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Enhanced external counterpulsation is a regenerative therapy

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1. ABSTRACT

Enhanced external counterpulsation (EECP) is used for the treatment of severe angina and heart failure in patients who are not candidates for revascularization. The clinical benefits of EECP extend well beyond the time period of any hemodynamic effects, but the cause of this prolonged effect is not understood. The prolonged clinical benefits suggest EECP could be a regenerative therapy. This study was performed to determine whether EECP increased circulating hematopoietic progenitor cells (HPCs) or endothelial progenitor cells (EPCs) and thus be a possible regenerative therapy. The proposed mechanism of the increase in regenerative circulating stem cells is the enhanced shear forces induced on the endothelial boundary by the flow reversal produced by the sequential inflation of the pneumatic cuffs during EECP therapy.

2. INTRODUCTION

2.1. History of enhanced external counterpulsation

Progenitor cells and their benefits in treating diseases were not considered during the development of external counterpulsation. The technology of enhanced external counterpulsation (EECP) has been evolving over the last five decades. The initial goal of this therapy was to provide circulatory assistance to patients who had compromised cardiac outputs. Initial studies were performed by re-infusing arterial blood during various phases of the cardiac cycle. These studies demonstrated improved cardiac output and increased coronary blood flow. The improvement was dependent on the timing of the re-infused blood (1). The technique was refined by introducing a balloon in the aorta that would inflate during diastole displacing blood, thus mimicking the infusion of

blood during diastole. This technique had added benefit of afterload reduction when the balloon was deflated during systole. The intra-aortic balloon pump is used routinely in patients with cardiogenic shock, angina, and heart failure (2). Since this technique was invasive an external non-invasive method was sought. The next logical evolution was to use external pressure on the extremities to squeeze blood back toward the heart. The technique was further refined in China (3. 4.). Technology development of dynamic valves, refinement of sensors that could detect the onset of diastole, and a compressed gas delivery system allowed a practical model to be developed. Further refinements included evaluation of the pressure maximum of the bladders that would augment diastolic flow similar to that of an intra-aortic balloon pump. The end result was a commercially available EECP device (Vasomedical, Inc., Westbury New York, USA) consisting of an air compressor, computer module, treatment table and a set of three pneumatic cuffs applied to each lower extremity (5). Continuous electrocardiographic monitoring was used to time inflation and deflation of the cuffs. The cuffs were inflated sequentially from calf to thigh during diastole, applying 250-300 mmHg of external pressure and deflated at end-diastole. The mystery of EECP therapy is the prolonged benefit after treatment. This paper will elucidate a possible mechanism for this improvement and suggest that there may be other mechanisms to achieve the same goal.

2.2. Hemodynamic effects of EECP

Other reviews of EECP have described the hemodynamic effects (6). The milking effect of sequential cuff inflations during diastole increases the central venous pressure from enhanced venous return and could result in pulmonary edema (5). The same milking of the arterial system results in augmentation of the diastolic pressure with increased coronary blood flow. The rapid collapse of the leg bladders decreases the afterload similar to an intra-aortic balloon pump, but with less efficiency since the afterload reduction is provided by release of external pressure on the arterial system instead of an active removal of intravascular volume (1. 6.). These hemodynamic changes can explain the short term benefit in patients suffering from low output syndromes, but does not address the prolonged improvement after the usual course of 35 treatments. An additional effect of EECP is the enhancement of shear force delivered to the venous and arterial endothelium. Shear force leads to deformation of the boundary layer between flowing blood and the vessel wall. Velocity of flowing blood at the wall boundary layer is zero. The thickness of the boundary layer depends on the viscosity, shear stress, and the velocity of the fluid. EECP on the arterial side causes a reversal of flow at the boundary layer and therefore imparts more wall shear. Flow is enhanced during systole due to the rapid runoff increasing wall shear. The venous side has augmentation of flow during systole and diastole increasing shear stress. We hypothesize that the increase in shear force related to EECP contributes to the prolonged benefit. The mechanism by which this occurs is unclear, but may be related to changes in circulating hematopoietic progenitor cells (HPCs) or endothelial progenitor cells (EPCs).

2.3. Progenitor cells

Progenitor cells are derived from the bone marrow, and can be found free in the circulation, adhering to vessel walls, or in stores within the endothelium and vessel walls. The bone marrow contains CD45+/CD34+ hematopoietic and CD45+/CD34- mesenchymal progenitor cells. The HPCs are associated with vasculogenesis and angiogenesis, which are processes seen after EECP therapy in an experimental model (7). EPCs are a subset of HPCs with a known potential to differentiate into endothelial cells. Circulating EPCs are rare and comprise less than 0.01% of mononuclear cells (8). Under normal conditions, there is a steady state production of these cells by the bone marrow, but with destruction of cardiomyocytes by ischemia or infarction, various chemotactic factors are released which may accelerate production of these cell lines in the bone marrow and facilitate entry from the endothelium into the circulation (9). Adult progenitor cells maintain cellular homeostasis, and replenish damaged or dying cells in tissue (10). These cells, capable of producing exact copies, divide indefinitely and differentiate into multiple cell lineages. Animal studies have shown progenitor cells injected into the myocardium can form new endothelium, smooth muscle cells and cardiomyocytes, which can lead to significant regeneration in damaged tissue (11). The Transplantation Of Progenitor Cells And Regeneration Enhancement in Acute Myocardial Infarction study showed that administration of progenitor cells after successful recanalization in acute myocardial infarction resulted in increased left ventricular contractility and improved myocardial perfusion (12). Likewise in a pilot study, patients with advanced coronary artery disease treated with CD34+ stem cells had trends toward improved perfusion and reduced symptoms (13). Stores of EPCs are present within vascular walls at the border between the smooth muscle and adventitial layer and have the capacity to differentiate into mature endothelial cells (14). EPCs in this intavascular location would not be affected by vascular shear forces, but cytokines released by circulating EPCs may increase the release and proliferation of these EPCs within the vascular wall (15). EPCs have been associated with increasing ventricular function after myocardial infarction, improved angiogenesis and re-endothelialization after vascular injury (12. 15. 16. 16.). Intracoronary infusion of EPCs improves coronary endothelial function, augments metabolism, and contributes to recruitment of hibernating myocardium (17). The number of circulating EPCs may be reduced in patients of advanced age and some studies demonstrate an age-dependent impairment of re-endothelialization and neoangiogenesis (18. 19.). Some have attributed this reduction in repair mechanisms to a decreased number of circulating EPCs (20. 21.), but other studies showed no age-related change in EPCs in healthy individuals without cardiovascular risk factors (22). Both the number and migratory activity of circulating EPCs are reduced in patients with coronary artery disease and vascular disease (23, 24). Reduction in the number of circulating EPCs may be related to impaired endothelial function and reduced levels of vascular endothelial growth factor (VEGF) (25). VEGF regulates differentiation of the hemangioblast into endothelial lineage cells and a low expression of VEGF has been reported to cause impairment

of angiogenesis (19). EECP has been shown to increase circulating levels of VEGF (26).

2.4. Proposed mechanism of action

Previous studies have documented the benefit of the hemodynamics changes associated with EECP, but have also shown that even with a small amount of hemodynamic change there was a substantial clinical benefit (6). Recruitment of collaterals to the ischemic myocardium and an increase in growth factors promoting angiogenesis are other postulated mechanisms of EECP. These proposed mechanisms invoke the role of shear stress on the endothelium as stimulus for releasing nitric oxide which has beneficial effects such as vasodilatation, plus antiinflammatory, antiproliferative and antithrombotic effects. It has been shown that EECP improves endothelial function, increases levels of nitric oxide, decreases inflammatory markers, and reduces arterial stiffness. Once EECP is discontinued the shear stress stimulus is removed and the favorable factors noted above are diminished. However, the patient remains improved and the clinical benefits which can last one to five years are still not explained. One possible explanation for the long-term benefit is that EECP is a regenerative therapy. Progenitor cells line the endothelium. The shear forces described above are forceful enough to dislodge these cells so they enter the circulation. These cells will find damaged endothelium, ischemic myocardium or apoptotic cells and will proliferate so the benefit extends well beyond the treatment period. The number of circulating stem cells has been correlated with vascular disease, age and, prognosis, thus increasing these circulating cells seems a reasonable target for therapy.

2.5. Clinical studies

Several studies have shown that the clinical benefits of EECP extend beyond the time period of any acute hemodynamic benefit. Patients treated with EECP in the Multicenter Study of Enhanced External Counterpulsation reported a reduction in angina episodes and decreased nitrate use beyond the duration of therapy (27). Other studies confirm these effects plus improved exercise time, quality of life scores and an increase in the time to the onset of ischemic ST depression (28). The Prospective Evaluation of Enhanced External Counterpulsation in Congestive Heart Failure study assessed the benefits of EECP in patients with mild-to-moderate heart failure (29). EECP improved exercise tolerance, quality of life, and functional classification, but without an accompanying increase in peak VO_2 and these improvements were sustained for 6 months in the active treatment group. Some of the long-term beneficial effects after EECP are also observed after exercise training. These effects may be due to decreased peripheral vascular resistance and improved heart rate responses. However, it is possible exercise may apply direct force on the endothelium and the bone marrow that could dislodge progenitor cells, resulting in increased circulating levels.

3. PILOT STUDY EVALUATING THE PROPOSED MECHANISM OF ACTION

3.1. Study patients

Nine patients were recruited from those referred for EECP. There were 8 men and 1 woman with a mean age 70.2 ± 10.2 years. All patients had limiting angina (Canadian Cardiovascular Society class II or greater), were receiving appropriate medical therapy and were not considered candidates for revascularization. To avoid the possibility of drug effects, no new medications were prescribed once the patient entered the protocol, and no adjustments in the dose of existing medications were permitted. Patients were encouraged to maintain the same activity level and dietary habits throughout the course of therapy. Patients were excluded if they had known or suspected malignancy, active thrombophlebitis, arrhythmias interfering with machine timing, pregnancy, anemia (hemoglobin < 9.0 gm/dl) or leukocytosis, or were unable to tolerate EECP therapy. This study was approved by the Institutional Review Board of our facility and all patients gave informed consent to participate.

3.2. Study design

Patients were treated with EECP by the standard protocol which consisted of 1 hour treatments administered 5 days per week for a total of 35 treatments.

3.2.1. Collection of blood samples

Blood was collected 5-7 days before EECP, immediately before the first treatment, and 7, 14 and 28 days after starting therapy. Peripheral blood samples (5 ml) were collected by standard venipuncture methods into a lavender-top collection tube (Vacutainer™ Becton Dickinson, Franklin Lakes New Jersey USA) containing di-potassium ethylene diamine tetraacetic acid. Blood samples were stored at a constant temperature of 4° C, and all processing completed within 12 hours of collection. A minimum of 4 aliquots of whole blood, each 100 µL were transferred to conical centrifuge tubes.

3.2.2. Isolation of leukocytes

The red blood cells in each sample were lysed in 0.15 M ammonium chloride lysis solution, which was prepared daily. The remaining white cells were sedimented (1200 x g, 1 min, 22 °C) and the supernatant decanted, after which the pellet was suspended in phosphate buffered saline (pH = 7.4) containing 2 mM ethylenediamine tetraacetic acid. The tubes were again centrifuged, the supernatant decanted, and the pellet resuspended. This process was repeated for 3 total washings of the remaining cells. The washed nucleated cells were stained with three fluorochromes using standard methods for each fluorochrome (BD Biosciences, San Jose California ,USA; Miltenyi Biotec, Auburn California, USA).

3.2.3. Labeling of CD clones

The CD45 clone was HI30 mouse $IgG_{1,k}$ and was conjugated with fluorescein isothiocyanate (BD Biosciences, San Jose California, USA). The CD34 clone was 8G12 mouse $IgG_{1,k}$ and was conjugated with peridinin-chlorophyll-protein (BD Biosciences San Jose California, USA). The CD133 clone was AC133 mouse IgG_1 and was conjugated with R-phycoerythrin (Miltenyi Biotec, Auburn California, USA). The cells were incubated with each flourochrome-immunoglobulin conjugate for 10 minutes. The tubes were centrifuged, and the supernatant decanted. The importance of labeling these cells is to find a

Table 1. Progenitor cells per 10,000 lymphocytes

	Number per 10,000 lymphocytes	
	CD45+/CD34+/CD133+(EPCs)	**CD45+/CD34+(HPCs)**
5-7 days before starting EECP	3.51 ± 1.18	8.08 ± 2.40
Before first treatment	3.67 ± 1.28	7.80 ± 2.43
p value	0.78	0.81

EECP = enhanced external counterpulsation; EPCs = endothelial progenitor cells; HPCs = hematopoetic progenitor cells.

Table 2. Progenitor Cells per 10,000 lymphocytes

	Number per 10,000 lymphocytes	
	CD45+/CD34+/CD133+ (EPCs)	**CD45+/CD34+ (HPCs)**
Baseline before treatment [1]	3.59 ± 1.18	7.93 ± 2.32
1 week	4.31 ± 1.17	9.49 ± 2.59
2 weeks	4.40 ± 1.21	9.73 ± 1.73
4 weeks	4.92 ±0.86[2]	10.91 ± 1.84[3]

EPCs = endothelial progenitor cells; HPCs = hematopoetic progenitor cells , [1]The baseline value used is the average of the value obtained 5-7 days before starting treatment and immediately before the first treatment. [2] $p = 0.014$ compared with baseline. [3] $p = 0.008$ compared with baseline.

population of cells that are primitive and function as stem cells that can differentiate into vascular tissue.

3.2.4. Flow cytometry

The stained cells were then resuspended in 2 ml of phosphate buffered saline, and cell fluorescence measured using a flow cytometer (FACSCalibur™ Becton Dickinson, Franklin Lakes New Jersey, USA) within 12 hours of staining. A total of 200,000 gated-events were acquired for each sample, including the sample with unstained cells. Analysis was performed using BD CellQuest™ Pro software (BD Biosciences, San Jose California, USA) using sequential gating and International Society of Hematotherapy and Graft Engineering (ISHAGE) methods as previously described (30). One aliquot containing unstained cells was used as a control to show that the sequential gating strategy eliminated all extraneous events. The number of CD45+/CD34+ cells (HPCs) and CD45+/CD34+/CD133+ cells (EPCs) present in each sample were determined (Figure 1). Results from each tube containing stained cells were averaged for each time period. Results are reported as the average number of progenitor cells per 10,000 lymphocytes for each time interval.

3.3. Statistical analysis

Differences in the number of progenitor cells obtained 5-7 days before treatment and at baseline before the first treatment were compared using a paired t test. The average of these two samples was used for comparison with the changes during EECP. Changes in the progenitor cell lines were tracked at the specified time intervals (1, 2, and 4, weeks after beginning therapy) to assess the time course of changes. Baseline values were compared with the 4 week values using a paired t-test. Results are shown as the mean ± 1 standard deviation with a p value ≤ 0.05 considered significant. Differences between functional class before and after treatment were compared using a Mann-Whitney test and expressed as medians.

3.4. Results

There were no significant differences in the counts for either progenitor cell line comparing the values for the samples obtained 5-7 days before treatment with the values obtained immediately before the first treatment (Table 1.) The number of EPCs at baseline and after 1, 2 and 4 weeks of therapy are shown in Table 2 and Figure 2. The total number of cells increased progressively during the 4 weeks of therapy. After 4 weeks, there was a significant increase in EPCs ($p = 0.014$ compared with the baseline value). Likewise, the number of HPCs also increased significantly ($p=0.008$ compared with baseline) during treatment (Table 2.). Patients were assessed using the Canadian Cardiovascular Society scale both before and after completing their course of EECP therapy. Before EECP, the median CCS class was 3.0 and decreased to 1.5 after 4 weeks of therapy ($p<0.01$)

4. DISCUSSION

4.1. Regenerative therapy is a new concept

Regenerative therapy is a new concept in the treatment of diseases. Historically, the heart was considered unable to regenerate, but recent studies have shown that cardiomyocytes can proliferate following ischemic events (31. 32). Various progenitor cells can migrate into damaged myocardium and form new myocytes or vasculature (33). Mild success has been obtained by injecting progenitor cells into regions of myocardial infarction with some improvement in function (32. 34). It is known that senescent cells are replaced daily in all the organs of the body. How this process occurs and its failure in certain diseased states such as myocardial infarction, stoke, and degenerative states is not understood.

4.2. Model of regenerative therapy

A model of regenerative therapy must be conceived, but the operational rules for such a model have not been proposed. Ideally, a model should be simple, compatible with a single cell origin as well as compatible with growth and development. Other biological processes should be consistent with the model. These processes include the interaction between self and environment "immunity", the location of organs in the body, scar formation and the failure to regenerate as well as the successful regeneration of the liver, blood, and other tissues which rapidly turnover. Finally disease states, pain, and aging should all be compatible with the model. Our proposed model of regeneration is based on a single

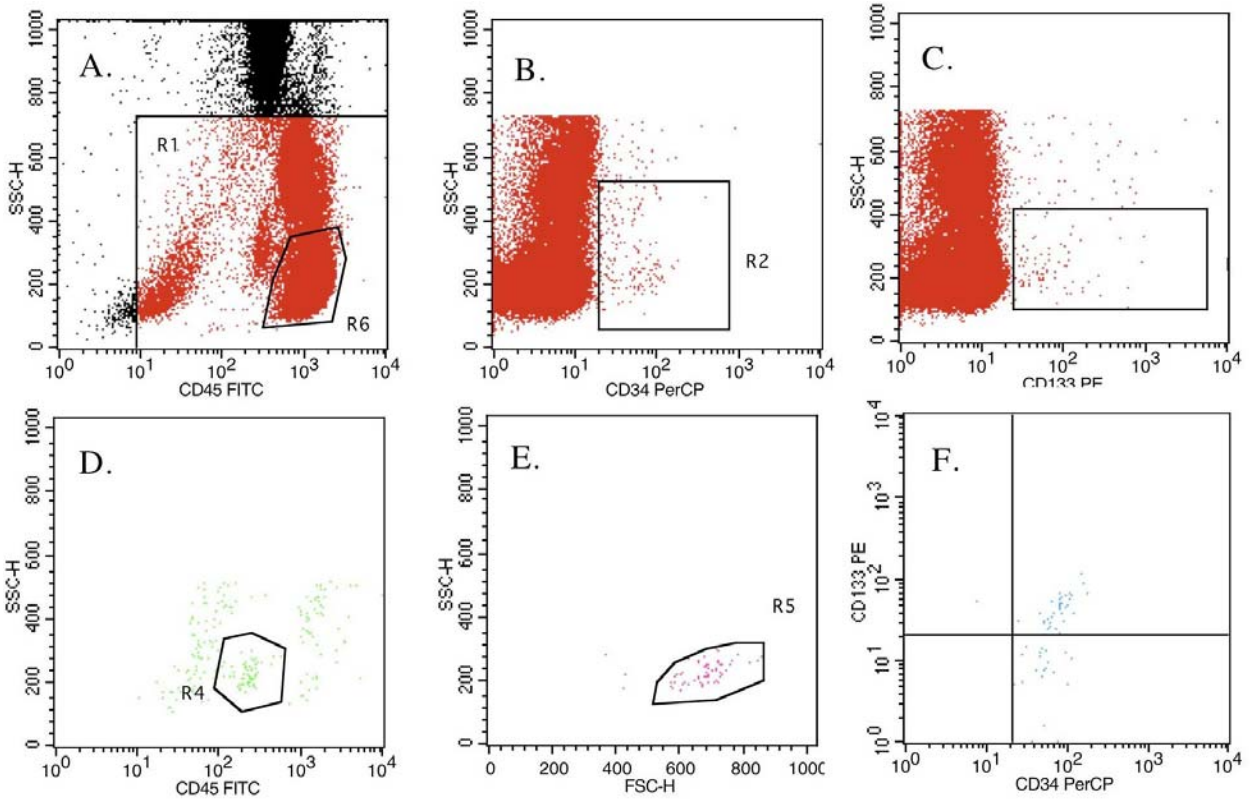

Figure 1. Sample of International Society of Hematotherapy and Graft Engineering (ISHAGE) sequential gating strategy. (A) CD45 versus Side Scatter (SSC). A total of 200,000 gated-events were acquired. Very small events were excluded. This plot was used to identify region 1 (CD45+ cells – $CD45^{bright}$ and $CD45^{dim}$) and region 6 (lymphocytes). (B) CD34 versus SSC gated to region 1 (plot A). This plot was used to identify region 2 (includes CD34+ cells). (C) CD133 versus SSC gated to region 1 (plot A). This plot was used to identify region 3 (includes CD133+ cells). (D) CD45 versus SSC gated to region 1 and either region 2 (plot B) or 3 (plot C). This plot was used to identify region 4 ($CD45^{dim}$ cells with low SSC). (E) Forward Scatter (FSC) versus SSC gated to region 4 (plot D). This plot was used to identify region 5 (cells that are similar in size to medium to large lymphocytes). Region 5 is sized based on the plot of cells from region 6. (F) CD34 versus CD133 gated to region 5 (plot E). This plot was used to classify cells based on CD34+ and CD133+ expression. All cells are CD45+.

progenitor cell that is a direct descendent of the original embryological cell formed after fertilization. The cells are propagated in the bone marrow and released into the general circulation where they nestle into the endothelium or organ niches (35). They randomly circulate until they detect and replace a senescent cell. The senescent cell during apoptosis has a shift in internal proteins that changes its polarity resulting in an electromagnetic attraction. The replacement cell then differentiates according to cell to cell information transfer at the level of the cellular membrane (36). If there is a failure of information transfer or a breakdown in the electromagnetic forces the default differentiation is a fibroblast. This regenerative model is really a model of regeneration and degeneration. Health is a state when regeneration is in balance with degeneration. Disease can occur if either regeneration or degeneration is out of balance. A moderator of this balance is inflammation/immunity that can tip the balance between degeneration and regeneration in both a favorable or unfavorable manner. For example, a wound is accompanied by inflammatory cells that release cytokines that will stimulate the bone marrow into greater production of progenitor cells. These same inflammatory cells may kill the progenitor cells or block the membrane transfer of information preventing proper differentiation.

4.3. Growth and development

This model of regeneration is compatible with embryological development demonstrating the necessity of the neurotube as an initial development that will electrically direct growth (37). Pre-natal, the patent foramen ovale and ductus arteriosus are necessary to allow circulation of the stem cells to body and brain bypassing the lungs. If there is an abnormality in the circulation such as a ventricular septal defect progenitor cells will be shifted toward the lungs and could explain the development of pulmonary hypertension due to pulmonary vascular proliferation. Stunting of growth in these children is reversed when the shunt decreases allowing more stem cells to reach the body. The location of the liver which receives most of the external toxins and requires the greatest regeneration is positioned in the body so the highest concentration of stem

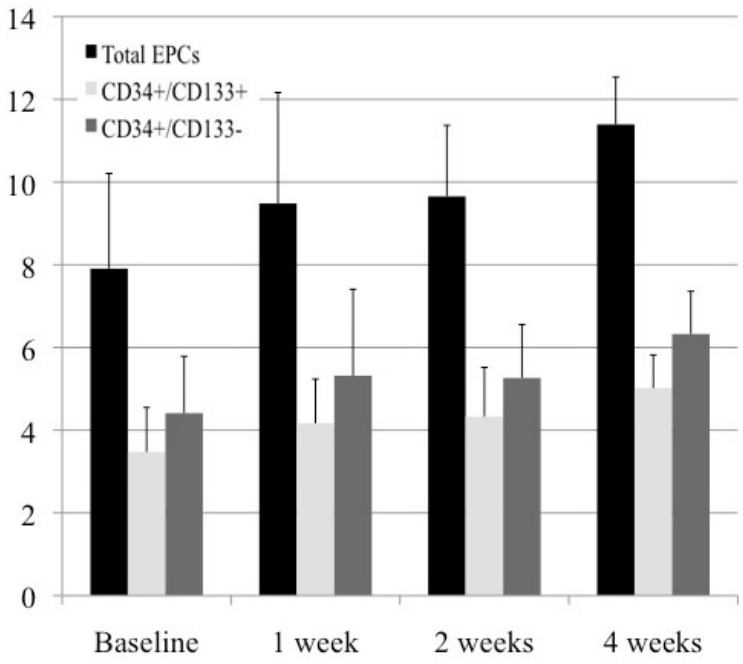

Figure 2. Changes in progenitor cells over time. Changes in hematopoetic progenitor cells (CD45+/CD34+, HPCs) and endothelial progenitor cells (CD45+/CD34+/CD133+, EPCs) over time are shown. The mean value is represented by the bar with 1 standard deviation shown. There was a significant increase in both cell lines after 4 weeks of therapy.

cells will be available. It receives circulation from the bone marrow and gets an extra boost into the hepatic veins every time the right atrium contacts. Regeneration is a steady state process; where as growth is a transient acceleration of the same process.

4.4. Definition of disease states based on model of regeneration

Using this model disease states can be defined as an imbalance between regeneration and degeneration, or a failure to differentiate. Cancer is a process where regeneration is unchecked and the cells have lost their ability to differentiate. Pulmonary hypertension is a regenerative disease with an unchecked proliferation of vascular progenitor cells. Pulmonary hypertension associated with liver cirrhosis is a regenerative disease possibly due to the lack of the liver's use of regenerative progenitor cells giving the pulmonary vasculature too many cells. Restenosis of coronary vessel treated by angioplasty is another example of regeneration being a detrimental disease process. The foremost degenerative process is age and can be defined as a reduction of circulating stem cells. Arthritis is a degeneration of cartilage at a greater rate than regeneration. Inflammation associated with diabetes, coronary disease, arthritis may be the fulcrum in determining the balance between health and disease (38). Vascular disease patients have endothelial dysfunction and fewer circulating progenitor cells (39). The inactivity associated with disease promotes degeneration over regeneration. Supporting evidence for this exists in the demonstration that exercise increases circulating progenitor cells and improves endothelial function (40. 41). Moreover, older individuals have more vascular disease than younger individuals and have fewer circulating progenitor cells (42. 43.). Decreased numbers of circulating endothelial progenitor cells are associated with greater cardiovascular death, cardiovascular events and hospitalizations (44).

4.5. Failure of regeneration

Acute myocardial infarction is the result of myocardial cell injury after an interruption of the blood supply. After an extended period of ischemia cardiac myocytes die and are replaced with fibroblasts. This failure to regenerate could be related to a lack of circulating stem cells into the infracted region due to a lack of vascular supply. The failure could also be due to damage of the electrical conducting system so that the electromagnetic signal to attract cells is lost or the communication between dying cells and progenitor cells is blocked by inflammation or blocking proteins. Cardiomyopathy associated with pacemakers or left bundle branch block and the improvement with bi-ventricular pacing can be explained by a breakdown in the electromagnetic attraction. The liver has always been a regenerative organ, but fails to do so after repeated insults and eventually becomes cirrhotic. The sick cells are likely no longer able to communicate to the progenitor cells with the default differentiation of the cells becoming fibroblasts.

4.6. Therapies promoting regeneration

The foremost therapy to promote regeneration is exercise. The mechanism for this benefit is an increase in circulating stem cells (40. 41.). A medical therapy

promoting circulation of stem cells is a "statin" drug (45). They also have been shown to increase circulating stem cells and this may explain their pleotrophic effects. A more direct method of increasing stem cells is to culture them outside of the body and then re-introduce them into the body by various methods. The methods that have been utilized include direct injection into peri-infarction areas by special catheters that sense electrical activity, direct injection to myocardial scar, injection into the infarct related vessel after revascularization, and intravenous injections. These therapies agree with the model presented that increasing the progenitor cells will promote regeneration by increasing the number of building blocks. Therapies that have had success but not necessarily attributed to this electromagnet attraction include bi-ventricular pacing and deep brain stimulation for Parkinson's disease and coma. Another device in development designed to increase contractility by providing diastolic low level current across the heart may also fulfill the requirement of attracting stem cells to regenerate the heart. Therapies to improve communication between dying cells and replacement cells are not available. However, the blocking of a protein produced in dementia may be an example of this therapy. Hypothermia by delaying apoptosis may allow time for the membrane transfer of information allowing regeneration to occur before communication is lost to inflammation.

4.7. Proposed therapies to promote regeneration

The therapies designed to regenerate organs will depend on the disease process. If an entire limb or organ is to be regenerated the regenerative process would need to mimic the transient accelerated process of growth and the mystery behind this accelerated growth process is not understood. Therapies to improve a chronic disease damaged organ can be developed that are consistent with the model presented. For success the model would predict that all requirements for regeneration are present which is circulating stem cells, electromagnetic signaling, membrane to membrane transfer of information, and inflammatory state that is conducive and not harmful to regeneration. Current therapies have not included all of these conditions but have met some of the requirements. Mass injection of cells into the infracted myocardium fulfills the first requirement. Injecting cells into an electrically active area fulfills the electromagnetic requirement. Timing of the therapy is critical to assure that some remnant of the cellular membrane is available for membrane to membrane transfer has only been observed and not clearly tested. Management of inflammation has not been actively pursued but it has been noted that female progenitor cells are less inflammatory than male cells and are more successful in regeneration. To successfully regenerate infracted myocardium the following multiple therapies could be considered: 1) revascularization to allow circulating stem cells to circulate into the infracted zone 2) increasing the number of cells into the infarction by direct injection, chemical recruitment, or increase in circulating stem cells by exercise, statins or other mechanical means 3) prevention of harmful inflammation by plasmapheresis of cytokines and, 4) providing electromagnetic signals by pacemaker. These series of therapies would accentuate the natural healing process. Current experimentation does not engage all of these steps and for this reason there has been only limited success in regenerating an infarction. In addition to the favorable hemodynamic effects of EECP, we propose that EECP increases circulating stem cells by mechanically releasing them from their storage in the endothelium. In addition our data, one recent study has shown similar results.

4.7.1 Confirmatory Study

4.7.1.1 Study patients

The study (46) enrolled twenty five patients with symptomatic coronary artery disease of which ten served as an age and gender matched control group. The average age was 69.7 years with 22 males and 13 females.

4.7.1.2. Study design

The control group received standard therapy while the treated group received standard therapy plus counterpulsation for 35 one hour treatments. The number of EPCs positive for CD34 and kinase insert domain receptor KDR were determined by flow cytometry and the number of colony-forming units assessed in a 7 day culture before after nine weeks.

4.7.1.3. Statistical analysis

Patients served as their own control. Changes between baseline and nine weeks in the control and treated group were assessed with the Wilcoxon matched-pairs signed-ranks test. Univariate correlations were performed using Spearman's correlation coefficient. All tests were two tailed. Differences of $p<0.05$ were considered significant.

4.7.1.4. Results

Counterpulsation improved anginal score from a median of 3.0 to 2.0. The EPC counts increased from a median of 10.2 to $17.8/10^5$ mononuclear cells and CFU increased from 3.5 to 11. These results were statistically significant. In the figures provided by this paper there was significant increase in both EPC colonies, CD34+/KDR cell while the control population remained flat during the same time period. The rise in cells is similar in magnitude to our pilot study. They also demonstrated an improvement in endothelial function by brachial artery flow-mediated dilatation (46).

4.8. Proposed therapies to promote degeneration

When regeneration is greater than degeneration (after the transient initial growth phase) diseases such as cancer pulmonary hypertension occur. Current therapies attempt to disrupt the cell cycle to prevent proliferation of these cells. Considering the model of regeneration disrupting the cell cycle would decrease the number of progenitor cells. Other novel therapies may be considered as suggested by the model of regeneration. One therapy would use electromagnetic signals to block or stimulate cells to disperse with electromagnetic fields. Finally the default setting could be turned on to drive the cells towards being a fibroblast.

4.9. Increasing circulating stems cells is a component of regenerative therapy

The above paragraphs are clearly speculative, but serve a purpose of proposing a model of biological regeneration. This model can serve to generate new

hypotheses and new therapies. Considering disease processes as an imbalance between regeneration and degeneration may suggest new novel therapies. Increasing circulating stem cells has been shown to be of at least limited benefit. Exercise will increase circulating stem cells and this may be the benefit of exercise in patients after myocardial infarction and in patients with congestive heart failure. Symptoms of angina, claudication, or congestive heart failure limits exercise capacity and this inactivity may cause a further reduction in circulating progenitor cells by less mechanical stimulation. Our pilot study and those of Barsheshet et al (44) demonstrate the effect of EECP on circulating progenitor cells. Our conclusions are that EECP increases circulating progenitor cells. This may occur because EECP causes a reversal of flow in the great vessels and this induces strong shear forces at the endothelial boundary layer. The shear forces mechanically release the stem cells from the endothelial surface into the circulation making them available for replacement of old endothelial cells, repairing damaged tissue and promoting regeneration. Our results suggest a possible mechanism for the long-lasting beneficial effects of EECP on myocardial ischemia and cardiac function. We demonstrate that circulating HPCs and EPCs increase during the course of EECP treatment. This could, in turn, result in a greater delivery of these progenitor cells to the myocardium and coronary vasculature resulting in regeneration demonstrated by improved ventricular performance and coronary blood flow.

5. SUMMARY AND FUTURE PERSPECTIVES

5.1. Review of possible mechanisms

Some of the long-term effects of EECP are proposed to be the result of increased coronary blood flow and improved endothelial function (20). Increased coronary blood flow velocity and pressure in vascular beds, including the coronary arteries, are noted with EECP therapy (21). The increased flow results in endothelial shear forces that stimulate the release of nitric oxide and reduce the release of endothelin-1 (47. 48. 49.). These effects improve coronary endothelial function favoring vasodilatation and myocardial perfusion, but plasma levels of nitric oxide increase only after several weeks of therapy. This delay may be due to required up-regulation in endothelial nitric oxide synthase, or the time that it takes for new progenitor cells to divided and replenish their previously lost function. Although the long-term beneficial effects may be due to increased nitric oxide synthesis, there may also be an increase in coronary collaterals (47. 48.). Finally, the increased entry of EPCs into the circulation during EECP could be related to mechanical dislodgement of adherent cells from the vessel wall, release from stores within the vessel wall, or increased production and release from bone marrow. This may occur as a result of a "milking effect" of the EECP on the lower extremities which causes diastolic reversal of flow through the circulatory system resulting in increased shear forces at the endothelial surface (28).

5.2. Future Perspectives

Although we have demonstrated an increase in progenitor cells during EECP, we cannot prove that this increase is responsible for the favorable long-term effects of EECP therapy. However, this possible mechanism warrants further evaluation and should focus on determining whether the progenitor cells released as a consequence of EECP therapy are found within the myocardium. A substantial hurdle to further investigation is the lack of an adequate animal model. There have been studies using modified EECP cuffs in a dog model, but this model is not ideal (7). In conclusion, the present study demonstrates an increase in progenitor cells during EECP therapy in patients with severe coronary artery disease. The mechanism whereby EECP causes this increase is unknown, but this observation could provide an explanation for the angiogenesis and improvements in left ventricular function observed with EECP and the long-lasting benefits following EECP therapy (50). By increasing circulating progenitor cells EECP could be considered a regenerative therapy and further proof of this concept may be warranted in the treatment of other degenerative diseases including vascular ulcers, peripheral neuropathy, Parkinson's disease, stoke, and myocardial infarction. Other methods of mechanically increasing circulating stems cells could also be investigated. The model of regeneration presented could serve as a new perspective on disease process and defining therapies as degenerative or regenerative therapies could better match disease with therapy. Finally the role of inflammation in the ability to fully regenerate should be intensely investigated.

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7. REFERENCES

1. W C Birtwell, R H Clauss, C Dennis, D E Harken, H S Soroff: The evolution of counterpulsation techniques. *Med Instrum* 10(5), 217-223 (1976)

2. Harry S Soroff, Herbert J Levine, Barry F Sachs, William C Birtwell, Ralph A Deterlini: Assisted Circulation: II. Effects of counterpulsation on left ventricular oxygen consumption and hemodynamics. *Circulation* 27, 722-731 (1963)

3. ZS Zheng, LQ Yu, SR Cai, H Kambic, TM Li, H Ma, PZ Chen, BJ Huang, Y Nose: New sequential external counterpulsation for the treatment of acute myocardial infarction. *Artif Organs*; 8(4), 470-477 1984

4. ZS Zheng, TM Li, H Kambic, GH Chen, LQ Yu, SR Cai, CY Zhan, YC Chen, SX Wo, GW Chen: Sequential external counterpulsation (SECP) in China. *Trans Am Soc Artif Intern Organs* 29, 599-603 (1983)

5. Aarush Manchanda, Ozlem Soran. Enhanced external counterpulsation and future directions. Step beyond

medical management for patient with angina and heart failure. *J Am Coll Cardiol* 50, 1523-31 (2007)

6. Piero O Bonetti, David R Holmes, Amir Lerman, Gregory W. Barsness: Enhanced external counterpulsation for ischemic heart disease: What's behind the curtain? *J Am Coll Cardiol* 41,1918-1925 (2003)

7. Guifu Wu, Zhimin Du, Chenghen Hu, Zhensheng Zheng, Chengyang Zhan, Hong Ma, Dianqiu Fang, Khan Ahmed, Roger Laham, John Hui, William Lawson: Angiogenic effects of long-term enhanced external counterpulsation in a dog model of myocardial infarction. *Am J Physiol Heart Circ Physiol* 290, H248-254 (2006)

8. Jonathan Hill, Mushabbar Syed, Andrew Arai, Tiffany Powell, Jonathan Paul, Gloria Zalos, Elizabeth Read, Hanh Khuu, Susan Leitman, McDonald Horne, Gyorgy Csako, Cynthia Dunbar, Myron Waclaw, Richard Cannon III: Outcomes and risks of granulocyte colony-stimulating factor in patients with coronary artery disease. *J Am Coll Cardiol* 46, 1643-1648 (2005)

9. Doreen Rosenstrauch, Gregor Poglajen, Nina Zidar, Igor Gregoric: Stem cell therapy for ischemic heart failure. *Tex Heart Inst J* 32, 339-347 (2005)

10. Nikos Werner, Stefan Junk, Ulrich Laufs, Andreas Link, Katrin Walenta, Michael Böhm, Georg Nickenig: Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ Res* 93, e17-24 (2003)

11. Donald Orlic, Jan Kajstura, Stefano Chimenti, Igor Jakoniuk, Stacie Anderson,Baosheng Li, James Pickel, Ronald McKay, Bernado Nadal-Ginard, David Bodine, Annarosa Leri, Piero Anversa: Bone marrow cells regenerate infarcted myocardium. *Nature* 410, 701-705 (2001)

12. Birgit Assmus, Volker Schächinger, Claudius Teupe, Martina Britten, Ralf Lehmann, Natascha Döbert, Frank Grünwald, Alexandra Aicher, Carmen Urbich, Hans Martin, Dieter Hoelzer, Stefanie Dimmeler, Andreas Zeiher: Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 106, 3009-3017 (2002)

13. Douglas Losordo, Richard Schatz, Christopher White, James Udelson, Vimal Veereshwarayya, Michelle Durgin, Kian Keong Poh, Robert Weinstein, Marianne Kearney, Muqtada Chaudhry, Aaron Burg, Liz Eaton, Lindsay Heyd, Tina Thorne, Leon Shturman, Peter Hoffmeister, Ken Story, Victor Zak, Douglas Dowling, Jay Traverse, Rachel Olson, Janice Flanagan, Donata Sodano, Toshinori Murayama, Atsuhiko Kawamoto, Kengo Fukushima Kusano, Jill Wollins, Frederick Welt, Pinak Shah, Peter Soukas, Takayuki Asahara, Timothy Henry: Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. *Circulation* 115, 3165-3172. (2007)

14. Elvin Zengin, Fariba Chalajour, Ursula Gehling, Wulf Ito, Hendrik Treede, Heidrun Lauke, Joachim Weil, Hermann Reichenspurner, Nerbil Kilic, Süleyman Ergün: Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 133, 1543-1551 (2006)

15. Atsuhiko Kawamoto, Tengis Tkebuchava, Jun-Ichi Yamaguchi, Hiromi Nishimura, Young-Sup Yoon, Charles Milliken, Shigeki Uchida, Osamu Masuo, Hideki Iwaguro, Hong Ma, Allison Hanley, Marcy Silver, Marianne Kearney, Douglas W. Losordo, Jeffrey Isner, Takayuki Asahara: Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. *Circulation* 107, 461-468 (2003)

16. Carl Sheridan, Deborah Rice, Paul Hiscott, David Wong, David Kent: The presence of AC133-positive cells suggests a possible role of endothelial progenitor cells in the formation of choroidal neovascularization. *Invest Ophthalmol Vis Science* 47, 1642-1645 (2006)

17. Sandra Erbs, Axel Linke, Volker Adams, Karsten Lenk, Holger Thiele, Klaus-Werner Diederich, Frank Emmrich, Regine Kluge, Kai Kendziorra, Osama Sabri, Gerhard Schuler, Rainer Hambrecht: Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study. *Circ Res* 97, 756-762 (2005)

18. Giuseppa Gennaro, Catherine Ménard, Sophie-Élise Michaud, Alain Rivard: Age-dependent impairment of reendothelialization after arterial injury: role of vascular endothelial growth factor. *Circulation* 107, 230-233 (2003)

19. Alain Rivard, Jean-Etienne Fabre, Marcy Silver, Dongfen Chen, Toyoaki Murohara, Marianne Kearney, Meredith Magner, Takayuki Asahara, Jeffrey Isner: Age-dependent impairment of angiogenesis. *Circulation* 99, 111-120 (1999)

20. Piero Bonetti, Gregory Barsness, Paul Keelan, Theresa Schnell, Geralyn Pumper, Jeffrey Kuvin, Robert Schnall, David Holmes, Stuart Higano, Amir Lerman: Enhanced external counterpulsation improves endothelial function in patients with symptomatic coronary artery disease. *J Am Coll Cardiol* 2003;41:1761-1768.

21. Andrew Michaels, Michel Accad, Thomas Ports, William Grossman: Left ventricuals systolic unloading and augmentation of intracoronary pressure and Doppler flow during enhanced external counterpulsation. *Circulation* 106,1237-1242 (2002)

22. Mien-Cheng Chen, Hon-Kan Yip, Chien-Jen Chen, Cheng-Hsu Yang, Chiung-Jen Wu, Cheng-I Cheng, Yen-Hsun Chen, Han-Tan Chai, Ching-Ping Lee, Hsueh-Wen Chang: No age-related change in circulating endothelial progenitor cells in healthy subjects. *Int Heart J* 47, 95-105 (2006)

23. Rebecca Gusic Shaffer, Sam Greene, Arash Arshi, Greg Supple, Andrew Bantly, Jonni Moore, Emile Mohler III: Flow cytometric measurement of circulating endothelial cells: the effect of age and peripheral arterial disease on baseline levels of mature and progenitor populations. *Cytometry* 70, 56-62 (2006

24. Robert Scheubel, Holger Zorn, Rolf-Edgar Silber Oliver Kuss, Henning Morawietz, Juergen Holtz, Andreas Simm: Age-dependent depression in circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting. *J Am Coll Cardiol* 42, 2073-2080 (2003)

25. Jonathan Hill, Gloria Zalos, Julian Halcox, William Schenke, Myron Waclawiw, Arshed Quyyumi, Toren Finkel: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 348, 593-600 (2003)

26. DP Casey, CR Conti, WW Nichols, CY Choi, MA Khuddus, RW Braith: Effect of enhanced external counterpulsation on inflammatory cytokines and adhesion molecules in patients with angina pectoris and angiographic coronary artery disease. *Am J Cardiol* 101, 300-302 (2008)

27. Rohit Arora, Tony Chou, Diwakar Jain, Bruce Fleishman, Lawrence Crawford, Thomas McKiernan, Richard Nesto: The multicenter study of enhanced external counterpulsation (MUST-EECP): effect of EECP on exercise-induced myocardial ischemia and anginal episodes. *J Am Coll Cardiol* 33, 1833-1840 (1999)

28. D Masuda , R Nohara , H Inada , T Hirai , C Li-Guang , H Kanda , M Inubushi , E Tadamura , M Fujita , S Sasayama: Improvement of regional myocardial and coronary blood flow reserve in a patient treated with enhanced external counterpulsation: evaluation by nitrogen-13 ammonia PET. *Jpn Circ* J 63, 407-411 (1999)

29. Arthur Feldman, Marc Silver, Gary Francis, Charles Abbottsmith, Bruce Fleishman, Ozlem Soran, Paul de Lame, Thomas Varricchione, for the PEECH Investigators: Enhanced external counterpulsation improves exercise tolerance in patients with chronic heart failure. *J Am Coll Cardiol* 48, 1199-1206 (2006)

30. D Barnett, G Janossy, A Lubenko, E Matutes, A Newland, J Reilly. Guideline for the flow cytometric enumeration of CD34+ haematopoietic stem cells. Prepared by the CD34+ haematopoietic stem cell working party. General Haematology Task Force of the British Committee for Standards in Haematology. *Clin Lab Haematol* 21, 301-308 (1999)

31. Piero Anversa, Jan Kajstura: Ventricular myocytes are not terminally differentiated in the adult mammalian heart. *Circ Res* 83, 1-14. (1998)

32. Antonio Beltrami, Konrad Urbanek, Jan Kajstura, Shao-Min Yan, Nicoletta Finato, Rossana Bussani, Bernardo Nadal-Ginard, Furio Silvestri, Annarosa Leri, Alberto Beltrami, Piero Anversa: Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* 344, 1750-7. (2001)

33. Abeel Mangi, N Noiseux, D Kong, M Rezvani: Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* 9, 1195-1201 (2003)

34. Gerd Meyer, Kai Wollert, Joachim Lotz, Jan Steffens, Peter Lippolt, Stephanie Fichtner, Hartmut Hecker, Arnd Schaefer, Lubomir Arseniev, Bernd Hertenstein, Arnold Ganser, Helmut Drexler: Intracoronary Bone Marrow Cell Transfer After Myocardial Infarction Eighteen Months' Follow-up Data From the Randomized, Controlled BOOST. *Circulation* 113, 1278-1294 (2006)

35. Carmen Urbich, Stefanie Dimmeler: Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 95, 343–353 (2004)

36. Catalin Toma, Mark Pittenger, Kevin Cahill, Barry Byrne, Paul Kessler. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 105, 93–98 (2002)

37. C D McCraig, A M Rajnicek, B Song, M Zhao: Has electrical growth cone guidance found its potential? *Trends Neurosci* (7):354-9 (2002)

38. Nikolaos Frangogiannis, Wayne Smith, Mark Entman. The inflammatory response in myocardial infarction. *Cardiovasc Res* 53, 31–47 (2002)

39. Mariuca Vasa, Stephan Fichtlscherer, Alexandra Aicher, Klaudia Adler, Carmen Urbich, Hans Martin, Andreas M. Zeiher, Stefanie Dimmeler: Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 89, e1-e7 (2001)

40. Jalees Rehman, Jingling Li, Lakshmi Parvathaneni, Gudjon Karlsson, Vipul Panchal, Constance J. Temm, Jo Mahenthiran, Keith March: Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. *J Am Coll Cardiol* 43, 2314-2318 (2004)

41. Ulrich Laufs, Nikos Werner, Andreas Link, Matthias Endres, Sven Wassmann, Kristina Jürgens, Eckart Miche, Michael Böhm, Georg Nickenig: Physical training increases endothelial progenitor cells, inhibits neointima formation and enhances angiogensis. *Circulation* 109, 220-226 (2004)

42. Robert Scheubel, Holger Zorn, Rolf-Edgar Silber, Oliver Kuss, Henning Morawietz, Juergen Holtz, Andreas Simm: Age-dependent depression in circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting. *J Am Coll Cardiol* 42, 2073-2080 (2003)

43. Christian Heiss, Stefanie Keymel, Ulrike Niesler, Jutta Ziemann, Malte Kelm, Christoph Kalka: Impaired progenitor cell activity in age-related endothelial dysfunction. *J Am Coll Cardiol* 45, 1441-1457 (2005)

44. Nikos Werner, Sonja Kosiol, Tobias Schiegl, Patrick Ahlers, Katrin Walenta, Andreas Link, Michael Böhm, Georg Nickenig: Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 353, 999-1007 (2005)

45. Mariuca Vasa, Stephan Fichtlscherer, Klaudia Adler, Alexandra Aicher, Hans Martin, Andreas Zeiher, Stefanie Dimmeler: Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* 103, 2885-90 (2001)

46. Alon Barsheshet, Hanoch Hod, Michael Shechter, Orna Sharabani-Yosef, Eti Rosenthal, Israel Barbash, Shlomi Matezky, Reshef Tal, Ariel G Bentancur, Ben-Ami Sela, Arnon Nagler, Jonathan Leor: The effects of external counter pulsation therapy on circulating endothelial progenitor cells in patients with angina pectoris. *Cardiology* 110, 160- 166 (2008)

47. Mateen Akhtar, Gui-Fu Wu, Zhi-Min Du, Zhen-Sheng Zheng, Andrew Michaels: Effect of external counterpulsation on plasma nitric oxide and endothelin-1 levels. *Am J Cardiol* 98, 28-30 (2006)

48. D Masuda, R Nohara, T Hirai, K Kataoka, L Chen, R Hosokawa, M Inubushi, E Tadamura, M Fujita, S Sasayama: Enhanced external counterpulsation improved myocardial perfusion and coronary flow reserve in patients with chronic stable angina. Evaluation by (13)N-ammonia positron emission tomography. *Eur Heart J* 22, 1451-1458 (2001)

49. Jaime Levenson, Marie Pernollet, Marie Iliou, Marie Devynck, Alain Simon: Cyclic GMP release by acute enhanced external counterpulsation. *Am J Hypertens* 19, 867-872 (2006)

50. Thomas Pettersson, Susanne Bondesson, Diodor Cojocaru, Ola Ohlsson, Angelica Wackenfors, and Lars Edvinsson. One year follow-up of patients with refractory angina pectoris treated with enhanced external counterpulsation. *BMC Cardiovasc Disord* 6, 1471-2261 (2006)

Abbreviations:EECP: Enhanced external counterpulsation. HPCs: hematopoetic progenitor cells. EPCs: endothelial progenitor cells

Key Words: Stem cells, Regeneration, EECP, Model of regeneration, Enhanced external counterpulsation

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