3.2 million in number before freezing. The removed explants were again aligned manually with the blunt sterile forceps so as to make sure that the stromal side is in contact with the surface of the tissue culture dish and cultured in the growth medium. Cultures were maintained in a humidified atmosphere with 5% CO2 at 37^0C. It is observed that approximately about 8. 8 million cells were harvested from the same passage zero from a total $6mm^3$ explants size of the UC, used for culturing cells.

In other words, the explants plated in the first plate were dislodged and replated in to second fresh plate for cell initiation at 60-70% confluency. After the second plate reaches 60-70% confluency, the same explants were manually dislodged from the second plate to the third one and so on to a maximum of four times. The cells from 3 dental Pulp samples were successfully isolated and compared which is tabulated in the below table 2. An average of $9.8X10^6$ cells was the yield from a total of $6mm^3$ dental pulp explants obtained from the extracted wisdom teeth.

TABLE 2

Sample	Total number of harvested cells from a total 6mm^2 explants (Dental Pulp)
1	8.8×10^6
2	11×10^6
3	9.6×10^6

Particularly this is advantageous because of their availability in unmanipulated state in homogeneous passage state of cell division, and potential for cell-based therapy.

The method allows the cells to adhere to the coated plastic petri dishes in spite of addition of further cell culture medium. Once the cells have adhered to the culture medium under gentle and constant temperature, the surface area for continued culturing is increased and also the final harvesting of cells is increased by the active detachment of the explants.