

VEGF 165 Gene Therapy for Patients with Refractory Angina: Mobilization of Endothelial Progenitor Cells

Clarissa G. Rodrigues^{1,2}, Rodrigo D.M. Plentz^{1,3}, Thiago Dipp¹, Felipe B. Salles^{1,3}, Imarilde I. Giusti¹, Roberto T. Sant'Anna¹, Bruna Eibel¹, Ivo A. Nesralla¹, Melissa Markoski¹, Nance N. Beyer^{1,4}, Renato A. K. Kalil³

Instituto de Cardiologia/Fundação Universitária de Cardiologia - Programa de Pós Graduação em Ciências da Saúde: Cardiologia¹, Porto Alegre, RS; Duke University Medical Center², Durham, North Carolina, USA; Universidade Federal de Ciências da Saúde de Porto Alegre³, Porto Alegre, RS; Universidade Federal do Rio Grande do Sul⁴, Porto Alegre, RS - Brazil

Abstract

Background: Vascular endothelial growth factor (VEGF) induces mobilization of endothelial progenitor cells (EPCs) with the capacity for proliferation and differentiation into mature endothelial cells, thus contributing to the angiogenic process.

Objective: We sought to assess the behavior of EPCs in patients with ischemic heart disease and refractory angina who received an intramyocardial injections of 2000 μ g of VEGF 165 as the sole therapy.

Methods: The study was a subanalysis of a clinical trial. Patients with advanced ischemic heart disease and refractory angina were assessed for eligibility. Inclusion criteria were as follows: signs and symptoms of angina and/or heart failure despite maximum medical treatment and a myocardial ischemic area of at least 5% as assessed by single-photon emission computed tomography (SPECT). Exclusion criteria were as follows: age > 65 years, left ventricular ejection fraction < 25%, and a diagnosis of cancer. Patients whose EPC levels were assessed were included. The intervention was 2000 μ g of VEGF 165 plasmid injected into the ischemic myocardium. The frequency of CD34+/KDR+ cells was analyzed by flow cytometry before and 3, 9, and 27 days after the intervention.

Results: A total of 9 patients were included, 8 males, mean age 59.4 years, mean left ventricular ejection fraction of 59.3% and predominant class III angina. The levels of EPCs on the 3th day after gene therapy were significantly higher than at baseline ($p=0,03$). However, on the 9th and 27th days after intervention, the levels were comparable to baseline.

Conclusion: We identified a transient mobilization of EPCs, which peaked on the 3th day after VEGF 165 gene therapy in patients with refractory angina and returned to near baseline levels on the 9th and 27th days. (Arq Bras Cardiol. 2013;101(2):149-153)

Keywords: Endothelial Cells; Capillary Permeability; Genetic Therapy; Vascular Endothelial Growth Factor A.

Introduction

Refractory angina is characterized by severe and persistent cardiac discomfort^{1,2} and is resistant to traditional cardiological treatments, including coronary artery bypass grafting (CABG), percutaneous coronary intervention (PCI), and maximum drug therapy^{1,2}. In the United States alone, there are approximately 300,000 to 900,000 cases of refractory angina; in addition, about 25,000 to 75,000 new patients are diagnosed each year³. Gene therapy, which has the potential to promote myocardial angiogenesis and collateral circulation in ischemic myocardium, may represent a possible treatment modality for these patients⁴.

Angiogenesis involves the mobilization of endothelial progenitor cells (EPCs), which are multipotent cells with the capacity to proliferate and differentiate into mature endothelial cells⁵. Gene therapy using vascular endothelial growth factor (VEGF) 165 appears to promote the mobilization of EPCs⁶, but very little is known about the behavior of such cells in patients with refractory angina who have undergone gene therapy with high doses of VEGF 165.

Most studies using VEGF 165 in patients with ischemic heart disease have been conducted in combination with other interventions such as CABG and PCI⁷⁻¹⁰. However, a class of patients exists who are not eligible for such interventions; therefore, these combined treatments are not an option for them^{1,2}. In such situations, gene therapy alone may be a possible solution, but till date, only a few studies have been conducted, with most of them using low doses of VEGF (125–250 μ g)^{9,11,12}. Therefore, the behavior of EPCs in patients receiving gene therapy with high doses of VEGF remains unclear.

Mailing Address: Renato Abdala Karam Kalil •

Av. Princesa Isabel, 370, Santana, Postal Code 90620-000, Porto Alegre, RS - Brazil

E-mail: kalil.pesquisa@gmail.com, editoracao-pc@cardiologia.org.br

Manuscript received October 29, 2012; revised October 29, 2012; accepted March 14, 2013

DOI: 10.5935/abc.20130128

The present study aimed to assess the behavior of EPCs in patients with ischemic heart disease and refractory angina who received an intramyocardial injection of 2000 μ g of VEGF 165 as the sole therapy.

Methods

Study Design

This was a subanalysis of a trial registered as ClinicalTrials.gov NCT 00744315⁴, which aimed to evaluate the safety, feasibility, and initial clinical effects of intramyocardial transthoracic administration of plasmid VEGF 165 on myocardial perfusion in patients with advanced coronary artery disease and refractory angina who did not qualify for percutaneous or surgical revascularization. The rationale and design of this trial have been described previously⁴. This was the first clinical trial that used gene therapy for patients with refractory angina in Latin America. The protocol was approved by the local Ethics Committee, and all patients provided written informed consent prior to participation.

Study Participants

Patients with advanced ischemic heart disease who were contraindicated for CABG or PCI as assessed by a cardiovascular surgeon and interventional cardiologist were eligible for enrollment. Inclusion criteria were as follows: signs and symptoms of angina and/or heart failure despite maximum medical treatment and a myocardial ischemic area of at least 5% as assessed by single-photon emission computed tomography (SPECT). Exclusion criteria were as follows: age > 65 years, left ventricular ejection fraction < 25%, and a cancer diagnosed. The subanalysis included only those patients whose EPC levels were assessed during the study.

Intervention

All patients were submitted to surgical intervention under general anesthesia at a center specializing in cardiovascular surgery. The heart was exposed by an incision of approximately 5 cm at the 4th or 5th left intercostal space according to the area to be treated. An extensive pericardiotomy was placed and was followed by pericardial repair. The area to receive the plasmid vector solution injection was identified by preoperative myocardial SPECT. Under direct vision, 10 points of the ischemic myocardium received a total of 2000 μ g of gene VEGF 165 plasmid, which was diluted in a total of 5 ml of saline solution, via a 25F butterfly injection needle. Prior to thoracography, a thoracic tube was placed and maintained for 12 h. Postoperative pain was managed using intercostal block and injectable analgesics.

Plasmid Vector

The plasmid backbone used was developed by one of our team members Sang W. Han at the Federal University of São Paulo and produced commercially by Excellion Technology (Brazil, Petropolis, RJ). The plasmid backbone of PEX-HV5 was composed of cytomegalovirus (CMV) intron 1 and its promoter with splicing signals. The human VEGF165 cDNA

was inserted between the CMV promoter and the bovine polyA sequence. This vector also contained a pUC origin and kanamycin resistance sequences for propagation in bacteria.

Outcomes

EPCs were analyzed using flow cytometry before and 3, 9, and 27 days after intervention. Mononuclear cells were separated from the peripheral blood using a Ficoll gradient (Ficoll-Paque, Invitrogen). Immunophenotyping was performed by specific staining with anti-CD34 (BD Biosciences) and anti-KDR (BD Biosciences) conjugated to the fluorophores fluorescein isothiocyanate (FITC) and phycoerythrin (PE), respectively. The CD34+ and KDR+ populations were analyzed in the FACSCalibur cytometer using CellQuest software in collaboration with the Laboratory of Immunogenetics at the Department of Genetics, Federal University of Rio Grande do Sul. The frequency of CD34+/KDR+ cells was analyzed in the lymphocyte gate in the peripheral blood mononuclear fraction and 100,000 events were counted. The results show the number of double-positive cells (n/100.000 cells).

Statistical Methods

Continuous variables are expressed as means and standard deviations and categorical variables as absolute and relative frequencies. The Friedman nonparametric test was used to compare the same variable at different times. Analyses were performed using the SPSS 18.0 statistical package.

Results

Patients

Of the 13 patients enrolled in the primary trial, 9 (8 males; mean age, 58.4 \pm 5.5 years; mean left ventricular ejection fraction, 59.3% \pm 8.8%) underwent assessment of EPCs and were included in the subanalysis. The patients workflow of the study is demonstrated in Figure 1.

Eight patients were hypertensive and 4 had diabetes. A majority of patients suffered from class III angina. All patients had previously undergone PCI, and 7 of them had also undergone CABG. These findings indicated the severity of ischemic disease in these patients (Table 1).

Outcomes

There were no deaths or recurrences. The therapy was demonstrated to be safe and feasible in this series of patients. Initial results showed a decrease in the severity of angina and intensity of myocardial ischemia, as previously described by our group⁴.

Analyses of EPCs demonstrated an increase in the number of cells on the 3th day after intervention compared to baseline (baseline, 42.7 \pm 19.2; day 3, 66.6 \pm 34.3; p = 0.03). However, the number of EPCs decreased to become comparable to baseline on days 9 and 27 (baseline, 42.7 \pm 19.2; day 9, 34.9 \pm 15.0; baseline, 42.7 \pm 19.2; day 27, 36.1 \pm 12.9; p = NS for both; Figure 2).

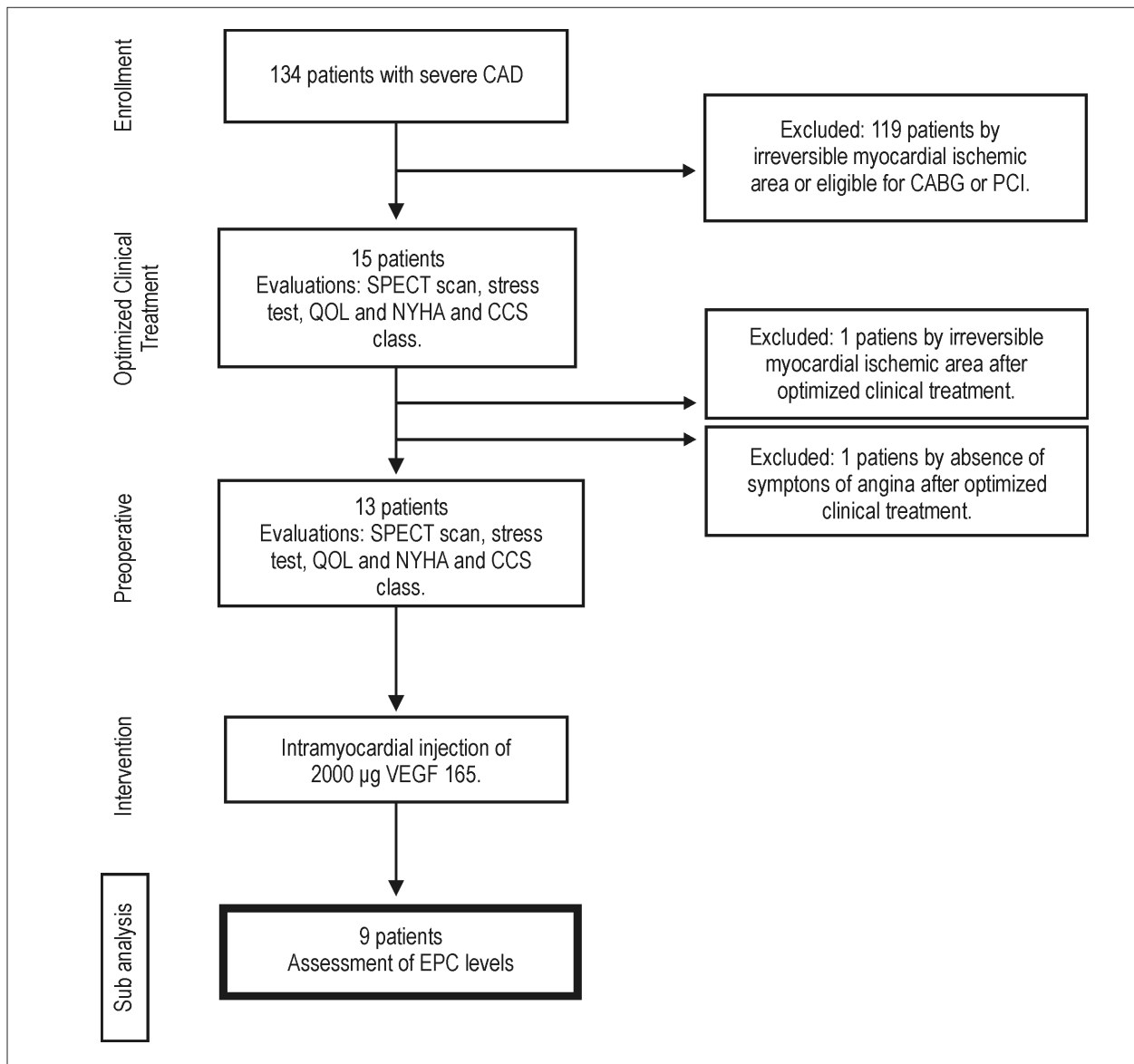


Figure 1 - Mobilization of Endothelial Progenitors Cells (EPCs). CAD: coronary artery disease; CABG: coronary artery bypass grafting; PCI: percutaneous coronary intervention; SPECT: single-photon emission computed tomography; QOL: quality of life; NYHA: New York Heart Association; CCS: Canadian Cardiology Society; VEGF: Vascular endothelial growth factor; EPC: endothelial progenitor cells.

Discussion

This study was the first, as per our knowledge, that assessed the mobilization of EPCs in patients with ischemic heart disease and refractory angina who underwent gene therapy with 2000 µg of VEGF 165. Our results showed a transient mobilization of EPCs, with peak on the 3th day after gene therapy.

A previous study evaluating mobilization of EPCs after gene therapy⁶ administered 250 µg of intramyocardial VEGF 165 to 13 patients with myocardial ischemia and reported a peak of EPCs during week 1 and week 4 after the intervention. However, besides using a substantially smaller dose of VEGF, their results of flow cytometry were

obtained based on histograms of 10,000 to 20,000 cells per sample, while we used the number of double-positive n/100.000 cells. Thus, making difficult the comparison between the trials.

EPCs have the ability to home in vascular injury sites and contribute to neoangiogenesis¹³. Mobilization of EPCs from the bone marrow in response to different signals has also been well described⁵. Previous studies have demonstrated the ability of VEGF to promote EPC mobilization *in vivo* and EPC differentiation *in vitro*^{14,15}. In addition, circulating EPCs have been shown to contribute to the neovascularization process¹⁶. The transient mobilization of EPCs after gene therapy as

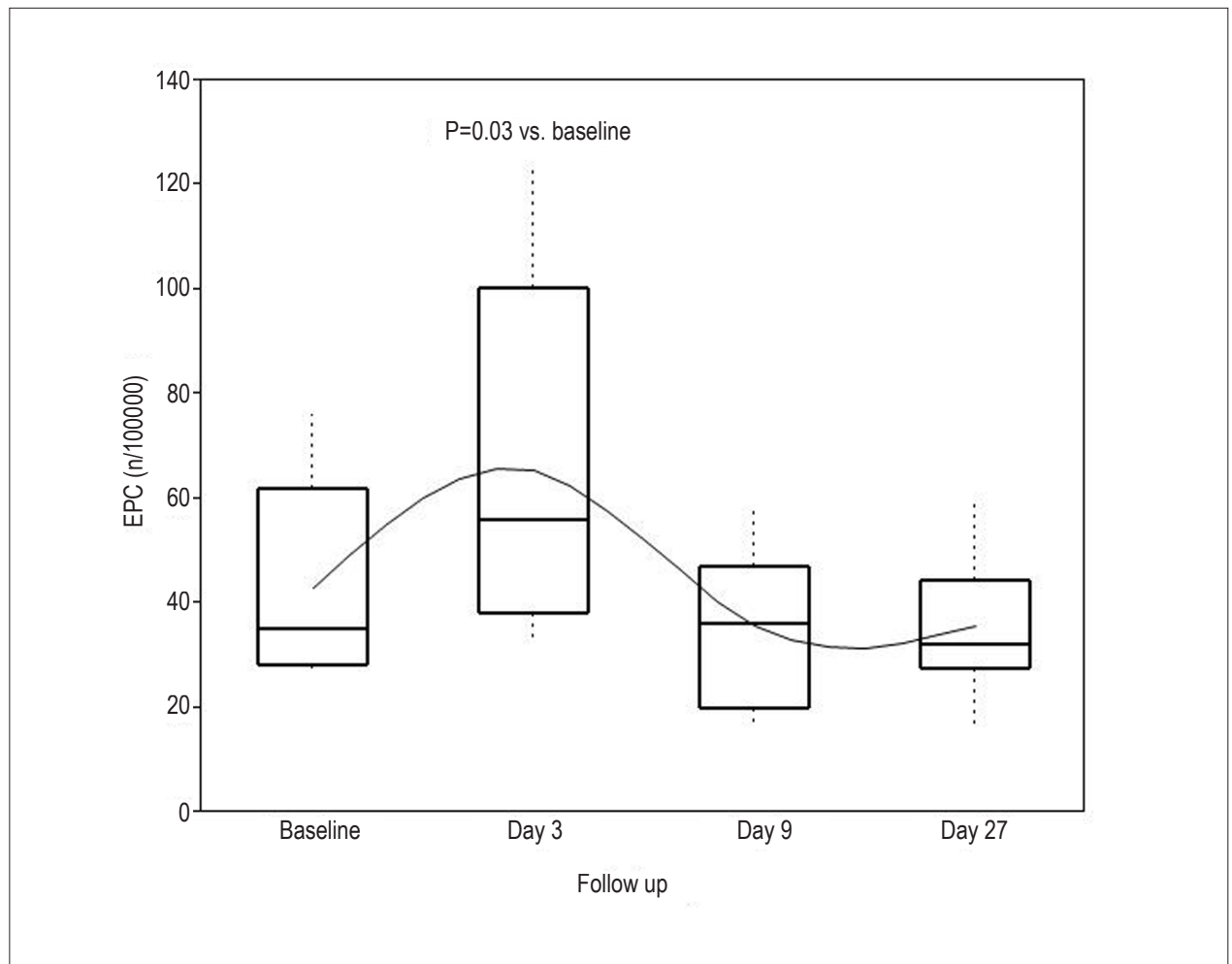
Table 1 - Demographic, clinical, and angiographic characteristics

Characteristics	N (%)
Male	8 (89)
Age*	58.4 ± 5.5
Left Ventricular Ejection Fraction*	59.3 ± 8.8
Hypertension	8 (89)
Diabetes	4 (44)
Angina Class (CCS)	
III	7 (78)
IV	2 (22)
Previous Miocardial Revascularization	
Percutaneous Coronary Intervention	9 (100)
Coronary Artery Bypass Grafting	7 (78)

*average and standard deviation. CCS: Canadian Cardiology Society.

observed in our study is in agreement with the findings of previous experimental and clinical studies⁶. VEGF expression after gene therapy seems to be transient⁶. Previous studies have shown a positive correlation between circulating EPCs and plasma VEGF levels^{6,14}, which may help explain the transient mobilization of EPCs. In addition, we suggest that permanent cell expression of VEGF and constant mobilization of EPCs after gene therapy are undesirable since such activity may increase the risk of tumor formation because of the possibility of uncontrolled cell multiplication. Therefore, it is desirable to achieve transient expression of VEGF and mobilization of EPCs, which is sufficient for the process of angiogenesis, formation of collateral circulation in ischemic myocardium, and with a subsequent improvement in myocardial perfusion. The results for mobilization of EPCs in this study were promising in this regard.

The small sample of patients may be thought to limit the power of the study; however, this sample size is derived from a phase I/II clinical trial⁴ to test the safety and

**Figure 2 - Patient workflow.**

feasibility of 2000 μg of VEGF 165 as the sole therapy for ischemic heart disease with refractory angina. Such studies aim to test the safety and feasibility of the procedure and provide initial clinical results, and they usually include small sample of patients for safety reasons. This study contributes to the understanding of EPC behavior and the myocardial angiogenic process after gene therapy with high doses of VEGF 165. Information obtained from this study can thus be used as a rationale for future phase II or III clinical trials.

Conclusion

We identified a transient mobilization of EPCs, which peaked on the 3th day and returned to baseline levels on the 9th and 27th days after gene therapy with 2000 μg of VEGF 165 in patients with refractory angina.

Author contributions

Conception and design of the research: Rodrigues CG, Plentz RDM, Dipp T, Salles FB, Sant'Anna RT, Nesralla IA, Beyer NN, Kalil RAK; Acquisition of data: Rodrigues CG, Dipp T, Salles FB,

Giusti II, Sant'Anna RT, Eibel B; Analysis and interpretation of the data: Rodrigues CG, Plentz RDM, Dipp T, Eibel B, Markoski M, Beyer NN, Kalil RAK; Statistical analysis: Rodrigues CG, Dipp T, Giusti II; Obtaining funding: Rodrigues CG, Plentz RDM, Dipp T, Nesralla IA, Markoski M, Beyer NN, Kalil RAK; Writing of the manuscript: Rodrigues CG, Salles FB, Eibel B; Critical revision of the manuscript for intellectual content: Plentz RDM, Giusti II, Sant'Anna RT, Nesralla IA, Markoski M, Beyer NN, Kalil RAK.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

This study was funded by FAPERGS.

Study Association

This article is part of the thesis of master submitted by Clarissa Garcia Rodrigues, from Instituto de Cardiologia do RG - Fundação Universitária de Cardiologia.

References

1. Bhatt AB, Stone PH. Current strategies for the prevention of angina in patients with stable coronary artery disease. *Curr Opin Cardiol*. 2006;21(5):492-502.
2. McGillion M, L'Allier PL, Arthur H, Watt-Watson J, Svorkdal N, Cosman T, et al. Canadian Cardiovascular Society. Recommendations for advancing the care of Canadians living with refractory angina pectoris: a Canadian Cardiovascular Society position statement. *Can J Cardiol*. 2009;25(7):399-401.
3. Yang EH, Barsness GW, Gersh BJ, Chandrakaran K, Lerman A. Current and future strategies for refractory angina. *Mayo Clin Proc*. 2004;78(10):1284-92.
4. Kalil RA, Salles FB, Giusti II, Rodrigues CG, Han SW, Sant'Anna RT, et al. VEGF gene therapy for angiogenesis in refractory angina: phase I/II clinical trial. *Rev Bras Cir Cardiovasc*. 2010;25(3):311-21.
5. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol*. 2003;23(7):1185-9.
6. Kalka C, Tehrani H, Lundenberg B, Vale PR, Isner JM, Asahara T, et al. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. *Ann Thorac Surg*. 2000;70(3):829-34.
7. Ruel M, Beanlands RS, Lortie M, Chan V, Camack N, deKemp RA, et al. Concomitant treatment with oral L-arginine improves the efficacy of surgical angiogenesis in patients with severe diffuse coronary artery disease: the Endothelial Modulation in Angiogenic Therapy randomized controlled trial. *J Thorac Cardiovasc Surg*. 2008;135(4):762-70.
8. Stewart DJ, Kutryk MJ, Fitchett D, Freeman M, Camack N, Su Y, et al. VEGF gene therapy fails to improve perfusion of ischemic myocardium in patients with advanced coronary disease: results of the NORTHERN trial. *Mol Ther*. 2009;17(1):1109-15.
9. Symes JF, Losordo DW, Vale PR, Lathi KG, Esakof DD, Maysky M, et al. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann Thorac Surg*. 1999;68(3):830-6.
10. Vale PR, Losordo DW, Milliken CE, Maysky M, Esakof DD, Symes JF, et al. Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. *Circulation*. 2000;102(9):965-74.
11. Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, et al. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*. 1998;98(25):2800-4.
12. Sarkar N, Rück A, Källner G, Y-Hassan S, Blomberg P, Islam KB, et al. Effects of intramyocardial injection of phVEGF-A165 as sole therapy in patients with refractory coronary artery disease – 12-month follow-up: angiogenic gene therapy. *J Intern Med*. 2001;250(5):373-81.
13. Urcich C, Dimmeler S. Endothelial progenitor cells. Characterization and role in vascular biology. *Circulation*. 2004;95(4):343-53.
14. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res*. 1999;85(3):221-8.
15. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med*. 1999;5(4):434-8.
16. Krenning G, van Luyn MJ, Harmsen MC. Endothelial progenitor cell- based neovascularization: implications for therapy. *Trends Mol Med*. 2009;15(4):180-9.