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Therapeutic potential of autologous bone marrow mononuclear cells preconditioned with Erythropoietin implantation in laser channels in patients with Cardiac arteria disease

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Abstract

The problem of incomplete myocardial revascularization for diffuse and distal lesions of the myocardium is still relevant. We assessed the clinical and instrumental long-term results of autologous bone marrow mononuclear cell (BM-MNCs) implantation in laser channels in ischemic heart disease with diffuse and distal coronary disease. In 2015-2018, 50 ischemic heart disease patients with diffuse and distal coronary arterial disease during coronary artery bypass grafting (CABG) underwent BM-MNCs short-term pre-treated with Erythropoietin implantation in laser channels (BM-MNCs group) and in 50 patients only CABG was done in the clinic of National Medical Research Center n.a. E.N. Meshalkin (Novosibirsk, Russia). Therapeutic potential of pre-treated BM-MNCs with Erythropoietin implantation was carried out at two weeks, six and twelfth months after surgery. Changes on morphofunctional properties of BM-MNCs after pre-treatment with Erythropoietin was carried out on basis phenotype, cell cycle, cell death, proliferation, migration, tube formation and cytokine production. In this study, we observed the presence in cellular graft of the hematopoietic stem cells (HSCs), and endothelial progenitor cells (EPCs) at the different stage of maturation/differentiation, and mesenchymal stem cells (MSCs). Precondition BM-MNCs with Erythropoietin increased number of HSCs carrying erythropoietin receptor (EpoR), and EPCs carrying CD184. Also, Epo detained CB34+ cells in a rest phase of cell cycle (G0G1). Condition media from BM-MNCs treated with Erythropoietin augment tube formation and wound healing by EA.hy 929. After six months postoperatively, the severity of angina and heart failure based NYHA functional class (NYHA FC) was significantly less in the BM-MNCs group than in control group (p=0.04). according to perfusion scintigraphy, there was a slight decrease of stable perfusion defects (SPD) in the early postoperative period. Left ventricular ejection fraction in BM-MNCs group have tendency to increase six months after treatment.

Аннотация

Проблема неполной реваскуляризации миокарда при диффузных и дистальных поражениях миокарда остается актуальной. Проведена оценка клинико-инструментальных отдаленных результатов имплантации аутологичных мононуклеарных клеток костного мозга (КМ-МНК) в лазерные каналы при ишемической болезни сердца с диффузной и дистальной ишемической болезнью сердца. В 2015-2018 годах, у 50 больных с ишемией сердца, обусловленной диффузным и дистальным коронарным атеросклерозом во время аортокоронарного шунтирования (АКШ) была проведена имплантация КМ-МНК предварительно проинкубированных с эритропоэтином в лазерные каналы (КМ-МНК группа) и у 50 больных проведено только АКШ на базе клиники Национального медицинского научно-исследовательского центра им. акад. Е. Н. Мешалкина (Новосибирск, Россия). Терапевтический потенциал имплантации предварительно обработанных КМ-МНК с эритропоэтином оценивали через две недели, шесть и двенадцатый месяцев после операции. Изменения морфофункциональных свойств КМ-МНК после предварительной обработки эритропоэтином осуществляли на основе фенотипа, клеточного цикла, гибели клеток, пролиферации, миграции, образования сосудисто-подобных структур и продукции цитокинов. В данном исследовании мы наблюдали наличие в клеточном трансплантате гемопоэтических стволовых клеток (ГСК) и эндотелиальных прогениторных клеток (ЭПК) на разной стадии созревания/дифференцировки, а также мезенхимальных стволовых клеток. Предварительная культивация КМ-МНК с эритропоэтином способствовала увеличению количества ГСК, несущих рецептор эритропоэтина, и ЭПК, несущих CD184. Кроме того, обработка клеток эритропоэтином удерживало CD34+ клеток в фазе покоя клеточного цикла (G0G1). Кондиционная среда от КМ-МНК проинкубированных с эритропоэтином увеличивало формирование сосудисто-подобных структур и заживление раневого дефекта монослоя EA.hy 929. Через полгода после операции тяжесть стенокардии и сердечной недостаточности по функциональному классу NYHA была достоверно меньше в группе КМ-МНК, чем в контрольной группе (P=0,04). По данным перфузионной сцинтиграфии в раннем послеоперационном периоде отмечено незначительное снижение стабильных перфузионных дефектов. Фракция выброса левого желудочка в группе КМ-МНК имеет тенденцию к увеличению через полгода после лечения.

Keywords: Coronary artery disease, erythropoietin, bone marrow mononuclear cells, intramyocardial injection, NYHA class, Borg class

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1. Introduction

In Cardiovascular diseases are the leading cause of morbidity, and mortality, and development of heart failure (1-2). The lack of capillary network and perfusion after acute myocardium infarction (AMI) caused oxygen and nutrients deficiency and leads to endothelial apoptosis, and an increase in the area of heart attack and left ventricular dysfunction. Also, after AMI, several processes occurred, including the irreplaceable loss of a part of cardiomyocytes, structural remodeling of a myocardium associated with inflammation, scar formation, and development of interstitial fibrosis in peri-infarction zone, and remodeling of blood supply of a myocardium, that are also the starting factors of heart failure development (3-4). Although pharmacological and surgical treatments can significantly improve patient outcomes, no treatment currently available is able to generate new contractile myocardium or revers ischemic myocardium, therefore new methods of therapy are necessary to repair myocardium function. In many reports the successful use of the stem cells to regenerate damaged myocardium in both animal and human AMI has been shown (5-6). Stem cell-based therapy is emerging as a potential therapeutic approach for damaged tissue regeneration and an important strategy for the treatment of heart failure (7-10). Implantation of autologous bone marrow stem cells into injured myocardium may be considered as a promising therapy for myocardium regeneration and ventricular contractility restoration (11-19). Analysis of obtained data from randomized clinical trials of intramyocardial bone marrow cells to treat ischemic heart disease showed increased left ventricular ejection fraction (LVEF), reduced left ventricular end-systolic volume (LVESV), and trend toward the decrease of left ventricular end-diastolic volume (LVEDV). The best efficacy of intramyocardial bone marrow cells injection observed on the left ventricular ejection fraction in patients with suitable for revascularization with coronary artery bypass grafting compared with patients unsuitable for revascularization (20). Numerous clinical trials have previously investigated the clinical outcomes in patients after myocardium infarction and reported significant efficacy in improving contractility and reducing infarction scar (13, 15, 18-19, 21). However, there was a problem of the limited efficacy: improvement of left-ventricular ejection fraction and reduction of infarction size, viability of stem cells. Under this situation, precondition of the adult stem cells before implantation would be a very feasible and safe way to augment the therapeutic efficacy. Erythropoietin (Epo), cytokine that controls erythropoiesis, also appears to have pleiotropic effects, such as anti-ischemic and anti-apoptotic properties, promotion of neovascularization, mobilization of EPCs, and enhancement of angiogenesis (22). The aim of this study was to investigate the therapeutic efficiency of intramyocardial administration of short-term preconditioning autologous bone marrow mononuclear cells in patients with cardiac arteria disease.

2. Material and Methods

2.1. Ethics Statement and Patients Characteristics

The Research involving humans was performed with the prior approval of the Ethics Committee of Institute of Clinical and Experimental Lymphology-Branch of National Research Centre Institute of Cytology and Genetics Siberian Division of Russian Academy of Sciences, and of the Ethics Committee of Meshalkin National Medical Research Center, Ministry of Health of Russian Federation, and all procedure was conducted in accordance with the principles and guidelines of the Declaration of Helsinki. All participants signed written informed consent prior to the study. One hundred ischemic heart disease patients with diffuse and distal coronary arteria disease (CAD) were enrolled from Meshalkin National Medical Research Center, Ministry of Health of Russian Federation (Novosibirsk, Russian Federation) in January 2016 – May 2018. Patients had to be at least 50 years old, suffering from chronic ischemic heart disease, and receiving constant state-of-the-art pharmacotherapy for at least 3 months prior to enrolment. 95% were men. All were in angina NYHA functional class II-III. Arterial hypertension was presented in 90%, peripheral atherosclerosis in 60%. The inclusion criteria were as follows: age ≥50 and <75 years; presence of coronary artery lesions; chronic ischemia heart failure; myocardial infarction within previous 12 months; New York Heart Association (NYHA) functional class II-III within last 6 months; systolic dysfunction with LVEF ≤35%; fixed perfusion defect on Tc-99m technetril single-photon emission computed tomography (SPECT). The exclusion criteria comprised the following factors: eligibility for percutaneous coronary intervention; eligibility for coronary artery bypass grafting; previous valve surgery; surgical remodeling of the left ventricle or cardiac resynchronization therapy; hemorrhagic symptoms; severe renal and liver dysfunction; malignancy. Patients who meet eligibility criteria will be scheduled for bone marrow harvest. Indications for surgery were: severe angina refractory to clinical antianginal therapy; diffuse coronary artery lesion of the distal bed or small coronary vessel diameter (less than 1 mm) or a viable (hibernating) myocardium. 50 patients received only coronary artery bypass grafting (CABG) – control group, 50 patients during CABG underwent autologous bone marrow mononuclear cells pre-treated with Epo implantation in laser channels – BM-MNCs group. Clinical and instrumental assessment of the therapeutic efficiency was carried out at two weeks, six and twelfth months after surgery. One of the main conditions of the operation was the presence of a viable myocardium in the revascularization area. In this regard, we analyzed two-staged myocardial scintigraphy with Tc-99

data for myocardial viability, as well as to assess the effectiveness of indirect revascularization. The status of the infarction was assessed on a 5-point scale: 4 – rule; 3 – ischemia (hibernating myocardium); 2 – small focal scarring; 1 – tripe; 0 –aneurysm. The main parameters were stable perfusion defect (SPD, %) and transient perfusion defect (TPD, %).

2.2 Human Bone Marrow Mononuclear Cells Morphofunctional Properties

Bone marrow samples of fifty patients with CAD were harvested for autologous transmyocardial implantation. Bone marrow aspirates were diluted with PBS and bone marrow mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation with Ficoll-Paque. The BM-MNCs were washed three times with 50 mL of PBS, counted, and viability testing was performed with trypan blue exclusion (≥ 95%), and used for the experiments. The 106 BM-MNCs/cm2 from patients with CAD were plated in PBS supplemented completed with 10% autologous serum and Epo (33.4 IU/mL of Recormon) for 1 hour. BM-MNCs phenotype was performed for CD18 (Integrin β2), CD29 (Integrin β1), CD31 (Platelet endothelial cell adhesion molecule-1, PECAM-1), CD34 (Glycoprotein), CD44 (Cell-surface glycoprotein), CD45 (Transmembrane glycoprotein), CD49a (Integrin alpha-1), CD54 (Inter-cellular adhesion molecule 1), CD62E (E-selectin), CD73 (68kDa GPI-anchored cell-surface protein with enzymatic and signal transduction activities), CD90 (GPI-anchored membrane glycoprotein of the Ig superfamily, Thy-1), CD105 (Endoglin, a major glycoprotein of human vascular endothelium), CD131(βIL-3 (AIC2A) and βc (AIC2B) cytokine receptor subunits), CD133(Promenin-1), CD146 (Cell surface glycoprotein MUC18), CD184 (CXC chemokine receptor, CXCR4), CD202b (Angiopoetin-1 receptor), KDR (Vascular endothelial growth factor receptor -2), and Erythropoietin-receptor (EpoR) expression. Cell cycle distribution were performed by using Propidium Iodide. The apoptosis assay was performed using an Annexin V-FITC/PI Apoptosis Detection kit. Proliferative potential of 2 x 105 BM-MNCs/well were done with Concanavalin A (10 μg/mL), Phytohemagglutinin A (10 μg/mL), Lipopolysaccharide (1 μg/mL), Epo (33.4 IU/mL of Recormon), and H2O2 (1, 3, and 5 mM) in DMEM plus 10% FCS, 0.3 mg/mL L-glutamine, 5 mM HEPES buffer), and 80 μg/mL of gentamycin during 72 hours in CO2-incubator, then cells were supplemented with fresh medium containing 5 mg/mL 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) and incubated for 4 hours at 37 0C. The formazan in viable cells was dissolved with 100 μL of dimethyl sulfoxide and determined by reading optical densities (OD) in microplate reader (Stat Fax 2100) at an absorption wave length of 570 nm. For enzyme-linked immunosorbent assay (ELISA), and wound healing test, and tubule-formation, media were collected from plates of BM-MNCs (CM-MNCs) after 72 hours of culture in DMEM plus 10% FCS, 0.3 mg/mL L-glutamine, and 80 μg/mL of gentamycin, and Epo (0 and 33.4 IU/mL of Recormon), aliquoted and storage at -70 0C before using in testing in vitro. IL-1β, TNF-α, TGF-β1, IL-6, IL-8, IL-10, PDGF-AB, VEGF, basal GFG, IGF-1, Epo, CXCL-12/SDF-1a, MMP-9, TIMP-1, and NO in CM-MNCs were determined by ELISA. NO inhibition assay was conducted using Griess reagent kit for nitrite determination. Wound healing was tested on EA.hy 926 cells (kindly gifted by Dr. C.J. Edgel, Caroline University, USA) scratched monolayer cultured or tubule-formation on In vitro angiogenesis assay kit in 100 μL of DMEM plus 0% or 10% FCS (negative and positive control), in mixture of 70% DMEM and 30% of CM-MNCs growth in the presence or absence of Epo (experimental wells) in CO2-incubator for 24 hours.

2.3 Indirect Method of Coronary Revascularization and Short -term Preconditioning Autologous Bone Marrow Mononuclear Cells with Erythropoietin Intramyocardial Implantation

In fifty patients with CAD after performed the distal anastomoses, 5-7 radially arranged blind laser channels in fifth points were formed by using laser surgical unit of LSP-IRE-Polus (Russia) with wavelength of 1.56 microns with a power of 15 watts. Laser was applied in pulsed mode, with a pulse duration of 20 ms and an interval between pulses of 20 ms. Channel length was determined by the size of scar area. Further, in order to create a closed cavity in the channel mouth, an n-stitch was superimposed, the 20 x 106 BM-MNCs pre-treated with Epo infusion was performed, and the n-stitch arose. The efficacy of treatment was assed in 2 weeks, 6 and 12 months by changes in the LVEF, LVESV, LVEDV by echocardiography and myocardial perfusion by ECG-synchronized SPECT using technetium (Tc-99m technetril) at rest and during pharmacological stress caused by intravenous administration of adenosine (0.14 mg/kg/min over 6 min). The results were assessed in 10 segments; score range in each segment from 0 to 4 (0 – normal activity, 4 – no activity). Also, efficiency of treatment was estimated using NYHA FC, and Borg dyspnea scale.

2.4 Statistical Analysis

All data analyses were performed by Statistica 10 statistical program. In this study, the normality of the distribution was determined by the w-Shapiro-Wilkes criterion, in tables the obtained data were presented as median with lower and upper quartiles (Me; LQ-UQ) with no less than three replicates for each experimental condition, the data were analyzed by Mann-Whitney U test for pairwise comparisons data, and correlation coefficient using the Spearman rank correlation (Rs). If p-value was less than 0.05, it was considered statistically significant.

3. Results and discussion

3.1. Morphofunctional properties of Bone Marrow Mononuclear Cells from Patients with CAD

Among mononuclear cells derived from bone marrow samples from patients with CAD we observed a higher number of hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs). So, among HSCs the number of CD45+/CD34- cells were more than 30%, CD45+/CD184+ cells more than 16%, and CD45+/EpoR+ more than 3%. Among EPCs the number of CD45-/CD34+ cells were more than 0.6%, CD34+/CD31+ cells more than 0.9%, CD34+/VEGFR2+ cells more than 1.25%, CD34+/CD133+ cells more than 0.95%, CD34+/CD131+ cells more than 1.15%, CD34+/CD184+ cells more than 2.6%, CD34+/EpoR+ cells more than 0.85%, and CD31+/CD184+ cells more than 5.5%. Among MSCs the number of CD73+/CD90+ cells were more than 1.3%, CD73+/CD105+ cells more than 3.7, and CD90+/CD105+ cells more than 0.9%. These findings indicated that BM-MNCs from patients with CAD contained heterogeneous stem cells populations. Information on BM-MNCs phenotypes changes after short-term conditioning with Erythropoietin was summarized in Table 1. Conditioning BM-MNCs with Epo significantly increased the number of HSCs expressed EpoR (CD45+/EpoR+ cells), and the number of EPCs expressed CD184 (CD31+/CD184+ cells), wherease the number of EPCs expressed CD133 (CD34+/CD133+ cells) significantly decreased. Also, we find a trend towards the decrese of the number of CD34+/CD133+ cells, CD34+/EpoR+ cells, CD34+/CD184+ and CD34+/CD131+ cells, and the trend towards the increase of the number of CD34+/VEGFR2+ cells and MSCs with CD73+/CD90+, CD73+/CD105+ and CD90+/CD105+ phenotype. We showed strong correlation between the number of CD34+/CD133+, and the number of CD34+/EpoR+ cells with the age of patients (R=0.52 and R=0.49, p ≤0.05). We found that pre-treatment of BM-MNCs with Epo significantly increased the number of CD34+ cells in subG0G1, G0G2 cell cycle phase, and significantly decreased the number of CD34+ cells in G2/M and S cell cycle phase (Table 2). Whereas, we did not observe any significant difference in the number of CD34+ cells in apoptosis and necrosis when we used an Annexin V-FITC/PI Apoptosis Detection kit, but we observed the trend towards the decrease of the number of CD34+ cells after pre-treatment with Epo on apoptosis/necrosis. Data of the effect of pre-treatment of BM-MNCs with Epo on expression of CD18, CD54, CD29, CD44, CD49a, CD62E, CD146, CD202b was summarized in Table 3. We found the trend towards the increase in the number of CD34+/CD54+, CD34+/CD18+, CD49a and CD202b cells among BM-MNCs after pre-treatment with Epo. Whereas, the number of CD18+/CD54+, CD29+, CD44+, CD62E+ and CD146+ cells tend to reduce among BM-MNCs after pre-treatment with Epo. BM-MNCs proliferation spontaneous or in the presence of stimulating factors or inductors of oxidative stress after pre-treatment with Epo was significantly reduced compared with basal and stimulated proliferation capacity of BM-MNCs without pre-treatment with Epo (Table 4). We

Table 1 | The effect of short-term preconditioning of the bone marrow mononuclear cells on the number of stem cells (%, Me; LQ-UQ). Эффект кратковременной экспозиции КМ-МНК с эритропоэтином на количество стволовых клеток.

Phenotype	*Basal*	*Epo -preconditioning*	*p-value*
Hematopoietic stem cells			
CD45+/EpoR+	4.76 (0.5-6.25)	10.91 (1.75-16.24)	0.01159
Endothelial progenitor cells			
CD34+/CD31+	0.94 (0.4-1.0)	0.92 (0.5-1.0)	0.41699
CD34+/CD133+	0.87 (0.5-1.06)	0.33 (0.1-0.7)	0.01052
CD34+/VEGFR2+	1.09 (0.5-1.3)	1.51 (0.1-2.5)	0.29870
CD34+/EpoR+	0.8 (0.4-0.9)	0.55 (0.3-0.55)	0.14634
CD34+/CD184+	2.46 (1.0-3.0)	1.69 (0.4-2.0)	0.19361
CD34+/CD131+	0.78 (0.2-1.45)	0.55 (0.2-0.5)	0.97278
CD31+/CD184+	6.13 (2.0-8.5)	11.48 (5.0-11.0)	0.02778
Mesenchymal stem cells			
CD73+/CD90+	0.85 (0.25-2.2)	1.15 (0.1-2.7)	0.96
CD73+/CD105+	1.75 (0.35-7.2)	2.0 (0.1-5.4)	0.91
CD90+/CD105+	0.5 (0.21-1.3)	0.95 (0.6-2.0)	0.34

Table 2 | The effect of short-term preconditioning of the bone marrow mononuclear cells on the cell cycle distribution and apoptosis/necrosis. Эффект краткосрочной экспозиции КМ-МНК с эритропоэтином на распределение клеток в фазах клеточного цикла и апоптоз/некроз

Parameters	*Basal*	*Epo - preconditioning*	*p-value*
Cell cycle			
subG0G1	5.58 (5.0-5.0)	6.12 (6.0-6.0)	0.011975
G0G1	78.25 (78.0-78.0)	85.29 (85.0-85.0)	0.001914
G2/M	15.85 (13.0-13.0)	7.1 (7.0-7.0)	0.000052
S	3.25 (3.0-3.0)	1.08 (1.0-1.0)	0.000052
Apoptosis/Necrosis			
Annexin V+/PI- (early apoptosis)	6.98 (1.7-10.0)	5.47 (0.85-5.0)	0.076424
Annexin V+/PI+ (apoptosis)	3.07 (0.1-3.6)	4.2 (0.6-2.95)	0.310768
Annexin V-/PI+ (necrosis)	2.72 (0.25-1.05)	1.58 (0.2-1.0)	0.966427

Table 3 | Effect of short-term preconditioning of the bone marrow mononuclear cells on the expression of adhesion molecules. Эффект краткосрочной экспозиции КМ-МНК на экспрессию молекул адгезии

Phenotype	*Basal*	*Epo -preconditioning*	*p-value*
CD34+/CD54+	1.77 (0.55-3.0)	2.0 (0.7-3.9)	0.67
CD34+/CD18+	1.4 (0.55-2.25)	1.7 (0.2-4.2)	0.52
CD18+/CD54+	59.47 (43.9-75.0)	54.27 (34.8-70.7)	0.94
CD29a+	97.27 (95.8-98.75)	89.2 (75.8-96.7)	0.83
CD44+	91.25 (84.6-97.9)	86.97 (64.5-99.8)	0.28
CD49a+	37.92 (22.65-53.2)	56.0 (51.1-63.4)	0.62
CD62E+	44.6 (21.95-67.25)	42.4 (8.4-73.7)	0.57
CD146+	6.85 (1.0-12.65)	5.9 (0.1-12.1)	0.94
CD202b+	13.65 (3.75-23.55)	17.63 (9.3-24.1)	1.0

established the trend to increase TNF-α, Epo, PDGF-AB and IGF-1production by BM-MNCs after pre-treatment with Epo (Table 5). Whereas, levels of IL-1β, IL-8, IL-10 and TGF -β1 production by BM-MNCs pre-treated with Epo did not change. Also, we did not establish significant differences between the basal levels of cytokines production by BM-MNCs and by BM-MNCs cultured in the presence of Epo ($p>0.05$). Conditioned media from BM-MNCs cultured with or without Epo stimulated vessel-like structures formation (Table 6). In the presence of CM from pre-treated with Epo BM-MNCs we observed increased wound healing (Figure 1) and tend to increase vessel-like structures formation (Figure 2).

3.2. Efficacy of intramyocardial administration of autologous bone marrow mononuclear cells pre-treated with Erythropoietin

Table 4 | Effect of short-term preconditioning of the bone marrow mononuclear cells on proliferative activities. Эффект краткосрочной экспозиции КМ-МНК с эритропоэтином на пролиферацию

Parameters	*Basal*	*Epo -conditioning*	*p-value*
Spontaneous	0.66 (0.58-0.68)	0.45 (0.4-0.45)	0.00072
Concanavalin A (10 μg/mL)	0.57 (0.56-0.6)	0.42 (0.36-0.46)	0.011384
Phytogemagglutinin A (10 μg/mL)	0.61 (0.6-0.67)	0.44 (0.37-0.46)	0.000083
Lipopolysaccharide (10 μg/mL)	0.69 (0.66-0.7)	0.49 (0.44-0.51)	0.00057
Hydrogen peroxide 1 μM	0.65 (0.57-0,76)	0.34 (0.32-0.36)	0.0000001
Hydrogen peroxide 3 μM	0.55 (0.51-0.57)	0.35 (0.3-0.42)	0.000541
Hydrogen peroxide 5 μM	0.48 (0.48-0.57)	0.37 (0.34-0.38)	0.048652
Erythropoietin (33.4 IU/mL)	0.69 (0.57-0.82)	0.5 (0.49-0.54)	0.006027

Six months after treatment, the severity of angina and heart failure based on NYHA FC significantly reduced in BM-MNCs group than in control group (0 ± 0 vs 1.6 ± 0.1, $p=0.01$). 12 months after surgery angina and heart insufficiency were at the same levels. Additionally, in BM-MNCs group we observed the increase in exercise tolerance from 321 m (285 – 385 m) to 356.5 m (325 – 420 m) during the 6 minutes walking test, and reduced Borg dyspnea scale

Table 5 | Effect of Erythropoietin on the cytokines production by bone marrow mononuclear cells. Эффект эритропоэтина на уровни продукции цитокинов КМ-МНК

Cytokine	*Basal (1)*	*Epo in culture media (2)*	*Epo -conditioning (3)*	*p-value*
IL-1β	22.3 (12.3-51.2)	34.25 (2.67-67.95)	26.8 (0.15-40.2)	0.93/0.54
TNF-α	7.04 (1.31-21.85)	7.9 (2.54-22.85)	10.3 (0.42-369.7)	0.97/0.69
IL-6	1615 (1208-1743)	1651 (1208.5-1776)	1560 (824-1666)	0.85/0.25
IL-8	1260 (1225-1320)	1270 (1164-1353)	1268 (1218-1280)	1.0/0.9
IL-10	164.15 (13.95-269)	62 (12.7-193.7)	13.9 (4.84-57)	0.65/0.39
Epo	240.9 (46.8-650.25)	843.7 (557.7-848.65)	561 (180.9-822.4)	0.07/0.06
PDGF-AB	97.5 (76,8-153.1)	95.4 (65.5-736.8)	66.8 (46.8-1240)	0.95/0.32
IGF-1	0.18 (0.15-0.21)	0.23 (0.18-0.28)	0.22 (0.16-0.25)	0.13/0.43
TGF-β1	7.7 (2.73-31.4)	2.36 (1.52-4.94)	5.7 (2.04021.2)	0.09/0.17
NO	1.1 (1-1.2)	1.05 (0.95-1.2)	1 (0.5-1.20	0.92/0.41

Table 6 | Effect of conditioned media from bone marrow mononuclear cells on vessel formation in vitro. Эффект кондиционных сред от КМ-МНК на формирование сосудисто-подобных структур

Hours	*Basal*	*Epo -conditioning*	*p-value*
Vessel length			
On 3 hours	20.58 (17.77-25.35)	19.78 (18.24-22.9)	0.849995
On 24 hours	43.3 (34.97-45.91)	41.43 (35.21-49.46)	0.969827
Vessel width			
On 3 hours	12,81 (10.05-16.98)	13.18 (12.02-14.08)	0.849995
On 24 hours	32.09 (27.24-33.53)	30.22 (23.94-38.31)	0.909654

the 6 minutes walking test, and reduced Borg dyspnea scale from 2 (2 -3) to 1 (0 -1). In BM-MNCs group we detected a slight decreased stable perfusion defects, reflecting irreversible scarring of the myocardium and partially hibernating myocardium, (7.9 ± 5.1% vs 7.4 ± 3.9% in control group and BM-MNCs group respectively). Intramyocardial implantation of BM-MNCs pre-treated with Epo additional to CABG increased left ventricule contractility (LVEDV 91 ± 45 mL and LVEF 63 ± 28% in control group vs LVEDV 83 ± 34 mL and LVEF 65 ± 25% in BM-MNCs group). There was an inversed correlation between the number of CD45+/EpoR+, the number of CD34+/EpoR+ cells, and NYHA (R=-0.49 and R=-0.43, p ≤0.05).

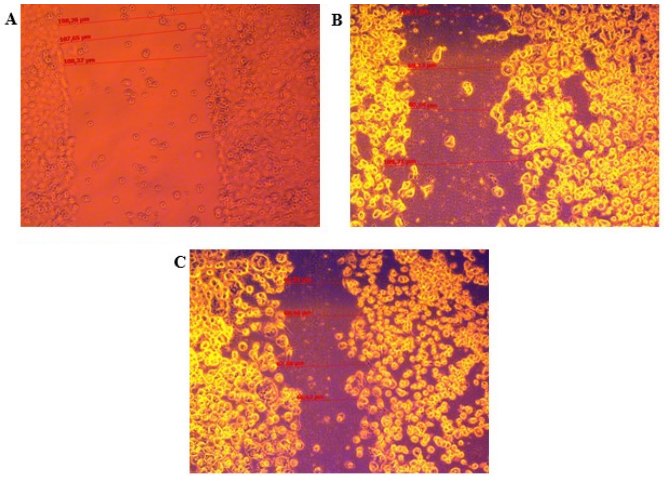

Figure 1 | Effect of condition media from BM-MNCs treated with Erythropoietin on EA.hy 929 wound healing. a, micrograph of a wound closure in media without growth factors at 24 hours (negative control). b, micrograph of a wound closure in media with 10% FSC at 24 hours (positive control). c, micrograph of a wound closure in media with 30% (v/v) conditioned media from Erythropoietin treated BM-MNCs at 24 hours. Эффект кондиционной среды от КМ-МНК проинкубированных с эритропоэтином на заживление раневого дефекта монослоя клеток EA.hy 929. a – закрытие раневого дефекта через 24 часа в культуральной среде без ростовых факторов (отрицательный контроль). b – закрытие раневого дефекта в присутствие 10% ЭТС через 24 часа (положительный контроль). c – закрытие раневого дефекта в присутствие 30% от объема кондиционной среды от КМ-МНК проинкубированных с эритропоэтином через 24 часа

4. Discussion

Ischemic heart failure (IHF) is an important cause of morbidity and mortality (13). The main reason of IHF development is myocardium infarction, because of the structural remodeling of a myocardium associated with inflammation, formation of scar, and development of interstitial fibrosis in peri-infarct zone, with the remodeling of blood supply of a myocardium (4). Bone marrow mononuclear cells contain EPCs and MSCs valuable in stem cell therapy for enhanced postischemic neovascularization (15, 18). Numerous clinical trials have previously investigated the clinical outcomes in patients after myocardium infarction and reported significant efficacy in improving contractility and reducing infarction scar (4, 13, 15, 18-19, 21). But the problem was in the limited efficacy: the improvement of left-ventricular ejection fraction and the reduction of infarction size, the viability of stem cells. Under this situation, precondition of the adult stem cells before implantation would be a very feasible and safe way to augment the therapeutic efficacy. Epo, cytokine that controls erythropoiesis, appears to have pleiotropic effects, such as

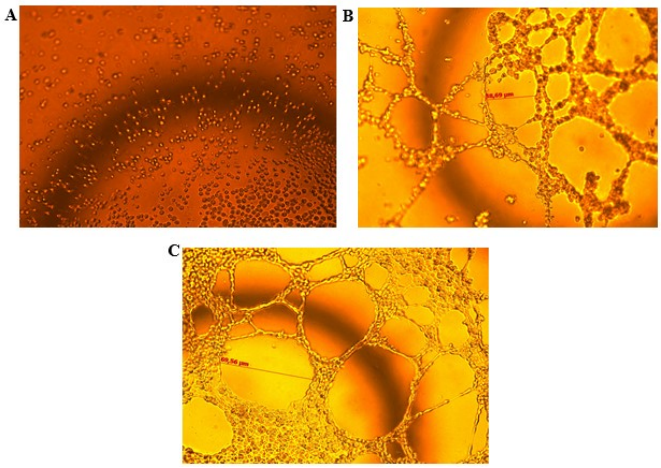

Figure 2 | Effect of condition media from BM-MNCs treated with Erythropoietin on EA.hy 929 vessel-like formation. A, - micrograph of a vessel-like formation by EA.hy 929 at 0 hour. B, - micrograph of a vessel-like formation by EA.hy 929 in presence of 30% (v/v) conditioned media from BM-MNCs with Erythropoietin 0 IU/mL at 24 hours (negative control). c, - micrograph of a vessel-like formation by EA.hy 929 in presence of 30% (v/v) conditioned media from BM-MNCs with Erythropoietin 33.4 IU/mL at 24 hours. Эффект кондиционной среды от КМ-МНК проинкубированных с эритропоэтином на формирование сосудисто-подобных структур клетками EA.hy 929. a – исходная картина (0 часов). b – формирование сосудистых образований клетками EA.hy 929 в присутствии 30% от объема кондиционной среды от КМ-МНК проинкубированных с 0 МЕ/мл эритропоэтина через 24 часа (отрицательный контроль). c – сосудо-образование клетками EA.hy 929 в присутствии 30% от объема кондиционной среды от КМ-МНК проинкубированных с 33,4 МЕ/мл эритропоэтина через 24 часа

anti-ischemic and anti-apoptotic properties, promotion of neovascularization, mobilization of EPCs, and enhancement of angiogenesis (22). This article presents the results of 6-12 months follow-up of patients who underwent coronary bypass surgery and transmyocardial laser revascularization, combined with implantation of autologous bone marrow mononuclear cells short-term pre-treated with Epo. We analyzed effect of short-term pre-treatment BM-MNCs with Epo on morphofunctional properties in vitro, and the clinical status of the patients, the dynamics of myocardium contractility, and local contractility of the left ventricule on echocardiography, as well as myocardium perfusion changes. Through this study we have showed the beneficial effect of in vitro Epo treatment to increase the vasculogenic potential of human bone marrow stem cells. First of all, we established that in bone marrow of patients with CAD there are three main stem cells populations (HSC, EPCs and MSCs). Moreover, BM-MNCs presented a higher number of 'early', and a lower number of 'late' EPCs (CD34-/CD133+, and CD34+/CD133- cells, respectively). Also, in BM-MNCs from patients with CAD there are 'mature' EPCs (CD34+/KDR+, and CD34+/CD31+ cells). Moreover, BM-MNCs from patients with CAD is characterized as a high proliferating cells, because 12% of CD34+ cells are in G2/M phase of cell cycle and spontaneous proliferation measured by MTT test was OD=0.66. Moreover, we observed that among CD34+ BM-MNCs there is very low number of apoptotic cells.

Ahmed and coworkers (2015), also Hur and coworkers (2004) demonstrated that EPCs expressed CD34 or VEGFR-2(KDR), and EPCs, which carried both surface markers (CD34+/KDR+ EPCs) is an independent predictor of clinical performance at cardiovascular pathology (23-24). About 1% of BM-MNCs were positive for EpoR and these cells were used to evaluate the specific effects of Epo treatment through its receptor. Epo treated BM-MNCs show the increase in HSCs bearing EpoR (increased in a 6.87-fold). Epo treatment also induced CD34+/CD133+ - EPCs decrease (on 30%) and increase CD31+/CD184+ ("homing-receptor") - EPCs (in a 5-fold). Epo treatment also leads to accumulation of CD34+ cells in G0G1 phase of cell cycle (on 8.97%). Epo treated BM-MNCs produced angiogenic factors, because addition of 30% (v/v) of the conditioned media from Epo treated BM-MNCs augment capillary-like structure formation by EA.hy 929, hybridoma cell line with a "mature" EPCs phenotype properties. Bone marrow cells can migrate from niches into blood stream through "homing" involve CXCR-4 (CD184), a specific receptor for the SDF-1/CXCL12, and CD144 to engraftment onto target organs, including ischemic tissues (25). The results of presented study show that a large number of HSCs and EPCs expressed CD184.

Moreover, Epo treatment of BM-MNCs increased a count of EPCs co-expressed CD31 and CD184 surface markers. It is well known, that EpoR is expressed on surface not only of erythroid progenitor cells, but also on other type of cells [16-19]. Epo maintains survival of different types of cells through interaction with EpoR (26). Epo binding with EpoR alone or in cooperation with CD131 leads to cytoprotective effect (27). Bennis and coworkers (2012) demonstrated that Epo in dosage of 5 IU/mL stimulates proliferative potential, migration, tubule-formation capacity, closure of wound made in monolayer of EPCs, and increased resistance of EPCs to oxidation stress (27). The unfavorable microenvironment present in the ischemic tissue might impair the effectiveness of stem cell transplantation. Additionally, the results of presented study shown that Epo treatment of BM-MNCs prevent cells from oxidative stress (proliferative activity of Epo treated BM-MNCs in the presence of H_2O_2). Kang and coworkers (2014) demonstrated that treatment of bone marrow stem cells with Epo increased the expression of pro-angiogenic factors, including IL-8, and IL-10, and b-FGF, and PDGF, and MMP -9, and levels of adhesion molecule such as integrin (28). Taken together, the presented study demonstrated that Epo has the potential to augment regenerative effect of BM-MNCs. CABG is preferred method for treatment of patients with ischemic heart disease, but sometimes coronary vessel diameter is insufficient for direct myocardium revascularization. Laser channels accompanied with stem cells implantation can improve the quality of life in patients with ischemic heart disease. Our data indicates that laser indirect revascularization with BM-MNCs pre-treated with Epo improves NYHA FC, exercise tolerance, dyspnea, and left ventricule contractility. Intramyocardial implantation of stem cells is used for restoring of ischemic myocardium microvasculature. There are no publications on pre-treated BM-MNCs with Epo and laser revascularization. It's known that pre-treatment with Epo of the organ injured and dysfunctionalized by hemorrhagic shock exerts tissue-protective effect as a result of mobilization of bone marrow endothelial progenitor cells (29). In animal model of myocardium infarction therapy with Epo and Granulocyte colony stimulating factor stabilized LVEF and improved LVEDV, and histopathology revealed increased areas of viable myocardium and vascular density (30). Authors evaluated therapeutic efficiency on animal model after systemic administration of Epo. But there is no information on the effect of the pre-treatment of bone marrow mononuclear cells with Epo and its implantation on laser channels into ischemic myocardium in human yet. Currently, in this article we have not studied enough patients, and so the results are contradictory. The small sample size, the number of BM-MNCs necessary for implantation, survival rate of implanted BM-MNCs do not allow to conclude that the pre-treatment with Epo BM-MNCs is really effective. Also, the effect of laser exposure on myocardium is not studied yet. We believe that our results suggest a cumulative effect of laser revascularization and short-term pre-treatment of bone marrow mononuclear cells, and they will improve the surgical treatment results of coronary arteria disease in the near period.

5. Concluding Remarks

Summarizing all the above, we can conclude that the short -term pre-treatment of bone marrow mononuclear cells with Epo increased count of EPCs with CD31+/CD184+ phenotype, increased a count of CD34+ cells in G0G1 phase of cell cycle, increased vasculogenic effect of conditioned media from Epo treated BM-MNCs on tube formation by EA.hy 929. Also, direct revascularization and combination of direct and indirect (laser channels) revascularization with Epo pre-treated bone marrow mononuclear cells implantation of the ischemic myocardium leads to myocardium perfusion improvement, and left ventricular contractility. Myocardial perfusion improvements at the early period after surgery is a result of direct revascularization, whereas at the long-term period it is a result of angiogenesis and vasculogenesis in the hibernate area, and the reduction of perfusion defects due to BM-MNCs implantation in laser channels. These findings strongly suggest that in vitro Epo treatment of BM-MNCs can be a feasible and effective method to augment the efficacy of BM-MNCs stem cells therapy for patients with CAD

Заключение

Показано, что кратковременная экспозиция костномозговых мононуклеаров с эритропоэтином способствует увеличению количества пула эндотелиальных прогениторных клеток с фенотипом CD31+/CD184+, количества CD34+ клеток в фазе G0G1 клеточного цикла и возрастанию васкулогенного потенциала кондиционных сред костномозговых мононуклеаров, предобработанных с эритропоэтином в модели in vitro образования сосудисто-подобных структур эндотелиальными клетками EA.hy 929. Кроме этого, прямая реваскуляризация и сочетание с прямой и непрямой (лазерные каналы) реваскуляризации, обусловленной имплантацией эритропоэтин предобработанных костномозговых мононуклеаров в ишемизированный миокард ведет к улучшению перфузии миокарда и сократимость левого желудочка. Параметры перфузии миокарда на ранние сроки после хирургического вмешательства являются следствием прямой реваскуляризации миокарда, тогда как на более поздних сроках наблюдения это следствие ангио- и васкулогенеза в гибернированной области миокарда, а также ответом на имплантацию костномозговых мононуклеаров в лазерные каналы. Полученные данные, указывают, что in vitro предобработка костномозговых мононуклеаров безопасный и эффективный способ увеличения терапевтического потенциала клеточной терапии стволовыми клетками больных с ишемической болезнью сердца

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Author Contribution

This work was carried out in equivalent collaboration of all authors.

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