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(54) Title: METHOD AND COMPOSITION FOR REPAIRING HEART TISSUE

(57) Abstract: The present invention is directed to the TVEMF-expansion of mammalian blood stem cells, preferably CD34+/CD38- cells, to compositions resulting from the TVEMF-expanded cells, and to a method of treating heart disease or repairing heart tissue with the compositions.

METHOD AND COMPOSITION FOR REPAIRING HEART TISSUE

FIELD OF THE INVENTION

The present invention is directed to a method of repairing and/or regenerating heart tissue, and a composition that will provide for such repair and/or regeneration.

BACKGROUND OF THE INVENTION

Regeneration of mammalian, particularly human, heart tissue has long been a desire of the medical community. For some tissues, repair of human tissue has been accomplished largely by transplantations of like tissue from a donor. Beginning essentially with the kidney transplant from one of the Herrick twins to the other and later made world famous by South African Doctor Christian Barnard's transplant of a heart from Denise Darval to Louis Washkansky on December 3, 1967, tissue transplantation became a widely accepted method of extending life in terminal patients.

Transplantation of mammalian tissue, from its first use, encountered major problems, primarily tissue rejection due to the body's natural immune system (Washansky lived only 18 days past the surgery). In order to overcome the problem of the body's immune system, numerous anti-rejection drugs (e.g. Imuran, Cyclosporine) were soon developed to suppress the immune system and thus prolong the use of the tissue prior to rejection. However, the rejection problem has continued creating the need for an alternative to tissue transplantation.

In recent years, researchers have experimented with the use of pluripotent embryonic stem cells as an alternative to tissue transplant. The theory behind the use of embryonic stem cells has been that they can theoretically be utilized to regenerate virtually any tissue in the body. The use of embryonic stem cells for tissue regeneration, however, has also encountered problems. Among the more serious of these problems are that transplanted embryonic stem cells have limited controllability, they sometimes grow into tumors, and the human embryonic stem cells that are available for research would be rejected by a patient's immune system (Nature, June 17, 2002: Pearson, "Stem Cell Hopes Double", news@nature.com, published online:21 June 2002). Further, widespread use of embryonic stem cells is so burdened with ethical, moral, and political concerns that its widespread use remains questionable.

The pluripotent nature of stem cells was first discovered from an adult stem cell found in bone marrow. Verfaille, C.M. et al., Pluripotency of mesenchymal stem cells derived from

adult marrow. *Nature* 417, published online 20 June; doi:10.1038/nature00900, (2002) cited by Pearson, H. Stem cell hopes double. news@nature.com, published online:21 June 2002; doi: 10.1038/news020617-11.

Boyse et al., U.S. Pat. No. 6,569,427 B1, discloses the cryopreservation and usefulness of cryopreserved fetal or neonatal blood in the treatment or prevention of various diseases and disorders such as anemias, malignancies, autoimmune disorders, and various immune dysfunctions and deficiencies. Boyse also discloses the use of hematopoietic reconstitution in gene therapy with the use of a heterologous gene sequence. The Boyse disclosure stops short, however, of expansion of cells for therapeutic uses. CorCell, a cord blood bank, provides statistics on expansion, cryopreservation, and transplantation of umbilical cord blood stem cells. "Expansion of Umbilical Cord Blood Stem Cells", Information Sheet Umbilical Cord Blood, CorCell, Inc. (2003). One expansion process discloses utilizing a bioreactor with a central collagen based matrix. Research Center Julich: Blood Stem Cells from the Bioreactor. Press release May 17, 2001.

Research continues in an effort to elucidate the molecular mechanisms involved in the expansion of stem cells. For example, the CorCell article discloses that a signal molecule named Delta-1 aids in the development of cord blood stem cells. Ohishi K. et al.: Delta-1 enhances marrow and thymus repopulating ability of human CD34+/CD38- cord blood cells. Clin. Invest. 110:1165-1174 (2002).

There is a need, therefore, to provide a method of repairing heart tissue that is not based on organ transplantation, or embryonic stem cell utilization.

SUMMARY OF THE INVENTION

The present invention is directed to a method for repairing heart tissue and replenishing heart cells, particularly by using a blood stem cell composition comprising TVEMF-expanded blood-derived adult stem cells, preferably TVEMF-expanded, and the body's ability to repair itself. A method of this invention for treating a mammal, preferably human, having need of heart repair comprises introducing to the mammal a therapeutically effective amount of blood derived expanded adult stem cells that have been TVEMF-expanded at least seven times the number of cells per volume as the number of cells per volume in the blood from which they were derived, where the TVEMF-expanded stem cells maintain their three-dimensional geometry and their cell-to-cell support and cell-to-cell geometry. The method includes such introduction within a

time period sufficient to allow the human body system to utilize the blood cells to effectively repair damaged heart tissue.

The present invention also relates in part to a blood stem cell composition for repairing heart tissue from a mammal, preferably human, preferably wherein said stem cells are TVEMF-expanded. The present invention also relates to blood stem cells from a mammal, preferably human, wherein said stem cells are in a number per volume that is at least 7 times greater than their source material (for instance, the blood source of the stem cells, prior to TVEMF expansion); and wherein the blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is the same essentially the same as stem cells of naturally-occurring (preferably source) blood. Such cells are preferably made by the TVEMF-expansion process described herein. The invention also relates blood stem cell compositions comprising these cells with other components added as desired, including pharmaceutically acceptable carriers, cryopreservatives, and cell culture media.

The present invention also relates to a process for preparing stem cells and stem cell compositions for repairing heart tissue by placing a blood mixture in a culture chamber of a TVEMF-bioreactor; and subjecting the blood mixture to a TVEMF and TVEMF-expanding the blood stem cells in the TVEMF bioreactor to prepare TVEMF-expanded blood stem cells and a stem cell composition. Preferably, the TVEMF applied to the cells is from about 0.05 to about 6.0 gauss. The present invention also relates to a method of cryopreserving the expanded stem cells by lowering their temperature to -120°C to -196°C for one year or longer, and raising the temperature thereafter to a temperature suitable for introducing the cells into a mammal.

Also comprised herein is a composition for the repair of heart tissue, and the use of such a composition and/or the expanded blood stem cells themselves in the preparation of a medicament for the repair or regeneration of heart tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings,

Figure 1 schematically illustrates a preferred embodiment of a culture carrier flow loop of a bioreactor;

Figure 2 is an elevated side view of a preferred embodiment of a TVEMF- bioreactor of the invention;

Figure 3 is a side perspective of a preferred embodiment of the TVEMF- bioreactor of Figure 2;
Figure 4 is a vertical cross sectional view of a preferred embodiment of a TVEMF- bioreactor;
Figure 5 is a vertical cross sectional view of a TVEMF- bioreactor;
Figure 6 is an elevated side view of a time varying electromagnetic force device that can house, and provide a time varying electromagnetic force to, a bioreactor;
Figure 7 is a front view of the device shown in Figure 6; and
Figure 8 is a front view of the device shown in Figure 6, further showing a bioreactor therein.

DETAILED DESCRIPTION OF THE DRAWINGS

In the simplest terms, a rotating TVEMF- bioreactor comprises a cell culture chamber and a time varying electromagnetic force source. In operation, a blood mixture is placed into the cell culture chamber. The cell culture chamber is rotated over a period of time during which a time varying electromagnetic force is generated in the chamber by the time varying electromagnetic force source. Upon completion of the period of time, the TVEMF-expanded blood mixture is removed from the chamber. In a more complex TVEMF- bioreactor system, the time varying electromagnetic force source can be integral to the TVEMF- bioreactor, as illustrated in Figures 2-5, but can also be adjacent to a bioreactor as in Figures 6-8. Furthermore, a fluid carrier such as cell culture media or buffer (preferably similar to that media added to a blood mixture, discussed below), which provides sustenance to the cells, can be periodically refreshed and removed. Preferred TVEMF- bioreactors are described herein.

Referring now to Figure 1, illustrated is a preferred embodiment of a culture carrier flow loop 1 in an overall bioreactor culture system for growing mammalian cells having a cell culture chamber 19, preferably a rotating cell culture chamber, an oxygenator 21, an apparatus for facilitating the directional flow of the culture carrier, preferably by the use of a main pump 15, and a supply manifold 17 for the selective input of such culture carrier requirements as, but not limited to, nutrients 3, buffers 5, fresh medium 7, cytokines 9, growth factors 11, and hormones 13. In this preferred embodiment, the main pump 15 provides fresh fluid carrier to the oxygenator 21 where the fluid carrier is oxygenated and passed through the cell culture chamber 19. The waste in the spent fluid carrier from the cell culture chamber 19 is removed and delivered to the waste 18 and the remaining cell culture carrier is returned to the manifold 17

where it receives a fresh charge, as necessary, before recycling by the pump 15 through the oxygenator 21 to the cell culture chamber 19.

In the culture carrier flow loop 1, the culture carrier is circulated through the living cell culture in the chamber 19 and around the culture carrier flow loop 1, as shown in Figure 1. In this loop 1, adjustments are made in response to chemical sensors (not shown) that maintain constant conditions within the cell culture reactor chamber 19. Controlling carbon dioxide pressures and introducing acids or bases corrects pH. Oxygen, nitrogen, and carbon dioxide are dissolved in a gas exchange system (not shown) in order to support cell respiration. The closed loop 1 adds oxygen and removes carbon dioxide from a circulating gas capacitance. Although Figure 1 is one preferred embodiment of a culture carrier flow loop that may be used in the present invention, the invention is not intended to be so limited. The input of culture carrier such as, but not limited to, oxygen, nutrients, buffers, fresh medium, cytokines, growth factors, and hormones into a bioreactor can also be performed manually, automatically, or by other control means, as can be the control and removal of waste and carbon dioxide.

Figures 2 and 3 illustrate a preferred embodiment of a TVEMF- bioreactor 10 with an integral time varying electromagnetic force source. Figure 4 is a cross section of a rotatable TVEMF-bioreactor 10 for use in the present invention in a preferred form. The TVEMF-bioreactor 10 of Figure 4 is illustrated with an integral time varying electromagnetic force source. Figure 5 also illustrates a preferred embodiment of a TVEMF- bioreactor with an integral time varying electromagnetic force source. Figures 6-8 show a rotating bioreactor with an adjacent time varying electromagnetic force source.

Turning now to Figure 2, illustrated in Figure 2 is an elevated side view of a preferred embodiment of a TVEMF-bioreactor 10 of the present invention. Figure 2 comprises a motor housing 111 supported by a base 112. A motor 113 is attached inside the motor housing 111 and connected by a first wire 114 and a second wire 115 to a control box 116 that has a control means therein whereby the speed of the motor 113 can be incrementally controlled by turning the control knob 117. The motor housing 111 has a motor 113 inside set so that a motor shaft 118 extends through the housing 111 with the motor shaft 118 being longitudinal so that the center of the shaft 118 is parallel to the plane of the earth at the location of a longitudinal chamber 119, preferably made of a transparent material including, but not limited to, plastic.

In this preferred embodiment, the longitudinal chamber 119 is connected to the shaft 118 so that the chamber 119 rotates about its longitudinal axis with the longitudinal axis parallel to the plane of the earth. The chamber 119 is wound with a wire coil 120. The size of the wire coil 120 and number of times it is wound are such that when a square wave current preferably of from 0.1mA to 1000mA is supplied to the wire coil 120, a time varying electromagnetic force preferably of from 0.05 gauss to 6 gauss is generated within the chamber 119. The wire coil 120 is connected to a first ring 121 and a second ring 122 at the end of the shaft 118 by wires 123 and 124. These rings 121, 122 are then contacted by a first electromagnetic delivery wire 125 and a second electromagnetic delivery wire 128 in such a manner that the chamber 119 can rotate while the current is constantly supplied to the coil 120. An electromagnetic generating device 126 is connected to the wires 125, 128. The electromagnetic generating device 126 supplies a square wave to the wires 125, 128 and coil 120 by adjusting its output by turning an electromagnetic generating device knob 127.

Figure 3 is a side perspective view of the TVEMF-bioreactor 10 shown in Figure 2 that may be used in the present invention.

Turning now to the rotating TVEMF-bioreactor 10 illustrated in Figure 4 with a culture chamber 230 which is preferably transparent and adapted to contain a blood mixture therein, further comprising an outer housing 220 which includes a first 290 and second 291 cylindrically shaped transverse end cap member having facing first 228 and second 229 end surfaces arranged to receive an inner cylindrical tubular glass member 293 and an outer tubular glass member 294. Suitable pressure seals are provided. Between the inner 293 and outer 294 tubular members is an annular wire heater 296 which is utilized for obtaining the proper incubation temperatures for cell growth. The wire heater 296 can also be used as a time varying electromagnetic force device to supply a time varying electric field to the culture chamber 230 or, as depicted in Figure 5, a separate wire coil 144 can be used to supply a time varying electromagnetic force. The first end cap member 290 and second end cap member 291 have inner curved surfaces adjoining the end surfaces 228, 229 for promoting smoother flow of the mixture within the chamber 230. The first end cap member 290, and second end cap member 291 have a first central fluid transfer journal member 292 and second central fluid transfer journal member 295, respectively, that are rotatably received respectively on an input shaft 223 and an output shaft 225. Each transfer journal member 294, 295 has a flange to seat in a recessed counter bore in an end cap member

290, 291 and is attached by a first lock washer and ring 297, and second lock washer and ring 298 against longitudinal motion relative to a shaft 223, 225. Each journal member 294, 295 has an intermediate annular recess that is connected to longitudinally extending, circumferentially arranged passages. Each annular recess in a journal member 292, 295 is coupled by a first radially disposed passage 278 and second radially disposed passage 279 in an end cap member 290 and 291, respectively, to first input coupling 203 and second input coupling 204. Carrier in a radial passage 278 or 279 flows through a first annular recess and the longitudinal passages in a journal member 294 or 295 to permit access carrier through a journal member 292, 295 to each end of the journal 292, 295 where the access is circumferential about a shaft 223, 225.

Attached to the end cap members 290 and 291 are a first tubular bearing housing 205, and second tubular bearing housing 206 containing ball bearings which relatively support the outer housing 220 on the input 223 and output 225 shafts. The first bearing housing 205 has an attached first sprocket gear 210 for providing a rotative drive for the outer housing 220 in a rotative direction about the input 223 and output 225 shafts and the longitudinal axis 221. The first bearing housing 205, and second bearing housing 206 also have provisions for electrical take out of the wire heater 296 and any other sensor.

The inner filter assembly 235 includes inner 215 and outer 216 tubular members having perforations or apertures along their lengths and have a first 217 and second 218 inner filter assembly end cap member with perforations. The inner tubular member 215 is constructed in two pieces with an interlocking centrally located coupling section and each piece attached to an end cap 217 or 218. The outer tubular member 216 is mounted between the first 217 and second inner filter assembly end caps.

The end cap members 217, 218 are respectively rotatably supported on the input shaft 223 and the output shaft 225. The inner member 215 is rotatively attached to the output shaft 225 by a pin and an interfitting groove 219. A polyester cloth 224 with a ten-micron weave is disposed over the outer surface of the outer member 216 and attached to O-rings at either end. Because the inner member 215 is attached by a coupling pin to a slot in the output drive shaft 225, the output drive shaft 225 can rotate the inner member 215. The inner member 215 is coupled by the first 217 and second 218 end caps that support the outer member 216. The output shaft 225 is extended through bearings in a first stationary housing 240 and is coupled to a first sprocket gear 241. As illustrated, the output shaft 225 has a tubular bore 222 that extends from a

first port or passageway 289 in the first stationary housing 240 located between seals to the inner member 215 so that a flow of fluid carrier can be exited from the inner member 215 through the stationary housing 240.

Between the first 217 and second 218 end caps for the inner member 235 and the journals 292, 295 in the outer housing 220, are a first 227 and second 226 hub for the blade members 50a and 50b. The second hub 226 on the input shaft 223 is coupled to the input shaft 223 by a pin 231 so that the second hub 226 rotates with the input shaft 223. Each hub 227, 226 has axially extending passageways for the transmittal of carrier through a hub.

The input shaft 223 extends through bearings in the second stationary housing 260 for rotatable support of the input shaft 223. A second longitudinal passageway 267 extends through the input shaft 223 to a location intermediate of retaining washers and rings that are disposed in a second annular recess 232 between the faceplate and the housing 260. A third radial passageway 272 in the second end cap member 291 permits fluid carrier in the recess to exit from the second end cap member 291. While not shown, the third passageway 272 connects through piping and a Y joint to each of the passages 278 and 279.

A sample port is shown in Figure 4, where a first bore 237 extending along a first axis intersects a corner 233 of the chamber 230 and forms a restricted opening 234. The bore 237 has a counter bore and a threaded ring at one end to threadedly receive a cylindrical valve member 236. The valve member 236 has a complimentarily formed tip to engage the opening 234 and protrude slightly into the interior of the chamber 230. An O-ring 243 on the valve member 236 provides a seal. A second bore 244 along a second axis intersects the first bore 237 at a location between the O-ring 243 and the opening 234. An elastomer or plastic stopper 245 closes the second bore 244 and can be entered with a hypodermic syringe for removing a sample. To remove a sample, the valve member 236 is backed off to access the opening 234 and the bore 244. A syringe can then be used to extract a sample and the opening 234 can be reclosed. No outside contamination reaches the interior of the TVEMF-bioreactor 10.

In operation, carrier is input to the second port or passageway 266 to the shaft passageway and thence to the first radially disposed 278 and second radially disposed passageways 279 via the third radial passageway 272. When the carrier enters the chamber 230 via the longitudinal passages in the journals 292, 294 the carrier impinges on an end surface 228, 229 of the hubs 227, 226 and is dispersed radially as well as axially through the passageways in

the hubs 227, 226. Carrier passing through the hubs 227, 226 impinges on the end cap members 217, 218 and is dispersed radially. The flow of entry fluid carrier is thus radially outward away from the longitudinal axis 221 and flows in a toroidal fashion from each end to exit through the polyester cloth 224 and openings in filter assembly 235 to exit via the passageways 266 and 289. By controlling the rotational speed and direction of rotation of the outer housing 220, chamber 230, and inner filter assembly 235 any desired type of carrier action can be obtained. Of major importance, however, is the fact that a clinostat operation can be obtained together with a continuous supply of fresh fluid carrier.

If a time varying electromagnetic force is not applied using the integral annular wire heater 296, it can be applied by another preferred time varying electromagnetic force source. For instance, Figures 6-8 illustrate a time varying electromagnetic force device 140 which provides an electromagnetic force to a cell culture in a bioreactor which does not have an integral time varying electromagnetic force, but rather has an adjacent time varying electromagnetic force device. Specifically, Figure 6 is a preferred embodiment of a time varying electromagnetic force device 140. Figure 6 is an elevated side perspective of the device 140 which comprises a support base 145, a cylinder coil support 146 supported on the base 145 with a wire coil 147 wrapped around the support 146. Figure 7 is a front perspective of the time varying electromagnetic force device 140 illustrated in Figure 6. Figure 8 is a front perspective of the time varying electromagnetic force device 140, which illustrates that in operation, an entire bioreactor 148 is inserted into a cylinder coil support 146 which is supported by a support base 145 and which is wound by a wire coil 147. Since the time varying electromagnetic force device 140 is adjacent to the bioreactor 148, the time varying electromagnetic force device 140 can be reused. In addition, since the time varying electromagnetic force device 140 is adjacent to the bioreactor 148, the device 140 can be used to generate an electromagnetic force in all types of bioreactors, preferably rotating.

In operation, during TVEMF- expansion, a TVEMF- bioreactor 10 of the present invention contains a blood mixture in the cell culture chamber. During TVEMF- expansion, the speed of the rotation of the blood mixture-containing chamber may be assessed and adjusted so that the blood mixture remains substantially at or about the longitudinal axis. Increasing the rotational speed is warranted to prevent wall impact. For instance, an increase in the rotation is preferred if the blood stem cells in the blood mixture fall excessively inward and downward on

the downward side of the rotation cycle and excessively outward and insufficiently upward on the upward side of the rotation cycle. Optimally, the user is advised to preferably select a rotational rate that fosters minimal wall collision frequency and intensity so as to maintain the blood stem cell three-dimensional geometry and their cell-to-cell support and cell-to-cell geometry. The preferred speed of the present invention is of from 5 to 120 RPM, and more preferably from 10 to 30 RPM.

The blood mixture may preferably be visually assessed through the preferably transparent culture chamber and manually adjusted. The assessment and adjustment of the blood mixture may also be automated by a sensor (for instance, a laser), which monitors the location of the blood stem cells within a TVEMF- bioreactor 10. A sensor reading indicating too much cell movement will automatically cause a mechanism to adjust the rotational speed accordingly.

Furthermore, in operation the present invention contemplates that an electromagnetic generating device is turned on and adjusted so that the square wave output generates the desired electromagnetic field in the blood mixture-containing chamber, preferably in a range of from 0.05 gauss to 6 gauss.

Preferably, the square wave has a frequency of about 2 to about 25 cycles/second, more preferably about 5 to about 20 cycles/second, for example about 10 cycles/second, and the conductor has an RMS value of about 1 to 1000 mA, preferably 1 to 6 mA. However, these parameters are not meant to be limiting to the TVEMF of the present invention, as such may vary based on other aspects of this invention. TVEMF may be measured for instance by standard equipment such as an EN131 Cell Sensor Gauss Meter.

As various changes could be made in rotating bioreactors subjected to a time varying electromagnetic force as are contemplated in the present invention, without departing from the scope of the invention, it is intended that all matter contained herein be interpreted as illustrative and not limiting.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention is related to a method of repairing, replenishing and regenerating heart tissue in humans. This invention may be more fully described by the preferred embodiment as hereinafter described, but is not intended to be limited thereto.

In the preferred embodiment of this invention, a method is described to prepare adult stem cells that can assist the body in repairing, replacing, regenerating heart tissue. Blood cells are removed from a patient. A subpopulation of these cells is currently referred to as adult stem cells. The blood cells, including adult stem cells, are placed in a bioreactor as described herein. The bioreactor vessel is rotated at a speed that provides for suspension of the blood cells to maintain their three-dimensional geometry and their cell-to-cell support and geometry. During the time that the cells are in the reactor, they may be fed nutrients, exposed to hormones, cytokines, or growth factors, and/or genetically modified, and toxic materials are preferably removed. The toxic materials typically removed are from blood cells comprising the toxic granular material of dying cells and the toxic material of granulocytes and macrophages. A subpopulation of these cells is expanded creating a large amount of cells. The expansion of the cells is controlled so that the cells expand at least seven times in a sufficient amount of time, preferably within seven days. The cells are then injected intravenously or directly into or immediately adjacent to the heart tissue to be repaired allowing the body's natural system to repair and regenerate the heart tissue. Further, in this method, blood stem cells can be manipulated to alter their curative characteristics, preferably by genetically modifying the cells.

The following definitions are meant to aid in the description and understanding of the defined terms in the context of the present invention. The definitions are not meant to limit these terms to less than is described throughout this application. Furthermore, several definitions are included relating to TVEMF – all of the definitions in this regard should be particularly considered to complement each other, and not construed against each other.

As used throughout this application, the term "adult stem cell" refers to a pluripotent cell that is undifferentiated and that may give rise to more differentiated cells. With regard to the present invention, an adult stem cell is preferably CD34+/CD38-. Adult stem cells are also known as somatic stem cells, and are <u>not</u> embryonic stem cells directly derived from an embryo.

As used throughout this application, the term "blood" refers to peripheral blood or cord blood, two primary sources of adult blood stem cells in a mammal. "Peripheral blood" is systemic blood; that is, blood that circulates, or has circulated, systemically in a mammal. The mammal is not meant to be a fetus. For the purposes of the present invention, there is no reason to distinguish between peripheral blood located at different parts of the same circulatory loop. "Cord blood" refers to blood from the umbilical cord and/or placenta of a fetus or infant. Cord

blood is one of the richest sources of stem cells known. The term "cord" is not meant in any way to limit the term "cord blood" of this invention to blood of the umbilical cord; the blood of a fetus' or infant's placenta is confluent with the blood of the umbilical cord. For the purposes of the present invention, there is no reason to distinguish between blood located at different parts of the same circulatory loop.

As used throughout this application, the term "blood cell" refers to a cell from blood; "peripheral blood cell" refers to a cell from peripheral blood; and "cord blood cell" refers to a cell from cord blood. Blood cells capable of replication may undergo TVEMF-expansion in a TVEMF-bioreactor, and may be present in compositions of the present invention.

As used throughout this application, the term "blood stem cell" refers to an adult stem cell from blood. Blood stem cells are adult stem cells, which as mentioned above are also known as somatic stem cells, and are not embryonic stem cells derived directly from an embryo. Preferably, a blood stem cell of the present invention is a CD34+/CD38- cell.

As used throughout this application, the term "blood stem cell composition", or reference thereto, refers to blood stem cells of the present invention, either (1) in a number per volume at least 7 times greater than the naturally-occurring blood source and having the same or very similar three-dimensional geometry and cell-to-cell geometry and cell-to-cell support as naturally-occurring blood stem cells, and/or (2) having undergone TVEMF-expansion, maintaining the above mentioned three-dimensional geometry and support. With the blood stem cells in a blood stem cell composition of this invention is a carrier of some sort, whether a pharmaceutically acceptable carrier, plasma, blood, albumin, cell culture medium, growth factor, copper chelating agent, hormone, buffer, cryopreservative, or some other substance. Reference to naturally-occurring blood is preferably to compare blood stem cells of the present invention with their original blood (i.e. peripheral, cord, mixed peripheral or cord, or other) source. However, if such a comparison is not available, then naturally-occurring blood may refer to average or typical characteristics of such blood, preferably of the same mammalian species as the source of the blood stem cells of this invention.

A "pharmaceutical blood stem cell composition" of this invention is a blood stem cell composition that is suitable for administration into a mammal, preferably into a human. Such a composition has a therapeutically effective amount of expanded (preferably TVEMF-expanded) blood stem cells. A therapeutically effective amount of expanded blood stem cells is (also

discussed elsewhere herein) preferably at least 1000 stem cells, more preferably at least 10^4 stem cells, even more preferably at least 10^5 stem cells, and even more preferably in an amount of at least 10^7 to 10^9 stem cells, or even more stem cells such as 10^{12} stem cells. Administration of such numbers of expanded stem cells may be in one or more doses. As indicated throughout this application, the number of stem cells administered to a patient may be limited to the number of stem cells originally available in source blood, as multiplied by expansion according to this invention. Without being bound by theory, it is believed that stem cells not used by the body after administration will simply be removed by natural body systems.

As used throughout this application, the term "blood mixture" refers to a mixture of blood/blood cells with a substance that helps the cells to expand, such as a medium for growth of cells, that may be placed in a TVEMF-bioreactor (for instance in a cell culture chamber). The "blood mixture" blood cells may be present in the blood mixture simply by mixing whole blood with a substance such as a cell culture medium. Also, the blood mixture may be made with a cellular preparation from blood, as described throughout this application, such as a "buffy coat," containing blood stem cells. Preferably, the blood mixture comprises CD34+/CD38- blood stem cells and Dulbecco's medium (DMEM). Preferably, about half of the blood mixture is a cell culture medium such as DMEM.

As used throughout this application, the term "TVEMF" refers to "Time Varying Electromagnetic Force". As discussed above, the TVEMF of this invention is a square wave (following a Fourier curve). Preferably, the square wave has a frequency of about 10 cycles/second, and the conductor has an RMS value of about 1 to 1000 mA, preferably 1 to 6 mA. However, these parameters are not meant to be limiting to the TVEMF of the present invention, as such may vary based on other aspects of this invention. TVEMF may be measured for instance by standard equipment such as an EN131 Cell Sensor Gauss Meter.

As used throughout this application, the term "TVEMF-bioreactor" refers to a rotating bioreactor to which TVEMF is applied, as described more fully in the Description of the Drawings, above. The TVEMF applied to a bioreactor is preferably in the range of 0.05 to 6.0 gauss, preferably 0.05-0.5 gauss. See for instance Figures 2, 3, 4 and 5 herein for examples (not meant to be limiting) of a TVEMF-bioreactor. In a simple embodiment, a TVEMF-bioreactor of the present invention provides for the rotation of an enclosed blood mixture at an appropriate gauss level (with TVEMF applied), and allows the blood cells (including stem cells) therein to

expand. Preferably, a TVEMF-bioreactor allows for the exchange of growth medium (preferably with additives) and for oxygenation of the blood mixture. The TVEMF-bioreactor provides a mechanism for growing cells for several days or more. Without being bound by theory, the TVEMF-bioreactor subjects cells in the bioreactor to TVEMF, so that TVEMF is passed through or otherwise exposed to the cells, the cells thus undergoing TVEMF-expansion. The rotation of the TVEMF-bioreactor during TVEMF-expansion is preferably at a rate of 5 to 120 rpm, more preferably 10 to 30 rpm, to foster minimal wall collision frequency and intensity so as to maintain the bloodstream cell three-dimensional geometry and cell-to-cell support and cell-to-cell geometry.

As used throughout this application, the term "TVEMF-expanded blood cells" refers to blood cells increased in number per volume after being placed in a TVEMF-bioreactor and subjected to a TVEMF of about 0.05 to 6.0 gauss. The increase in number of cells per volume is the result of cell replication in the TVEMF-bioreactor, so that the total number of cells increase. The increase in number of cells per volume is expressly not due to a simple reduction in volume of fluid, for instance, reducing the volume of blood from 70 ml to 10 ml and thereby increasing the number of cells per ml.

As used throughout this application, the term "TVEMF-expanded blood stem cells" refers to blood stem cells increased in number per volume after being placed in a TVEMF-bioreactor and subjected to a TVEMF of about 0.05 to 6.0 gauss. The increase in number of stem cells per volume is the result of cell replication in the TVEMF-bioreactor, so that the total number of stem cells in the bioreactor increase. The increase in number of stem cells per volume is expressly not due to a simple reduction in volume of fluid, for instance, reducing the volume of blood from 70 ml to 10 ml and thereby increasing the number of stem cells per ml.

As used throughout this application, the term "TVEMF-expanding" refers to the step of cells in a TVEMF-bioreactor replicating (splitting and growing) in the presence of TVEMF in a TVEMF-(rotating) bioreactor. Blood stem cells (preferably CD34+/CD38- stem cells) preferably replicate without undergoing further differentiation, so that all or substantially all CD34+/CD38- stem cells expanded according to this invention replicate, but do not differentiate, during their time in a bioreactor. "Substantially all" is meant to refer to at least 70%, preferably at least 80%, more preferably at least 90%, even more preferably at least 95%, even more

preferably at least 97%, and most preferably at least 99% of CD34+CD38- cells do not differentiate such that they are no longer CD34+/CD38- during TVEMF-expansion.

As used throughout this application, the term "TVEMF-expansion" refers to the process of increasing the number of blood cells in a TVEMF-bioreactor, preferably blood stem cells, by subjecting the cells to a TVEMF of about 0.05 to about 6.0 gauss. Preferably, the increase in number of blood stem cells is at least 7 times the number per volume of the original blood source. The expansion of blood stem cells in a TVEMF-bioreactor according to the present invention provides for blood stem cells that maintain, or have the same or essentially the same, three-dimensional geometry and cell-to-cell support and cell-to-cell geometry as blood stem cells prior to TVEMF-expansion. Other aspects of TVEMF-expansion may also provide the exceptional characteristics of the blood stem cells of the present invention. Not to be bound by theory, TVEMF-expansion not only provides for high concentrations of blood stem cells that maintain their three-dimensional geometry and cell-to-cell support and geometry. Not to be bound by theory, TVEMF may affect some properties of stem cells during TVEMF-expansion, for instance up-regulation of genes promoting growth, or down regulation of genes preventing growth. Overall, TVEMF-expansion results in promoting blood stem cell growth but not differentiation.

As used throughout this application, the term "TVEMF-expanded cell" refers to a cell that has been subjected to the process of TVEMF-expansion.

Throughout this application, the terms "repair", "replenish" and "regenerate" are used. These terms are not meant to be mutually exclusive, but rather related to overall tissue repair.

Throughout this application, reference to the repair of heart tissue, treatment of heart disease, treatment of heart condition, are not meant to be exclusive but rather relate to the objective of overall tissue repair where improvement in tissue results from administration of stem cells as discussed herein. While the present invention is directed in part to heart diseases or conditions that are symptomatic, and possibly life-threatening, the present invention is also meant to include treatment of minor repair, and even prevention/prophylaxis of heart disease/condition by early introduction of expanded stem cells, before symptoms or problems in the mammal's (preferably human's) health are notice.

As used throughout this application, the term "toxic substance" or related terms may refer to substances that are toxic to a cell, preferably a blood stem cell; or toxic to a patient. In

particular, the term toxic substance refers to dead cells, macrophages, as well as substances that may be unique or unusual in blood (for instance, sickle cells in peripheral blood, maternal urine or waste in cord blood, or other tissue or waste). Other toxic substances are discussed throughout this application. Removal of toxic substances from blood is well-known in the art, in particular art relating to the introduction of blood products to a patient.

As used throughout this application, the term "apheresis of bone marrow" refers to inserting a needle into bone and extracting bone marrow. Such apheresis is well-known in the art.

As used throughout this application, the term "autologous" refers to a situation in which the donor (source of blood stem cells prior to expansion) and recipient are the same mammal. The present invention includes autologous heart tissue repair and replenishment.

As used throughout this application, the term "allogeneic" refers to a situation in which the donor (source of blood stem cells prior to expansion) and recipient are not the same mammal. The present invention includes allogeneic heart tissue repair and replenishment.

As used throughout this application, the term "CD34+" refers to the presence of a surface antigen (CD34) on the surface of a blood cell. CD34 protein is present on the surface of hematopoietic stem cells in all states of development.

As used throughout this application, the term "CD38-" refers to the lack of a surface antigen (CD38) on the surface of a blood cell. CD38 is not present on the surface of stem cells of the present invention.

As used throughout this application, the term "cell-to-cell geometry" refers to the geometry of cells including the spacing, distance between, and physical relationship of the cells relative to one another. For instance, TVEMF-expanded stem cells of this invention stay in relation to each other as in the body. The expanded cells are within the bounds of natural spacing between cells, in contrast to for instance two-dimensional expansion containers, where such spacing is not kept.

As used throughout this application, the term "cell-to-cell support" refers to the support one cell provides to an adjacent cell. For instance, healthy tissue and cells maintain interactions such as chemical, hormonal, neural (where applicable/appropriate) with other cells in the body. In the present invention, these interactions are maintained

within normal functioning parameters, meaning they do not for instance begin to send toxic or damaging signals to other cells (unless such would be done in the natural blood environment).

As used throughout this application, the term "three-dimensional geometry" refers to the geometry of cells in a three-dimensional state (same as or very similar to their natural state), as opposed to two-dimensional geometry for instance as found in cells grown in a Petri dish, where the cells become flattened and/or stretched.

For each of the above three definitions, relating to maintenance of cell-to-cell support and geometry and three dimensional geometry of stem cells of the present invention, the term "essentially the same" means that normal geometry and support are provided in TVEMF-expanded cells of this invention, so that the cells are not for instance changed in such a way as to be disfunctional, unable to repair tissue or toxic or harmful to other cells.

Other statements referring to the above-defined terms or other terms used throughout this application are not meant to be limited by the above definitions, and may contribute to the definitions. Information relating to various aspects of this invention is provided throughout this application, and is not meant to be limited only to the section to which it is contained, but is meant to contribute to an understanding of the invention as a whole.

The present invention is directed to providing TVEMF-expanded blood stem cells for repairing, replenishing and regenerating heart tissue. This invention may be more fully described by the preferred embodiment(s) as hereinafter described, but is not intended to be limited thereto.

Operative Method – Preparing a TVEMF-expanded blood stem cell composition

In a preferred embodiment of this invention, a method is described for preparing TVEMF-expanded blood stem cells that can assist the body in repairing, replacing and regenerating heart tissue.

In this preferred method, blood is collected from a mammal, preferably a primate mammal, and more preferably a human, for instance as described throughout this application and as known in the art, and preferably via a syringe as well known in the art. Blood may be collected expanded immediately and used, or cryopreserved in expanded or unexpanded form

for use. Blood would only be removed from a human in an amount that would not be threatening to the subject. Preferably, about 10 to about 500 ml blood is collected; more preferably, 100-300 ml, even more preferably, 150-200 ml. The collection of blood according to this invention is not meant to be limiting, but can also include for instance other means of directly collecting mammalian blood, pooling blood from one or more sources, indirectly collecting blood for instance by acquiring the blood from a commercial or other source, including for instance cryopreserved peripheral or cord blood from a "blood bank", or blood otherwise stored for later use.

Typically, when directly collected from a mammal, blood is drawn into one or more syringes, preferably containing anticoagulants. The blood may be stored in the syringe or transferred to another vessel. Blood may then be separated into its parts; white blood cells, red blood cells, and plasma. This is either done in a centrifuge (an apparatus that spins the container of blood until the blood is divided) or by sedimentation (the process of injecting sediment into the container of blood causing the blood to separate). Second, once the blood is divided with the red blood cells (RBC) on the bottom, white blood cells (WBC) in the middle, and the plasma on top, the white blood cells are removed for storage. The middle layer, also known as the "buffy coat" contains the blood stem cells of interest; the other parts of the blood are not needed. For some blood banks, this will be the extent of their processing. However, other banks will go on to process the buffy coat by removing the mononuclear cells (in this case, a subset of white blood cells) from the WBC. While not everyone agrees with this method, there is less to store and less cryogenic nitrogen is needed to store the cells.

Another method for separating blood cells is to subject all of the collected blood to one or more (preferably three) rounds of continuous flow leukapheresis in a separator such as a Cobe Spectra cell separator. Such processing will separate blood cells having one nucleus from other blood cells. The stem cells are part of the group having one nucleus. Other methods for the separation of blood cells are known in the art.

It is preferable to remove the RBC from the blood sample. While people may have the same HLA type (which is needed for the transplanting of stem cells), they may not have the same blood type. By removing the RBC, adverse reactions to a stem cell transplant can be minimized. By eliminating the RBC, therefore, the stem cell sample has a better chance of being compatible with more people. RBC can also burst when they are thawed, releasing free

hemoglobin. This type of hemoglobin can seriously affect the kidneys of people receiving a transplant. Additionally, the viability of the stem cells are reduced when RBC rupture.

Also, particularly if storing blood cryogenically or transferring the blood to another mammal, the blood may be tested to ensure no infectious or genetic diseases, such as HIV/AIDS, hepatitis, leukemia or immune disorder, is present. If such a disease exists, the blood may be discarded or used with associated risks noted for a future user to consider.

In still another embodiment of this invention, blood cells may be obtained from a person needing heart repair or from a donor not in need of repair. Prior to collection, the donor may be treated with G-CSF 6 ng/kg every 12 hr over 3 days and then once on day 4. In a preferred method, a like amount of GM-CSF is also administered. Blood is then collected from the donor, and PBCs may be separated by subjecting the donor's total blood volume to 3 rounds of continuous-flow leukapheresis through a separator, such as a Cobe Spectra cell separator.

In still another embodiment of this invention, blood cells may be obtained from a donor. Prior to collection, the donor is treated with G-CSF (preferably in an amount of 0.3ng to 5ug, more preferably 1 ng/kg to 100ng/kg, even more preferably 5 ng/kg to 20 ng/kg, and even more preferably 6 ng/kg) every 12 hr over 3 days and then once on day 4. In a preferred method, a like amount of GM-CSF is also administered. Other alternatives are to use GM-CSF alone, or other growth factor molecules, interleukins. Blood is then collected from the donor, and may be used whole in a blood mixture or first separated into cellular parts as discussed throughout this application, where the cellular part including stem cells (CD34+/CD38-) is used to prepare the blood mixture to be expanded. Cells may be separated, for instance, by subjecting the donor's total blood volume to 3 rounds of continuous-flow leukapheresis through a separator, such as a Cobe Spectra cell separator. Preferably, the expanded stem cells are reintroduced into the same donor, where the donor is in need of heart tissue repair as discussed herein. However, allogeneic introduction may also be used, as also indicated herein. Other pre-collection administrations will also be evident to those skilled in the art.

Preferably, red blood cells are removed from the blood and the remaining cells including blood stem cells are placed with an appropriate media in a TVEMF-bioreactor (see "blood mixture") such as that described herein. In a more preferred embodiment of this

invention, only the "buffy coat" (which includes blood stem cells, as discussed throughout this application) described above is the cellular material placed in the TVEMF-bioreactor. Other embodiments include removing other non-stem cells and components of the blood, to prepare different blood preparation(s). Such a blood preparation may even have, as the only remaining blood component, CD34+/CD38- blood stem cells. Removal of non-stem cell types of blood cells may be achieved through negative separation techniques, such as but not limited to sedimentation and centrifugation. Many negative separation methods are well-known in the art. However, positive selection techniques may also be used, and are preferred in this invention. Methods for removing various components of the blood and positively selecting for CD34+/CD38- are known in the art, and may be used so long as they do not lyse or otherwise irreversibly harm the desired blood stem cells. For instance, an affinity method selective for CD34+/CD38- may be used. Preferably, a "buffy coat" as described above is prepared from blood, and the CD34+/CD38- cells therein separated from the buffy coat for TVEMF-expansion.

The collected blood, or desired cellular parts as discussed above, must be placed into a TVEMF-bioreactor for TVEMF-expansion to occur. As discussed above, the term "blood mixture" comprises a mixture of blood (or desired cellular part, for instance blood without red blood cells, or preferably CD34+/CD38- blood stem cells isolated from blood) with a substance that allows the cells to expand, such as a medium for growth of cells, that will be placed in a TVEMF-bioreactor. Cell culture media, media that allow cells to grow and expand, are well-known in the art. Preferably, the substance that allows the cells to expand is cell culture media, more preferably Dulbecco's medium. The components of the cell media must, of course, not kill or damage the stem cells. Other components may also be added to the blood mixture prior to or during TVEMF-expansion. For instance, the blood may be placed in the bioreactor with Dulbecco's medium and further supplemented with 5% (or some other desired amount, for instance in the range of about 1% to about 10%) of human serum albumin. Other additives to the blood mixture, including but not limited to growth factor, copper chelating agent, cytokine, hormone and other substances that may enhance TVEMF-expansion may also be added to the blood outside or inside the bioreactor before being placed in the bioreactor. Preferably, the entire volume of a blood collection from one individual (preferably human blood in an amount of about 10 ml to about 500 ml, more preferably about

100 ml to about 300 ml, even more preferably about 150 to about 200 ml blood) is mixed with a cell culture medium such as Dulbecco's medium (DMEM) and supplemented with 5% human serum albumin to prepare a blood mixture for TVEMF-expansion. For instance, for a 50 to 100 ml blood sample, preferably about 25 to about 100 ml DMEM/5% human serum albumin is used, so that the total volume of the blood mixture is about 75 to about 200 ml when placed in the bioreactor. As a general rule, the more blood that may be collected, the better; if a collection from one individual results in more than 100 ml, the use of all of that blood is preferred. Where a larger volume is available, for instance by pooling blood (from the same or different source), more than one dose may be preferred. The use of a perfusion TVEMF-bioreactor is particularly useful when blood collections are pooled and TVEMF-expanded together.

A copper chelating agent of the present invention may be any non-toxic copper chelating agent, and is preferably Penicillamine or Trientine Hydrochloride. More preferably, the Penicillamine is D(-)-2-Amino-3-Mercaptor-3-Methylbutanic Acid (Sigma-Aldrich), dissolved in DMSO and added to the blood mixture in an amount of about 10 ppm. The copper chelating agent may also be administered to a mammal, where blood will then be directly collected from the mammal. Preferably such administration is more than one day, more preferably more than two days, before collecting blood from the mammal. The purpose of the copper chelating agent, whether added to the blood mixture itself or administered to a blood donor mammal, or both, is to reduce the amount of copper in the blood prior to TVEMF-expansion. Not to be bound by theory, it is believed that the decrease in amount of available copper may enhance TVEMF-expansion.

The term "placed into a TVEMF-bioreactor" is not meant to be limiting – the blood mixture may be made entirely outside of the bioreactor and then the mixture placed inside the bioreactor. Also, the blood mixture may be entirely mixed inside the bioreactor. For instance, the blood (or a cellular portion thereof) may be placed in the bioreactor and supplemented with Dulbecco's medium and 5% human serum albumin either already in the bioreactor, added simultaneously to the bioreactor, or added after the blood to the bioreactor.

A preferred blood mixture of the present invention comprises the following: CD34+/CD38- stem cells isolated from the buffy coat of a blood sample; and Dulbecco's medium which, with the CD34+/CD38- cells, is about 150-250 ml, preferably about 200 ml

total volume. Even more preferably, G-CSF (Granulocyte-Colony Stimulating Factor) is included in the blood mixture. Preferably, G-CSF is present in an amount sufficient to enhance TVEMF-expansion of blood stem cells. Even more preferably, the amount of G-CSF present in the blood mixture prior to TVEMF-expansion is about 25 to about 200 ng/ml blood mixture, more preferably about 50 to about 150 ng/ml, and even more preferably about 100 ng/ml.

The TVEMF-bioreactor vessel (containing the blood mixture including the blood stem cells) is rotated at a speed that provides for suspension of the blood stem cells to maintain their three-dimensional geometry and their cell-to-cell support and cell-to-cell geometry. Preferably, the rotational speed is 5-120 rpm; more preferably, from 10-30 rpm. These rotational speeds are not intended to be limiting; rotational speed will depend at least in part on the type of bioreactor and size of cell culture chamber and sample placed therein. During the time that the cells are in the TVEMF-bioreactor, they are preferably fed nutrients and fresh media (for instance, DMEM and 5% human serum albumin; see above discussions of fluid carriers), exposed to hormones, cytokines, and/or growth factors (preferably G-CSF); and toxic materials are removed. The toxic materials removed from blood cells in a TVEMF-bioreactor include toxic granular material of dying cells and toxic material of granulocytes and macrophages. The TVEMF-expansion of the cells is controlled so that the cells preferably expand (increase in number per volume) at least seven times. Preferably, blood stem cells (with other cells, if present) undergo TVEMF-expansion for at least 4 days, preferably about 7 to about 14 days, more preferably about 7 to about 10 days, even more preferably about 7 days. TVEMF-expansion may continue in a TVEMF-bioreactor for up to 160 days. While TVEMF-expansion may occur for even longer than 160 days, such a lengthy expansion is not a preferred embodiment of the present invention.

Preferably, TVEMF-expansion is carried out in a TVEMF-bioreactor at a temperature of about 26°C to about 41°C, and more preferably, at a temperature of about 37°C.

One method of monitoring the overall expansion of cells undergoing TVEMF-expansion is by visual inspection. Blood stem cells are typically dark red in color. Preferably, the medium used to form the blood mixture is light or clear in color. Once the bioreactor begins to rotate and the TVEMF is applied, the cells preferably cluster in the center of the bioreactor vessel, with the medium surrounding the colored cluster of cells.

Oxygenation and other nutrient additions often do not cloud the ability to visualize the cell cluster through a visualization (typically clear plastic) window built into the bioreactor. Formation of the cluster is important for helping the stem cells maintain their three-dimensional geometry and cell-to-cell support and cell-to-cell geometry; if the cluster appears to scatter and cells begin to contact the wall of the bioreactor vessel, the rotational speed is increased (manually or automatically) so that the centralized cluster of cells may form again. A measurement of the visualizable diameter of the cell cluster taken soon after formation may be compared with later cluster diameters, to indicate the approximate number increase in cells in the TVEMF-bioreactor. Measurement of the increase in the number of cells during TVEMF expansion may also be taken in a number of ways, as known in the art for conventional bioreactors. An automatic sensor could also be included in the TVEMF-bioreactor to monitor and measure the increase in cluster size.

The TVEMF-expansion process may be carefully monitored, for instance by a laboratory expert, who may check cell cluster formation to ensure the cells remain clustered inside the bioreactor and will increase the rotation of the bioreactor when the cell cluster begins to scatter. An automatic system for monitoring the cell cluster and viscosity of the blood mixture inside the bioreactor may also monitor the cell clusters. A change in the viscosity of the cell cluster may become apparent as early as 2 days after beginning the TVEMF-expansion process, and the rotational speed of the TVEMF-bioreactor may be increased around that time. The TVEMF-bioreactor speed may vary throughout TVEMF-expansion. Preferably, the rotational speed is timely adjusted so that the cells undergoing TVEMF-expansion do not contact the sides of the TVEMF-bioreactor vessel.

Also, a laboratory expert may, for instance once a day, during TVEMF-expansion, or once every two days, manually (for instance with a syringe) insert fresh media and preferably other desired additives such as nutrients and growth factors, as discussed above, into the bioreactor, and draw off the old media containing cell wastes and toxins. Also, fresh media and other additives may be automatically pumped into the TVEMF-bioreactor during TVEMF-expansion, and waste automatically removed.

Blood stem cells may increase to at least seven times their original number about 7 to about 14 days after being placed in the TVEMF-bioreactor and TVEMF-expanded. Preferably, the TVEMF-expansion occurs for about 7 to 10 days, and more preferably about 7

days. Measurement of the number of stem cells does not need to be taken during TVEMF-expansion therefore. As indicated above and throughout this application, TVEMF-expanded blood stem cells of the present invention have the same or essentially the same three-dimensional geometry and cell-to-cell support and cell-to-cell geometry as naturally-occurring, non-TVEMF-expanded blood stem cells.

Upon completion of TVEMF-expansion, the cellular material in the TVEMF-bioreactor comprises the stem cells of the present invention, in a composition of the present invention. Various substances may be removed from or added to the composition for further use. Another embodiment of the present invention relates to an ex vivo mammalian blood stem cell composition that functions to assist a body system or tissue to repair, replenish and regenerate tissue, for example, the tissues described throughout this application. The composition comprises TVEMF-expanded blood stem cells, preferably in an amount of at least seven times the number per volume of blood stem cells per volume as in the blood from which it originated. For instance, preferably, if a number X of blood stem cells was placed in a certain volume into a TVEMF-bioreactor, then after TVEMF-expansion, the number of blood stem cells in the TVEMF-bioreactor will be at least 7X (barring removal of cells during the expansion process). While this at-least-seven-times-expansion is not necessary for this invention to work, this expansion is particularly preferred for therapeutic purposes. For instance, the TVEMF-expanded cells may be only in amount of 2 times the number of blood stem cells in the naturally-occurring blood, if desired. Preferably, TVEMF-expanded cells are in a range of about 4 times to about 25 times the number per volume of blood stem cells in naturally-occurring blood. The present invention is also directed to a composition comprising blood stem cells from a mammal, wherein said blood stem cells are present in a number per volume that is at least 7 times greater than naturally-occurring blood from the mammal; and wherein the blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is the same or similar to or essentially the same as stem cells of the naturally-occurring blood. A composition of the present invention may include a pharmaceutically acceptable carrier; including but not limited to plasma, blood, albumin, cell culture medium, growth factor, copper chelating agent, hormone, buffer or cryopreservative. "Pharmaceutically acceptable carrier" means an agent that will allow the introduction of the stem cells into a mammal, preferably a human. Such carrier may include substances

mentioned herein, including in particular any substances that may be used for blood transfusion, for instance blood, plasma, albumin; also, saline or buffer (preferably buffer supplemented with albumin), preferably from the mammal to which the composition will be introduced. The term "introduction" of a composition to a mammal is meant to refer to "administration" of a composition to an animal. Preferably, administration of stem cells of the present invention to a mammal is performed intravenously. However, other forms of administration may be used, as are well-known in the art. In particular, for instance injection directly into the heart or tissue near the heart may be used, to bring the stem cells as close as possible to the site of damage. For instance, for treatment of a heart attack, myocardial infarction, preferably a stem cell composition having few to no cells other than stem cells are injected directly into heart muscle. Even more preferably, such injection occurs with an acceptable amount G-CSF, for instance in an amount of 0.3ng to 5ug, more preferably 1 ng/kg to 100ng/kg, even more preferably 5 ng/kg to 20 ng/kg, and even more preferably 6 ng/kg. Administration of stem cells may occur with pharmaceutically carriers as described in the general state of the art. "Acceptable carrier" generally refers to any substance the blood stem cells of the present invention may survive in, i.e. that is not toxic to the cells, whether after TVEMF-expansion, prior to or after cryopreservation, prior to introduction (administration) into a mammal. Such carriers are well known in the art, and may include a wide variety of substances, including substances described for such a purpose throughout this application. For instance, plasma, blood, albumin, cell culture medium, buffer and cryopreservative are all acceptable carriers of this invention. The desired carrier may depend in part on the desired use

Other expansion methods known in the art (none of which use TVEMF) do not provide an expansion of blood stem cells in the amount of at least 7 times that of naturally-occurring blood while still maintaining the blood stem cells three-dimensional geometry and cell-to-cell support. TVEMF-expanded blood stem cells have the same or essentially the same, or maintain, the three-dimensional geometry and the cell-to-cell support and cell-to-cell geometry as the blood from which they originated. The composition may comprise TVEMF-expanded blood stem cells, preferably suspended in Dulbecco's medium or in a solution ready for cryopreservation. The composition is preferably free of toxic granular material, for example, dying cells and the toxic material or content of granulocytes and macrophages. The

composition may be a cryopreserved composition comprising TVEMF-expanded blood stem cells by decreasing the temperature of the composition to a temperature of from -120^{0}C to -196^{0}C and maintaining the cryopreserved composition at that temperature range until needed for therapeutic or other use. As discussed below, preferably, as much toxic material as is possible is removed from the composition prior to cryopreservation.

Another embodiment of the present invention relates to a method of regenerating heart tissue with a pharmaceutical composition of TVEMF-expanded blood stem cells, either having undergone cryopreservation or soon after TVEMF-expansion is complete. The cells may be introduced into a mammalian body, preferably human, for instance injected intravenously or directly into the tissue to be repaired, allowing the body's natural system to repair and regenerate heart tissue. Preferably, the composition to be introduced into the mammalian body is free of toxic material and other materials that may cause an adverse reaction to the administered TVEMF-expanded blood stem cells. The cells are readily available for treatment or research where such treatment or research requires the individual's blood cells, especially if a disease has occurred and cells free of the disease are needed. For a person in need of heart tissue repair later in life, stored expanded peripheral blood or cord blood may be useful. Cord blood is especially desired if a child is predisposed to developing a heart condition or otherwise needing heart tissue repair.

Example I— Actual TVEMF-Expansion of Cells in a TVEMF Bioreactor

Peripheral blood was collected and peripheral blood cells expanded as shown in Table 1, and described below.

A) Collection and maintenance of cells

Human peripheral blood (75 ml; about 0.75 x 10^6 cells/ml) was collected from 15 human donors by syringe as above; blood collected from 10 donors was suspended in 75ml Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY) supplemented with 20% of 5% human albumin (HA), 100 ng/ml recombinant human G-CSF (Amgen Inc., Thousand Oaks, CA), and 100 ng/ml recombinant human stem cell factor (SCF) (Amgen) to prepare a blood mixture. Part of each blood sample was set aside

as a "control" sample. The peripheral blood mixture was placed in a TVEMF-bioreactor as shown in Figures 2 and 3 herein. TVEMF-expansion occurred at 37°C, 6% CO_2, with a normal air O_2/N ratio. The TVEMF-bioreactor was rotated at a speed of 10 rotations per minute (rpm) initially, then adjusted as needed, as described throughout this application, to keep the peripheral blood cells suspended in the bioreactor. A time varying current of 6mA was applied to the bioreactor. The square wave TVEMF applied to the peripheral blood mixture was about 0.5 Gauss. (frequency: about 10 cycles/sec). Culture media in the peripheral blood mixture in the TVEMF-bioreactor was changed/freshened every one to two days. At day 10, the cells were removed from the TVEMF-bioreactor and washed with PBS and analyzed. The results are as set forth in Table 1. Control data refers to a sample of human peripheral blood that has not been expanded; Expanded Sample refers to the respective control sample after TVEMF-expansion.

Table 1

Control 1	Cell Count 300,000	Viability 98%
Control 2	Cell Count 325,000	Viability 100%
Control 3	Cell Count 350,000	Viability 98%
Control 4	Cell Count 300,000	Viability 98%
Control 5	Cell Count 315,000	Viability 99%
Control 6	Cell Count 320,000	Viability 98%
Control 7	Cell Count 310,000	Viability 98%
Control 8	Cell Count 340,000	Viability 100%
Control 9	Cell Count 300,000	Viability 98%
Control 10	Cell Count 320,000	Viability 98%
Expanded Sample 1	Cell Count 3,000,000 Corresponding CD34+ increase: yes	Viability 99%
Expanded Sample 2	Cell Count 3,500,000 Corresponding CD34+ increase: yes	Viability 100%
Expanded Sample 3	Cell Count 3,750,000 Corresponding CD34+ increase: yes	Viability 98%
Expanded Sample 4	Cell Count 3,250,000 Corresponding CD34+ increase: yes	Viability 98%
Expanded Sample 5	Cell Count 3,450,000 Corresponding CD34+ increase: yes	Viability 100%
Expanded Sample 6	Cell Count 3,400,000 Corresponding CD34+ increase: yes	Viability 98%
Expanded Sample 7	Cell Count 3,200,000 Corresponding CD34+ increase: yes	Viability 98%
Expanded Sample 8	Cell Count 3,500,000 Corresponding CD34+ increase: yes	Viability 100%
Expanded Sample 9	Cell Count 3,150,000 Corresponding CD34+ increase: yes	Viability 98%
Expanded Sample 10	Cell Count 3,500,000 Corresponding CD34+ increase: yes	Viability 99%

As may be seen from Table 1, TVEMF-expansion of peripheral blood cells resulted in roughly a 10-fold increase in the number of cells over 10 days, as compared to non-expanded control, with a corresponding increase in CD34+ cells. The culture media where the cells were growing was changed/freshened once every 1-2 days.

B) Analysis of TVEMF-expanded cells

Total cell counts of Control and Expanded Samples were obtained with a counting chamber (a device such as a hemocytometer used by placing a volume of either the control cell suspension or expanded sample on a specially-made microscope slide with a microgrid and counting the number of cells in the sample). The results of the total cell counts in Control samples and in Expanded Samples after 10 days of TVEMF-expansion are shown in Table 1.

The indication of corresponding CD34+ increase in Table 1 was determined as follows: CD34+ cells of the Expanded Samples were separated from other cells therein with a Human CD34 Selection Kit (EasySep positive selection, StemCell Technologies), and counted with a counting chamber as indicated above and confirmed with FACScan flow cytometer (Becton-Dickinson). CFU-GEMM and CFU-GM were counted by clonogenic assay. Cell viability (where a viable cell is alive and a non-viable cell is dead) was determined by trypan blue exclusion test. The answer of "yes" in all Expanded Samples indicates that the number of CD34+ cells increased in amounts corresponding to the total cell count.

C) Increase in amount of hematopoietic colony-forming cells

Incubation of the donors' peripheral blood cells in this TVEMF-expansion tissue culture system significantly increases the numbers of hematopoietic colony-forming cells.

As determined in a separate assay, a constant increase in the numbers of CFU-GM (up to 7-fold) and CFU-GEMM (up to 9-fold) colony-forming cells is observed up to day 7 with no clear plateau.

D) Increase in CD34+ cells

Incubation of MNCs from normal donors in this TVEMF-expansion tissue culture system significantly increases the numbers of CD34+ cells. As determined in a separate assay, the average number of CD34+ cells increased 10-fold by day 6 of culture and plateaus on that same day.

Operative Method-Repair of Heart Tissue

The following describes an illustrative procedure for repairing heart tissue in a human. Fifteen patients with severe ischemic heart failure and no other option for standard revascularization therapies will be identified to participate in the procedure. Patients will be enrolled sequentially, with the first 10 patients assigned to a treatment group and the last 5 patients to a control group. All patients will be placed on maximally tolerated medical therapy at time of enrollment. The following inclusion criteria will be required for patient enrollment: (1) chronic coronary artery disease with reversible perfusion defect detectable by single-photon emission computed tomography (SPECT); (2) left ventricular (LV) ejection fraction (EF) <40%; (3) ineligibility for percutaneous or surgical revascularization, as assessed by coronary arteriography; and (4) signed, informed consent. Patients will not be enrolled in the study if any one of the following exclusion criteria are met: (1) difficulty in obtaining vascular access for percutaneous procedures; (2) previous or current history of neoplasia or other comorbidity that could impact the patient's short-term survival; (3) significant ventricular dysrhythmias (sustained ventricular tachycardia); (4) LV aneurysm; (5) unexplained abnormal baseline laboratory abnormalities; (6) bone tissue with abnormal radiological aspect; (7) primary hematologic disease; (8) acute myocardial infarction within 3 months of enrollment in the study; (9) presence of intraventricular thrombus by 2D Doppler echocardiogram; (10) hemodynamic instability at the time of the procedure; (11) atrial fibrillation; or (12) any condition that would place the patient at undue risk.

Baseline evaluation in the treatment group will include a complete clinical evaluation (history and physical), laboratory evaluation (complete blood count, blood chemistry, C-reactive protein [CRP], brain natriuretic peptide [BNP], creatine kinase [CK]-MB and troponin serum levels), exercise stress test with ramp treadmill protocol, 2D Doppler echocardiogram, dipyridamole SPECT perfusion scan, and 24-hour Holter monitoring.

The control group will undergo the above-mentioned baseline evaluation except for 24-hour Holter monitoring, CK-MB, and troponin serum levels.

Patients in the treatment group will have serum CRP, complete blood count, CK, troponin, and BNP levels measured and an ECG performed just before the procedure. Immediately after the procedure, another ECG and 2D Doppler echocardiogram will be performed, and 24-hour Holter monitoring will be begun. Serum CRP, CK, and troponin levels will also be assessed at 24 hours. Patients are monitored for 48 hours after the injection procedure.

TVEMF-expanded blood stem cells prepared for instance according to Example I will be exhaustively washed with heparinized saline containing 5% human serum albumin and filtered for instance through 100 μm nylon mesh to remove cell aggregates. The cells will be resuspended in saline with 5% human serum albumin for injection as a pharmaceutical TVEMF-expanded blood stem cell composition. A small fraction of the composition will be used for cell counting and viability testing with trypan blue exclusion. Cell viability is expected to be >98%, similar to the results shown in Table 1.

A high correlation between granulocyte-macrophage colony-forming units and $CD45^{lo}CD34+$ cells is seen. Fibroblast colony-forming assay may be done as previously described to determine the presence of putative progenitor mesenchymal lineages. Bacterial and fungal cultures of the composition will be performed to ensure it is negative.

The following antibodies will be available, either biotinylated or conjugated with fluorescein isothiocyanate (Pharmingen); phycoerythrin (PE), or PerCP: anti-CD45 as a pan-leukocyte marker (clone HI30), anti-CD34 as a hematopoietic progenitor marker (clone HPCA-II), anti-CD3 as a pan-T-cell marker (clone SK7), anti-CD4 as a T-cell subpopulation marker (clone SK3), and anti-CD8 as a T-cell subpopulation marker (clone SK1) from Becton Dickinson; anti-

CD14 as a monocyte marker (clone TUK4), anti-CD19 as a pan-B-cell marker (clone SJ25-C1), and anti-CD56 as an NK-cell marker (clone NKI nbl-1), from Caltag Laboratories (Burlingame, Calif); and anti-HLA-DR (MHC-II, clone B8.12.2) from Beckman-Coulter. The biotinylated antibodies may be revealed with Streptavidin PECy7 (Caltag Laboratories). Three-color immunofluorescence analysis may be used for the identification of leukocyte populations in total nucleated bone marrow cell suspensions. After staining, erythrocytes will be lysed with a Becton Dickinson lysis buffer solution according to the manufacturer's instructions, or similar solution, and CD45 antibody used to assess the percentages of leukocytes in each sample. Data acquisition and analyses may be performed on a fluorescence-activated cell sorter such as Calibur with CellQuest 3.1 software (Becton Dickinson).

In the cell-injection treatment group, patients will be taken to the cardiac catheterization laboratory ~1 hour before the anticipated arrival of the pharmaceutical TVEMF-expanded blood stem cell composition from the laboratory. Left heart catheterization with biplane LV angiography will be performed. Subsequently, electromechanical mapping (EMM) of the left ventricle will be performed as previously described. The general region for treatment will be selected by matching the area identified as ischemic by previous SPECT perfusion imaging. The electromechanical map will then be used to target the specific treatment area by identifying viable myocardium (unipolar voltage $\geq$6.9mV) within that region. Areas associated with decreased mechanical activity (local linear shortening <12%, indicating hibernating myocardium) will be preferred.

A NOGA injection catheter may be prepared by adjusting the needle extension at 0° and 90° flex and by placing 0.1 cc of the pharmaceutical TVEMF-expanded blood stem cell composition expanded stem cells to fill the needle dead space. The injection catheter tip will be placed across the aortic valve and into the target area, and each injection site will be carefully evaluated before the cells are injected. Before an injection of cells into the LV wall, the following criteria has to be met: (1) perpendicular position of the catheter to the LV wall; (2) excellent loop stability (<4 mm); (3) underlying voltage >6.9mV; and (4) presence of a premature ventricular contraction on extension of the needle into the myocardium. Fifteen injections of 0.2 cc will be delivered to each patient in the treatment group with an expected amount of total cells of about 14 million cells/0.2 cc. The number of stem cells to be preferably introduced is discussed throughout this

application, and is most preferably about 10^7 to 10^9 stem cells. The control group may receive injections without any stem cells. All patients, both treated and control, will undergo noninvasive follow-up evaluations at 2 months.

The predicted Vo_2max will be used to tailor the patient workload. Treadmill speed will initially be 0.5 mph, and inclination will be 0% to 10% with a planned duration of 10 minutes of exercise. The echocardiographic data will be analyzed. Images may be stored digitally and analyzed offline. The end-systolic volume (ESV), end-diastolic volume (EDV), and EF will be measured according to standard protocols.

Dipyridamole stress and resting SPECT imaging will be performed with the same stress procedure at baseline and at follow-up. Approximately 740 MBq of technetium-99m sestamibi will be injected at rest and after stress, with dipyridamole infusion at a rate of 142 μg/kg of body weight per minute infused for 4 minutes. One hour later, SPECT imaging will be initiated, using a 15% window centered over the 140-keV photopeak. Acquisitions will be performed with a 1-detector gamma camera (Ecam, Siemens), acquiring 32 projections over 180° (right anterior oblique 45° to left posterior oblique 45°) (low-energy, high-resolution collimation; 64x64 matrixes; and 35 seconds per projection). Short-axis and vertical and horizontal long-axis tomograms of the left ventricle may be extracted from the reconstructed transaxial tomograms by performing coordinate transformation with appropriate interpolation. No attenuation or scatter correction is applied. Quantitative SPECT analysis will be performed for instance on an ICON workstation computer (Siemens) or similar setup. The analysis will be performed with the use of a completely automated software package, with the exception of a quality-control check to verify the maximum count circumferential profiles. In brief, processing parameters, including the apical and most basal tomographic short-axis slices, the central axis of the LV chamber, and a limiting radius for myocardial count search, will be automatically derived. Short-axis tomograms will then be sampled by using a maximum-count circumferential profile sampling technique with a cylindrical approach for sampling the body of the left ventricle and a spherical approach for sampling the LV apex. Comparisons are made to sex-matched normal limits. Polar map displays and quantitative values will then be generated to indicate stress myocardial perfusion defect extent and severity.

Patients in the control group will not undergo NOGA mapping or repeat LV angiograms at late follow-up to avoid unnecessary risk.

Patients in the treatment group will have 4-month invasive follow-up evaluations consisting of LV angiograms and EMM. LV angiography may be performed through the femoral approach with the use of a 5F pigtail catheter. All angiograms are obtained in 2 planes—a 30° right anterior oblique view and a 60° left anterior oblique view—during a period of stable sinus rhythm. Ventricular volume is not measured during or after a premature beat. A 40-mm sphere is used as a calibration device.

EMM is performed according to established criteria with a fill threshold of 15 mm. After the acquisition of points, post-processing analysis will be performed with a series of filters (moderate setting) to eliminate inner points, points that do not fit the standard stability criteria (location stability <4 mm, loop stability <6 mm, and cycle length variation <10%), points acquired during ST-segment elevation, and points not related to the left ventricle (e.g., those in the atrium).

The total procedural time for mapping and injection will be about 81±19 minutes. Electromechanical maps may comprise an average of 92±16 points. Patients will receive an average of 15 ±2 cell composition injections in a mean 2±0.7 segments (6 inferior, 14 lateral, 2 anterior, and 5 septal). Each injection of 14 million cells will be delivered in a volume of 0.2 cc.

It is expected that 2-3% (about 400,000/mm^2) of injected cells will be hematopoietic progenitor cells ($CD45^{lo}CD34+$). Similarly, about 0.1% (about 15,000/mm^2) of injected cells are expected to be early hematopoietic progenitor cells ($CD45^{lo}CD34+HLA^-DR^-$) and about 25 to 30% (about 4,000,000/mm^2) injected cells are expected to be CD4+ T-cells (CD45+CD3+CD4+). About 15% of injected cells (about 2,200,000/mm^2) are expected to be CD8+ T-cells (CD45+CD3+CD8+), and about 2% of injected cells (about 1,600,000/mm^2) are expected to be B cells (CD45+CD19+). About 10% of injected cells (about 1,400,000/mm^2) are expected to be monocytes (CD45+CD14+) and about 1-2% of injected cells (about 150,000/mm^2) are expected to be NK cells (CD45+CD56+).

Results expected from these experiments are that patients in the treatment group will experience less heart failure and fewer anginal symptoms at the 2-month follow-up when compared with the control group, by both New York Heart Association (NYHA) and Canadian Cardiovascular Society Angina Score (CCSAS) distribution. Baseline exercise test variables (METs and Vo_2max) will be similar for the 2 groups. There will be a significant increase, however, in METs and Vo_2max at follow-up in the treatment group. NYHA classis will be cut in half after treatment with TVEMF-expanded stem cells but will remain the same without expanded stem cells. CCSAS is also expected to be less than half after treatment than before treatment but virtually unchanged for non-treated patients. Vo_2max is expected to increase by approximately 35% with treatment but will be virtually unchanged without treatment. Echocardiogram, ESV, volume will decrease by approximately 15% with treatment but will increase without treatment. SPECT, total reversible defect will decrease by approximately 80% with treatment but will increase without treatment.

On EMM, segmental analysis will reveal a significant mechanical improvement of the injected segments. Significant improvement in mechanical function at the injection site will be shown.

It may thus be seen that significant heart repair is accomplished by the treatment discussed herein. If the TVEMF-expanded stem cells are intravenously inserted, similar results are expected to be achieved, although the time period for repair may be longer.

Experiments conducted on animal models or other situations where heart tissue repair is desired are expected to provide for a showing, upon histological or pathological analysis, or other analysis as desired, of the repair of heart tissue with this invention.

Operative Method - Cryopreservation

As mentioned above, blood is collected from a mammal, preferably a human. Red blood cells, at least, are preferably removed from the blood. The blood stem cells (with other cells and media as desired) are placed in a TVEMF-bioreactor, subjected to a time varying electromagnetic force and expanded. If RBCs were not removed prior to TVEMF-expansion, preferably they are removed after TVEMF-expansion. The TVEMF-expanded cells may be

cryogenically preserved. Further details relating to a method for the cryopreservation of TVEMF-expanded blood stem cells, and compositions comprising such cells are provided herein and in particular below.

After TVEMF-expansion, the TVEMF-expanded cells, including TVEMF-expanded blood stem cells, are preferably transferred into at least one cryopreservation container containing at least one cryoprotective agent. The TVEMF-expanded blood stem cells are preferably first washed with a solution (for instance, a buffer solution or the desired cryopreservative solution) to remove media and other components present during TVEMF-expansion, and then preferably mixed in a solution that allows for cryopreservation of the cells. Such solution is commonly referred to as a cryopreservative, cryopreservation solution or cryoprotectant. The cells are transferred to an appropriate cryogenic container and the container decreased in temperature to generally from -120°C to -196°C, preferably about –130°C to about –150°C, and maintained at that temperature. Preferably, this decrease in temperature is done slowly and carefully, so as to not damage, or at least to minimize damage, to the stem cells during the freezing process. When needed, the temperature of the cells (about the temperature of the cryogenic container) is raised to a temperature compatible with introduction of the cells into the human body (generally from around room temperature to around body temperature), and the TVEMF-expanded cells may be introduced into a mammalian body, preferably human, for instance as discussed throughout this application.

Freezing cells is ordinarily destructive. Not to be bound by theory, on cooling, water within the cell freezes. Injury then may occur by osmotic effects on the cell membrane, cell dehydration, solute concentration, and ice crystal formation. As ice forms outside the cell, available water is removed from solution and withdrawn from the cell, causing osmotic dehydration and raised solute concentration that may eventually destroy the cell. (For a discussion, see Mazur, P., 1977, Cryobiology 14:251-272.)

Different materials have different freezing points. Preferably, a blood stem cell composition ready for cryopreservation contains as few contaminating substances as possible, to minimize cell wall damage from the crystallizaton and freezing process.

These injurious effects can be reduced or even circumvented by (a) use of a cryoprotective agent, (b) control of the freezing rate, and (c) storage at a temperature sufficiently low to minimize degradative reactions.

The inclusion of cryopreservation agents is preferred in the present invention. Cryoprotective agents which can be used include but are not limited to a sufficient amount of dimethyl sulfoxide (DMSO) (Lovelock, J. E. and Bishop, M. W. H., 1959, Nature 183:1394-1395; Ashwood-Smith, M. J., 1961, Nature 190:1204-1205), glycerol, polyvinylpyrrolidine (Rinfret, A. P., 1960, Ann. N.Y. Acad. Sci. 85:576), polyethylene glycol (Sloviter, H. A. and Ravdin, R. G., 1962, Nature 196:548), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe, A. W., et al., 1962, Fed. Proc. 21:157), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender, M. A., et al., 1960, J. Appl. Physiol. 15:520), amino acid-glucose solutions or amino acids (Phan The Tran and Bender, M. A., 1960, Exp. ***Cell*** Res. 20:651), methanol, acetamide, glycerol monoacetate (Lovelock, J. E., 1954, Biochem. J. 56:265), and inorganic salts (Phan The Tran and Bender, M. A., 1960, Proc. Soc. Exp. Biol. Med. 104:388; Phan The Tran and Bender, M. A., 1961, in Radiobiology, Proceedings of the Third Australian Conference on Radiobiology, Ilbery, P. L. T., ed., Butterworth, London, p. 59). In a preferred embodiment, DMSO is used. DMSO, a liquid, is nontoxic to cells in low concentration. Being a small molecule, DMSO freely permeates the cell and protects intracellular organelles by combining with water to modify its freezability and prevent damage from ice formation. Adding plasma (for instance, to a concentration of 20-25%) can augment the protective effect of DMSO. After addition of DMSO, cells should be kept at 0°C or below, since DMSO concentrations of about 1% may be toxic at temperatures above 4°C. My selected preferred cryoprotective agents are, in combination with TVEMF-expanded blood stem cells for the total composition: 20 to 40% dimethyl sulfoxide solution in 60 to 80% amino acid-glucose solution, or 15 to 25% hydroxyethyl starch solution, or 4 to 6% glycerol, 3 to 5% glucose, 6 to 10% dextran T10, or 15 to 25% polyethylene glycol or 75 to 85% amino acid-glucose solution. The amount of cryopreservative indicated above is preferably the <u>total</u> amount of cryopreservative in the entire composition (not just the amount of substance added to a composition).

While other substances, other than blood cells and a cryoprotective agent, may be present in a composition of the present invention to be cryopreserved, preferably cryopreservation of a TVEMF-expanded blood stem cell composition of the present invention occurs with as few other substances as possible, for instance for reasons such as those discussed regarding the mechanism of freezing, above.

Preferably, a TVEMF-expanded blood stem cell composition of the present invention is cooled to a temperature in the range of about –120°C to about –196°C, preferably about –130°C to about –196°C, and even more preferably about –130°C to about –150°C.

A controlled slow cooling rate is critical. Different cryoprotective agents (Rapatz, G., et al., 1968, Cryobiology 5(1):18-25) and different cell types have different optimal cooling rates (see e.g. Rowe, A. W. and Rinfret, A. P., 1962, Blood 20:636; Rowe, A. W., 1966, Cryobiology 3(1):12-18; Lewis, J. P., et al., 1967, Transfusion 7(1):17-32; and Mazur, P., 1970, Science 168:939-949 for effects of cooling velocity on survival of peripheral cells (and on their transplantation potential)). The heat of fusion phase where water turns to ice should be minimal. The cooling procedure can be carried out by use of, e.g., a programmable freezing device or a methanol bath procedure.

Programmable freezing apparatuses allow determination of optimal cooling rates and facilitate standard reproducible cooling. Programmable controlled-rate freezers such as Cryomed or Planar permit tuning of the freezing regimen to the desired cooling rate curve. Other acceptable freezers may be, for example, Sanyo Modl MDF-1155ATN-152C and Model MDF-2136ATN –135C, Princeton CryoTech TEC 2000. For example, for blood cells or CD34+/CD38- cells in 10% DMSO and 20% plasma, the optimal rate is 1 to 3°C /minute from 0°C to -200°C.

In a preferred embodiment, this cooling rate can be used for the cells of the invention. The cryogenic container holding the cells must be stable at cryogenic temperatures and allow for rapid heat transfer for effective control of both freezing and thawing. Sealed plastic vials (e.g., Nunc, Wheaton cryules) or glass ampules can be used for multiple small amounts (1-2 ml), while larger volumes (100-200 ml) can be frozen in polyolefin bags (e.g., Delmed) held between metal plates for better heat transfer during cooling. (Bags of bone marrow cells have been successfully frozen by placing them in -80°C freezers that, fortuitously, gives a cooling rate of approximately 3°C /minute).

In an alternative embodiment, the methanol bath method of cooling can be used. The methanol bath method is well suited to routine cryopreservation of multiple small items on a large scale. The method does not require manual control of the freezing rate nor a recorder to monitor the rate. In a preferred aspect, DMSO-treated cells are precooled on ice and transferred to a tray containing chilled methanol that is placed, in turn, in a mechanical

refrigerator (e.g., Harris or Revco) at -130°C. Thermocouple measurements of the methanol bath and the samples indicate the desired cooling rate of 1 to 3°C /minute. After at least two hours, the specimens will reach a temperature of -80°C and may be placed directly into liquid nitrogen (-196°C) for permanent storage.

After thorough freezing, TVEMF-expanded stem cells can be rapidly transferred to a long-term cryogenic storage vessel (such as a freezer). In a preferred embodiment, the cells can be cryogenically stored in liquid nitrogen (-196°C) or its vapor (-165°C). The storage temperature should be below –120°C, preferably below –130°C. Such storage is greatly facilitated by the availability of highly efficient liquid nitrogen refrigerators, which resemble large Thermos containers with an extremely low vacuum and internal super insulation, such that heat leakage and nitrogen losses are kept to an absolute minimum.

The preferred apparatus and procedure for the cryopreservation of the cells is that manufactured by Thermogenesis Corp., Rancho Cordovo, CA, utilizing their procedure for lowering the cell temperature to below -130°C. The cells are held in a Thermogenesis plasma bag during freezing and storage.

Other freezers are commercially available. For instance, the "BioArchive" freezer not only freezes but also inventories a cryogenic sample such as blood or cells of the present invention, for instance managing up to 3,626 bags of frozen blood at a time. This freezer has a robotic arm that will retrieve a specific sample when instructed, ensuring that no other examples are disturbed or exposed to warmer temperatures. Other freezers commercially available include, but are not limited to, Sanyo Model MDF-1155 ATN-152C and Model MDF-2136 ATN-135C, and Princeton CryoTech TEC 2000.

After the temperature of the TVEMF-expanded blood stem cell composition is reduced to below -120 °C, preferably below -130 °C, they may be held in an apparatus such as a Thermogenesis freezer. Their temperature is maintained at a temperature of about -120 °C to -196 °C, preferably -130 °C to -150 °C. The temperature of a cryopreserved TVEMF-expanded blood stem cell composition of the present invention should not be above -120°C for a prolonged period of time.

Cryopreserved TVEMF-expanded blood stem cells, or a composition thereof, according to the present invention may be frozen for an indefinite period of time, to be thawed

when needed. For instance, a composition may be frozen for up to 18 years. Even longer time periods may work, perhaps even as long as the lifetime of the blood donor.

When needed, bags with the cells therein may be placed in a thawing system such as a Thermogenesis Plasma Thawer or other thawing apparatus such as in the Thermoline Thawer series. The temperature of the cryopreserved composition is raised to room temperature. In another preferred method of thawing cells mixed with a cryoprotective agent, bags having a cryopreserved TVEMF-expanded blood stem cell composition of the present invention, stored in liquid nitrogen, may be placed in the gas phase of liquid nitrogen for 15 minutes, exposed to ambient air room temperature for 5 minutes, and finally thawed in a 37°C water bath as rapidly as possible. The contents of the thawed bags may be immediately diluted with an equal volume of a solution containing 2.5% (weight/volume) human serum albumin and 5% (weight/volume) Dextran 40 (Solplex 40; Sifra, Verona, Italy) in isotonic salt solution and subsequently centrifuged at 400 *g* for ten minutes. The supernatant would be removed and the sedimented cells resuspended in fresh albumin/Dextran solution. See Rubinstein, P. et al., Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc. Natl. Acad. Sci.* 92:10119-1012 (1995) for Removal of Hypertonic Cryoprotectant; a variation on this preferred method of thawing cells can be found in Lazzari, L. et al., Evaluation of the effect of cryopreservation on ex vivo expansion of hematopoietic progenitors from cord blood. *Bone Marrow Trans.* 28:693-698 (2001).

After the cells are raised in temperature to room temperature, they are available for research or regeneration therapy. The thawed TVEMF-expanded blood stem cell composition may be introduced directly into a mammal, preferably human, or used in its thawed form for instance for desired research. The solution in which the thawed cells are present may be completely washed away, and exchanged with another, or added to or otherwise manipulated as desired. Various additives may be added to the thawed compositions (or to a non-cryopreserved TVEMF-expanded blood stem cell composition) prior to introduction into a mammalian body, preferably soon to immediately prior to such introduction. Such additives include but are not limited to a growth factor, a copper chelating agent, a cytokine, a hormone, a suitable buffer or diluent. Preferably, G-CSF is added. Even more preferably, for humans, G-CSF is added in an amount of about 20 to about 40 micrograms/kg body weight, and even more preferably in an amount of about 30 micrograms/kg body weight. Also, prior

to introduction, the TVEMF-expanded blood stem cell composition may be mixed with the mammal's own, or a suitable donor's, plasma, blood or albumin, or other materials that for instance may accompany blood transfusions. The thawed blood stem cells can be used for instance to test to see if there is an adverse reaction to a pharmaceutical that is desired to be used for treatment or they can be used for treatment.

While the FDA has not approved use of expanded blood stem cells for regeneration of tissue in the United States, such approval appears to be imminent. Direct injection of a sufficient amount of expanded blood stem cells should be able to be used to repair and regenerate heart tissue.

A TVEMF-expanded blood stem cell composition of the present invention should be introduced into a mammal, preferably a human, in a "therapeutically effective" amount, sufficient to achieve tissue repair or regeneration, or to treat a desired disease or condition. Preferably, at least 20 ml of a TVEMF-expanded blood stem cell composition having 10^7 to 10^9 stem cells per ml is used for any treatment, preferably all at once, in particular where a traumatic injury has occurred and immediate tissue repair needed. This amount is particularly preferred in a 75-80 kg human. The amount of TVEMF-expanded blood stem cells in a composition being introduced into a mammal depends in part on the number of cells present in the source blood material (in particular if only a fairly limited amount is available). A preferred range of TVEMF-expanded blood stem cells introduced into a patient may be, for instance, about 10 ml to about 50 ml of a TVEMF-expanded blood stem cell composition having 10^7 to 10^9 stem cells per ml, or potentially even more. While it is understood that a high concentration of any substance, administered to a mammal, may be toxic or even lethal, it is unlikely that introducing all of the TVEMF-expanded blood stem cells, for instance after TVEMF-expansion at least 7 times, will cause an overdose in TVEMF-expanded blood stem cells. Where blood from several donors or multiple collections from the same donor is used, the number of blood stem cells introduced into a mammal may be higher. Also, the dosage of TVEMF-cells that may be introduced to the patient is not limited by the amount of blood provided from collection from one individual; multiple administrations, for instance once a day or twice a day, or once a week, or other administration time frames, may more easily be used. Also, where a tissue is to be treated, the type of tissue may warrant the use of as many

TVEMF-expanded blood stem cells as are available, or the use of a smaller dose. For instance, liver may be easiest to treat and may require fewer stem cells than other tissues.

It is to be understood that, while the embodiment described above generally relates to cryopreserving TVEMF-expanded blood stem cells, TVEMF-expansion may occur after thawing of already cryopreserved, non-expanded, or non-TVEMF-expanded, blood stem cells. Also, if cryopreservation is desired, TVEMF-expansion may occur both before and after freezing the cells. Blood banks, for instance, have cryopreserved compositions comprising blood stem cells in frozen storage, in case such is needed at some point in time. Such compositions may be thawed according to conventional methods and then TVEMF-expanded as described herein, including variations in the TVEMF-process as described herein. Thereafter, such TVEMF-expanded blood stem cells are considered to be compositions of the present invention, as described above. TVEMF-expansion prior to cryopreserving is preferred, for instance as if a traumatic injury occurs, a patient's blood stem cells have already been expanded and do not require precious extra days to prepare.

Also, while not preferred, it should be noted that TVEMF-expanded blood stem cells of the present invention may be cryopreserved, and then thawed, and then if not used, cryopreserved again. Prior to the cells being frozen, are preferably TVEMF-expanded (that is, increased in number, not size). The cells may also be expanded after being frozen and then thawed, even if already expanded before freezing.

Expansion of blood stem cells may take several days. In a situation where it is important to have an immediate supply of blood stem cells, such as a life-or-death situation or in the case of a traumatic injury, especially if research needs to be accomplished prior to reintroduction of the cells, several days may not be available to await the expansion of the blood stem cells. It is particularly desirable, therefore, to have such expanded blood stem cells available from birth forward in anticipation of an emergency where every minute in delaying treatment can mean the difference in life or death.

Also, it is to be understood that the TVEMF-expanded blood stem cells of the present application may be introduced into a mammal, preferably the source mammal (mammal that is the source of the blood), after TVEMF-expansion, with or without cryopreservation. However, such introduction need not be limited to only the source mammal (autologous); the TVEMF-expanded cells may also be transferred to a different mammal (allogenic).

Also, it is to be understood that, while blood is the preferred source of adult stem cells for the present invention, adult stem cells from bone marrow may also be TVEMF-expanded and used in a manner similar to blood stem cells in the present invention. Bone marrow is not a readily available source of stem cells, but must be collected via apheresis or some other expensive and painful method.

The present invention also includes a method of researching heart tissue, for instance in relation to a heart disease or condition. The method may include, for instance, introducing a blood stem cell composition into a test system for the disease state. Such as system may include, but is not limited to, for instance a mammal having the disease, an appropriate animal model for studying the disease or an in vitro test system for studying the disease. TVEMF-expanded blood stem cells may be used for research for possible cures for diseases relating to the heart.

During the entire process of expansion, preservation, and thawing, blood stem cells of the present invention maintain their three-dimensional geometry and their cell-to-cell support and cell-to-cell geometry.

While preferred embodiments have been herein described, those skilled in the art will understand the present invention to include various changes and modifications. The scope of the invention is not intended to be limited to the above-described embodiments.

Claims

I claim:

1. A method of repairing heart tissue comprising the step of administering to a mammal a therapeutically effective amount of a pharmaceutical blood stem cell composition comprising expanded blood stem cells in a number per volume that is at least 7 times greater than naturally-occurring blood, and wherein the blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as stem cells of naturally-occurring blood.

2. A method of repairing heart tissue comprising the step of administering to a mammal a therapeutically effective amount of a pharmaceutical blood stem cell composition comprising TVEMF-expanded blood stem cells in a number per volume that is at least 2 times greater than naturally-occurring blood, and wherein the blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as stem cells of naturally-occurring blood.

3. The method according to claim 2, wherein the number of TVEMF-expanded blood stem cells per volume is at least 7 times greater.

4. The method of claim 3, wherein the administering step comprises the administration of the pharmaceutical blood stem cell composition into at least one of the mammal's peripheral blood stream, tissue adjacent to the heart, or heart tissue.

5. The method of claim 3, wherein the pharmaceutical blood stem cell composition further comprises at least one of human GM-CSF and human G-CSF.

6. The method of claim 3, wherein the mammal is human.

7. The method of claim 3, further comprising, prior to the administering step, the steps of:
   a. placing a blood mixture in a culture chamber of a TVEMF-bioreactor;

b. subjecting the blood mixture to a TVEMF and TVEMF-expanding the blood stem cells in the TVEMF-bioreactor until the number per volume of TVEMF-expanded blood stem cells is more than 7 times the number per volume of blood stem cells placed in the TVEMF-bioreactor; and

c. mixing the TVEMF-expanded cells with an acceptable pharmaceutical carrier to form a pharmaceutical blood stem cell composition.

8. The method of claim 7, further comprising removing toxic material from the TVEMF-expanded cells.

9. The method according to claim 7, wherein said TVEMF is about 0.05 to about 6.0 gauss.

10. The method according to claim 7, further comprising the step of collecting blood prior to placing the blood mixture in a TVEMF-bioreactor, wherein the blood is collected from an autologous source.

11. The method according to claim 7, further comprising the step of collecting blood prior to placing the blood mixture in a TVEMF-bioreactor, wherein the blood is collected from an allogeneic source.

12. The method according to claim 11, further comprising the step of collecting blood prior to placing the blood mixture in a TVEMF-bioreactor, wherein the blood is collected from at least one of a mammal, a blood bank, a hospital and a cryopreserved blood sample.

13. The method of claim 7, wherein the blood mixture comprises CD34+/CD38- blood stem cells separated from other blood components.

14. The method of claim 7, wherein the blood mixture comprises a buffy coat separated from other blood components.

15. The method of claim 7, wherein the blood mixture is free of red blood cells.

16. The method of claim 2, wherein the therapeutically effective amount of TVEMF-expanded blood stem cells to be administered to the mammal is about 20 ml of about 10^7 to about 10^9 stem cells/ml.

17. A pharmaceutical blood stem cell composition for repairing heart tissue of a mammal comprising expanded blood stem cells in a number per volume that is at least 7 times greater than naturally-occurring blood, and wherein the blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as stem cells of naturally-occurring blood.

18. A pharmaceutical blood stem cell composition for repairing heart tissue of a mammal comprising TVEMF-expanded blood stem cells in a number per volume that is at least 2 times greater than naturally-occurring blood, and wherein the blood stem cells have a three-dimensional geometry and cell-to-cell support and cell-to-cell geometry that is essentially the same as stem cells of naturally-occurring blood.

19. The pharmaceutical blood stem cell composition according to claim 18, wherein the number of TVEMF-expanded blood stem cells per volume is at least 7 times greater.

20. The composition according to claim 19, wherein the composition further comprises at least one pharmaceutically acceptable carrier selected from the group consisting of plasma, blood, albumin and saline with 5% human serum albumin.

21. Use of the composition of claims 17 to 20 in the preparation of a medicament for the repair of heart tissue.

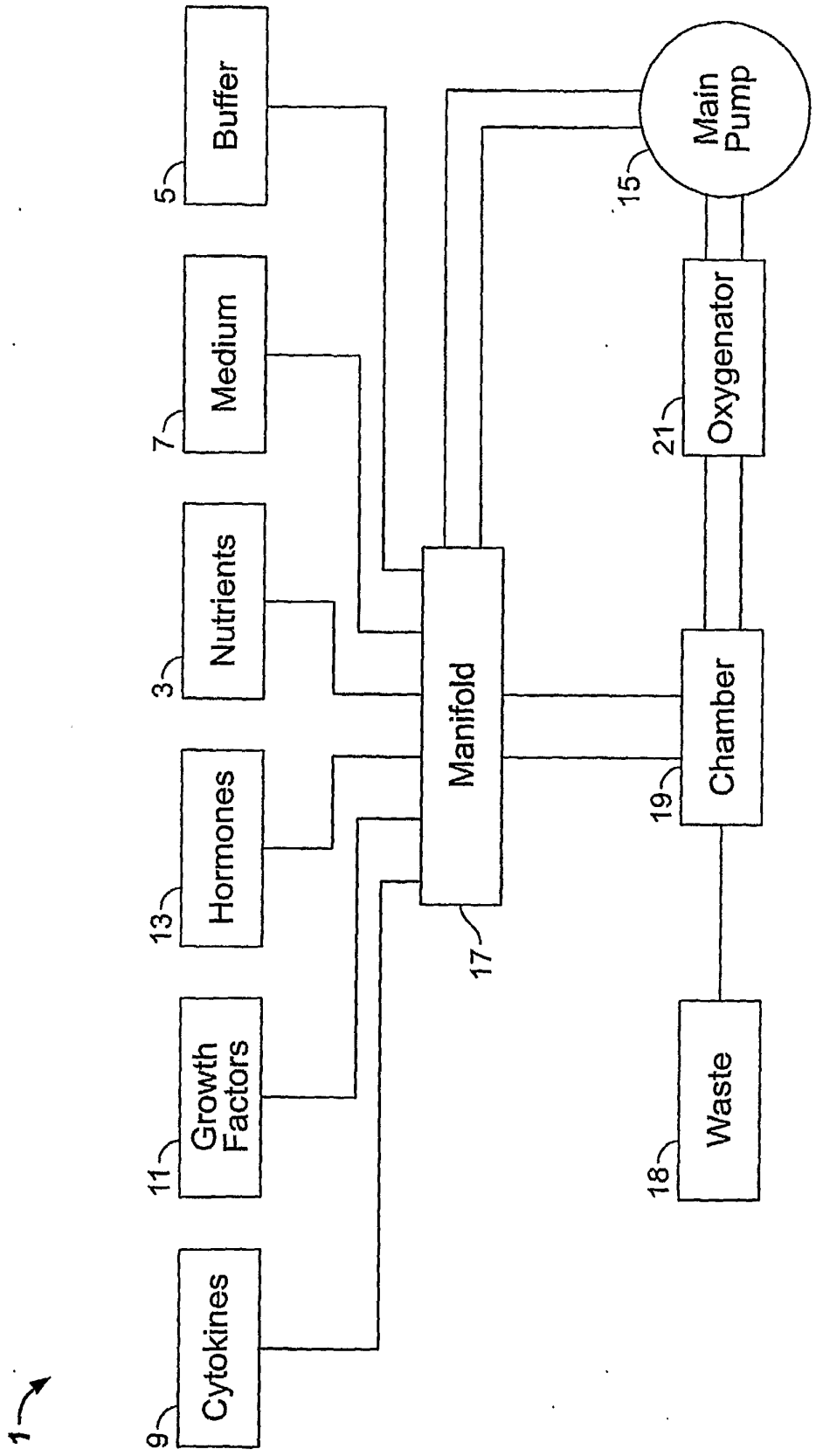
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FIG. 1

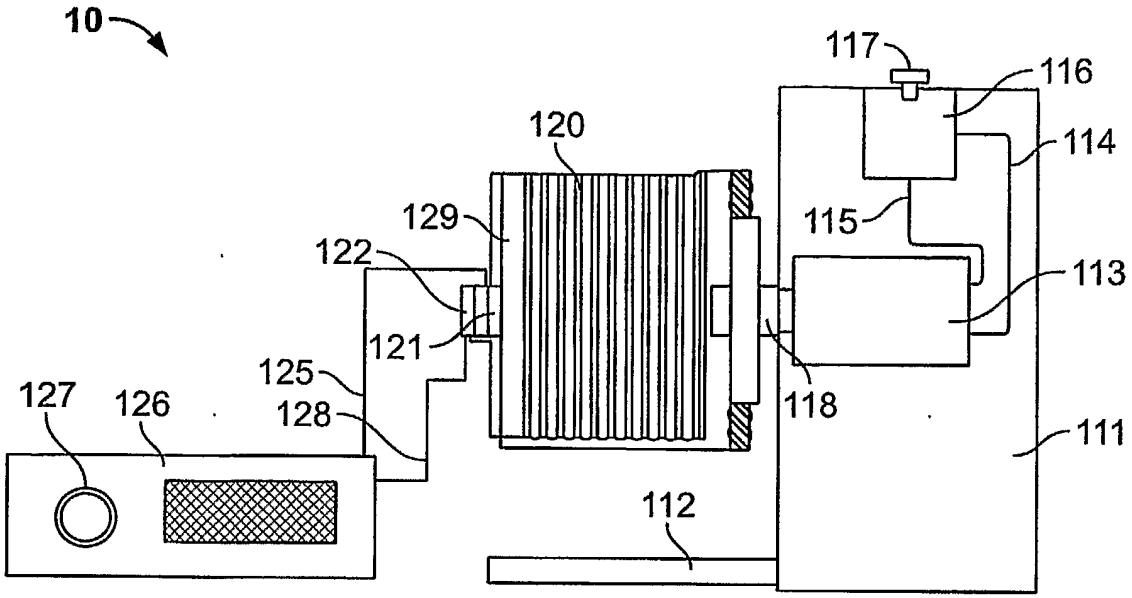
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FIG. 2

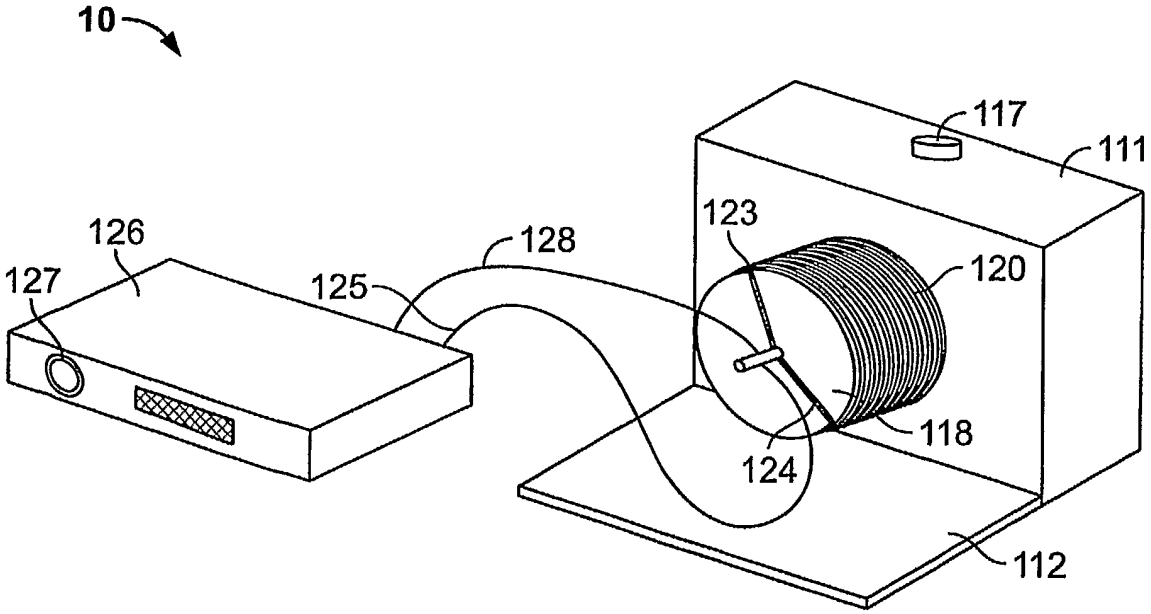
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FIG. 3

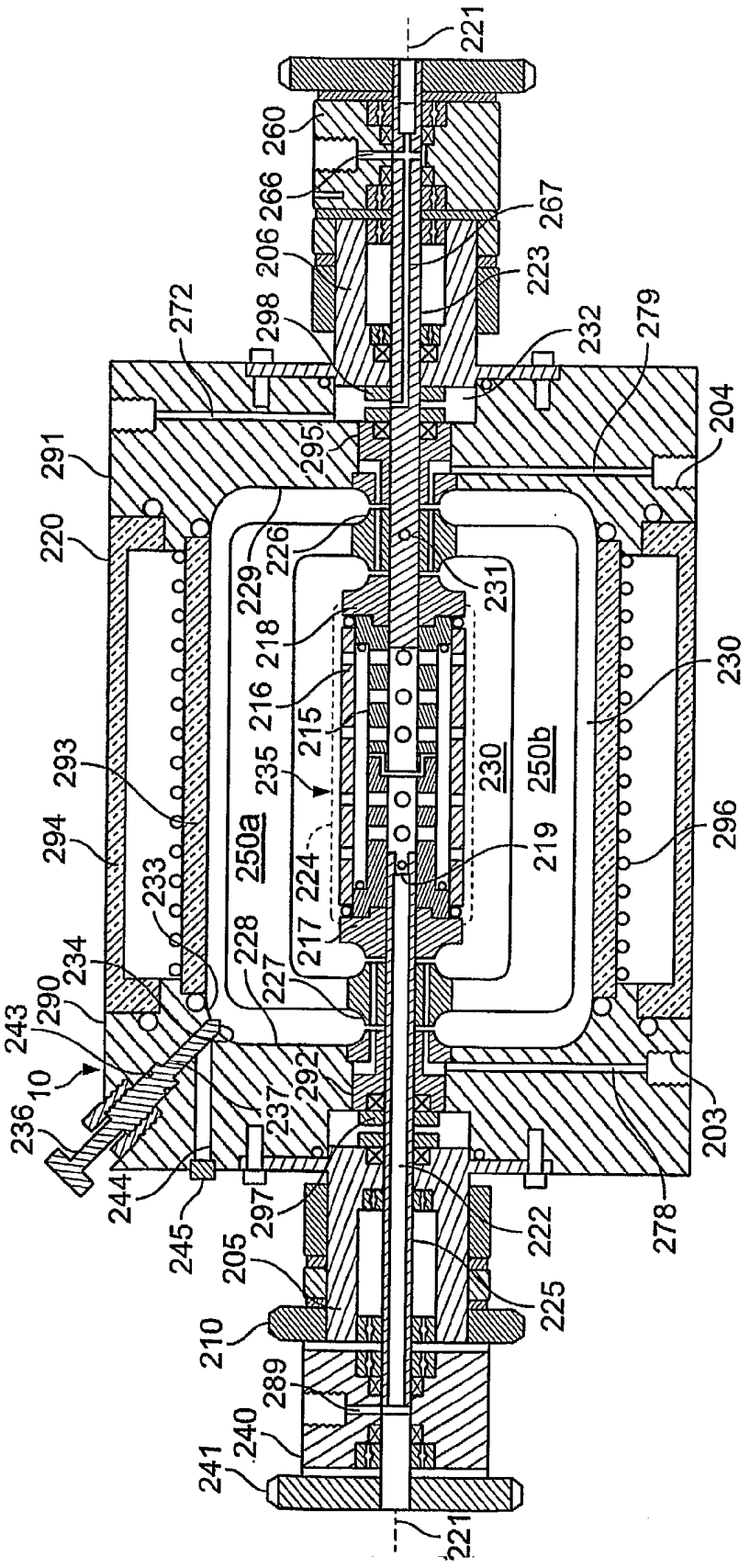
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FIG. 4

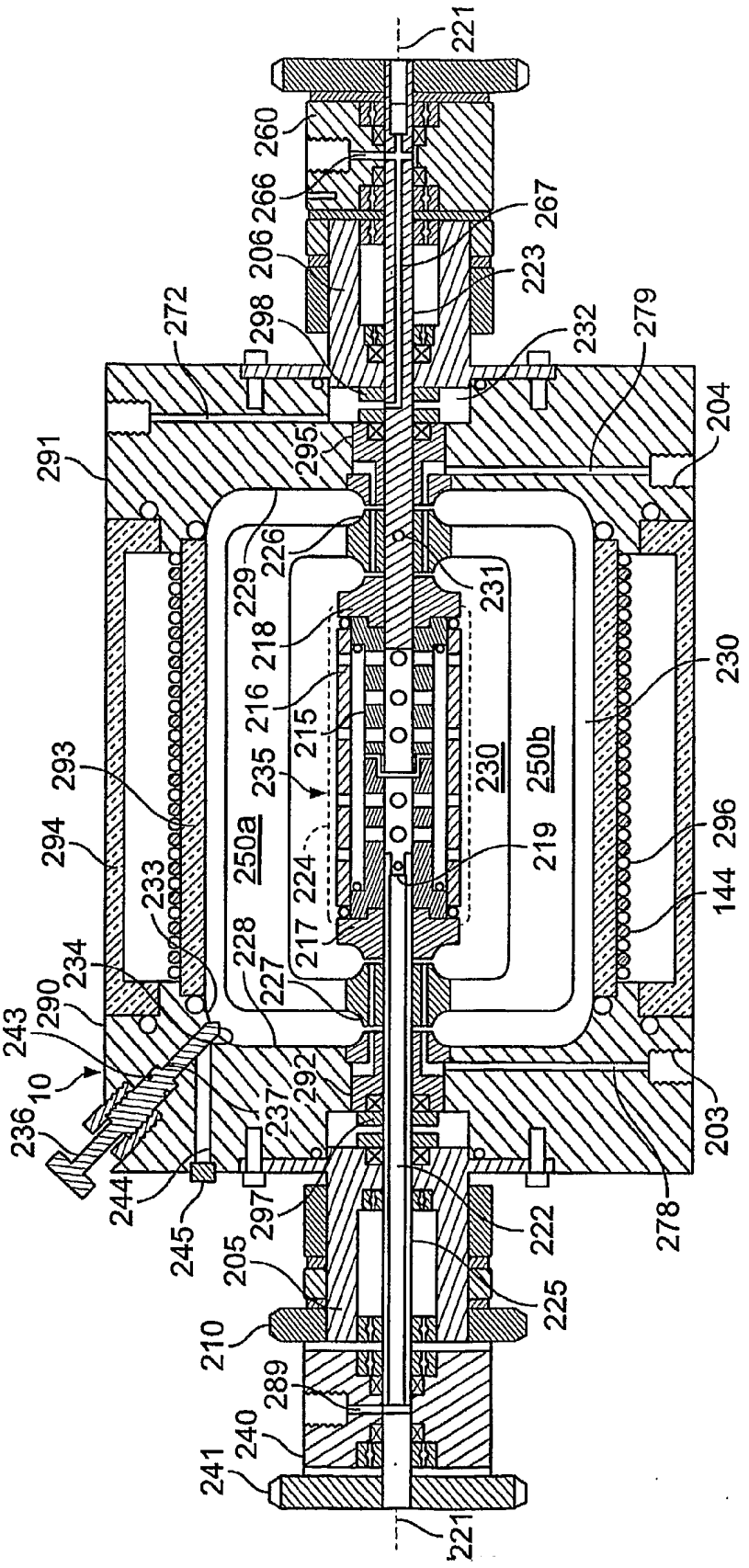
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FIG. 5

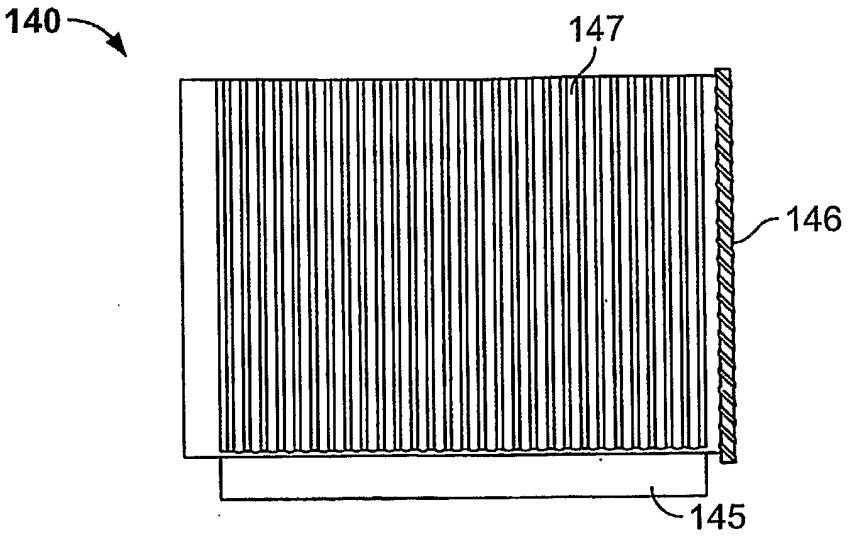

FIG. 6

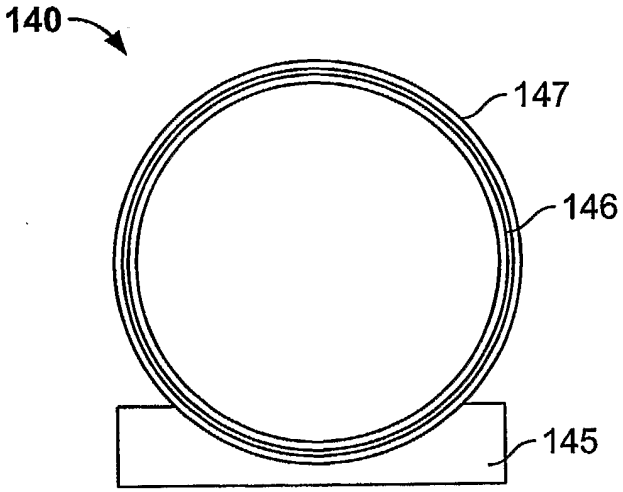

FIG. 7

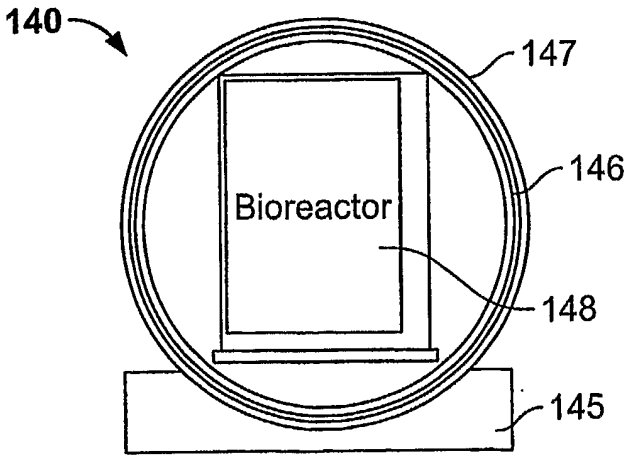

FIG. 8