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$VEGF_{165}$ Gene Therapy Improves Left Ventricular Function and Exercise Capacity in Diabetic Rats after Myocardial Infarction: Impact on Mortality Rate

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Abstract

Despite the increased amount of evidence about the benefits of human vascular endothelial growth factors by plasmid ($pHuVEGF_{165}$) based gene transfer after an ischemic event, the effects of $pHuVEGF_{165}$ therapy in diabetic hearts after myocardial infarction (MI) remains poorly investigated. We evaluated the effects of intramyocardial $pHuVEGF_{165}$ injection on left ventricular (LV) morphometry, function and blood flow, maximal oxygen consumption (VO_2max), and the total mortality rate of diabetic rats after MI. Male Wistar rats were divided into control (C), myocardial infarction+saline injection (I+SAL), myocardial infarction+$pHuVEGF_{165}$ injection (I+VEGF), diabetes+myocardial infarction+saline injection (DI+SAL), and diabetes+myocardial infarction+$pHuVEGF_{165}$ injection (DI+VEGF). MI was induced after 15 days of streptozotocin diabetes induction. One day after MI, the animals received $pHuVEGF_{165}$ or saline intramyocardial injection. LV function and maximal oxygen consumption (VO_2max) were evaluated at the initial injection and 30 days after injections. MI area evaluation showed an additional reduction in DI+VEGF (8±1%) in comparison with group I+VEGF (31±3%). Improvement in systolic function, evaluated invasively and noninvasively, lung wet/dry weight ratio, and VO_2max were observed in both $pHuVEGF_{165}$ injected groups. Consequently, mortality rate was reduced in I+VEGF (19%) and DI+VEGF (12.5%) when compared with I+SAL (48%) and DI+SAL (37.5%) groups. In conclusion, $pHuVEGF_{165}$ therapy resulted in reduced MI area, stabilization and maintenance of left ventricular function, increased VO_2 max, and reduced mortality in MI animals, diabetic or not. These results highlight the importance of continuing experimental studies and controlled clinical trials of gene therapy for ischemic cardiomiopathy associated with the pathological conditions of diabetes.

Keywords: Diabetes; Myocardial infarction; $pHuVEGF_{165}$ injection; LV function; VO_2max; Mortality

Introduction

The diabetes in developed countries is approaching epidemic proportions and has been associated with an increased risk of cardiovascular abnormalities and death from cardiovascular causes [1]. More specifically, diabetes mellitus increases the frequency and severity of myocardial infarction (MI) in persons with diabetes [2,3] compared with age-matched persons without diabetes. Furthermore, these individuals have a 2- to 3-fold greater risk of developing congestive heart failure after MI and suffer from increased mortality after acute MI [4].

Although clinical studies have clearly established a correlation between diabetes mellitus and increased severity of MI, very little is currently known regarding the pathophysiological mechanisms involved in the response of the diabetic heart to ischemic injury. In the experimental setting, our group has evidenced that diabetic rats have an imbalance in the oxidative stress profile [5], changes in the cellular survival pathway [6], reduced expression of the cardiac calcium handling proteins and ventricular function [7] at different times after MI.

Gene transfer constitutes an alternative strategy for accomplishing therapeutic angiogenesis in patients with limb and myocardial ischemia. Transient myocardial overexpression of human vascular endothelial growth factors A (huVEGF) by adenovirus or plasmid (pHuVEGF) based gene transfer induces angiogenesis both experimentally and in patients with myocardial ischemia [8]. In fact, small and unblinded gene therapy studies of intramyocardial delivered genes encoding $VEGF\text{-}A_{165}$ or $VEGF\text{-}A_{121}$ have been performed in patients with severe coronary artery diseases, and results have been encouraging, demonstrating both clinical improvement and evidence of angiogenesis [9,10]. However, despite the catastrophic complications of MI in those with diabetes, the effects of $pHuVEGF_{165}$ therapy associated with these 2 pathological conditions have been poorly investigated. This study was undertaken to test the hypothesis that intramyocardial $pHuVEGF_{165}$ injection in diabetic rats undergoing MI improves left ventricular (LV) dysfunction and maximal exercise capacity, and consequently this positive adaptation could impact survival. The present investigation was designed to evaluate the effects of intramyocardial $pHuVEGF_{165}$ injection on (i) LV morphometry and function; (ii) maximal oxygen consumption (VO_2max); and (iii) total mortality rate of diabetic rats after MI.

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Methods

Production of $pHuVEGF_{165}$

The human vascular endothelial growth factor plasmid ($pHuVEGF_{165}$), a transient and nonviral vector that expresses the $VEGF_{165}$ gene transcriptionally regulated by the cytomegalovirus promoter/enhancer, was obtained from Genentech (San Francisco, CA, USA) and introduced into XL1-Blue *Escherichia coli* by standard heat-shock transformation. Three clones of transformed cells were analyzed with different restriction enzymes, and one of them was selected for further cloning. The resulting plasmid was extracted with the PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, USA), which allows the isolation of a large amount of DNA (0.5-1 mg). Plasmid integrity was analyzed by electrophoresis in agarose gel stained with ethidium bromide, and DNA was quantified by spectrophotometry at 260-280 nm [11,12].

Experimental design

Experiments were performed in adult male Wistar rats (~290g) from the Animal House of the University of São Paulo, São Paulo, Brazil. Rats were fed standard laboratory chow and water *ad libitum*. The animals were housed in collective polycarbonate cages in a temperature-controlled room (22°C) with a 12-hour dark-light cycle (light 07:00-19:00h). The experimental protocol was approved by the institutional animal care and use committee of the Medical School of the University of São Paulo, and this investigation was conducted in accordance with previously described [13]. Fifty days after diabetes induction or buffer injection, the animals underwent surgical occlusion of the left coronary artery, which resulted in MI. One day after MI surgery, echocardiographic analyses were used to measure the MI area and the ejection fraction (EF). Only animals with EF<55% and MI area>30% were kept in the experiment (30% were excluded). The rats received either $pHuVEGF_{165}$ or saline intramyocardial injection. Maximal oxygen consumption (VO_2max) was measured one day after the injections (two days after MI), and the animals were followed for 30 days. After this period, echocardiographic and VO_2max evaluations, as well as invasive left ventricular function and blood flow measurements were realized, as observed in (Figure 1). Rats were randomly assigned to control (C, n=16), myocardial infarction + saline injection (I+SAL, n=16), myocardial infarction + $pHuVEGF_{165}$ injection (I+VEGF, n=16), diabetes + myocardial infarction + saline injection (DI+SAL, n=16) and diabetes + myocardial infarction + $pHuVEGF_{165}$ injection (DI+VEGF, n=16). The whole sample (n=80) was evaluated for mortality, an assessment that was started after $pHuVEGF_{165}$ or saline intramyocardial injection, excluding the influence of anesthesia or stress from the surgical procedure. Additional evaluations were performed in 8 other animals from each group.

Diabetes induction

Experimental diabetes was induced by intravenous injection of 50 mg/kg STZ (Sigma Chemical Co., St. Louis, MO) dissolved in citrate buffer (pH 4.2). Rats were fasted overnight before STZ injection. Control rats were injected with buffer only (10 mM citrate buffer, pH 4.5). Forty-eight hours after STZ injection, diabetes was confirmed by blood glucose levels above 200 mg/dL [7].

Myocardial infarction

Fifteen days after diabetes induction or buffer injection, anesthetized rats (80mg/kg ketamine and 12mg/kg Xylazine, i.p.) underwent surgical occlusion of the left coronary artery or sham, which resulted in myocardial infarction, as previously described elsewhere [5,7,14]. Briefly, a left intercostal thoracotomy was performed, the third intercostal space dissected, and the heart exposed. The left anterior descending coronary artery was occluded with a single nylon (6.0) suture at approximately 1 mm distal to the left atrial appendage. The sham rats (C group) underwent the same procedures except that myocardial ischemia was not induced. The chest was closed with silk suture. The animals were maintained in the ventilator until recovery. All rats received antibiotics (penicillin, 20,000 U) and Tramadol (20 mg/kg, every 6 h).

Intramyocardial injection

A left intercostal thoracotomy was newly performed in the third intercostal space, and the heart exposed. In the VEGF groups (I+VEGF and DI+VEGF), the rats received 50 µg of plasmid dissolved in 75 µl saline. A suture was made in the middle area of the MI to make a landmark for the injection. Plasmid was injected intramyocardially with an insulin syringe (0.3 mm in diameter) at 5 locations into the peri-infarction region along the border of the MI at the level of the suture. Aspiration without retrograde blood filling of the syringe was used as a sign that the needle was in the myocardium but not in the left ventricular cavity [11,12].

Noninvasive evaluation of left ventricular function: echocardiographic measurements

Echocardiography was performed by a double-blinded observer, under the guidelines of the American Society of Echocardiography, 1 day (initial evaluation) after MI and 30 days (final evaluation) after $pHuVEGF_{165}$ or saline intramyocardial injection. Rats were anesthetized (80 mg/kg ketamine and 12 mg/kg xylazine), and images were obtained with a 10-14 MHz linear transducer in a SEQUOIA 512 (ACUSON Corporation, Mountain View, CA) for measurement of morphometric parameters: left ventricular (LV) mass (corrected by body weight) and LV end-diastolic diameter (LVEDD); systolic function parameters: ejection fraction (EF) and velocity of circumferential fiber shortening (VCF); diastolic function parameters: LV isovolumetric relaxation time (IVRT), and peak E deceleration time (EDT) corrected by

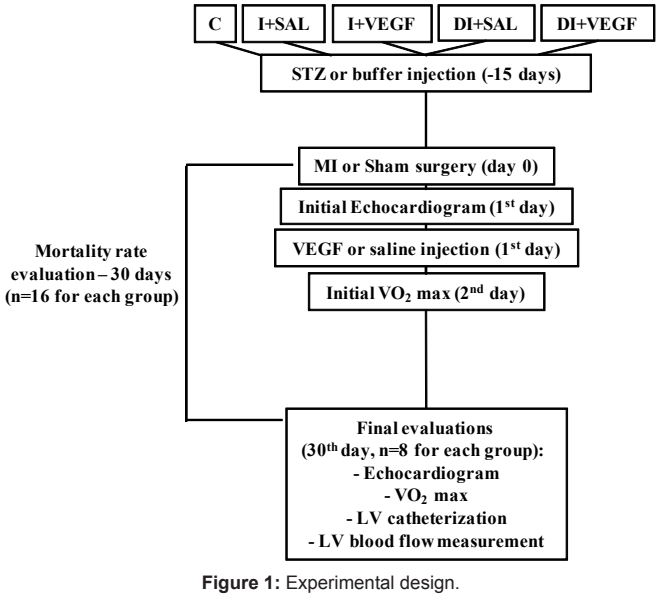

Figure 1: Experimental design.

the square root of the R-R interval, because EDT and IVRT are HR dependent; and global function: myocardial performance index (MPI). Echocardiographic parameters were measured as described in detail elsewhere [5,7,14].

Myocardial infarction area determinations

The MI area was evaluated by echocardiography 1 day (initial evaluation) after MI and 30 days (final evaluation) after $pHuVEGF_{165}$ or saline intramyocardial injection. The infarction area was delimited taking into account the movement of LV walls, by the observation of longitudinal, apical, and transversal views of the LV. Regions with systolic thickness under normal, as well as portions with paradoxal movement, were considered as infarcted. The infarcted area (%) was thus determined by the ratio of these regions by the total area of LV walls [5,7,14]. To confirm the echocardiography, the MI area was also analyzed after all other evaluations (30 days after $pHuVEGF_{165}$ or saline injections) by dissecting the fibrous scar from the remaining LV muscle. The outlines of both fragments were drawn on graph paper, and their areas were measured by the cross-point method [15]. The interventricular septum was considered part of the left ventricle.

Maximal oxygen consumption (VO_2max)

VO_2max was measured at 2 and 31 days after $pHuVEGF_{165}$ or saline intramyocardial injection. The VO_2 max was determined by analysing expired gas during a progressive exercise ramp protocol, with 3 m/minute increments every 3 minutes and no grade until exhaustion. Gas analysis was performed using an oxygen (S-3A/I) analyzer (Ametek, Pittsburgh, PA, USA). The VO_2 was calculated using the measured flow through the metabolic chamber, the expired fraction of effluent oxygen, and the fraction of oxygen in room air, as described elsewhere [14,16].

Left ventricular catheterization and microspheres infusion

LV function was also measured invasively in anesthetized rats (pentobarbital sodium, 40 mg/kg) one day after the last VO_2max evaluation. One catheter of PE-50 was inserted into the right carotid artery and advanced into the LV. Ventricular pressure signals were measured with a transducer and conditioner (Blood Pressure XDCR, Kent© Scientific, USA) and digitally recorded (5 min) with a data acquisition system (WinDaq, 2-kHz, DATAQ, Springfield, OH). The recorded data were analyzed on a beat-to-beat basis to quantify changes in LV pressure. The following indices were obtained: heart rate (HR), LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and maximum rate of LV pressure rise and fall (+dP/dt and -dP/dt), as previously described [7,14]. After LV pressure basal records, yellow (150.000) 15 mm Dye-Trak microspheres (Triton Technology, San Diego, CA, USA) were infused to blood flow measurements in the LV. Microspheres infusion and processing were performed as described previously [14].

Statistical analysis

Data are reported as means ± SEM. After confirming that all continuous variables were normally distributed using the Kolmogorov-Smirnov test, statistical differences between the groups were obtained by two-way and repeated measures ANOVA followed by the Student-Newman-Keuls post-test. Pearson's correlation was used to study the association between different parameters. The survival curve was estimated by the Kaplan-Meier method and compared by the Log-rank (Mantel-cox) Test. All tests were two-sided, and the significance level was established at $p<0.05$. Statistical calculations were performed using SPSS version 12.0.

Results

Animals

At the beginning of the protocol, body weight was similar between the groups (~290 ± 15g). After 30 days of pHuVEGF or saline injection, STZ-diabetic rats had a reduced body weight (DI+SAL: 232 ± 7 and DI+VEGF: 294 ± 12 g) compared with nondiabetic rats (I+SAL: 349 ± 9 and I+VEGF: 415 ± 12 g). Furthermore, the groups that were injected with VEGF (I+VEGF and DI+VEGF) displayed increased body weight compared with their respective saline injected groups (I+SAL and DI+SAL). Glycemia was increased in STZ-diabetic rats (DI+SAL: 400 ± 27 and DI+VEGF: 371 ± 37 mg/dL) in comparison with nondiabetic rats (I+SAL: 90 ± 3 and I+VEGF: 96 ± 3 mg/dL).

Myocardial infarction area and lung wet/dry weight ratio

The MI akinetic area (MI area), measured by echocardiography, was similar between infarcted groups at the beginning of the protocol (Table 1). However, after 30 days of pHuVEGF injection, I+VEGF and DI+VEGF had MI area reduction in comparison with saline injected animals (I+SAL and DI+SAL). In addition, DI+SAL and DI+VEGF had a reduction of MI area compared with that in I+SAL and I+VEGF groups, respectively. Parallel to this procedure, we also evaluated the MI area by millimeter graph paper stamps (MGPS). Corroborating the final echocardiographic data, I+VEGF and DI+VEGF had reduced MI area in comparison with saline injected animals (I+SAL and DI+SAL) and displayed a positive correlation ($r=0.9$, $p<0.0001$) between these two MI area measurements.

The lung wet/dry weight ratio was increased in all experimental groups in relation to that in the C group (Table 1). DI+SAL had an additional increase in comparison with I+SAL. However, I+VEGF and DI+VEGF animals displayed reduced lung wet/dry weight ratio compared with their respective saline injected groups. Furthermore, it is important to highlight that DI+VEGF had an additional reduction in relation to I+VEGF group, as observed in (Table 1).

Maximal oxygen consumption (VO_2max)

VO_2max data are presented in (Figure 2). At the initial evaluation, all experimental rats had a reduction in VO_2max compared with the C group. Nevertheless, at the final evaluation, I+VEGF and DI+VEGF evidenced an increase in VO_2max in comparison with respective saline injected controls (I+SAL and DI+SAL), and were not different from that in the C animals.

Parameters	C	I+SAL	I+VEGF	DI+SAL	DI+VEGF
MI area by Echocardiogram					
Initial (%)	----	40±3	39±2	41±3	40±1
Final (%)	----	41±3	31±3#†	32±2#†	8±1#‡•
MI area by MGPS					
Final (%)	----	46±5	34±2†	35±1†	12±2‡•
Lung Wet/Dry Weight Ratio	1.7±0.1	5.1±0.1*	4.0±0.1*†	6.0±0.5*†	3.3±0.2*‡•

Values are expressed as mean ± SEM. MGPS – millimeter graph paper stamps. # $p<0.05$ vs. initial evaluation; * $p<0.05$ vs. C; † $p<0.05$ vs. I+SAL; ‡ $p<0.05$ vs. DI+SAL; • $p<0.05$ vs. I+VEGF; (n=8 for each group).

Table 1: Myocardial infarction area determinations and lung wet/dry weight ratio in control (C), myocardial infarction + saline (I+SAL), myocardial infarction + VEGF (I+VEGF), diabetes + myocardial infarction + saline (DI+SAL), and diabetes + myocardial infarction + VEGF (DI+VEGF) groups.

Left ventricular function: noninvasive and invasive evaluations

The noninvasive left ventricular function parameters are shown in Table 2. Initial evaluation, performed 1 day after the MI surgery, showed that LV mass, IVRT, EDT, and MPI were similar between the experimental groups. In contrast, increased LV chamber (LVEDD), reduced EF, and VCF were observed in the MI groups, STZ-diabetic (DI+SAL and DI+VEGF) or nondiabetic (I+SAL and I+VEGF), compared with the C group at the initial evaluation.

Final echocardiographic evaluation showed that LV mass was not different between the groups; however, I+SAL and DI+SAL animals had an increase in this parameter compared with their initial evaluation. LVEDD was increased in I+SAL, I+VEGF, and DI+SAL compared to that in the C group; however, DI+VEGF group was not different than that in the C group. Moreover, diastolic dysfunction was present in I+SAL, I+VEGF, DI+SAL, and DI+VEGF groups compared with C rats, as evidenced by IVRT. All experimental groups displayed reduced systolic function evaluated by EF and VCF in relation to the C group. However, pHuVEGF injected groups (I+VEGF and DI+VEGF) had attenuation of EF in relation to the respective saline injected controls. Additionally, EF was increased in the DI+SAL group in comparison with the I+SAL, and MPI was increased in the I+SAL group compared with the C group, indicating impairment in the global ventricular function in these animals (Table 2).

Invasive LV function data, performed after the final echocardiographic evaluation and shown in Table 3, demonstrated that the LVSP was reduced in all experimental groups compared with that in the C group. Furthermore, STZ-diabetic animals (DI+SAL and DI+VEGF) had additional impairment in LVSP compared with nondiabetic animals. LVEDP was markedly increased in both I+SAL and DI+SAL rats compared with C rats. However, the I+VEGF group had attenuated LVEDP compared with the I+SAL group. HR was reduced in diabetic groups (DI+SAL and DI+VEGF) compared to that in the C and I+SAL groups.

Ventricular function was also estimated by +dP/dt (inotropic index) and -dP/dt (lusitropic index). Experimental groups exhibited systolic and diastolic dysfunction evaluated by +dP/dt and –dP/dt compared with the C group. However, it is important to highlight that VEGF injected animals (I+VEGF and DI+VEGF) had increased inotropic and lusitropic indexes compared with saline-injected controls (I+SAL and DI+SAL) (Table 3).

Invasive LV function data paralleled echocardiography findings, as observed by the positive correlation obtained between LVEDD and LVEDP (r=0.75; p<0.007), suggesting that increased diastolic diameter was associated with higher LVEDP values. In addition, a positive correlation was also obtained between EF and +dP/dt (r=0.80; p=0.005).

Parameters		C	I+SAL	I+VEGF	DI+SAL	DI+VEGF
Morphometric						
LV mass (g/kg)	Initial	1.02±0.02	1.06±0.01	1.05±0.04	1.04±0.01	1.05±0.05
	Final	1.06±0.03	1.15±0.02#	1.09±0.01	1.14±0.03#	1.09±0.04
LVEDD (cm)	Initial	0.65±0.01	0.75±0.01*	0.76±0.02*	0.77±0.03*	0.76±0.01*
	Final	0.67±0.03	0.98±0.02#*	0.79±0.02*†	0.88±0.03#*†	0.74±0.02
Systolic						
EF (%)	Initial	74±2	48±3*	47±2*	51±3*	52±3*
	Final	71±1	31±2#*	53±3*†	44±3*†	53±2*‡
VCF (circ/s) (10^{-4})	Initial	56±3	38±2*	37±3*	38±3*	39±1*
	Final	53±4	30±3#*	39±3*	29±2#*	38±3*
Diastolic						
IVRT (ms)	Initial	1.81±0.07	1.81±0.09	1.79±0.08	1.94±0.08	1.90±0.09
	Final	1.77±0.05	2.00±0.07*	1.95±0.05*	2.10±0.03*	1.93±0.05*
EDT (ms)	Initial	1.87±0.11	1.97±0.10	1.99±0.12	1.83±0.09	1.81±0.07
	Final	1.81±0.15	2.05±0.15	2.02±0.10	2.10±0.07#	2.01±0.04#
Global Function						
MPI	Initial	0.39±0.01	0.46±0.03	0.45±0.01	0.44±0.03	0.45±0.01
	Final	0.43±0.02	0.55±0.02#*	0.47±0.02†	0.48±0.01†	0.42±0.02

Values are expressed as mean ± SEM. LV mass – Left ventricular mass corrected by body weight; LVEDD – Left ventricular end-diameter during diastole; EF - Ejection fraction; VCF - Velocity of circumferential fiber shortening; EDT– Peak E deceleration time; IVRT– Left ventricular isovolumetric relaxation time; MPI - Myocardial performance index. # p<0.05 vs. initial evaluation; * p<0.05 vs. C; † p<0.05 vs. I+SAL; ‡ p<0.05 vs. DI+SAL (n=8 for each group)

Table 2: Initial and final echocardiographic measurements in control (C), myocardial infarction + saline (I+SAL), myocardial infarction + VEGF (I+VEGF), diabetes + myocardial infarction + saline (DI+SAL), and diabetes + myocardial infarction + VEGF (DI+VEGF) groups.

Parameters	C	I+SAL	I+VEGF	DI+SAL	DI+VEGF
LVSP (mm Hg)	134±5	113±4*	120±6*	93±4*†	94±4*‡
LVEDP (mm Hg)	5±0.3	19±2.1*	10±0.6†	14±3.2*	10±0.8
HR (bpm)	352±12	342±6	350±9	305±5*†	307±10*•
+dP/dt (mm Hg/sec)	9441± 430	4642±457*	6900±430*†	4702±325*	7207±261*‡
-dP/dt (mm Hg/sec)	-7188±160	-3218±471*	-5327±476*†	-3523±260*	5208±245*‡

Values are expressed as mean ± SEM. LVSP – left ventricle systolic pressure; LVEDP – left ventricle end diastolic pressure; HR – heart rate; +dP/dt and –dP/dt - maximum rate of LV pressure rise and fall. * p<0.05 vs. C; † p<0.05 vs. I+SAL; ‡ p<0.05 vs. DI+SAL; • p<0.05 vs. I+VEGF; (n=8 for each group).

Table 3: Invasive left ventricular function in control (C), myocardial infarction + saline (I+SAL), myocardial infarction + VEGF (I+VEGF), diabetes + myocardial infarction + saline (DI+SAL), and diabetes + myocardial infarction + VEGF (DI+VEGF) groups.

Left ventricle blood flow

As observed in Figure 3, myocardial infarction reduced left ventricle blood flow in I+SAL and DI+SAL when compared with the C animals. However, pHuVEGF injected groups (I+VEGF and DI+VEGF) displayed an increase of left ventricle blood flow if compared with the saline injected groups.

Mortality rate

Total mortality rate evaluation (Kaplan-Meier survival curve) showed that I+SAL (7 deaths among 16 animals, 43.8% mortality), I+VEGF (3 deaths among 16 animals, 18.8% mortality), DI+SAL (6 deaths among 16 animals, 37.5% mortality) and DI+VEGF (2 deaths among 16 animals, 12.5% mortality) groups had higher mortality rates compared with C (no deaths). However, VEGF-injected animals had an increased survival rate compared with saline-injected controls, as observed in Figure 4.

Discussion

The main findings of the present study are i) MI area was reduced after 30 days of $pHuVEGF_{165}$ intramyocardial injection in nondiabetic and STZ-diabetic animals, being associated with improvement of LV blood flow; however, STZ-diabetic animals had an additional reduction in MI area and LV remodeling in comparison with nondiabetic animals; ii) LV function was improved in both I+VEGF and DI+VEGF animals, evaluated noninvasively and invasively; iii) maximal oxygen consumption was increased in $pHuVEGF_{165}$ injected rats, assuming values similar to that in the controls. Moreover, it is possible that these positive adaptations have had an influence in the reduced mortality rate observed in the $pHuVEGF_{165}$ injected groups.

Myocardial infarction area and LV remodeling

In the present study, MI area was reduced in STZ-diabetic and nondiabetic infarcted animals after 30 days of $pHuVEGF_{165}$ intramyocardial injection. In addition, as a reflex of MI area reduction, the lung wet/dry weight ratio, an important index of pulmonary congestion, was also reduced in injected animals. Since the study of Yanagisawa-Miwa et al. [17] where the authors demonstrated in a canine experimental MI model that the intracoronary injection of basic fibroblast growth factor improved cardiac systolic function and reduced infarct size, the effects of angiogenic growth factor injection on MI has been reported. Later, VEGF injection was shown to increase myocardial blood flow in porcine hearts [18]; thus growth factors have been proposed as potential tools for infarct size reduction and blood flow improvement [19]. In this sense, Samuel et al. [20] demonstrated that the combination of gene therapy coexpressing VEGF and angiopoietin-1 significantly reduced the ventricular remodeling, evidenced by the significant reduction in the collagenous fibrotic tissue, in diabetic and nondiabetic infarcted animals. The authors documented that the reduction in the fibrotic tissue may have occurred by the angiogenesis and vessel maturation induction in the treated rats.

A variety of studies have investigated the effects of VEGF to induce angiogenesis. This fact may be a possible explication to the MI area reduction in our study, as well as the LV remodeling frequently observed after MI, that was blunted by the $pHuVEGF_{165}$ intramyocardial injection. In this sense, we also observed that LV blood flow was increased in VEGF injected animals evaluated by colored microspheres infusion. It has been previously reported in a canine model of ischemic cardiomiopathy, with myocardial perfusion analysis through scintillography, in which the gene encoding the green

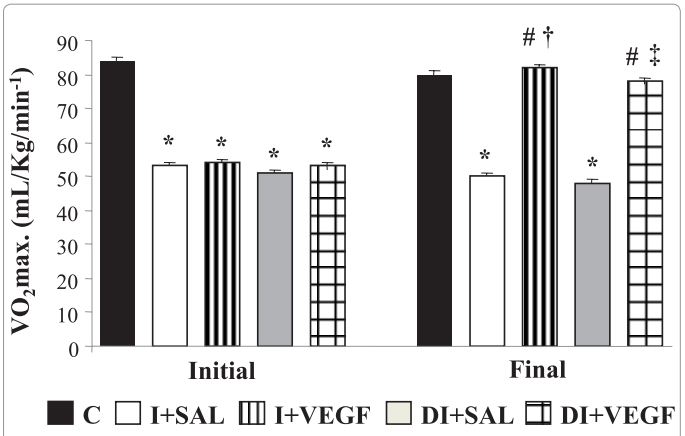

Figure 2: Maximal oxygen consumption (VO_2max) evaluated 2 and 31 days after $pHuVEGF_{165}$ or saline intramyocardial injection in control (C), myocardial infarction + saline (I+SAL), myocardial infarction + VEGF (I+VEGF), diabetes + myocardial infarction + saline (DI+SAL), and diabetes + myocardial infarction + VEGF (DI+VEGF) groups. # p<0.05 vs. initial evaluation; * p<0.05 vs. C; † p<0.05 vs. I+SAL; ‡ p<0.05 vs. DI+SAL; (n=8 for each group).

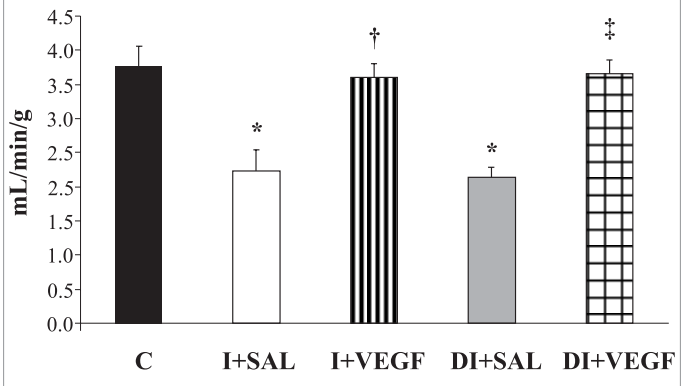

Figure 3: Left ventricle blood flow in control (C), myocardial infarction + saline (I+SAL), myocardial infarction + VEGF (I+VEGF), diabetes + myocardial infarction + saline (DI+SAL), and diabetes + myocardial infarction + VEGF (DI+VEGF) groups. * p<0.05 vs. C; † p<0.05 vs. I+SAL; ‡ p<0.05 vs. DI+SAL; (n=8 for each group).

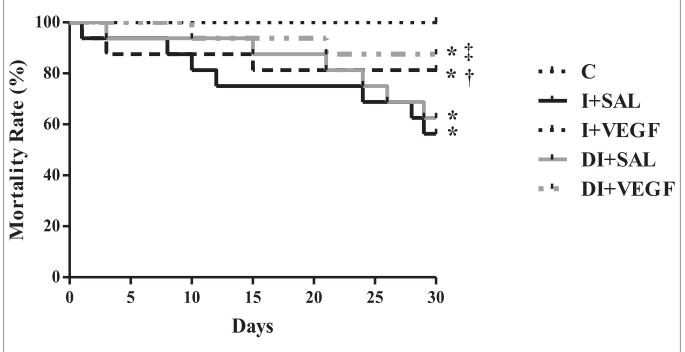

Figure 4: Mortality rates, as estimated by Kaplan-Meier survival curves for the control (C), myocardial infarction + saline (I+SAL), myocardial infarction + VEGF (I+VEGF), diabetes + myocardial infarction + saline (DI+SAL), and diabetes + myocardial infarction + VEGF (DI+VEGF) groups. * p<0.05 vs. C; † p<0.05 vs. I+SAL; ‡ p<0.05 vs. DI+SAL.

fluorescent protein was successfully transfected [21]. With a similar model, myocardial angiogenesis was induced by transmural injection of plasmodial $VEGF_{165}$ in MI areas of dogs [22].

Studies in experimental models of myocardial ischemia involving medium-size mammals have shown that VEGF is arteriogenic, supporting their use in promoting arteriole growth in severe coronary disease [23]. Other roles have also been suggested for VEGF, such as the ability to induce cardiomyocyte cytokinesis, as revealed by cardiomyocyte hyperplasia in an experimental model for ischemia [24]. Furthermore, in the present work, we observed an additional reduction in MI area in diabetic animals compared to nondiabetic animals, treated with $pHuVEGF_{165}$ or not. The data for MI area in the noninjected animals corroborates previous findings of our group where diabetic animals had reduced heart fibrosis and reduced infarct size [6,7] after 7 and 30 days of MI. In accordance with Malfitano et al. [6], it is possible that the improvement in MI area may have been the final pathway promoted by a positive balance in regulatory genes related to programmed cell survival, reduced inflammatory cytokines, and increased utilization of glucose as an energy substrate.

The favorable results obtained in experimental studies, and the absence of adverse effects related to the use of $pHuVEGF_{165}$ in initial clinical trials, indicate the potential clinical use of this therapeutic approach. In a clinical study, the authors showed that high doses of $pHuVEGF_{165}$ improve myocardial perfusion in patients with known severe coronary artery disease and provided evidence of a dose-dependent effect [25]. However, it is important to emphasize that injection of expressive doses (500μg) may induce vascular malformation, as observed in MI rats by Schwarz et al. [26].

LV function and maximal oxygen consumption

To further investigate if the changes in LV remodeling in the $pHuVEGF_{165}$ injected animals were responsible by functional positive adaptations, we evaluated the invasive and noninvasive LV function, as well as VO_2 max in the experimental groups. Previous findings of our group had observed that LV dysfunction was attenuated in STZ-diabetic rats after different experimental times of MI [6,7]. In fact, in the present investigation, we evidenced better LV function evaluated invasively and noninvasively in DI+SAL in comparison with I+SAL rats. These finding suggest that experimental diabetes is associated with the activation of endogenous cardioprotective mechanisms, a better profile of calcium handling proteins [7], attenuating the remaining LV dysfunction in the DI+SAL group.

After $pHuVEGF_{165}$ injection, the experimental animals had an accentuated improvement in ejection fraction, +dP/dt, -dP/dt, and LVEDP in relation to the noninjected animals, showing that the positive LV remodeling observed in these groups culminated in an attenuation of the LV dysfunction. In this sense, Giordano et al. [27] found in the cardiac VEGF-deficient mouse model a thinner LV wall and a lower LV ejection fraction compared with those in normal mice. Rottbauer et al. [28] observed that VEGF increased myocardial contractility and coronary blood flow in embryonic hearts.

Although we did not analyze the gene or protein expression of VEGF, it is possible that the delivery of VEGF was successful, since that in the present investigation LV blood flow was increased in VEGF injected animals. In this sense, we corroborated with studies in humans [29] and animals [28,30], where have been documented a stabilization or improvement in ventricular function. In addition, experimental observations also indicate that VEGF exerts a direct effect on cardiomyocyte contractility after its interaction with VEGF receptor-1 and the consequent activation of PLCγ1 [28]. Taken together, these observations suggest that VEGF might have protective or regenerative functions that extend far beyond its angiogenic activity, improving global cardiac function, as observed in the present investigation.

Reduction in VO_2max has been classically reported in diabetic [31] or ischemic heart failure patients [32], and in both [33] due to cardiac and peripheral damage. In fact, Guazzi et al. [34] studying noninsulin dependent diabetic patients in New York Heart Association functional class II to III chronic heart failure, due to idiopathic or ischemic cardiac disease, observed that lung volumes and oxygen uptake were significantly more reduced compared to that in nondiabetic heart failure patients. Thus, the coexistence of diabetes seems to trigger a series of additional metabolic, functional, and hemodynamic impairments inherent to heart failure that culminate in more representative exercise intolerance. In contrast, in the present study, STZ-diabetes was not an additional condition for the impairment in VO_2max, because I+SAL and DI+SAL had similar reductions in this variable. It is possible that the experimental period utilized in our protocol was the determinant of this similarity between the groups.

Furthermore, in the present investigation, $pHuVEGF_{165}$ injected animals displayed an increase in VO_2max at the end of the protocol compared with the saline injected rats. In this sense, if the classical view of VO_2 assumes that this variable is limited under most circumstances by the ability of cardiovascular and respiratory systems working together to provide both a delivery system (of O_2) and a removal system (of CO_2) from the tissues [35], we can believe that the improvement in VO_2 in I+VEGF and DI+VEGF groups is related to better ventricular function observed in these animals. Although we did not evaluate the cardiac output in the present study, it is possible that the increase in left ventricular function is associated with the improvement of this variable and, consequently, better VO_2max. In fact, Rolim et al. [36] previously showed that the reduction in VO_2 response during incremental exercise in an ischemic model of heart failure is closely related to the decreased cardiac output response, largely caused by depressed stroke volume kinetics.

Study Limitations

Some possible limitations of this study deserve comments. First, in the present investigation we did not perform molecular and cellular evaluations related to the beneficial effects of $pHuVEGF_{165}$ injection in the myocardium of the studied animals. However, this study provides a range of *in vivo* evaluations of the positive effects of VEGF gene therapy in the main parameters that are usually clinical determinants of mortality after an ischemic event. Furthermore, we measured the LV blood flow in experimental animals, providing a possible mechanism associated with the improvement of LV function and reduction of MI area in VEGF injected animals. Secondly, since the experimental rats started the protocol with similar values of MI area, left ventricular function, and VO_2max, the prediction of survival (or mortality) based on these parameters becomes difficult and is limited to methods used in this study.

Conclusions

In this animal model of diabetes and ischemic heart failure, $pHuVEGF_{165}$ injected after MI surgery resulted in reduced MI area, stabilization and maintenance of left ventricular function, improvement of LV blood flow, and increased VO_2 max. These positive adaptations induced considerable improvement in the survival rate

in MI animals, diabetic or not. These results stress the importance of continuing experimental studies and controlled clinical trials of gene therapy for ischemic cardiomiopathy associated with the pathological condition of diabetes.

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