

TITLE:

A method of inducing bone formation by ex-vivo osteoblast culturing for implantation

5 FIELD OF INVENTION:

The present invention relates to a method of inducing bone formation, particularly by osteoblast implantation.

BACKGROUND OF INVENTION:

10 Osteoblasts are known to be large cells in control of the synthesis and mineralization of bone formation and the subsequent remodeling of the same. The process of bone formation from osteoblasts is known as osteogenesis and involves three basic steps: (a) Synthesis of extracellular organic matrix (osteoid), (b) Matrix mineralization leading to the formation of bone, (c) Remodeling of bone by the
15 process of resorption and reformation. Bone remodeling is a continuing process of synthesis and destruction that gives bone its mature structure and maintains the normal structure of bone. Now, destruction, or resorption of bone occurs by virtue of large cells called osteoclasts.

20 The conventional methods used for replacement of defect bones in bone associated diseases or during circumstances of new bone formation due to breakage or injury, are numerous. Depending on the case, certain scenarios can prove to be very challenging, especially the realignment of the bone.

Autologous bone graft of various types is available, which provide good mechanical and biological properties but factors like donor site morbidity, molding

challenges, limited operative time for graft harvest and the limited volume in terms of quantity available is a great concern.

Again, allograft, which involves the transplant of a tissue, is one where the donor is of a different genetic makeup. Although allograft mitigates a few issues associated with autologous bone graft as there is no donor site morbidity and there is no limitation with respect to the quantity, few other problems are involved, which could be the sterilization process which weakens the bone rejection reactions due to a different genetic make-up or the graft may transmit infections such as hepatitis and HIV/AIDS.

Bone generation by virtue of growth factors such as BMPs (Bone morphogenic proteins) which is yet another method wherein the quantitative replacement of defective bone is considerably less to provide predictable results.

Therefore, the above-mentioned problems associated with the existing methods for replacement of defect bones need to be alleviated.

OBJECTIVES OF THE INVENTION:

1. It is an object of the invention to induce bone formation using osteoblast cells.
2. It is another object of the invention to provide a method of mimicking natural bone formation, which delivers a highly accelerated process as compared to already existing methods.
3. It is another object of the invention to provide a minimally invasive method and a timesaving process as opposed to erstwhile known methods.
4. It is a further object of the invention to prevent donor site morbidity and provide a considerable volume of osteoblasts for bone formation.

SUMMARY:

The present disclosure addresses the existing problems associated with methods of inducing bone formation and provides an implantation method comprising an *ex-vivo* osteoblast culturing. Again, *ex-vivo* culturing can be defined as culturing in the external environment without alteration in the natural conditions and parameters. The *ex-vivo* culture, which leads to the formation of the active substance to be injected at the site of bone formation, further comprises the steps of isolation of osteo-progenitor cells, differentiation of osteo-progenitor cells into osteoblasts, expansion culture, cell culture harvest and wash and filling and packaging. This invention particularly accelerates the bone formation process apart from being a minimally invasive method. Thus in one aspect, the present invention refers to a method of inducing bone formation by *ex-vivo* osteoblast culturing for implantation, comprising isolating osteo-progenitor cells from a harvest of a subject; differentiation of the osteo-progenitor cells into osteoblasts; expansion of the osteoblasts for proliferation into an osteoblast culture; and harvesting and washing of the osteoblast growth culture. In another aspect, the present invention refers to an active substance is formed by means of the *ex-vivo* osteoblast culturing for implantation as disclosed herein.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to a method of inducing bone formation by osteoblast implantation in the area of defect or a condition, which requires the same.

This inventive method, which is an osteoblast implantation method, is broadly constructed in a way that the method comprises steps for inducing bone formation.

Therefore, the broad steps involved in osteoblast implantation is an *ex-vivo* culture of live osteoblasts. Furthermore, in the following description the steps are described in detail:

The *ex-vivo* culturing follows the harvesting of bone marrow cells from a subject. Harvesting involves as the name suggests harvesting of bone marrow from the posterior-superior iliac crest or sternum of the Subject. Furthermore, the subject there is the receiver of the induced bone formation.

In order for the ex-vivo culture to be carried out, a biopsy kit needs to be shipped out of the place where the harvest was conducted. The biopsy kit is pre-conditioned prior to shipment and is transported under strict monitoring of temperature between the ranges of 2-8 degrees Celsius during transport. In addition, it has to be ensured that the collected bone marrow is aseptically transferred into sterile biopsy collection medium.

The ex-vivo osteoblast culturing, involves the formation of the active substance. Active substance can be defined as the substance, which is injected at the site of induced bone formation. The process which leads to the formation of the active substance further comprises the steps of:

- Isolation of osteo-progenitor cells, differentiation of osteo-progenitor cells in to osteoblasts, expansion culture, cell culture harvest, washing and filling.

As mentioned above, the ex-vivo culturing starts with the isolation of osteo-progenitor cells. Osteo-progenitor cells are mesenchymal cells that differentiate into an osteoblast as collagen is secreted in the process to harden the bone structure. The rate of differentiation of bone marrow cells in to osteo-progenitor may be controlled by virtue of endothelial cells. Ideally, the osteo-progenitor cells should be maintained at a pre-osteoblastic stage at the site of inducing of bone formation thereby avoiding mineral deposition within the vessel. After the transfer from the vessels to the site of induction rapid differentiation of mature osteoblasts should occur.

In accordance with the invention, the isolation procedure in day one involves the collection of bone marrow with the biopsy collection medium in centrifuge tubes. The biopsy collection medium containing biopsy is checked with respect to sterility, cell viability, cell count, cell characterization, clarity and color as per standard protocol. The collected sample is washed several times with a washing medium under aseptic conditions. Unwanted debris and adipose (fat) layer is

removed after the centrifugation at 1700 rpm for time duration of 7 minutes by suction. The lyses of red blood cells are allowed and nucleated marrow cells are separated.

After which the separated nucleated marrow cells are washed with DMEM culture medium (Dulbecco's Modified Eagle's Medium) and filtered through 40- μ filter to remove the debris. The cells are counted by using a hemocytometer. The homogenous cell suspension is seeded in tissue culture flasks along with culture medium.

Differentiation of osteo-progenitor cells in to osteoblasts, which is a 3-5 day process, is the ensuing process. In ideal conditions, after two days of culture, spent medium is removed and fresh differentiation medium is added for the differentiation of osteo-progenitor cells in to osteoblasts. Osteoblasts play the pivotal role of inducing the formation of the trabecular bone, which is a type of osseous tissue that in turn forms the bones.

The third sub-step following the differentiation procedure is the expansion of the osteoblasts in to an osteoblast growth culture, which requires approximately more than forty days starting from the initial day of isolation.

In this step, the culture flasks are transferred to a CO₂ incubator tuned to specific conditions, the temperature range being 37-38 degrees Celsius. The other conditions are maintained at 5 % CO₂ and 80% humidified atmosphere. Furthermore, the culture flasks get replenished with fresh culture medium at regular intervals. In such cases, the average interval taken is two to three days. It is ensured that the cultured flasks are regularly inspected.

When the cultures reach confluence, the cells are dissociated from the flask surface and sub-cultured in fresh tissue culture flasks until the appropriate number (not less than 48 million cells) of expanded cells has been reached, thereby repeating the process till the culture is expanded in terms of the required number of cells.

Quality control checks are an essential practice in laboratory methods and therefore the spent medium is pooled from every flask and sampled for sterility and mycoplasma tests. Mycoplasma tests are performed using the PCR method. Cell Characterization is performed by flow cytometry test checking bone alkaline phosphate and collagen type I molecular markers, which are the basic criteria to study the cell surface expression. Here, CD 44+ and 151+ are osteoblast markers, which are used to characterize cultured cells obtained at various stages. This test is followed by an Alizarin red stain test, which is performed to check the calcium deposition induced by these cells to confirm they are osteoblast in nature.

After the desired completion of expansion in to the osteoblast growth culture, the cells are detached and collected. The collected cells are centrifuged at 1400 rpm at an ambient temperature of 22-28 degree Celsius for about five-six minutes and washed with DMEM (Dulbecco's Modified Eagle's Medium) thoroughly. The cells obtained at the end of this stage are considered the active substance, which is the substance to be injected at the site of induction of bone.

In order to transport the active substance to the site where the bone needs to be induced, the harvested cells are filled in sterile vials and sealed effectively under aseptic conditions. The temperature to be maintained while transporting the same should be in the range of 2-8 degrees Celsius.

The active substance should be used within 72 hours from the date of manufacture of the same. In addition, the active substance should be allowed to settle down and solidify for 7-8 min before wound suture for effective regeneration of the defective bone.

Advantageously, it has been observed that on completion of ninety days, there is approximately ninety percent of bone regeneration while the conventional bone grafting is able to achieve sixty-five percent of bone regeneration.

Therefore by way of example as mentioned above the present invention has considerable advantages over conventional bone grafts and addresses the issues existing in the prior art.

5

10

15

20

I claim:

1. A method of inducing bone formation by ex-vivo osteoblast culturing for implantation, comprising:

- isolating osteo-progenitor cells from a harvest of a subject;
- differentiating the osteo-progenitor cells into osteoblasts;
- expanding the osteoblasts for proliferation into an osteoblast culture;
- selecting of osteoblasts expressing both CD44+ and CD151+ cell surface markers; and
- harvesting and washing of the selected osteoblasts;

resulting in live osteoblasts not less than 48 million cells.

2. The method as claimed in claim 1, wherein the harvest of the subject is from a posterior-superior iliac crest or sternum.

3. The method as claimed in claim 1, wherein the isolation of osteo-progenitor cells includes separating of nucleated bone marrow cells.

4. The method as claimed in claim 3, wherein the separation of nucleated bone marrow cells is followed by washing of the nucleated bone marrow cells with DMEM culture medium.

5. The method as claimed in claim 1, wherein the differentiation step includes replenishing of differentiation medium in culture flasks.

6. The method as claimed in claim 1, wherein the expansion step further includes transferring of culture flasks to a CO₂ incubator at a temperature range of 37-38 degree Celsius.

7. The method as claimed in claim 6, wherein the CO₂ incubator is conditioned at a range of 5 % CO₂ content.

8. The method as claimed in claim 6, wherein the CO₂ incubator is humidified at 80 %.

9. The method as claimed in claim 1, wherein the osteoblast culture harvest step further includes centrifuging the harvested osteoblast culture at a range of 1300-1500 rpm for five-six minutes.

10. The method as claimed in claim 9, wherein the centrifugation is followed by washing of the osteoblast culture with DMEM (Dulbecco's Modified Eagle's Medium).