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(54) **Title**: A PROCESS FOR PREPARING STEM CELL BASED FORMULATIONS

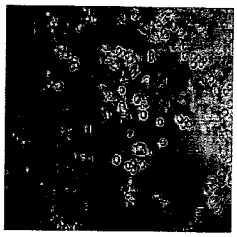

Fig 2

(57) **Abstract**: The present invention discloses a process for preparing a formulation comprising stem cells the process comprises the steps of: subjecting a bone marrow aspirate to centrifugation to separate supernatant and cellular fraction comprising stem cells; isolating the stem cells; subjecting the supernatant to a plurality of centrifugation steps wherein following each centrifugation step the supernatant is freezed and thawed rapidly to remove cellular matter and obtain an acellular supernatant; reconstituting the stem cells with acellular supernatant. The formulation thus prepared comprises stem cells suspended in an acellular bone marrow supernatant which is devoid of any other type of contaminating cells such as T cells or cellular component, however it would have the extrinsic factors present in the bone marrow. The formulation is in a ready to use form for clinical application for use in stem cell based therapies.

WO 2010/140162 A2

TITLE

A PROCESS FOR PREPARING STEM CELL BASED FORMULATIONS.

FIELD OF THE INVENTION

The present invention relates to a process for preparing a formulation comprising mesenchymal and /or hematopoietic stem cells. More particularly the present invention relates to a formulation comprising mesenchymal and /or hematopoietic stem cells for stem cell based therapies and process for preparing such formulation.

BACKGROUND OF THE INVENTION

Adult bone marrow is a rich source of mesenchymal as well as hematopoietic stem cells. These stem cells of adult origin have been shown to have a very high potential to treat degenerative diseases. Theoretically, stem cells can divide without limit to replenish other cells. When a stem cell divides, each new cell has the potential to either remain as a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell. Adult mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) that are undifferentiated with multipotency are typically isolated from bone marrow aspirate and cultured in vitro with established culture conditions. The expanded cell culture is counted for the density and the cells are transplanted at the chosen site for tissue specific grafting.

In conventional methods of using bone marrow for stem cell based therapies, whole or fresh bone marrow has been either used directly or with a matrix material to produce bone marrow graft for transplantation. Since whole bone marrow being a physiological fluid, it was readily acceptable by the patient's body in case of

autologus grafting. Using whole or fresh bone marrow had its disadvantages since this treatment greatly relied on the native levels of MSC and/or HSC in the patient which may be depleted at times. Moreover, even at relatively normal native levels of MSC and HSC, these cells are relatively scarce in fresh bone marrow so the potential of the whole bone marrow for use in treatment is thereby limited. Thus, the biggest technical hurdle to the clinical application of stem cells is still the small number of cells that can be isolated from any adult tissue. With the advent of tissue culture techniques instead of using whole bone marrow, MSC's and HSC's were isolated from bone marrow aspirate, enriched by growing and expanding them in artificial medium and then combined with suitable medium or matrix material to produce bone marrow graft. This way, it was possible to achieve higher levels of MSC or HSC in the same volume of graft and increase the potential for the use of MSC's and/or HSC's.

It was however still difficult to predict the efficacy of the transplantation treatment since little was known about stem cell based therapies and presentation of stem cells particularly in clinically ready and acceptable forms. There is no single factor that is shown to be affecting the efficacy of the transplantation outcome. There are several studies that use extremely high numbers of MSC's and/or HSC's for treatment however there are no studies on efficacy vs number of stem cells transplanted (Antonio Giordano et al., Journal of Cellular Physiology, vol 211, 2007 pages 27-35, Lazarus et al., Bone marrow transplantation, 1995, vol 16, pages 557-564) . Some of the studies reveal the complex nature of the mechanisms by which extrinsic factors regulate signal transduction and cell-fate decisions of

hematopoietic stem cells (Cheng C. Zhang et al, Curr Opin Hematol. 2008 July; 15(4): 307–311). Many cell culture experiments have shown that HSCs respond to multiple cytokines and that the fate of an HSC – self renewal, apoptosis, mobilization from the niche, formation of differentiated progeny cells – depends on multiple cytokines, adhesion proteins, and other signals. Bone marrow comprises a mix of cell types which include nucleated stem cells, red blood cells, platelets, etc, as well acellular components such as proteins, fats and naturally available growth factors. The presence of cell types other than stem cells such as red blood cell, macrophages etc. may lead to complications at the site of treatment due to uncontrolled interactions of these cells with the host tissue. The unwanted cellular presence in the transplantable material may affect the efficacy and dilute the outcome. It has been demonstrated by the Birgit Assmus et al that contaminating RBCs affects the functionality of isolated bone marrow cells and determines the extent of left ventricular ejection fraction recovery after intracoronary BMC infusion in patients with acute myocardial infarction (J Am Coll Cardiol, 2010; 55:1385-1394). Further, it has been suggested in one of the study that human autologous serum is advantageous in a point of decrease in risk of virus or bacterial infection and foreign protein contamination and enhancement of proliferation of hMSCs rather than with the conventional bovine serum (Noriyoshi Mizuno et al, Cell biology international. 01/07/2006; 30(6):521-4).

Thus, while the cellular components as discussed could pose problems in transplantation treatment by causing unwanted interferences, the naturally

available proteins and growth factors would ensure viability and functionality of the stem cells after grafting.

There is therefore a need to provide a process for preparing a formulation comprising mesenchymal stem cells and/or hematopoietic stem cells and naturally available proteins and growth factors present in bone marrow but having minimal or no other cellular components, the formulation being suitable for stem cell based therapies.

SUMMARY OF THE INVENTION

Therefore, it is an object of the present invention to provide a process for preparing a formulation comprising stem cells supplemented with naturally available proteins and growth factors present in bone marrow but no other cellular components and the formulation being in a ready to use form for clinical application for use in stem cell based therapies.

Accordingly, in one aspect the present invention provides a process for preparing a formulation comprising stem cells and an acellular bone marrow supernatant, the process comprising the steps of:

i. subjecting a bone marrow aspirate to centrifugation to separate supernatant and cellular fraction comprising stem cells;

ii. isolating the stem cells;

iii. subjecting the supernatant to a plurality of centrifugation steps wherein following each centrifugation step the supernatant is freezed and thawed rapidly to remove cellular matter and obtain an acellular supernatant.

iv. reconstituting the stem cells with acellular supernatant.

It is further object of the present invention to provide formulation comprising stem cells supplemented with naturally available proteins and growth factors present in bone marrow but no other cellular components, the formulation being suitable for stem cell based therapies.

Thus, in another aspect of the present invention there is provided a formulation comprising of stem cells and an acellular bone marrow supernatant for use in stem cell based therapies.

In a related aspect the present invention provides a formulation comprising of a hematopoietic stem cells and/or mesenchymal stem cells and an acellular bone marrow supernatant in a ready to use form for clinical applications.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 & 2: Phase contrast photomicrographs (10X) showing the colonies of cultured progenitor cells.

Figure 3 & 4: Phase contrast photomicrographs (10X) of the samples of the acellular bone marrow supernatant before and after culturing revealing no evidence of cells or cellular matter.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to a process for preparing the formulation comprising stem cells for use in stem cell based therapies and the formulation prepared by such process. The process comprises the steps of: subjecting a bone marrow aspirate to centrifugation to separate supernatant and cellular fraction comprising

stem cells; isolating the stem cells; subjecting the supernatant to a plurality of centrifugation steps wherein following each centrifugation step the supernatant is freezed and thawed rapidly to remove cellular matter and obtain an acellular supernatant; reconstituting the stem cells with acellular supernatant.

Stem cells can be broadly classified into two categories based on the source from which they are obtained viz. embryonic stem cells and Adult stem cells. Stem cells used in the present invention are preferably but not limited to stem cells belonging to the class of adult stem cells such as hematopoietic stem cells and mesenchymal stem cells. In one embodiment mesenchymal and hematopoietic stem cells isolated from Bone marrow are used in the present invention.

For use in the present invention process, the bone marrow aspirate may be a fresh bone marrow aspirate, aspirated under sterile condition from a suitable site preferably iliac crest which is a rich source of bone marrow.

The bone marrow aspirate is subjected to centrifugation, at about 500g - 1500g, preferably at about 800g for 5 mins to 30 mins to pellet cellular matter. The pellet so obtained after centrifugation is subjected to a suitable density gradient centrifugation for further separation of mesenchymal stem cells, hematopoietic stem cells and other cell types present in the aspirate. The bone marrow supernatant remaining at the top is collected, frozen and stored at about -20 degree centigrade for further use.

Density gradient centrifugation can be performed using any suitable gradient medium such as ficoll. The cellular fraction obtained is added on top of the gradient medium and centrifuged at about 500g - 1500g preferably for atleast 10 mins at a

temperature of about 20 degree centigrade to recover a buffy coat fraction comprising mesenchymal stem cells, hematopoietic stem cells and other mono nuclear cells which are then resuspended in a suitable medium and plated for isolation and expansion of specific types of stem cells.

Isolation and expansion of the mesenchymal stem cells and hematopoietic stem cells may be carried out by any known methods using suitable medium and culture conditions. In one embodiment, the buffy coat fraction obtained is suspended in Dulbeco's modified Eagle medium having F12 (DMEM-F12) with 15% fetal bovine serum. For isolation, the part of the cell suspension can be plated in a same medium and allowed to grow and expand in humidified incubators having a favorable concentration of CO_2 for isolating mesenchymal stem cells, and part of the cell suspension containing mono nucleated cells is plated in a granulocyte-macrophage colony stimulating factor rich medium and allowed to grow and expand in humidified incubators having a favorable concentration of CO_2 for isolating hematopoietic stem cells. After sufficient time of adherence period, the used culture medium may be replaced with fresh medium, preferably at regular intervals, till the cells reached the confluence.

The stem cells expanded *in-vitro* using cell culture techniques are detached using suitable detachment medium and washed to using suitable buffer such as phosphate saline buffer. After adjusting the cell density, they are suspended in the saline buffer medium.

The frozen supernatant from the earlier step is retrieved after about 18 to 24 hours, thawed rapidly and left at room temperature for atleast 10 mins to sediment. The

thawed solution is subjected to centrifugation multiple times, preferably twice to sediment any additional particulate suspension or debris left in the supernatant. Each centrifugation step is followed by freezing the supernatant at atleast -20 degree centigrade followed by rapid thawing in the same order. This helps in denaturing any cellular matter present in the supernatant particularly the T cells which can interfere during the treatment and would lead to immune response and rejection of the stem cell transplant. Particulate matter obtained from the denatured cells is removed in the subsequent centrifugation steps. The acellular supernatant so obtained devoid of any cellular matter and particulate suspension is then frozen at about -20 degree centigrade and stored till required for reconstitution of the stem cells.

Suspension containing the expanded stem cell is added to the requisite quantity of acellular bone marrow supernatant, to obtain formulation ready for clinical application. It may also be possible to dilute the acellular bone marrow supernatant with saline preferably in the ratio of 1:1 to adjust the requisite volume. Preferably the stem cell count in the formulation is 1-1.5 x 10^6 cells.

The present invention in another embodiment also provides the formulation comprising stem cells and an acellular bone marrow supernatant for the use in the stem cell based therapies. The present invention preferably provides a formulation comprising hematopoietic stem cells and/or mesenchymal stem cells and an acellular bone marrow supernatant in a ready to use form for clinical applications.

The process as provided by the present invention advantageously helps in preparing the formulation comprising of mesenchymal stem cells and /or hematopoietic stem

cells along with acellular components of bone marrow and devoid of any particulate suspension, impurities more particularly contamination with other cell types such as red blood cells and T, positively influencing the viability of the stem cells and reduced immune rejection on count of absence of T cells. Further, the formulation of the present invention comprising mesenchymal stem cells and /or hematopoietic stem cells suspended in acellular supernatant of bone marrow rather than conventionally used saline or artificial suspension medium would have the presence of multiple cytokines, adhesion proteins, to have the positive effect on the stem cell self renewal, apoptosis, mobilization from the niche and formation of differentiated progeny cells. This translates into better engraftment and reduced time of healing. While the present invention has been described herein with respect to the various embodiments these are exemplary only, it will be apparent to one ordinary skilled in the art that many modifications, improvements and sub combinations of the various embodiments, and variations can be made without departing from the spirit and the scope of the present invention.

EXAMPLES

Example 1

Preparation of stem cell formulation:

20 ml of bone marrow aspirate obtained under sterile conditions from posterior iliac crests was taken to the biosafety cabinet and centrifuged at 1,000g for 10 minutes at 4°C. The bone marrow supernatant was separated for further processing.

The Pellet from the above step was added onto 25 ml of Ficoll (density, 1.073 g/mL) at a density of 5-10 x 10^6 cells/ml. Gradients were centrifuged at 1000g for 20 minutes at 20°C and recovered mononuclear cells (MNCs) were resuspended in Dulbecco's modified Eagle medium, F12 (DMEM-F12) with 15% fetal bovine serum. 20 mL of this cell suspension was plated in a 175 cm2 flask for MSCs isolation while 5,000 MNCs were plated in granulocyte-macrophage colony-stimulating factor rich medium for HSC colonies to grow and 1-2 x 10^6 CD34+ cells were transferred in to a fresh tube for formulation.

MSCs were cultured in 175 cm2 flask in humidified incubators with 5% CO_2. After 48 hours of adherence period, the used culture medium was replaced with fresh batch, followed by media change every 2 days. 85-90% confluent and adherent cells in the flask were detached with 0.05% trypsin-EDTA and washed with sterile Phosphate Buffer Saline (sPBS) twice. Phase contrast microscopic evaluation (10X) at the end of respective culture period showed the colonies of progenitor cells grown (Fig 1 & 2). The density of the pellet was enumerated and 1-1.5 x 10^6 cells dense pellet suspension was transferred to a fresh tube for preparing the formulation.

The bone marrow supernatant collected from the 1st step before the separation of pellet comprising the MSCs or HSCs was frozen at -70^{0}C. After a day, the frozen solution was thawed rapidly and left at room temperature for 15-20 minutes to sediment. The thawed solution was centrifuged at 1,000g for 8 minutes at 20^{0}C and the upper supernatant was frozen twice again at -70^{0}C with the intermediate thawing and centrifuging steps in order to make it acellular and devoid of any

particulate suspensions. The processed upper acellular bone marrow supernatant solution was stored at -20^{0}C until required for reconstituting the MSCs or HSCs.

To 1ml of the thawed acellular bone marrow supernatant solution were added either MSCs or HSCs counted and transferred to the fresh tubes in order to formulate the stem cells formulation ready for clinical application. Depending on the weight of the patients, the numbers of MSCs or HSCs were formulated for transplantation with proportionately acellular bone marrow supernatant solution.

Example 2

Evaluation of acellular nature of the bone marrow supernatant:

The processed upper acellular bone marrow supernatant solution obtained from Example 1 was studied under the phase contrast microscope for cellular content. 250 µl of this acellular bone marrow supernatant solution was cultured in a 25 cm^2 tissue culture flask with 8 ml of DMEM (Dulbecco's Minimum Essential Medium) with 10% of FBS (Fetal Bovine Serum) for 21 days with alternate day replacement of spent medium at 37 ^{0}C and 5% CO_2. Phase contrast microscopic examination of the samples before and after culturing revealed no evidence of cells (Fig 3 & 4).

CLAIMS

1. A process for preparing a formulation comprising stem cells and an acellular bone marrow supernatant, the process comprising the steps of:
   i. subjecting a bone marrow aspirate to centrifugation to separate supernatant and cellular fraction comprising stem cells;
   ii. isolating the stem cells;
   iii. subjecting the supernatant to a plurality of centrifugation steps wherein following each centrifugation step the supernatant is freezed and thawed rapidly to remove cellular matter and obtain an acellular supernatant.
   iv. reconstituting the stem cells with acellular supernatant.

2. The process as claimed in claim 1 wherein the stem cells are mesenchymal stem cells, hematopoietic stem cells and/or their combination.

3. The process as claimed in claim 1 wherein the centrifugation of the bone marrow aspirate is carried out at about 500g - 1500g, for 5 mins to 30 mins to pellet cellular matter.

4. The process as claimed in claim 1 wherein the stem cells are isolated by subjecting the cellular matter pellet to density gradient centrifugation in a suitable gradient medium.

5. The process as claimed in claim 4 wherein the density gradient centrifugation is carried out at about 500g - 1500g, for atleast 10 minutes, at a temperature of about 20 degree centigrade.

6. The process as claimed in claim 1 wherein the supernatant is freezed at atleast -20 degree centigrade.

7. The process as claimed in claim 1 wherein the stem cells are expanded in a culture media before reconstituting with acellular supernatant.

8. The process as claimed in claim 1 wherein the stem cells count is adjusted to 1 to 1.5 X 10^6 cells before reconstituting with acellular supernatant.

9. The process as claimed in claim 1 wherein the acellular supernatant for reconstituting stem cells is undiluted.

10. The process as claimed in claim 1 wherein the acellular supernatant for reconstituting stem cells is diluted with saline in the ratio of 1:1.

11. A formulation comprising stem cells and an acellular bone marrow supernatant for use in stem cell based therapy.

12. The formulation as claimed in claim 11 wherein the stem cells are mesenchymal stem cells, hematopoietic stem cells and/or their combination.

13. The formulation as claimed in claim 11 wherein the stem cells count is 1 – 1.5 X 10^6 cells.

14. The formulation as claimed in claim 11 wherein the acellular supernatant is undiluted.

15. The formulation as claimed in claim 11 wherein the acellular supernatant is diluted with saline in the ratio of 1:1.

Fig. 1

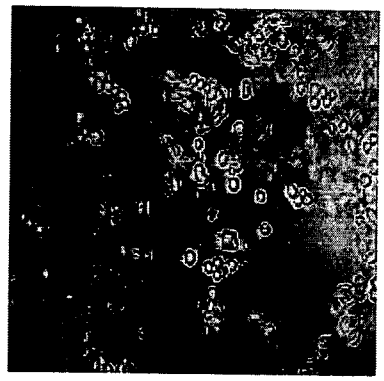

Fig 2

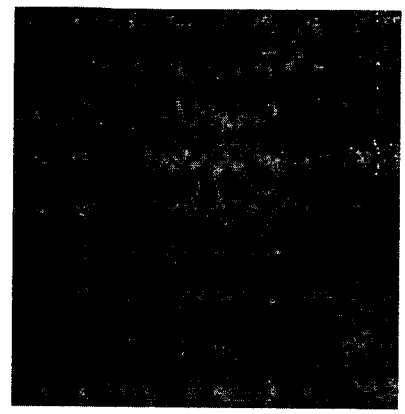

Fig 3

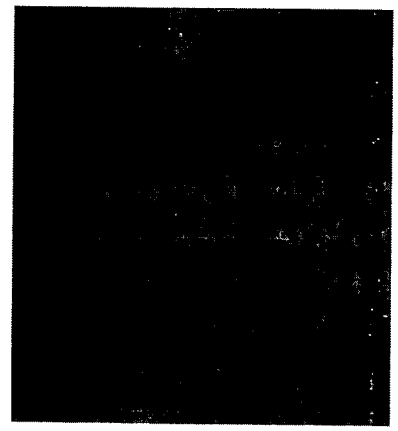

Fig 4