



Gene therapy for refractory angina and cell therapy for heart failure: experience of a Brazilian research group

Roberto Tofani Sant'Anna¹ · Bruna Eibel¹ · Melissa Medeiros Markoski² · Clarissa Garcia Rodrigues³ · Felipe Borsu de Salles¹ · Imarilde Inês Giusti¹ · Ivo Abrahão Nesralla¹ · Nance Beyer Nardi^{1,4} · Renato Abdala Karam Kalil¹

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Abstract

Cell therapy has shown impressive effects in experimental cardiomyopathy models. To a lesser extent, gene therapy has also been studied. In both cases, translation to clinical therapy has been disappointing. This paper is intended to describe the experience and achievements of a multicenter working group located in Porto Alegre, southern Brazil, in experimental and translational research projects for cell-based and gene therapy methods in the treatment of dilated and ischemic cardiomyopathies. The results of preclinical and clinical studies showed that bone marrow mononuclear stem cells indeed have an effect in improving myocardial perfusion and contractile function, but the overall results are poorly translated to the clinical level. Gene therapy studies with direct myocardial injections of naked VEGF 165 plasmid showed improvement in myocardial perfusion and function in animal models. A randomized clinical trial found that this method is safe and improved myocardial perfusion, but the benefits disappeared after 1 year. An animal experiment associating VEGF 165 with angiopoietin was undertaken in mini pigs to extend the durability of that therapy. In conclusion, our efforts to better understand the mechanisms and functions of gene and cell-based therapies in cardiology resulted in significant findings and propose a future look at cell-free therapeutic approaches.

Introduction

Heart failure (HF) is a leading cause of morbidity and mortality, affecting at least 26 million people worldwide [1]

These authors contributed equally: Roberto Tofani Sant'Anna, Bruna Eibel

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✉ Renato Abdala Karam Kalil
kalil.renato@gmail.com

- ¹ Instituto de Cardiologia do Rio Grande do Sul, Av Princesa Isabel 395, 90620-001 Porto Alegre, RS, Brazil
- ² Universidade Federal de Ciências da Saúde de Porto Alegre, Rua Sarmiento Leite 245, 90050-170 Porto Alegre, RS, Brazil
- ³ Global Research and Innovation Network – GRINN, Rua Doutor João Colin 1285, Sala 03, 89204-001 Joinville, SC, Brazil
- ⁴ Universidade Luterana do Brasil, Av Farroupilha, 8001, 92425-900 Canoas, RS, Brazil

and with an estimated cost of \$108 billion per annum in 2012 [2]. Since the population is ageing, HF burden will likely increase over the next decades. Although reports indicate that mortality for HF is decreasing, it remains higher than for other lethal diseases, such as many forms of cancer [3]. The unadjusted 1-year mortality after hospitalization for HF reaches 32% [4]. Treatment of end stage HF (e.g., heart transplant and mechanical circulatory assist devices) is complex and expensive, particularly for less developed countries.

Current treatment for HF can improve symptoms and ameliorate prognosis, but it should be viewed as palliative since the central problem of cardiac tissue loss is not addressed [5]. Loss of cardiomyocytes and fibrosis are features of all end-stage heart diseases. The injured myocardium, whether scarred or not, does not resolve clinically [6]. The existence of endogenous cardiac stem cells, known as c-kit+ cells, was proposed in the early 2000s by Anversa and his group [7], leading to the development of numerous clinical trials. Later work by various research teams, however, have casted doubt on the c-kit+ cells ability to produce new tissue, and more recently Anversa and

coauthors had papers retracted or with “expression of concern” by the publishers [8].

Initial cell therapy studies focused on the capacity of stem cells of different sources to regenerate damaged myocardium. Bone marrow contains different types of stem cells and is easy to harvest, becoming the preferred cell source for preclinical and clinical studies. In sequence, adipose tissue-derived stem cells [9] were also successfully applied for myocardial regeneration. The curative potential of stem cells led to fast translational efforts, and the clinical application of bone marrow cells in patients with ischemic cardiomyopathy occurred within a year after publication of experimental data [10, 11].

Coronary artery disease is the most common cause of HF [12]. Advances in pharmacological and interventional treatment led to a decrease in mortality [13], but a significant proportion of patients are not amenable to conventional revascularization and suffer from refractory angina [14, 15]. Gene therapy represents an alternative therapy that aims to stimulate angiogenesis in the ischemic myocardium in order to minimize angina symptoms and possibly increase life expectancy and quality of life for these patients. In this context, gene therapy using vascular endothelial growth factor (VEGF) could represent a new therapeutic option [16, 17]. In 1998, the first clinical trial using gene therapy with VEGF 165 was conducted for patients with refractory angina [18] and was closely followed by randomized trials using signaling proteins to promote angiogenesis [19].

Cell and gene therapy both promise a new era in the treatment of HF and related cardiac diseases through multiple pathways [20, 21]. Clinical trials have been published in the last 15 years; however, despite initial enthusiasm, they have failed to meet their potential, so that cell and gene therapy are not part of current guideline-oriented therapy for HF [22]. During this period, our research group developed several protocols in both cell and gene therapy, including experimental and clinical studies, and more recently focused on limitations of translational medicine. In this article, we summarize our research in the light of current advances in the field of regenerative medicine.

The Rio Grande do Sul Institute of Cardiology is an academic hospital managed by the University Foundation of Cardiology and affiliated with the Federal University of Health Sciences of Porto Alegre (Universidade Federal de Ciências da Saúde, UFCSPA), located in Porto Alegre, capital of the State of Rio Grande do Sul, the southernmost state in Brazil. Since 1997, the surgery group has been motivated by reports on molecular and cellular therapeutic strategies for myocardial ischemia and failure in experimental models, as well as some initial clinical reports of those effects in the clinical setting. The group then started experimental projects on gene transfection of green

fluorescent protein (GFP) into healthy canine myocardium. The positive results led to experimental models of myocardial infarction, which opened the way for obtaining the support of government agencies' grants.

In 2009, the group coordinated a statewide research network of 12 centers, supported by CNPq and FAPERGS, with cell therapy studies in several *in vitro* and *in vivo* systems. A significant finding related to the clinical trial on cell-based therapy for dilated cardiomyopathy (DCM), as detailed below, was the demonstration that the transitory contractility improvement induced by stem cell injection occurred not only in the wall where the cells were injected, but also in the whole myocardium, which was thought to represent one of the first clinical evidences of the paracrine effect of this therapy.

Parallel to cell therapy, the group has been intensively involved in gene therapy. After the first canine myocardium gene transfection tests detailed below, experiments evolved and culminated with a research at the clinical level. In 2004 the group participated in a nationwide network on gene therapy, with a randomized clinical trial, which was, to the best of our knowledge the first clinical trial on gene therapy in Latin America. The trial evaluated the effects of gene therapy-induced angiogenesis for myocardial revascularization in ischemic heart disease, the results of which were presented at the American Heart Association's 2011 Meeting and published [23].

Research has continued since then, mostly in experimental and *in vitro* models, with several publications, as described below. There is an ongoing clinical series of cell therapy for DCM in young patients, which was motivated by the fact that the only patient with permanent improvement in the first clinical trial was a teenager and associated with the fact that the impressive animal results were all done in young animals. In this setting, we have also investigated the effects of aging on stem cell performance. Figure 1 presents a roadmap of experimental and clinical studies that our group conducted in the field of cell and gene therapy.

Dilated cardiomyopathy

DCM is a frequent cause of HF and is the most common indication for heart transplantation [24]. The true prevalence of DCM is not fully known and is probably underestimated due to the insidious onset of the disease and the existence of asymptomatic cases [25]. A survey of 156,013 patients hospitalized with HF in the US found a 41% prevalence of non-ischemic cardiomyopathy [26]. Of these, hypertension was the most common cause (48%), followed by idiopathic etiology (31%). Familial DCM is responsible for 30–50% of the cases [27, 28] and, within these, 40% of patients have an identifiable genetic mutation.

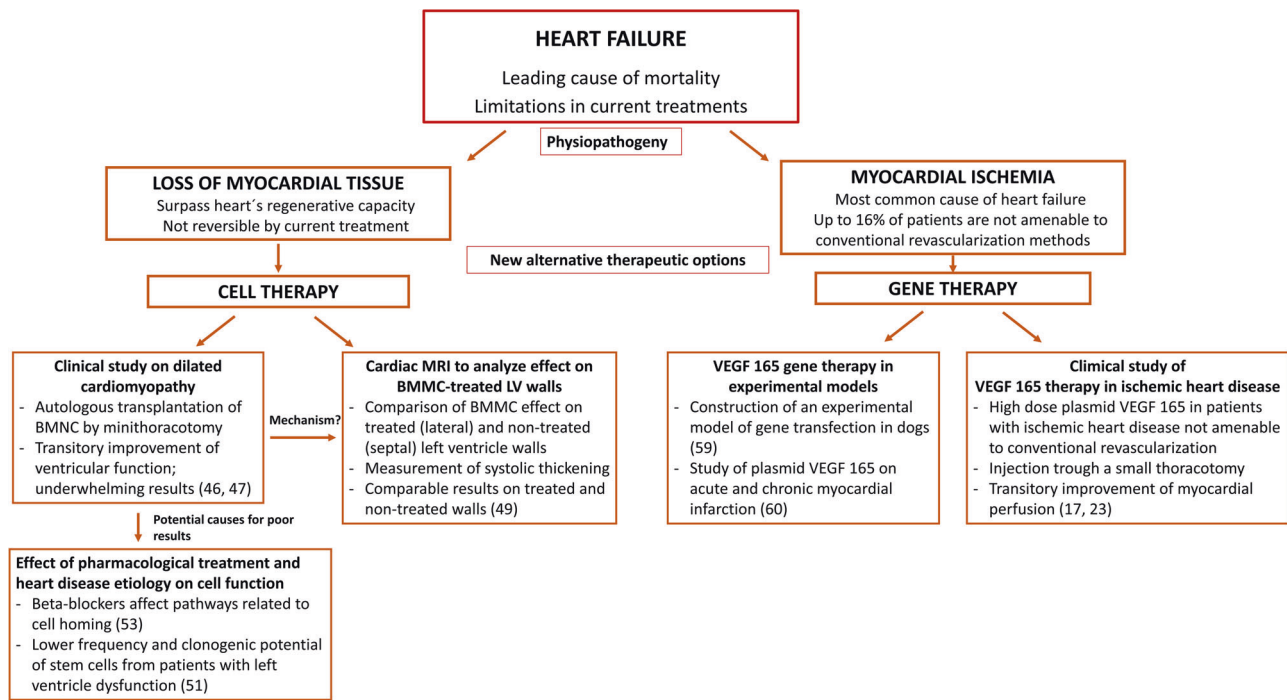


Fig. 1 Roadmap of experimental and clinical studies conducted by our group in the field of cell and gene therapy of cardiovascular diseases. Numbers refer to published articles for each study

Guideline-directed medical and device therapies, including implantable cardioverter-defibrillator and cardiac resynchronization, are beneficial in DCM. Death is caused by disease progression and pump failure in two-thirds of patients with DCM [29, 30]. In the remainder, the mechanism is sudden death related to cardiac arrhythmias. Clinical studies indicated that patients with DCM have a better prognosis than patients with ischemic heart disease when adjusted for confounding factors and comorbidities, albeit with variations according to specific etiologies or gene mutations [29].

Regardless of the initial cardiac insult, HF progresses as a result of overexpression of compensatory mechanisms that ultimately exert deleterious effects on the heart and circulation. Numerous so called “neurohormonal mechanisms” have been described in HF, including activation of renin–angiotensin–aldosterone and sympathetic nervous systems, production of inflammatory mediators and cardiomyocytes apoptosis. Pharmacological treatments aimed at inhibition of these activation mechanisms have drastically improved the prognosis of HF. However, current treatments are incapable of restoring lost cardiac tissue, so that in advanced cases they are able to halt disease’s progression and control symptoms, but seldom revert cardiac dysfunction.

There are few investigations regarding DCM in experimental and clinical studies of cell therapy, and there are important considerations to examine before translating

results from ischemic to non-ischemic causes of HF. DCM is a phenotype resulting from different pathological processes, and animal models are limited to specific etiologies, such as toxic cardiomyopathy induced by doxorubicin infusion [30], Chagas disease [31], and immune myocarditis models [32].

Nagaya et al. [33] published the first experimental work regarding cell therapy in DCM, in which a rat model of DCM was used. Five weeks after immunization, mesenchymal stem cells (MSC) or vehicle was injected into the myocardium. MSC transplantation increased capillary density and decreased the collagen volume fraction in the myocardium, resulting in increased left ventricular maximum dP/dt. Psaltis et al. [34] evaluated the effect of MSC injection in an ovine model of anthracycline-induced DCM. Transendocardial allogeneic MSC or placebo were injected under electromechanical mapping guidance. MSC attenuated ventricle dilatation and stabilized the ejection fraction. Histologically, there was less fibrosis with cell therapy and increased density of cardiomyocytes and myocardial arterioles, despite modest cellular engraftment.

Chronic stable conditions, such as DCM, lack the adequate microenvironment for cell homing. Intracoronary injection is associated with a small percentage of transplanted cells that are effectively being retained in cardiac tissue, which could limit results when that route is used for cell therapy in the absence of an appropriate signaling for cell homing [35]. Few clinical or experimental studies

compared different forms of cell delivery. Vrtovec et al. [36] compared intracoronary and transendocardial cell delivery in a randomized study with 40 DCM patients. At 6 months, the left ventricular ejection fraction (LVEF) and exercise capacity increased in both groups, but the increase was greater in the group treated by transendocardial cell delivery [36].

Cell therapy studies

Our studies started with cell therapy in cardiology after experimental work from Orlic et al. [10], in which bone marrow-derived c-kit⁺ mononuclear cells were used to regenerate cardiac function in an experimental model of acute myocardial infarction (AMI). Two injections of $0.15\text{--}1 \times 10^5$ Lin-c-kit⁺ cells were made into the myocardium adjacent to the infarct region. The treatment induced the formation of new cardiac myocytes and coronary blood vessels, which led to an increase in left ventricle end-diastolic pressure. The same group demonstrated that bone marrow mononuclear cells (BMMC), mobilized by stem cell factor and granulocyte-colony stimulating factor, homed to the infarcted region and promoted myocardial repair, resulting in a significant tissue regeneration 27 days after AMI [37].

Subsequent experiments carried out by other groups failed to accurately reproduce these results, as recently reviewed [38]. The mechanism through which functional improvement is obtained was questioned and is still a matter of debate. Later work refuted the hypothesis of transdifferentiation by demonstrating the presence of little to none of transplanted cells a few weeks after injection, pointing to a paracrine mechanism involving the secretion of growth factors, microRNAs (miRNAs), and exosomes by transplanted cells as being responsible for cardiac repair [39]. Fusion of bone marrow cells with resident cardiac cells has also been refuted as a potential mechanism for cardiac regeneration [40].

In clinical trials, the most commonly used cells are bone marrow-derived stem or progenitor cells derived from the patient's own bone marrow. Bone marrow is a heterogeneous tissue containing multiple cell populations with a small proportion of progenitor cells ($\approx 1\%$), which includes stem cell of hematopoietic origin, MSCs and endothelial progenitor cells (EPCs). Initial clinical trials showed safety of BMMC in AMI and chronic myocardial ischemia [11, 41]. BMMC were associated with improving myocardial blood flow with enhancement of regional and global left ventricular function. This led to several phase I and II randomized trials of cell therapy in patients with HF, AMI, and chronic ischemic heart disease, most of which used BMMC. Randomized, placebo-controlled trials, however,

demonstrated unimpressive results in AMI [41] and chronic ischemic HF [42]. Recently, Gyöngyösi et al. [43] reviewed the different meta-analyses from trials that evaluated potential benefits of cell therapies for treatment of HF and AMI. The largest meta-analyses included 1907 patients with HF and 2307 patients with AMI. Most meta-analyses agreed that the benefit of cell therapy in these conditions is still inconclusive and impaired by statistical underpower. Authors also noted pitfalls in trial design and inconsistencies in reporting trial results.

Our experience in the use of bone marrow cell therapy for DCM

For our studies, we opted to use BMMCs collected from iliac bone and isolated with Ficoll-Hypaque. BMMC isolation is relatively easy, fast, and inexpensive, albeit potentially limited by the small proportion of stem cells. Experimental data pointed to a superior regenerative capacity of single clonally purified MSC in AMI [44], yet the use of MSC requires cell culture, which in clinical conditions is more complex and expensive.

We hypothesized that injection of the cells in a limited ventricular area could affect the entire chamber performance. Thoracotomy is a safe procedure, even in patients with advanced HF, as experience with epicardial implantation of the left ventricular leads indicates [45]. Initially, we evaluated the safety and viability of autologous transplantation of BMMC by minithoracotomy in a case series [46]. Nine patients with class III/IV HF due to DCM and severe left ventricular dysfunction (ejection fraction $<35\%$) received $9.6 \pm 2.6 \times 10^7$ BMMC. Cell injections were spread across 20 sites in the ventricular free wall, which was accessed by a 5 cm-length thoracotomy in the fifth left intercostal space.

Echocardiograms and cardiac magnetic resonance imaging (CMRI) were used to compare cardiac function before and after cell transplantation. There were no major procedure-related complications. Patients had significant symptomatic improvement according to NYHA functional class, and there were no deaths through the 1st year of follow-up. Echocardiograms demonstrated significant increases in LVEF and decreases in percent left ventricular (LV) fiber shortening. When evaluated by MRI, LV parameters showed a non-significant variation. Table 1 summarizes the findings of this trial along with other relevant clinical studies from our group.

In this small study, the results showed that intramyocardial transplantation of BMMC in DCM was feasible and safe. There were early improvements in symptoms and LV performance, which regressed during follow-up, although symptomatic improvement was preserved. As pointed out earlier, cell retention in the myocardium is low and limited

Table 1 Most relevant clinical studies of cell and gene therapy from our group

Population	Therapy	Design	Results	Conclusions	Ref
9 patients with chronic class III/IV HF, LVEF \leq 35%, DCM	$9.6 \pm 2.6 \times 10^7$ BMNC	Case series	- No procedure-related complications - Increase in LVEF % (echocardiogram) in the 2 months evaluation, with regression to baseline values after 12 months - Sustained symptomatic improvement during 1-year follow-up	Procedure safety Improved functional capacity (placebo effect?) Positive transient effect on ventricular function	[46]
30 patients with chronic class III/IV HF, LVEF \leq 35%, DCM randomized	Treated group: 20 patients, 1.06 ± 10^8 BMNC Control group: 10 patients, clinical follow-up	Randomized trial	- No change of LVEF % (MRI) between baseline values and 9 months follow-up, both in treated and control group - No difference in MRI results between groups - Treated group: Improvement in quality of life (Minnesota), but not on 6-Minutes-Walk Test results	No sustained benefit of BMNC to left ventricular function Symptomatic improvement could be related to a placebo effect	[47]
13 patients with chronic class III/IV HF, refractory angina	2000 μ g of a plasmid expressing VEGF 165	Case series	- Transitory increase in myocardial perfusion at the 3rd-month SPECT under stress and at the 6th month under rest - 1 year after, there were improvements in treadmill test steps, oxygen consumption, QOL scores, and CCS classes	Feasible and safe. Improvements in treadmill test steps, oxygen consumption and Minnesota quality of life questionnaire. A transitory increase in myocardial perfusion	[23]

We used the same surgical technique in all trials, which consisted of an approximately 5 cm incision anterolaterally in the fifth left intercostal space and injection of cell/gene vectors by direct visualization

in time. Most experimental data use short-term models and without concomitant HF, thus medium- and long-term effect analyses are lacking. Patients included had chronic HF and were stable on maximum tolerated HF therapy before the procedure, but as a case series this small study suffered from the limitations of lacking a control group [46].

We further studied the effect of BMNC in a randomized clinical trial [47]. Thirty nonischemic DCM patients with LVEF $<35\%$ were randomized at a 1:2 ratio into two groups, control and treated, which received 1.06 ± 10^8 BMNC through mini-thoracotomy. There was no intervention in the control group. Assessment was carried out through clinical evaluation as well as a 6-min walk test, CMRI imaging and echocardiogram. The results are summarized in Table 1 and Fig. 2. In short, both the BMNC and placebo groups maintained left ventricular function during follow-up. Patients in the BMNC group had significant improvement in NYHA heart failure class and in the Minnesota Living with Heart Failure Questionnaire, but no improvement in functional capacity measured by the 6-min walk test. Differences observed within the BMNC group could be due to a placebo effect or low statistical power. In this trial, we observed a high mortality after the procedure (4 deaths in the first 30 days) and another 3 patients died during 1-year follow-up, two of which were sudden and potentially arrhythmia related.

Table 2 presents a summary of published results of randomized clinical trials for cell therapy in DCM. The discrepancy of results could be explained by different routes of cell delivery, cell types, and cell numbers. Vrtovec et al. utilized a pure population of stem cells (CD34+ cells) delivered in regions of perfusion defects as evaluated by myocardial scintigraphy [36], which could account for the greater benefit observed in the trial. A metaanalysis by Lu et al. [48], which grouped seven randomized trials, associated bone marrow cell therapy with a decrease in mortality rate, significant LVEF improvement within 6 months, and significantly improved LVEF after mid-term (6–12 months) follow-up, but there was no significant benefit in the 6-min walk test.

Effect of BMNC transplantation in untreated left ventricle walls

Contractility changes were observed in areas that were treated (free wall) and untreated (septal wall) with BMNC in selected patients who showed significant ventricular improvement after free wall-only intramyocardial stem cells injection [49]. A relative LVEF improvement greater than 15% was observed in 7 (46.7%) of the 15 patients with DCM that received $9.6 \pm 2.6 \times 10^7$ BMNC, injected at ten points distributed over the left ventricular free wall. These

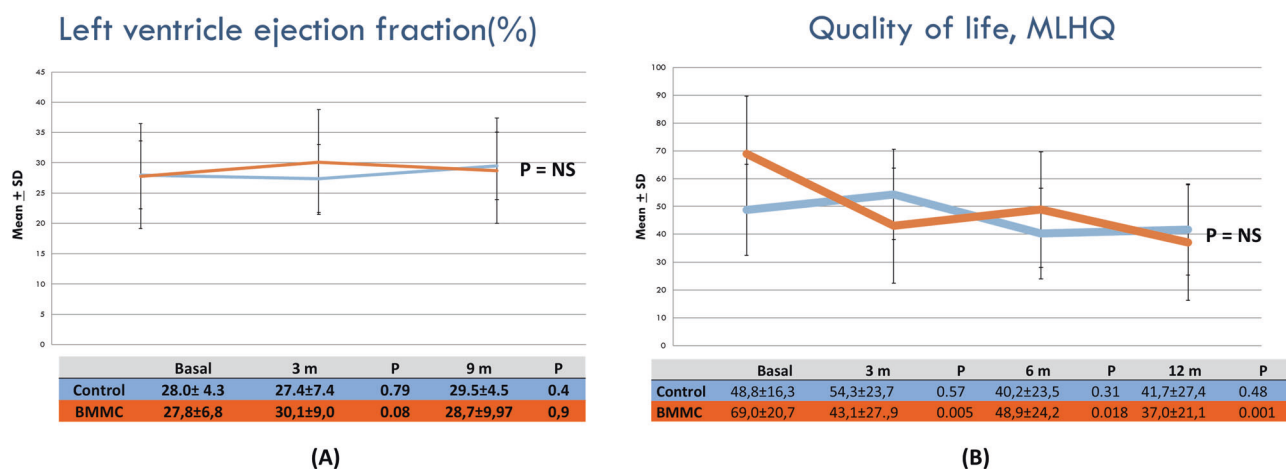


Fig. 2 Comparison of left ventricle ejection fraction, assessed by MRI (a), and quality of life, evaluated by Minnesota Living with Heart Failure Questionnaire (b), between 20 patients that received $9.6 \pm 2.6 \times 10^7$ BMMC and 10 control patients

Table 2 Summary of randomized trials of cell therapy in dilated cardiomyopathy

Study	Number of patients ^a	Cell type	Number of cells	Route of delivery	Follow up	Blind, placebo Cled	Outcome
ABCD trial [67]	45T, 40C	BMNC	$1.68 \pm 0.96 \times 10^8$	Intracoronary	28 ± 9 months	No	↑ LVEF ↑ NYHA
IMPACT-DCM [68]	18T, 11C	Cultured BMNC	$0.35 - 2.95 \times 10^8$	Intramyocardial (thoracotomy or NOGA)	1 year	No	↔ LVEF ↔ NYHA
Vrtovec et al. [36]	55T, 55C	CD34+	$1.13 + 0.26 \times 10^8$	Intracoronary	5 years	No	↑ LVEF ↑ 6MWD ↓ Mortality
INTRACELL [47]	19T, 10C	BMNC	$1.06 + 0.43 \times 10^8$	Intramyocardial (thoracotomy)	9–12 months	No	↔ LVEF ↔ NYHA ↑ Mortality
MiHeart [69]	61T, 54C	BMNC	$2.36 (1.76 - 2.95) \times 10^8$	Intracoronary	1 year	Yes	↔ LVEF ↔ Mortality
REGENERATE – DCM [70]	15T, 15C	BMNC	1.06×10^8	Intracoronary	1 year	Yes	↑ LVEF ↑ NYHA

All trials included patients with symptomatic heart failure and LVEF <40%

^aT treated, C control

patients were selected for further contractility study based on cardiac MRI. We measured the systolic thickening of the septal (untreated) and free (treated) walls before injection and at 3 months postoperatively.

Mean thickening increased from 0.46 to 1.23 mm (an absolute increase of 0.77 ± 1.3 mm and relative increase of 167.4%) in the systolic septal wall, and from 1.13 to 1.87 mm (an absolute increase of 0.74 ± 1.5 mm and relative increase of 65.5%) in the free wall. Comparison of the rate of absolute or relative systolic thickening between the two walls ($p = 0.866$ and 1.0, respectively) showed no difference. This study suggests that cell therapy can exert a global effect on cardiac function, despite injection in a limited area. These results imply the existence of a diffuse (i.e.,

paracrine) mechanism of action (video on Supplementary Material). Also, regional improvement of contractile function could attenuate the neurohormonal effect of HF, thus leading to a more global response, although this hypothesis was not directly addressed.

Effect of pharmacological treatment and heart disease etiology on cell function

The unimpressive clinical results with cell therapy have multiple complementary explanations. First, preclinical studies generally employ healthy animals in which the condition to be investigated is experimentally induced. When compared to autologous cells from patients with

cardiovascular disease and multiple comorbidities, tissue sources from these animals have different population in terms of frequency and therapeutic potential [50]. Second, clinical patients receive other forms of treatment, that could directly affect cell therapy through specific mechanisms.

We conducted an investigation of three cell compartments: endothelial stem/progenitor cells (EPCs), hematopoietic stem/progenitor cells (HSCs), and mesenchymal stem/progenitor cells of patients with ischemic or valvular heart disease. The study included 36 patients referred for open-heart surgery. Before the operation, sternal bone marrow samples were harvested and analyzed using flow cytometry and colony assays [51].

A reduced frequency of stem cells in both groups of patients (ischemic and valvular) was observed. When the clinical attributes potentially affecting the frequency and function of EPCs and HSC were evaluated, we found the following associations: lower clonogenic potential for EPCs and LV dysfunction was associated with increased frequency of CD34+KDR+ cells and use of aspirin; lower clonogenic potential for HSCs was associated with hemoglobin level <12 g/dL; and increased frequency of CD34+CD38- cells was associated with use of β -blockers. We were unable to analyze clinical and laboratory characteristics potentially affecting MSC, which had a low frequency in the samples [52]. Furthermore, patients with higher surgical risk or ischemic disease severity, as assessed by the Euro-SCORE and SYNTAX score respectively, had a lower frequency of CD34+KDR+ cells.

Our group studied the effects of beta-blockers on cell homing in an experimental setting of hypertension [53] and in patients who underwent heart surgery [54]. Beta-blockers are one of the pillars of HF treatment [55]; they prevent cardiac remodeling through multiple mechanisms, including reduction of heart metabolic demand, preventing cardiomyocytes hypertrophy, and death and controlling arrhythmias. They also reduce mortality across different etiologies and subgroups of patients with HF [55].

We administrated beta-blockers for 30 days to spontaneously hypertensive rats and analyzed interference with a major chemokine of cell homing and the expression of its receptors, as well as GRK-2, G-protein-coupled receptor (GPCR) desensitization proteins, β -arrestins (β 1-AR and β 2-AR), and a downstream molecule involved in transcription. The study drugs were shown to modulate these proteins, with a strong effect of metoprolol and propranolol on the expression of β 1-AR ($p = 0.0102$) and β 2-AR ($p = 0.0034$). These drug interactions may interfere with the normal functioning of these pathways and greatly affect the body's response, as in tissue regenerative and renewal capacity, which in turn may interfere with the response to cell therapy [53].

Gene therapy applied to ischemic cardiomyopathy

Gene therapy, which has the potential to promote myocardial angiogenesis and collateral circulation in ischemic myocardium, may represent a possible treatment modality for these patients [20]. Angiogenesis involves the mobilization of EPCs, which are multipotent cells with the capacity to proliferate and differentiate into mature endothelial cells [22]. Gene therapy is not intended to replace an abnormal gene, but regulates the expression of useful proteins, increasing their DNA contents. Its effectiveness depends on the gene vector and method of administration used [9]. VEGF functions both as an important marker of endothelial damage and as the mediator of repair. It encourages the maintenance, mobilization, and recruitment of EPCs from bone marrow [5]. The angiogenic potential of VEGF stimulates the endothelial production of nitric oxide through activation of nitric oxide synthase (eNOS).

The concept of therapeutic angiogenesis in humans progressed to the point of phase I clinical trials in patients with ischemic cardiomyopathy. Clinical trials of VEGF 165 gene therapy as the sole treatment for patients with refractory angina have used low gene vector doses (125–400 μ g) [18, 56]. Higher doses have been used in some studies, although in combination with well-established techniques of myocardial revascularization, such as CABG and PCI [16, 57, 58]. Such trials were able to demonstrate the safety and feasibility of this type of therapy, but benefits over myocardial perfusion and functional capacity were small and inconsistent.

Our experience in the use of gene therapy

Our first experimental study in this area proposed assessing the transfection of the gene that encodes the GFP through direct injection into healthy canine myocardium. Cells transfected and non-transfected with the pREGFP plasmid showed differences by fluorescence microscopy, with mild fluorescence in the cardiac fibers of the control group and overt EGFP expression in transfected myocardial cells, showing that transfection of the EGFP gene in healthy canine myocardium was effective [59].

In a second study, we evaluated coronary angiogenic response to transmural injection of a plasmid encoding VEGF 165 in AMI zones using a similar canine model [60]. The heart of 11 dogs was exposed and AMI was induced by occlusion of the diagonal branch of the anterior descending coronary artery. For each of ten selected points in the infarction area and its peripheral zone, 1-ml injections of saline solution (control group: 5 dogs) or of plasmid encoding VEGF 165 solution (200 μ g/ml) (VEGF group: 6 dogs) were introduced. Technetium myocardial scintigraphy

was performed immediately after animal recovery and 14 days later to evaluate the myocardial perfusion. Fourteen days after coronary ligation, the ischemic area evaluated by SPECT demonstrated recovery of ischemic area in both groups, reflecting the abundant collateral circulation present in canine circulation. Histologic evaluation of the peripheral area of AMI indicated a larger number of vessels in the VEGF group when compared to controls, which resulted mainly from an increase in the number of capillaries. In the canine model of chronic myocardial infarction, intramyocardial injection of plasmid VEGF 165 resulted in preservation of the LVEF, which was contrary to the control group where the LVEF showed continuous decline during the experiment [60].

Between 2009 and 2011, our group conducted a clinical trial (NCT 00744315) with gene therapy using a plasmid expressing the VEGF 165 isoform for patients with refractory angina [17, 23]. The aim of this study was to assess safety and feasibility and to evaluate the results, both clinical and on myocardial perfusion, of gene therapy with 2000 µg of a plasmid expressing the VEGF 165 isoform in patients with advanced ischemic heart disease and refractory angina. Concerning myocardial perfusion, there was no difference in stress and differential ischemia SPECT scores during the previous optimal drug treatment period. However, an increase in the rest ischemia SPECT score was observed. Three months after VEGF 165 gene therapy, analyses of ischemia SPECT scores showed a reduction in the number of ischemic segments under stress and at rest. There was no difference in the differential score in the same period. Six months after intervention, there was a significant difference only in the rest ischemia score. One year after gene therapy, the ischemia scores were similar to the preoperative values.

In respect to functional capacity, 1 month after VEGF 165 gene therapy, there was improvement in the number of steps performed in the treadmill test, which was maintained at 1 year after intervention. Oxygen consumption in the patients was improved 3 months after intervention, and this effect remained at 1 year after gene therapy. During the optimal drug treatment period, there was no difference in the number of steps or in oxygen consumption in the treadmill tests.

Gene therapy with the VEGF 165 plasmid was shown to be safe and feasible in this patient group. Three months after gene therapy, the clinical results demonstrated an improvement in the intensity of myocardial perfusion in the treated areas. One year after treatment, the ischemia scores were similar to the preoperative levels, but no patients had worsening of ischemia during this period. Improvements in quality of life and classes of angina and HF were observed in the same 1-month period after gene therapy and were maintained for 1 year after treatment [17, 23].

Also, two studies analyzing blood samples from these patients demonstrated mobilizations of important cell homing markers. The first one [61] analyzed the frequency of CD34+/KDR+ cells (EPCs) by flow cytometry before and 3, 9, and 27 days after gene therapy. The number of EPCs on day 3 was significantly higher than that at baseline ($p = 0.03$); however, on days 9 and 27 this frequency was similar to baseline. We identified a transient mobilization of EPCs, which peaked on day 3 after VEGF 165 gene therapy in patients with refractory angina and returned to near baseline levels on days 9 and 27 [61].

In the second study [62], blood samples were subjected to systemic analysis of protein expression by ELISA. The levels of proinflammatory IL-6 and ET-1 were increased on day 3 after gene therapy, and VEGF was increased on day 9. The mobilization of EPCs and TNF- α on day 9 showed a strong positive correlation, indicating pro-inflammatory status. Expression of angiogenic molecules and mobilization of EPCs are time-dependent, influenced by chronic inflammatory processes and continuous pharmacological treatment [62].

Following these results, our group led another experimental study to investigate the safety and hemodynamic and tissue improvement ability of gene therapy combining VEGF and angiopoietin 1 (Ang 1) in a mini pig model [63]. The treatment resulted in the improvement of ventricular function following ischemic cardiomyopathy in mini pigs, when compared to the results of the other treatment groups. The therapy with VEGF and the combination of VEGF with Ang 1 promoted recovered function of the myocardium, characterized by reduced akinetic area and induction of neovascularization [63].

Conclusions

Taken as a whole, our results with gene therapy approaches for heart diseases showed that gene therapy mobilizes stem cells for the circulation. An improvement in myocardial perfusion and function has been demonstrated in animal myocardial infarction models treated with direct myocardial injections of naked VEGF 165 plasmid. A randomized clinical trial found that improved myocardial perfusion was clinically demonstrated by myocardial scintigraphy and that the method is safe, but that the improved myocardial perfusion diminishes in a few months. An animal experiment associating VEGF 165 with angiopoietin was undertaken in mini pigs, as a tentative experiment to extend the durability of that therapy.

Regarding cell therapy for cardiovascular diseases, our results in preclinical and clinical studies show that the impressive effects of BMSC in improving myocardial perfusion and contractile function in animal models is not

translated to the clinical level, where they are smaller, sometimes achieving statistical significance, but with questionable clinical importance. Similar results have been observed in the large number of stem cell trials for cardiovascular disease [64] and have been explained by multiple factors involving methodology, individual patient factors, and even fraudulent practice [8]. In our experience, investigation into the mechanisms mediating the effects observed showed that cell function is affected by beta-blocker drugs, which are widely used in cardiology; cell function diminishes with age, which could explain, in part, the clinical results, considering that experimental animals are always young. Based on the finding that effects are improved in young patients, a clinical pediatric series of cell therapy for DCM is underway. MSC-derived exosomes are attracting much attention for their potential to treat cardiovascular diseases, among others [65]. We have shown this effect on human islets isolated for transplantation therapy of Type 1 diabetes, for instance [66]. Research continues and a future look at cell-free therapy research is foreseen.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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