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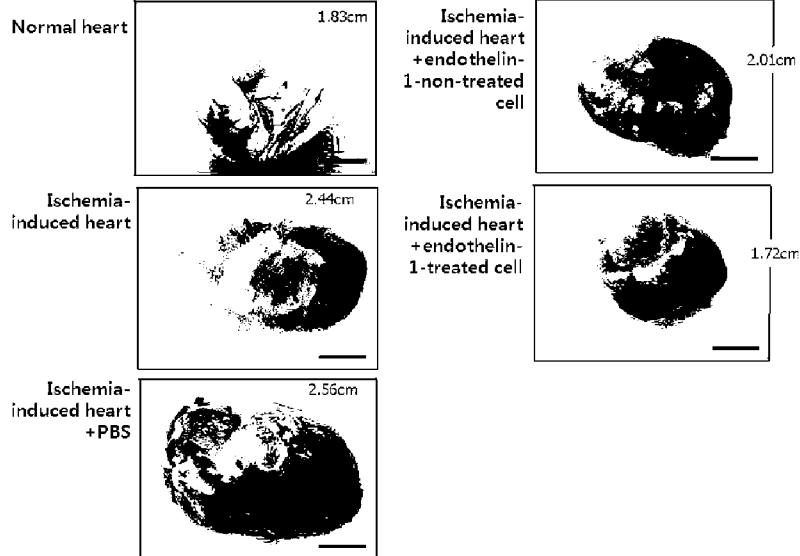
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(54) Title: METHOD FOR INCREASING STEM CELL ACTIVITY AND STEM CELLS PRODUCED THEREBY

[Fig. 7]



(57) Abstract: Disclosed is a method for increasing the activity of human stem cells. The method is characterized by treating a culture medium with endothelin-1. Also, the highly active human stem cells prepared by the method and cell therapeutics comprising the highly active human stem cells are provided. Human mesenchymal stem cells of various origins can be activated by the method, and thus resulting highly active human mesenchymal stem cells can be developed as effective cell therapeutics.

Description

Title of Invention: METHOD FOR INCREASING STEM CELL ACTIVITY AND STEM CELLS PRODUCED THEREBY

Technical Field

- [1] The present invention relates to a method for increasing stem cell activity, stem cells produced thereby, and cell therapeutics comprising the highly active stem cells.

Background Art

- [2] A stem cell is a generic name for an undifferentiated type of body cell found in the tissue of embryos, fetuses and adults, which is characterized by the ability to differentiate into a diverse range of specialized cell types. Stem cells can differentiate into specific cells under a differentiation stimulus (e.g., the environment). Also, stem cells can self-renew to produce more stem cells as differentiated cells divide into more cells. Stem cells have differentiation plasticity such that different environments or stimuli cause stem cells to differentiate into different specialized cell types.
- [3] According to potency, stem cells may be classified into pluripotent, multipotent and unipotent cells. Pluripotent stem cells can differentiate into nearly all cells. Among them are embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS). Adult stem cells show multipotency or unipotency.
- [4] ES cells are isolated from the inner cell mass of blastocysts and have the potency to differentiate into cells of any tissue. ES cells can remain undifferentiated after numerous cycles of cell division and give rise during development to germ cells with inheritance into the next generation, unlike adult stem cells (Thomson et al., Science, 282: 1145-1147, 1998; Reubinoff et al., Nat. Biotechnol., 18: 399-404, 2000).
- [5] Human ES cells are cultures of cells isolated from the inner cell mass (ICM) of a human blastocyst formed in early embryogenesis. Human ES cells currently existing over the world are those that have been isolated from frozen embryos after in vitro fertilization. In spite of various attempts for utilizing the pluripotency of human ES cells in cell therapeutics, the likelihood of oncogenesis and immune rejection still remains as a high barrier to overcome.
- [6] A solution to these problems that has recently been rising is induced pluripotent stem cells (iPS cells). iPS cells are a type of pluripotent stem cell artificially derived from a differentiated adult somatic cell by reprogramming. To date, iPS cells have been reported to be almost identical to natural pluripotent stem cells such as ES cells, in terms of the expression of certain stem cell genes and potency. iPS cells avoid the issue of immune rejection unlike ES cells because they are derived entirely from the patient, but the risk of oncogenesis with iPS cells is still a task to be solved.

- [7] As an alternative, mesenchymal stem cells are being promoted because they exhibit immunomodulatory effects and no risk of oncogenesis. Mesenchymal stem cells are multipotent stem cells that can differentiate into a variety of cell types, including adipocytes (fat cells), osteoblasts (bone cells), chondrocytes (cartilage cells), myoblasts (muscle cells), neuroblasts (nerve cells), and cardiomyocytes (myocardial cells), and are known to have the function of modulating immune responses.
- [8] Mesenchymal stem cells may be isolated from various tissues such as the bone marrow, cord blood, adipose tissue, etc., but are not sufficiently defined because cell surface markers are somewhat different from one to another according to the origin from which the mesenchymal stem cells are derived. On the whole, if they can differentiate into osteoblasts, chondrocytes and myoblasts, have a whirl morphology, and express the surface markers CD73(+), CD105(+), CD34(-) and CD45(-), the stem cells are defined as mesenchymal stem cells.
- [9] In this context, mesenchymal stem cells of different genetic origins and/or backgrounds do not significantly differ from one to another in terms of their definition, i.e. that of a mesenchymal stem cell, but are typically different from each other in terms of in vivo activity. Further, when mesenchymal stem cells are used as exogenous cell therapeutics, a limited pool of mesenchymal stem cells does not allow many choices for available options.
- [10] In addition, the minimal number of mesenchymal stem cells necessary for them to be used as cell therapeutics in the regenerative medicine and/or cell therapy is 1×10^9 cells. In practice, the minimal number is increased in consideration of experiments for setting proper conditions and determining criteria. The supply of mesenchymal stem cells in such quantities from various origins requires at least ten in vitro passages. In this case, however, the cells are aged and deformed so that they may be unsuitable for use as cell therapeutics.
- [11] In full consideration of the environment, increasing the activity of mesenchymal stem cells so that the number of applied cells can be reduced is a task of the art that puts them to practical use as regenerative medicine or as cell therapeutics.

Disclosure of Invention

Technical Problem

- [12] Accordingly, the present invention has been made keeping in mind the above problems occurring in the prior art, and an object of the present invention is to provide a method for preparing active mesenchymal stem cells which satisfy the efficiency of cell therapeutics required by regenerative medicine and cell therapy, thereby overcoming the limited efficiency of conventional human mesenchymal stem cells of various origins and increasing the practical applicability of mesenchymal stem cells as

cell therapeutics.

Solution to Problem

- [13] In order to accomplish the above objects, the present invention provides a method for increasing the activity of human mesenchymal stem cells. The method is characterized by the treatment of a culture medium for mesenchymal stem cells with endothelin-1. Also, the present invention provides active human stem cells prepared by the method, and cell therapeutics comprising the active stem cells.

Advantageous Effects of Invention

- [14] The *in vivo* activity of human mesenchymal stem cells, one of the most effective cell therapeutics can be increased according to the present invention. Also, the present invention provides a standardized method for increasing the activity of a broad spectrum of mesenchymal stem cells irrespective of origin and culture condition. Ultimately, the active mesenchymal stem cells prepared by the method of the present invention contribute to the effectiveness of mesenchymal stem cells as cell therapeutics and to the practical use of cell therapeutics in the treatment of intractable diseases such as cardiovascular and nervous system diseases.

Brief Description of Drawings

- [15] FIG. 1 shows the effect of endothelin-1 on the growth of mesenchymal stem cells.
[16] FIG. 2 shows the effect of endothelin-1 on the cell cycle of mesenchymal stem cells.
[17] FIG. 3 shows the effect of endothelin-1 on the secretion ability of mesenchymal stem cells at a VEGF RNA level.
[18] FIG. 4 shows the effect of endothelin-1 on the secretion ability of mesenchymal stem cells at a VEGF protein level.
[19] FIG. 5 shows the specific expression of endothelin receptor A in mesenchymal stem cells.
[20] FIG. 6 shows changes in VEGF expression after treatment with the endothelin receptor A-specific blocker BQ123.
[21] FIG. 7 shows sizes of the heart excised from ischemic cardiovascular rat models injected with mesenchymal stem cells treated with endothelin-1.
[22] FIG. 8 shows data of echocardiography conducted on ischemic cardiovascular rat models injected with mesenchymal stem cells treated with endothelin-1.

Best Mode for Carrying out the Invention

- [23] The present invention pertains to a method for increasing the activity of mesenchymal stem cells of various genetic backgrounds and/or origins, characterized by the addition of endothelin-1 to a culture medium for the mesenchymal stem cells.
[24] Leading to the present invention, intensive and thorough research into the gene expression levels of the human mesenchymal stem cells which exhibited the highest *in*

vivo activity when human mesenchymal stem cells different in *in vivo* activity were subject to DNA array, resulted in the finding that endothelin-1 functions to increase the activity of mesenchymal stem cells.

- [25] Endothelin-1 is a 21-amino acid peptide produced primarily in the vascular endothelium. The initial gene product of 212 amino acids, called prepro ET-1, is enzymatically cleaved into a 38-amino acid big ET-1. Subsequently, its C-terminal region is cleaved off by endothelin converting enzyme (ECE) to generate biologically active ET-1. Endothelin-1 has aroused the interest of many researchers due to its potential and constant vasoconstriction activity. Endothelin-1 is produced in vascular smooth cells, but mainly by vascular endothelial cells.
- [26] The biosynthesis of endothelin-1 is promoted by various factors including thrombin, angiotensin II, vasopressin, transforming growth factor β , tumor necrosis factor α , hypoxia and oxidized LDL, but suppressed by an endothelium-derived relaxing factor (EDRF, that is, nitric oxide). Also, the expression of endothelin-1 is induced by weak shear stress (force against the vessel in the direction of blood flowing), but down-regulated by strong shear stress.
- [27] Ischemic heart disease is a disease characterized by ischemia to the heart muscle, usually due to the stenosis or closure of the coronary arteries resulting in functional impediment or necrosis of the myocardium. There is a lot of data showing the implication of endothelin-1 in the pathogenesis of ischemic heart disease. For example, blood endothelin-1 levels are increased in patients with acute myocardial infarction. In addition, cardiomyocytes of the heart which undergoes ischemia-reperfusion were observed to increase in the population of endothelin receptors and to produce and secrete endothelin-1 at an accelerated rate. Also, increased contractile reactivity of the coronary artery to endothelin-1 was observed. Such data indicates that endothelin-1 may play a role in the pathogenesis of myocardial infarction. Under the assumption that endothelin-1 might have an influence on the size of myocardial infarction, many experiments were done to examine whether endothelin antagonists could decrease the size of infarction. However, there coexist contrary results that are reported to have and not have antagonistic effects.
- [28] In consideration of the above-mentioned endothelin-1 activity, the present inventors changed the concept of use of endothelin-1 by treating human mesenchymal stem cells with endothelin to afford active stem cells which are therapeutically useful in the treatment of ischemic myocardial infarction. Accordingly, the present invention has significance in that endothelin-1 finds a novel application of activating mesenchymal stem cells so that utility in cell therapy of the cells can be improved.
- [29] In accordance with an aspect thereof, the present invention provides a method for increasing the activity of human mesenchymal stem cells, comprising pre-treating a

culture medium for human mesenchymal stem cells with endothelin-1.

- [30] No limitations are imparted to the genetic background and/or origin of the human mesenchymal stem cells used in the present invention. For example, mesenchymal stem cells derived from human cord blood may be used.
- [31] During the culture of human mesenchymal stem cells, pre-treatment with endothelin-1 was examined for effect on the activity of human mesenchymal stem cells, in detail, its effect on the growth and cell cycle of mesenchymal stem cells. Mesenchymal stem cells treated with endothelin-1 were observed to outnumber a control which had not been treated with endothelin-1 (see FIG. 1). As for the cell cycle, the cells in S phase (synthesis) are increased upon pre-treatment with endothelin-1 (see FIG. 2).
- [32] These results indicate that the method of the present invention can increase the number of mesenchymal stem cells during ex vivo culturing, with the concomitant prevention of aging the cells. When mesenchymal stem cells are sub-cultured to achieve the cell population necessary for regenerative medicine and cell therapy, pre-treatment with endothelin-1 can not only retard the aging of the cells, but also reduce the number of the passage.
- [33] In addition, the effect that endothelin-1 has on the secretion of mesenchymal stem cells was examined. Both RNA and protein levels of VEGF (vascular endothelial growth factor), which is an important paracrine factor in the treatment of cardiovascular diseases, were observed to increase upon pre-treatment with endothelin-1 (see FIG. 3 and FIG. 4). The increased level of VEGF was proven to be attributable to endothelin-1. In this regard, first, endothelin receptor A was found to be specifically expressed in mesenchymal stem cells while endothelin receptor B was not (FIG. 5). After being treated with BQ 123, a receptor A-specific blocker, the mesenchymal stem cells were examined for VEGF expression level. As a result, the level of VEGF in the cells was decreased at both the RNA and protein level (FIG. 6). These results clearly demonstrate that the increase in VEGF level at both the RNA and protein level is attributable to endothelin-1.
- [34] Next, the effect of endothelin-1 on in vivo activity of mesenchymal stem cells was evaluated. An ischemic cardiovascular rat model was used for an in vivo activity assay. As used herein, the term "ischemic cardiovascular rat model" is intended to refer to a rat model with a cardiovascular disease induced by ligating the coronary artery of the heart. In the present invention, mesenchymal stem cells were pretreated with endothelin-1 and cultured before injection into the cardiac muscle of the rat model. The endothelin-1-treated model was found to have a heart significantly smaller than that of the control (see FIG. 7), indicating that endothelin-1 significantly suppresses the fibrosis-induced heart enlargement which is caused as cardiomyocytes dying upon ischemic heart disease (FIG. 7).

- [35] In addition, four weeks after injection with mesenchymal stem cells, the ischemic model was subjected to electrocardiography to evaluate the in vivo activity of mesenchymal stem cells. In detail, left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular end-fractional shortening (LVFS), and left ventricular end-ejection fraction (LVEF) were arithmetized to analyze improvements in the disease. LVFS is defined as $LVEDD - LVESD / LVEDD$ while LVEF is calculated by $LVEDD^2 - LVESD^2 / LVEDD^2$. Greater improvements are obtained as LVEDD and LVESD becomes smaller or as LVFS and LVEF become larger. In the endothelin-1-treated group, the improvement indices LVFS and LVEF were measured to be much higher than in the control (see FIG. 8).
- [36] From the results, it is apparent that endothelin-1 increases the activity of human mesenchymal stem cells. Hence, the present invention can be used to increase in vivo activity of human mesenchymal stem cells.
- [37] Therefore, the present invention provides a method for preparing of highly active human mesenchymal stem cells, comprising: culturing human mesenchymal stem cells in an adherent pattern in a culture medium; and adding endothelin-1 to the culture medium. Also, the present invention provides mesenchymal stem cells of high activity prepared by the method. Having high activity, the mesenchymal stem cells prepared by the method of the present invention can be used in a smaller number in the regenerative medicine and cell therapy to achieve a desired effect than can conventional stem cells. In addition, the present invention provides cell therapeutics comprising the active stem cells as an active ingredient.
- [38] Further, the present invention provides a kit for the preparation of active mesenchymal stem cells. The kit may comprise α -MEM, FBS and endothelin-1. The use of the kit may be a standard method for increasing the activity of human mesenchymal stem cells irrespective of their origin and culture condition.

Mode for the Invention

- [39] The following examples may provide a better understanding of the present invention and are set forth to illustrate, but are not to be construed as limiting the present invention.
- [40]
- [41] **[EXAMPLES]**
- [42]
- [43] For use in the present invention, human cord blood-derived mesenchymal stem cells were obtained from MEDIPOST. The cells were selected by conducting an experiment to identify human mesenchymal stem cells. The cells were found to uniformly express the positive markers (CD29, CD44, CD73, CD105, CD166, HLA-ABC) at a rate of

95% or higher and the negative markers (CD34, CD45, HLA-DR) at a rate less than 5% and have multipotency before identification as "human cord blood-derived mesenchymal stem cells".

[44]

[45] **EXAMPLE 1. Assay of Human Mesenchymal Stem Cells for In vitro Activity**

[46]

[47] Mesenchymal stem cells pre-treated with or without endothelin-1 were assayed for in vitro activity, as illustrated in detail below.

[48]

[49] **(1) Effect on Growth**

[50] Mesenchymal stem cells were cultured in an adherent pattern in α -MEM (Minimum Essential Medium, Invitrogen) supplemented with 10% FBS (fetal bovine serum) ($5 \times 10^5/60$ @dish). After culturing for 24 hrs, the cells were further incubated for 72 hrs with 10 nM endothelin-1.

[51] Trypsinization separated the cells into individuals, followed by cell counting with a hemacytometer. The results are shown in FIG. 1. The same mesenchymal stem cells, except for treatment without endothelin-1, were used as a control.

[52] As seen in FIG. 1, the endothelin-1-treated group actively proliferated compared to the control.

[53]

[54] **(2) Effect on Cell Cycle**

[55] Mesenchymal stem cells were cultured in an adherent pattern in α -MEM supplemented with 10% FBS ($5 \times 10^5/60$ @dish). After culturing for 24 hrs, the cells were further incubated for 72 hrs with 10 nM endothelin-1.

[56] Trypsinization separated the cells into individuals, followed by flow cytometry (FACS) with PI, dye for staining nuclear DNA, to count the cells in each phase of the cell cycle. The results are shown in FIG. 2. The same mesenchymal stem cells, except for being untreated with endothelin-1, were used as a control.

[57] As seen in FIG. 2, treatment with endothelin-1 increased by two-fold the number of the cells in the S phase (synthesis), which indicates cell activation and proliferation, while the number of the cells in the rest phases G0-G1 was reduced.

[58]

[59] **(3) Effect on Secretion**

[60] An examination was made of the effect of endothelin-1 on the secretion of VEGF, a paracrine factor which plays an important role in the therapeutic improvement of cardiovascular diseases. First, the effect was evaluated at the RNA level by real-time PCR. In detail, VEGF-specific primers were synthesized. Separately, cDNA was synthesized from RNA which was previously isolated 1, 6, 24 and 72 hrs after treatment

with endothelin-1. Real-time PCR was performed in the presence of the primers with the cDNA serving as a template. As seen in FIG. 3, the endothelin-1-treated group started to significantly increase in VEGF RNA level starting from one hour after treatment, as compared to the control.

[61] An experiment was also conducted to evaluate the effect at the protein level. In detail, the culture media of the RNA test groups were harvested and ELISA was used to quantitatively analyze their secreted VEGF. As seen in FIG. 4, significantly higher amounts of VEGF were secreted from the endothelin-1-treated group than from the control, with the gap therebetween increasing over time.

[62]

[63] These data indicate that the VEGF RNA levels elevated by treatment with endothelin-1 lead to elevated VEGF protein levels and are well maintained.

[64]

[65] In addition, an experiment was conducted to examine whether the increase in VEGG level resulted from the binding of endothelin-1 to an endothelin receptor.

[66] First, the expression of endothelin receptors A and B were investigated for human mesenchymal stem cells. In this regard, primers specific for endothelin receptor A and B were synthesized while RNA was isolated to prepare cDNA therefrom. PCR was performed using the cDNA as a template with the primers. The results are shown in FIG. 5. For this, GAPDH was used as a control. Out of endothelin receptors A and B, as seen in FIG. 5, only receptor A was expressed in mesenchymal stem cells.

[67] On the basis of the results obtained above, a blocker to endothelin receptor A was applied to the mesenchymal stem cells which were then monitored for change in VEGF expression in RNA and protein levels. In detail, the cells were treated with 10 μ M BQ123 (cyclo(d-a-aspartyl-l-propyl-d-valyl-l-leucyl-d-tryptophyl, Sigma), a typical endothelin receptor A blocker, 30 min before the addition of 10 nM endothelin-1 to the culture medium. After incubation for 24 hrs, the cells were examined for VEGF level at the RNA and protein levels. For comparison, the cells treated with endothelin-1, but without BQ123 were used as a control. The results are shown in FIG. 6.

[68] As is apparent from the data of FIG. 6, the RNA and protein levels of VEGF which were up-regulated by endothelin-1 were decreased by the endothelin receptor A blocker, BQ123.

[69]

[70] Consequently, the onset of the up-regulation of VEGF RNA and protein levels is attributed to the binding of endothelin-1 to an endothelin receptor, demonstrating that endothelin-1 is involved in the regulation of the secretion ability of mesenchymal stem cells.

[71]

[72] **EXAMPLE 2: Assay of Human Mesenchymal Stem Cells for In vivo Activity**

[73]

[74] Mesenchymal stem cells treated with and without endothelin-1 were assayed for in vivo activity.

[75] For this, ischemic cardiovascular rat models were employed. The models were rats which suffered from heart ischemia induced by ligating the coronary artery of the heart.

[76] The mesenchymal stem cells treated with endothelin-1 were injected at a density of 1×10^5 cells/rat to the animal models. On four weeks after injection, they were subjected to echocardiography to analyze clinical improvements in the disease. After echocardiography, the rats were sacrificed, and the hearts were excised therefrom and compared with regard to size. For comparison, the rats which were injected with PBS after the induction of ischemic injury were used as a control for the groups injected with mesenchymal stem cells (treated with and without endothelin-1). Also, the rats which were injected with neither cells nor PBS after the introduction of ischemic injury were used as a control for the cell- or PBS-injected groups. Each group was composed of at least five rats.

[77] The comparison of the size of the heart is shown in FIG. 7. As seen, the hearts excised from the endothelin-1-treated group (ischemia-induced heart + endothelin-1-treated cells) was observed to be significantly smaller than those of the non-treated control (ischemia-induced heart + endothelin-1-non-treated cells), but similar in size to normal hearts. As a rule, heart ischemia induces the death of cardiomyocytes, giving rise to the enlargement of the heart (see ischemia-induced heart of FIG. 7). Therefore, a relatively small heart indicates a therapeutic improvement of the heart disease.

[78] As is apparent from the data of FIG. 8 which were arithmetized from the echocardiography results, significantly high levels of LVFS and LVEF, which are indices indicating therapeutic improvement, were detected in the endothelin-1-treated group. In consideration of the fact that when the heart is exposed to ischemia, the heart wall becomes thin due to the fibrosis thereof and the heart loses motility and undergoes volumetric expansion, with the concomitant reduction of LVFS and LVEF, the heart injected with endothelin-1-treated cells restrained the typical progresses upon exposure to ischemia, including the thinning of the heart wall, the loss of motility, and volumetric enlargement.

[79] The results obtained above demonstrate that the endothelin-1-treated mesenchymal stem cells can be used as cell therapeutics which brings about an excellent improvement in heart disease, compared to the mesenchymal stem cells treated without endothelin-1.

[80]

[81] Consequently, as described hitherto, the present invention can be used to increase the in vivo activity of human mesenchymal stem cells, one of the most effective cell therapeutics. Also, the present invention provides a standardized method for increasing the activity of a broad spectrum of mesenchymal stem cells irrespective of origin and culturing conditions. In the end, the active mesenchymal stem cells prepared by the method of the present invention contribute to the effectiveness of mesenchymal stem cells as cell therapeutics and to the practical use of cell therapeutics.

[82]

[83] Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

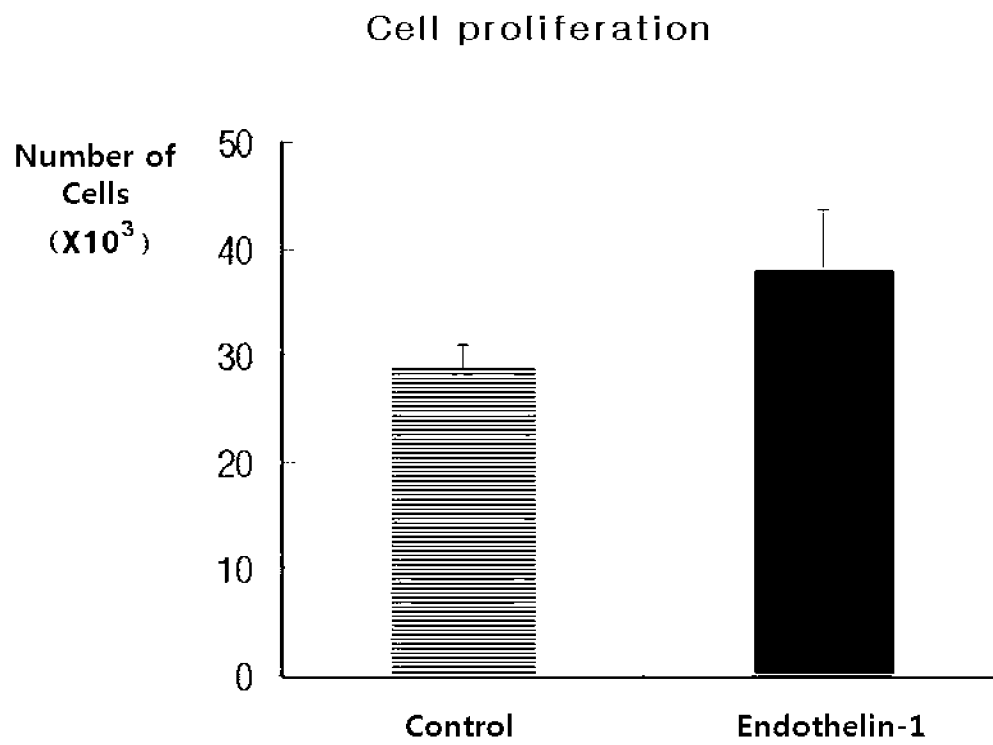
Industrial Applicability

[84] The present invention relates to a method for increasing the activity of human stem cells, the highly active human stem cells prepared by the method and cell therapeutics comprising the highly active stem cells. Ultimately, the active mesenchymal stem cells prepared by the method of the present invention contribute to the effectiveness of mesenchymal stem cells as cell therapeutics and to the practical use of cell therapeutics in the treatment of intractable diseases such as cardiovascular and nervous system diseases.

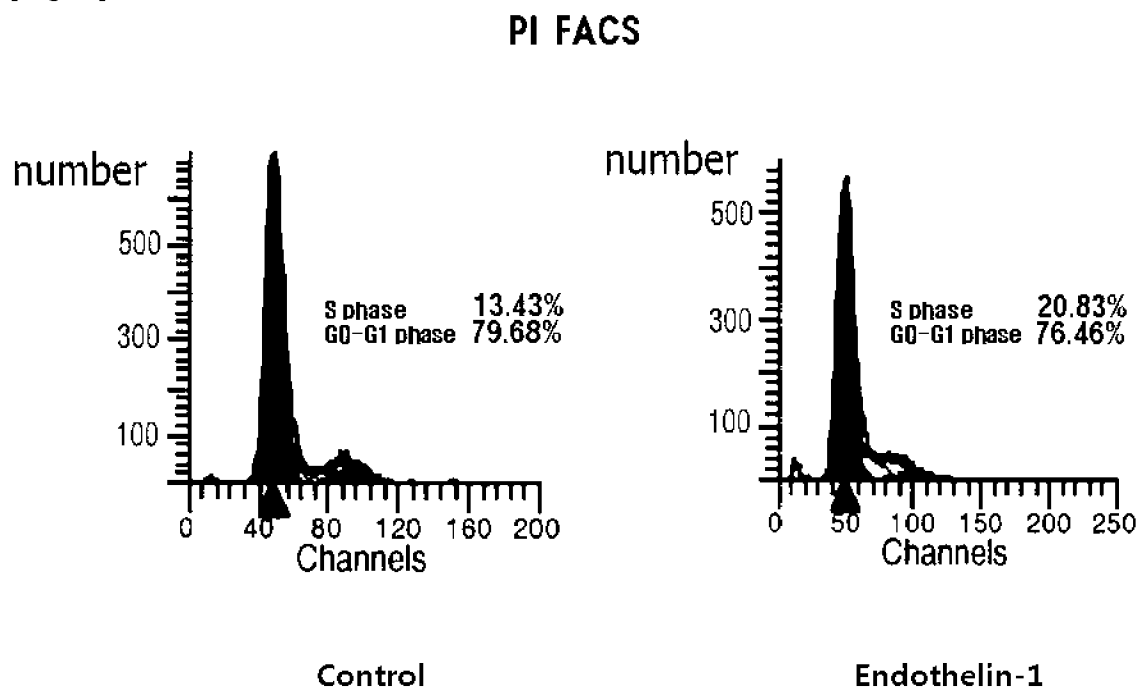
Claims

- [Claim 1] A method for preparing active human mesenchymal stem cells, comprising:
a) culturing human mesenchymal stem cells in an adherent pattern in a culture medium; and
b) adding endothelin-1 to the culture medium.
- [Claim 2] The method of claim 1, wherein the culture medium is α -MEM (Minimum Essential Medium) supplemented with 10% FBS (Fetal Bovine Serum).
- [Claim 3] The method of claim 1, wherein the endothelin-1 is added after the mesenchymal stem cells are cultured for 24 hours in an adherent pattern.
- [Claim 4] The method of claim 1, wherein the human mesenchymal stem cells are human cord blood-derived mesenchymal stem cells.
- [Claim 5] Active human mesenchymal stem cells, prepared by the method of one of claims 1 to 4.
- [Claim 6] Cell therapeutics comprising the active human mesenchymal stem cells of claim 5.
- [Claim 7] A kit for preparing active human mesenchymal stem cells, comprising:
a) α -MEM (Minimum Essential Medium);
b) FBS (fetal bovine serum); and
c) endothelin-1.

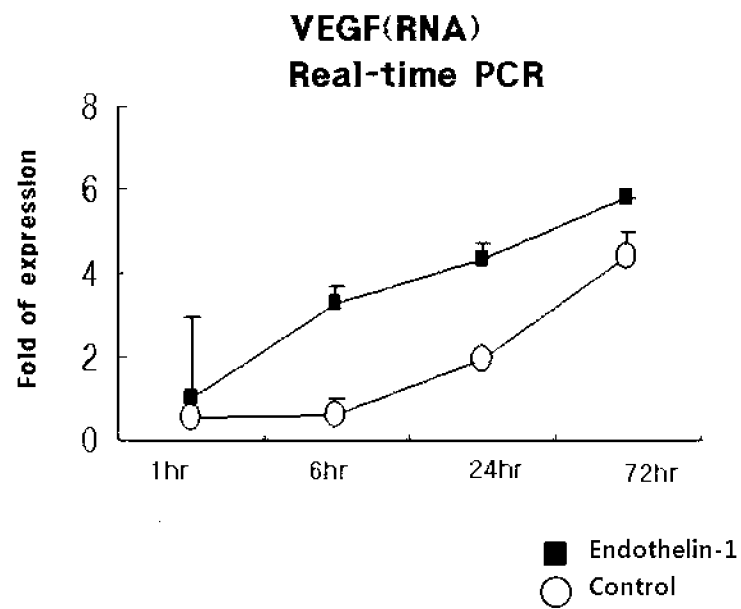
[Fig. 1]



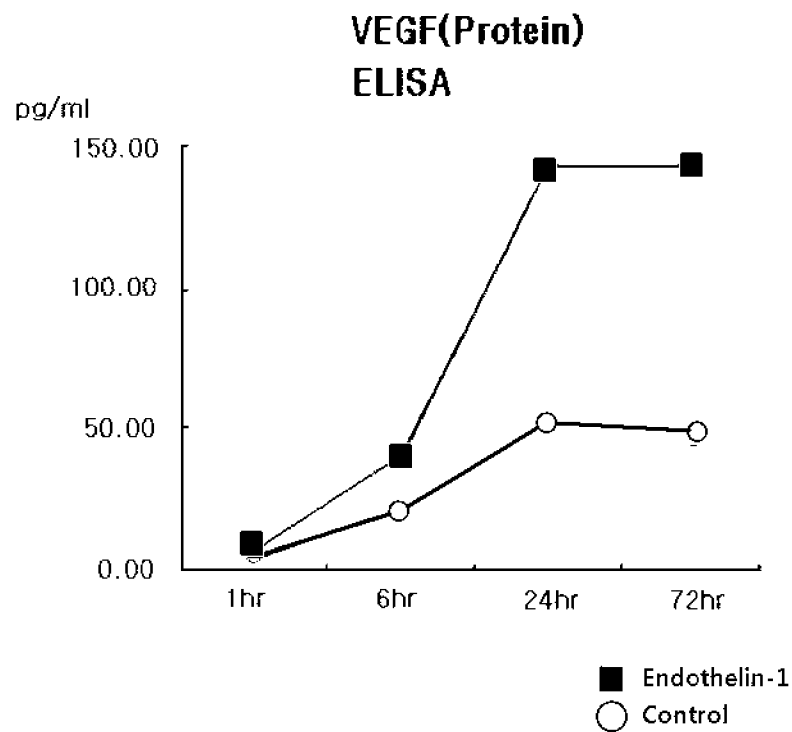
[Fig. 2]



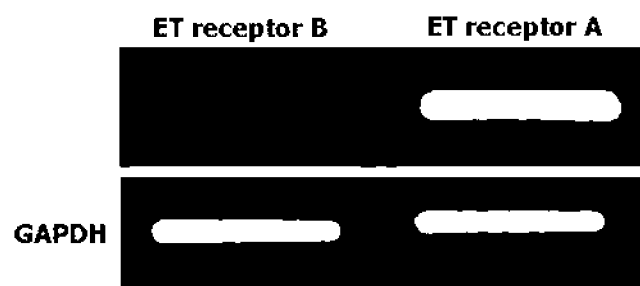
[Fig. 3]



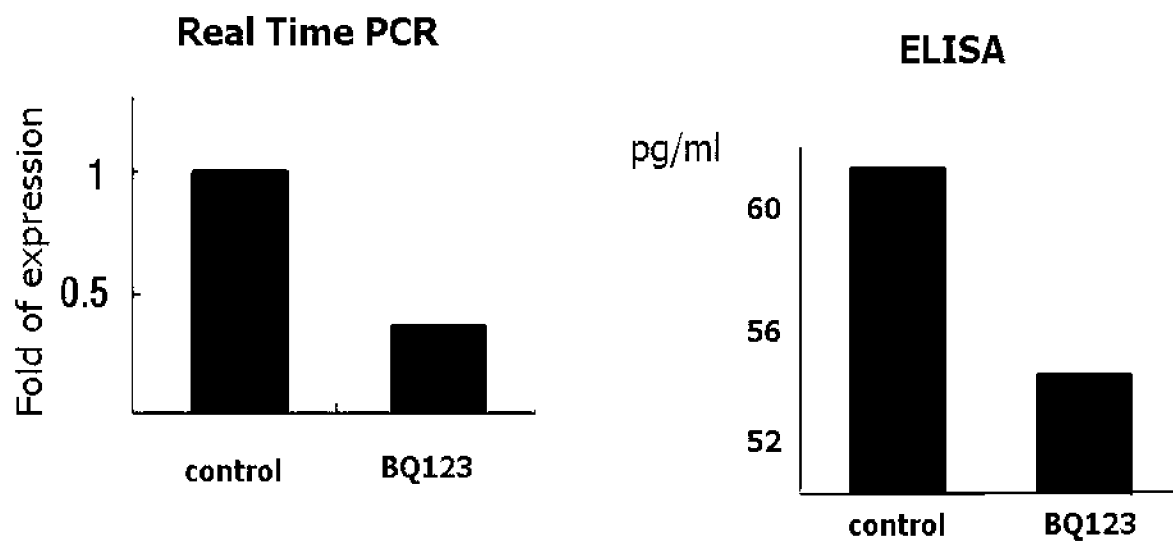
[Fig. 4]



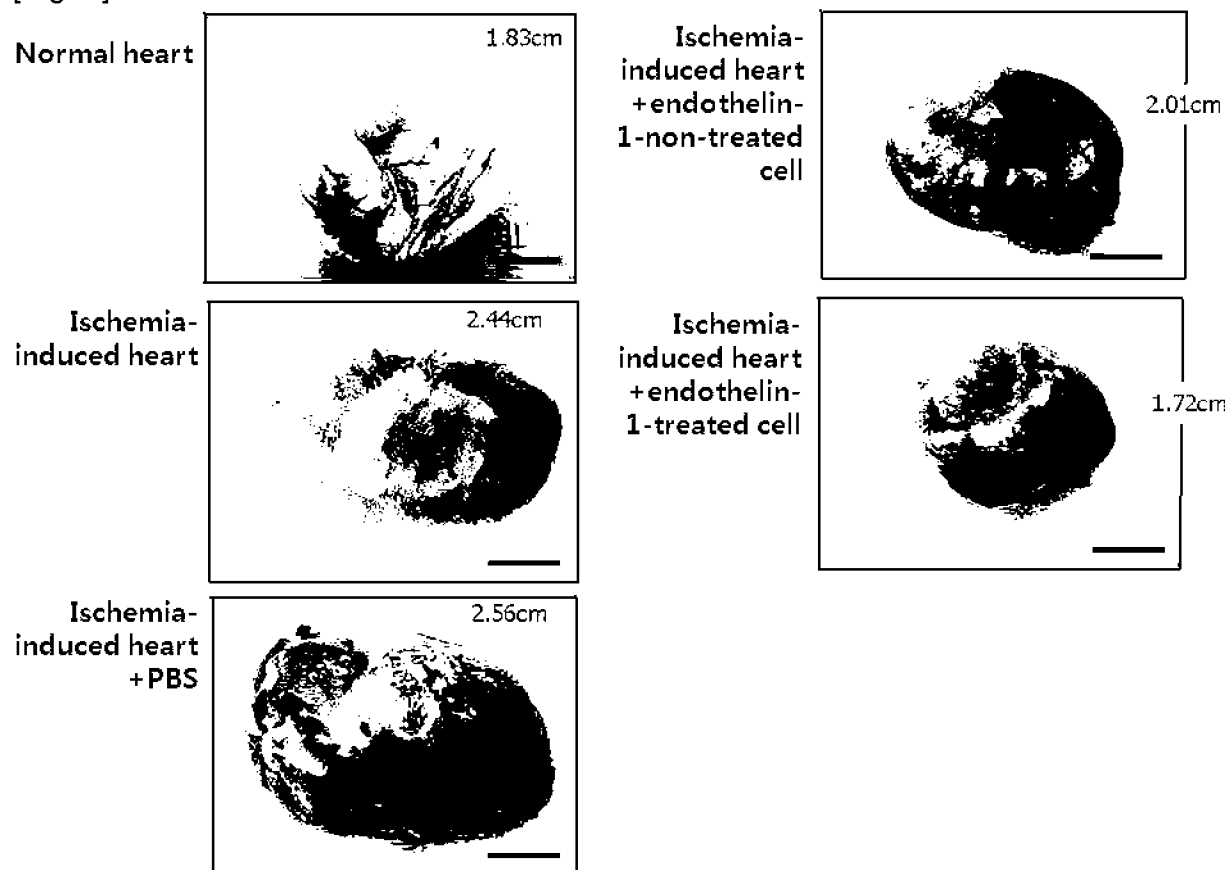
[Fig. 5]



[Fig. 6]

VEGF

[Fig. 7]



[Fig. 8]

