Departamento de Farmacia y Tecnología Farmacéutica

Facultad de Farmacia

Universidad de Navarra

TESIS DOCTORAL

"Novel tissue engineering strategies based on the combination of polymeric devices and adult stem cells for cardiac repair"

Paula Díaz Herráez

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Trabajo presentado por Paula Díaz Herráez para obtener
el Grado de Doctor

Fdo. Paula Díaz Herráez
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FACULTAD DE FARMACIA

Departamento de Farmacia y Tecnología Farmacéutica

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> Que el presente trabajo, titulado "**Novel tissue engineering strategies based on the combination of polymeric devices and adult stem cells for cardiac repair"**, presentado por DÑA PAULA DÍAZ HERRÁEZ para optar al grado de Doctor en Farmacia, ha sido realizado bajo su dirección en el Departamento de Farmacia y Tecnología Farmacéutica, en colaboración con el grupo de D. FELIPE PRÓSPER del Área de Terapia Celular del Centro de Investigación Médica Aplicada. Considerando finalizado su trabajo autorizan su presentación a fin de que pueda ser juzgado y calificado por el Tribunal correspondiente.

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La educación es el pasaporte hacia el futuro, el mañana pertenece a aquellos que se preparan para él en el día de hoy.

Education is our passport to the future, for tomorrow belongs to the people who prepare for it today.

Malcolm X (American politician, 1925-1965)

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Abbreviations:

ADSC Adipose-derived stem cells
BMSC Bone marrow derived stem cells
BSA Bovine serum albumin
CDC Centers for Disease Control and Prevention
CPC Cardiac progenitor cell
CSC Cardiac stem cells
CT Clinical trial
cTnT Troponin T
CVD Cardiovascular disease
DCM Dichloromethane
DDS Drug delivery system
Dex Dextran
DES Drug eluting stents
DMSO Dimethylsulfoxide
DS Degree substitution
ECM Extracellular matrix
EE Encapsulation efficiency
EF Ejection fraction
EPO Erythropoietin
ESC Embryonic stem cells
FDA Food and Drug Administration
FGF Fibroblast growth factor
G-CSF Granulocyte colony stimulating factor
GF Growth factor
GFP Green fluorescent protein
GM-CSF Granulocyte-macrophage colony stimulating factor
GRAS Generally recognized as safe
HA Hyaluronic acid
HE Hematoxylin-eosin
HGF Hepatocyte growth factor
HRP Horseradish peroxidase
HSA Human serum albumin
IGF-1 Insulin like growth factor-1
IC Intracoronary
IM Intramyocardial
iPS Induced pluripotent stem cells
IV Intravenous
LV Left ventricle
LVEF Left ventricular ejection fraction
MI Myocardial infarction
MP Microparticle
MSC Mesenchymal stem cell
MTS Cell proliferation assay
NF Nanofiber
NP Nanoparticle
NRG Neuregulin
PBS Phosphate buffered solution

PDGF Platelet derived growth factor
PDL Poly-D-lysine
PEG Poly (ethylene glycol)
PGCL Poly-glycolide-co-caprolactone
PIGF Placental growth factor
PLA Polylactic acid
PLCL Poly(lactic-co-Ɛ-caprolactone)
PLGA Poly lactic co-glycolic acid
PLLA Poly-L-lactic acid
PVA Polyvinyl alcohol
SC Subcutaneous
SC Stem cell
SD Standard deviation
SDF Stromal cell-derived factor-1
SEM Scanning electron microscopy
α-SMA Smooth muscle actine
SVF Stromal vascular factor
TA Tyromine
TBS Tris buffered solution
TE Tissue engineering
TGF-β Transforming growth factor-β
TROMS Total recirculation one machine system
VEGF Vascular endothelial growth factor

INTRODUCTION

CARDIAC DRUG DELIVERY

Introduction:

CARDIAC DRUG DELIVERY

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Abstract:

Myocardial infarction is the leading cause of death worldwide. Since classical therapies produce more palliative than regenerative effects, extensive research has been performed to find an effective cure. New therapies, like growth factor and cell therapies, are arousing great interest. The clinical trials performed until now, although promising, have demonstrated that their efficiency is limited due to some drawbacks, such as short protein half-life or low cell survival rate. With a view to reducing or eliminating their limitations, the interest in combining these therapies with drug delivery systems (DDS) has increased over the last few decades. In this chapter, the studies performed over the last ten years using DDS in animal models of myocardial infarction have been reviewed in order to assess the possible benefits produced by the combination of DDS with protein and/or cell therapies in regeneration after myocardial infarction. Finally, the conclusions drawn from all these studies and the future trends under investigation that can be explored to achieve further improvement in the area of infarcted heart regeneration are discussed.

1 Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide. In fact, 17.3 million people died from CVDs in 2008, representing 30% of all global deaths. It is estimated that almost 25 million people will die from CVDs by 2030 (available in http://www.who.int/mediacentre/factsheets/fs317/en/index.html). Although the death rate due to CVD is high, a similar number of people survive. Nevertheless, those patients who survive may still face a difficult recovery process. The ongoing complications that result from CVD greatly contribute to the economic burden on the health-care system and on society as a whole. For example, the direct cost of CVD in the United States was $312.6 billion in 2009 (Go *et al*., 2013) and in 2010, the cost in health-care expenditures and loss of productivity amounted to nearly $444 billion (available in CDCs The Million Hearts Initiative, 2012).

CVD is caused by disorders of the heart and blood vessels. CVD includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. Of all of these, heart attack is the most important disorder, being responsible for 7.3 million deaths each year (available in http://www.who.int/mediacentre/factsheets/fs317/en/index.html). A heart attack, also called myocardial infarction (MI), is usually caused by a coronary artery occlusion that produces loss of blood flow in a specific heart region. Artery blockage is mainly due to the combination of a blood clot and an atheroma, formed by a thrombotic and an atherosclerotic process respectively (Fig. 1). The final consequence is the ischemia and hypoxia of the surrounding area. The lack of oxygen causes the death of the cardiac cells, called myocytes, which become apoptotic and/or necrotic over MI progression, generating the infarcted area (Kurrelmeyer *et al*., 1998). The extent of the damage depends on the blockage location and on the time since the MI was caused (Fig. 1). Interestingly, there is a transitional step in which heart cells lay between the normal well-vascularized and the necrotic/ischemic myocardium. During that period of time, myocytes are still alive and allow several treatments to avoid irreversible heart tissue loss. If blood flow is restored early enough, much of the heart muscle that could have been damaged might ultimately survive. This is why MI is a medical emergency, and treatment should be given urgently. The quicker the blood flow is restored, the better

the outlook.

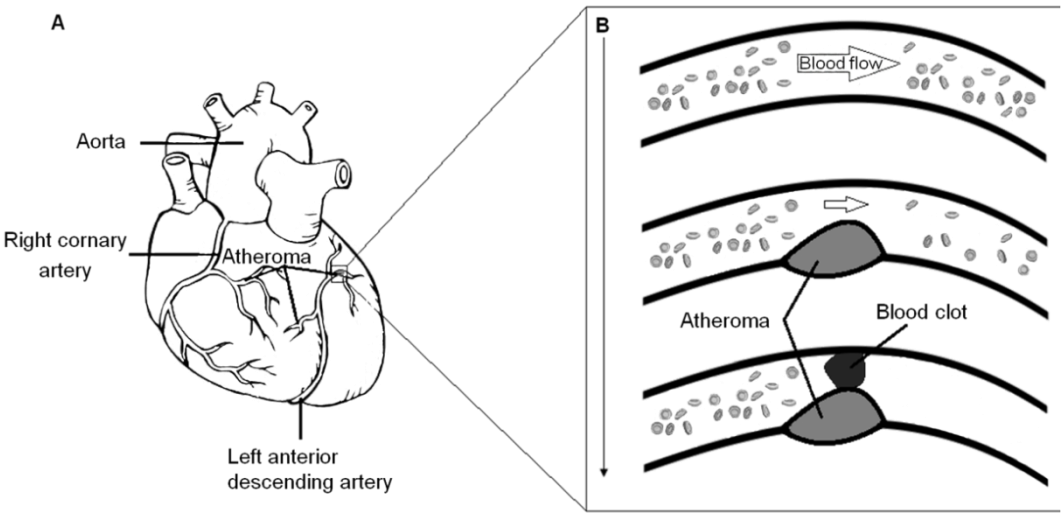

Figure 1: Myocardial infarction scheme: (A) Heart with several branch arteries blocked, (B) Section of a coronary artery which shows the steps during an obstruction process.

1.1 Current therapies and their limitations

Nowadays there are several options available for those patients suffering from MI. The conventional therapies include:

Pharmacological treatments: drug therapy is an important component of long-term care following MI. Common medication used for MI treatment includes the use of angiotensin converting enzyme inhibitors, beta-blockers, diuretics and vasodilators. These drugs reduce left ventricular filling pressure and volume, allowing cardiac remodeling process control. Other pharmacological treatments are based on antiplatelet agents, such as aspirin or clopidogrel, used to prevent clotting in patients who have had a heart attack. Moreover, antiplatelet drugs can also improve the short and long-term outcomes of patients treated with coronary stents (Scott *et al*., 2008).

Balloon angioplasty: is a percutaneous intervention were blocked coronary arteries are reopened by inflating a tiny balloon inside the blockage, compressing the fatty plaque against the artery walls and widening the vessel.

Stent: in this strategy a tiny metal mesh tube or stent, often inserted during angioplasty, is used as a scaffold to help the artery to keep open. The assembly is pushed into the narrowed artery, were the balloon is inflated, expanding the stent. The balloon is then deflated and withdrawn. After several weeks the artery heals around the stent.

Coronary bypass: in this type of surgery, one or more blocked coronary arteries are

bypassed by a length of blood vessels grafted from patient´s chest, legs or arms, with the aim of restoring normal blood flow.

Heart transplant: this procedure is only performed after all other options have been exhausted.

These classical approaches are useful in mitigating the symptoms and have reduced the MI mortality rate. However, cardiac dysfunction remains an issue, due to inadequate heart healing after ischemia (Sy and Davis, 2010). Several factors, including contractile cell loss, inflammatory response, cardiac hypertrophy and lack of suitable cues for progenitor cells, cause fibrosis in the heart and cardiac function loss. To date, medical and interventional treatments for MI are not able to regenerate the tissue or restore heart function. Moreover, current treatments are either highly invasive or rely on continuous administration of several drugs. Their beneficial effects are only observed when large doses are administered, which is frequently accompanied by side effects. Regarding angioplasty, acute occlusion in the treated vessel, as well as restenosis, occur in 30-40% of lesions. The use of bare metal stents reduced restenosis incidence to 25-30%, and the percentage was further reduced by drug eluting stents (DES), falling to less than 10% in initial clinical trials (CT). These results led to the use of DES in more than 85% of all coronary interventions. However, in-stent thrombosis or blood clot formation occurs more frequently in DES as compared to bare metal stents. The most definite solution is perhaps a fully biodegradable scaffolding device that does not leave any struts after drug elution has occurred (Onuba *et al*., 2011).

Finally, for some patients, the only option is organ transplantation (Formiga *et al*., 2012), although it has numerous drawbacks, such as the donor waiting list or the immunosuppressive regimen to prevent rejection.

In conclusion, although therapeutic advances have led to significant improvements in the outcomes of MI patients, multiple aspects for treating this pathology remain challenging. Therefore, additional strategies to rescue and regenerate the myocardium are needed.

2 New therapeutics strategies for cardiovascular diseases

Clinical and translational researches have advanced the available therapeutic options for MI management, and patients have their best survival rates ever. However, the current limitations of conventional therapies have led to an increase in the efforts to

develop new strategies. The advent of new molecular and cellular targets, together with advances in genomic and proteomic technologies, have accelerated the discovery of novel pharmaceutical compounds able to regenerate the heart. This emerging class of substances has high specificity and potency, and includes proteins, gene therapies, siRNAs, cell-based therapies or small molecules, among others (Meng and Hoang, 2012).

In this section, we revise the therapies based on proteins and cells, since these strategies are the ones that have shown the most encouraging results so far.

2.1 Protein therapy:

At present, there are numerous protein candidates for MI treatment. The most promising ones are: vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), placental growth factor (PIGF), granulocyte colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), neuregulin (NRG), insulin like growth factor-1 (IGF-1), transforming growth factor-β (TGF-β), erythropoietin (EPO), platelet derived growth factor (PDGF) and stromal cell-derived factor 1-α (SDF) (Segers and Lee, 2010). These growth factors (GF) have aroused interest due to their specific biological functions and roles in MI heart regeneration (Fig. 2). They have the potential to induce: 1) angiogenesis, 2) chemotaxis, differentiation and proliferation of stem cells, 3) reduction of apoptosis, 4) stimulation, survival and proliferation of cardiomyocytes, 5) cardiac muscle development and 6) reduction of remodeling. The current applications of these GFs in CT are detailed next.

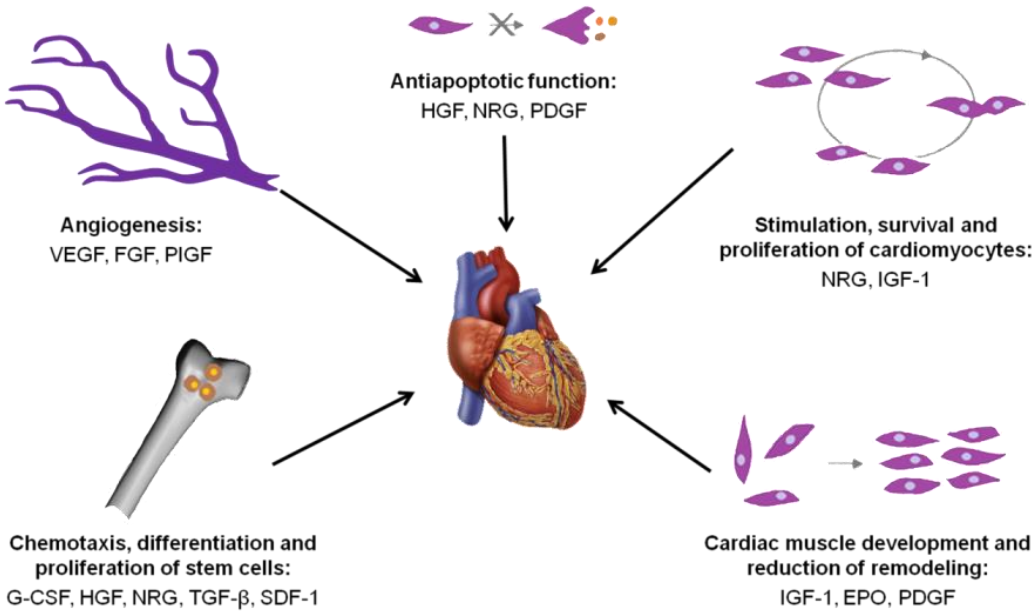

Figure 2: Scheme of protein candidates for myocardial infarction treatment indicating their specific biological function and roles in heart regeneration.

2.1.1 Clinical trials with proteins:

The first CT using therapeutic proteins for cardiac repair involved human FGF-1 (Table 1). In this first study, 40 coronary heart disease patients were included. All of them were treated with bypass surgery, and 20 of them also received FGF-1 intramyocardial (IM) injection. A dense capillary network next to the FGF-1 injection area, as well as a local blood supply increase, were observed after 12 weeks (Schumacher *et al.*, 1998). At the moment, the ongoing ACORD CT Phase II (Clinicaltrials.gov identifier NCT00117936), with an estimated enrollment of 120 patients, is being performed in order to test vessel growth stimulation around the blocked coronary arteries after IM injection of FGF-1 at different dose rates (0, 2, 20 and 40 µg/kg).

Several CTs have been performed to date with FGF-2 (Laham *et al.*, 1999 and 2000; Unger *et al.*, 2000; Udelson *et al.*, 2000). The results of phase I FGF-2 trials confirmed treatment safety and feasibility, and suggested a benefit when applied to ischemic cardiac patients. Based on these results, the FIRST study was then conducted. This phase II CT included 337 patients with coronary artery disease. They administered intracoronary (IC) FGF-2 in a single bolus at different doses (0, 3 or 30 µg/kg). No improvement was detected until day 90 and only a trend toward symptomatic

improvement was observed. However, this benefit disappeared at day 180, due to the continued improvement observed in the placebo group (Simons *et al*., 2002).

Table 1: Current clinical trials using growth factors for the treatment of myocardial infarction.

YEAR	GF	ADM. ROUTE	N	REF
1998	FGF-1	IM	20	Schummacher *et al*., 1998.
-			estimated 120	ClinicalTrials.gov identifier NCT00117936
1999	VEGF	IV	28	Gibson *et al.*, 1999.
2000		IC	14	Hendel *et al*., 2000.
2001			15	Henry *et al*., 2001.
2003		IC/IV	178	Henry *et al*., 2003.
1999	FGF-2	IM	24	Laham *et al*., 1999.
2000		IC	25	Unger *et al*., 2000.
			52	Laham *et al*., 2000.
		IC/IV	59	Udelson *et al*., 2000.
2002		IC	337	Simons *et al*., 2002.
2001	G-CSF	IC/SC	21	Seiler *et al.*, 2001.
2005		SC	20	Valgimigli *et al*., 2005.
			50	Ince *et al*., 2005.
			14	Zbinden *et al*., 2005.
2006			114	Zohlnhofer *et al*., 2006.
			78	Ripa *et al*., 2006.
2009			52	Meier *et al*., 2009.
2010			60	Achilli *et al*., 2010.
			44	Engelmann *et al*., 2010.
	G-CSF + sitagliptin	SC	100	Theiss *et al*., 2010.
2009	EPO	IV	44	Tang *et al*., 2009.
2010			529	Voors *et al*., 2010.
2006	long-acting EPO	IV	22	Lipsic *et al*., 2006.
2009	pHGF	IV	49	Wang *et al*., 2009.
2010	NRG	IV	15	Jabbour *et al*., 2011.
			44	Gao *et al*., 2010
-			331	ClinicalTrials.gov identifier NCT01131637
		SC	estimated 120	ClinicalTrials.gov identifier NCT01214096
			120	ClinicalTrials.gov identifier NCT01251406
		IV	estimated 50	ClinicalTrials.gov identifier NCT01258387
			146	ClinicalTrials.gov identifier

				NCT01439789
			14	ClinicalTrials.gov identifier NCT01439893
			estimated 1600	ClinicalTrials.gov identifier NCT01541202

VEGF was considered a very promising GF to achieve neovascularization due to the results obtained in preclinical studies. In a phase I trial with 28 patients, intravenous (IV) VEGF administration showed improvement in myocardial perfusion and in collateral density (Gibson *et al*., 1999). Two phase I trials have been performed using IC VEGF administration, concluding the safety and tolerability of the treatment and a dose-dependent effect (Hendel *et al*., 2000; Henry *et al*., 2001). The first large CT with VEGF was the VIVA trial which enrolled 178 patients who received different doses of VEGF administered IV or IC. Despite previously demonstrated beneficial effects, this CT did not show any significant improvement beyond placebo by day 60 and 120. The only significant difference was found by day 120 in the high dose group which showed a reduced number of angina events, indicating an improvement in patients' quality of life (Henry *et al*., 2003).

Several CTs with G-CSF have been conducted in the last decade (Zbinden *et al*., 2005; Valgimigli *et al*., 2005). FIRSTLINE-AMI was a trial in which 25 out of 50 patients were randomly assigned to receive a 10 µg/kg daily subcutaneous (SC) dose for 6 days. This treatment promoted mononuclear CD^{34+} cell mobilization, which correlated with better ventricular function preservation and less remodeling (Ince *et al*., 2005). In the context of the first results obtained in small CTs, the REVIVAL-2 study was conducted. 114 patients were included, and half of them received an SC daily dose of 10 µg/kg of G-CSF for 5 days, while the rest received placebo. Although stem cell mobilization was significant, it did not have any impact on infarct size, left ventricular function or coronary restenosis (Zohlnhöfer *et al*., 2006). The same results were observed in the STEMMI trial performed in 78 patients (Ripa *et al.*, 2006). In spite of these negative results, more CTs with G-CSF have been conducted since then (Meier *et al*., 2009; Achilli *et al*., 2010; Theiss *et al*., 2010; Engelmann *et al*., 2010).

In a CT with EPO, no improvement in left ventricular ejection fraction (LVEF) was observed 4 months after the long-acting glycoprotein IV administration (Lipsic *et al*., 2006). Next, in a different trial with acute MI patients, angiogenesis signaling protein

expression in peripheral blood mononuclear cells was increased (Tang *et al*., 2009). Finally, a large phase II CT, with 529 patients, failed to improve LVEF after 6 weeks (Voors *et al*., 2010).

In the last three years, NRG, a therapeutic protein that has shown great promise in preclinical animal models, has been tested in numerous undergoing CTs. When it was first administered to patients, it appeared to favor hemodynamic effects (Jabbour *et al*., 2011). In a phase II trial, it was also demonstrated to improve cardiac function and reduce ventricular remodeling (Gao *et al*., 2010). Now, larger CTs have been launched to confirm treatment efficacy (Clinicaltrials.gov identifiers NCT01131637, NCT01214096, NCT01251406, NCT01258387, NCT01541202).

2.1.2 Lessons from clinical trials with proteins:

Taking an overview of CT data, some interesting conclusions can be outlined. A main limitation of protein therapy is the half-life of the different proteins. This is because GF are labile molecules that are degraded in a very short period of time, ranging from some minutes to a few hours, when directly administered into the organism. Therefore, to obtain a more sustained effect over time, many administrations or the use of systems that protect GFs from degradation would be required. This fact justifies the efforts to incorporate therapeutic proteins into delivery systems that protect them from degradation and that allow their sustained release. This point will be extensively discussed in next sections.

Another drawback concerns the CT design. Studies involving small populations tend to release positive data. But when they are scaled up no therapeutic benefit can be demonstrated. A better small trial design will help to determine the optimal treatment conditions for larger studies before these are performed. Also, in most of the studies, short-term results do not seem to correlate with long-term effects. For these reasons, patients should be followed up for longer periods of time to obtain more valuable information about the treatment.

Thus, after more than 10 years of CTs with therapeutic proteins, information about several GFs has been accumulated. This will help us to choose the adequate protein or combination of proteins to treat the patients in the future. For instance, VEGF promotes neovascularization but its effect is not powerful enough to affect cardiac function. Thus, in the future, it could be combined with other factors that promote cell recruitment, such as EPO.

Another aspect to take into account is that each patient is different, and thus have different requirements. Bearing in mind that most of the GFs are implicated in the acute process, they are always administered to patients at this stage. Nevertheless, NRG is a better candidate to treat not only acute patients, but also chronic patients, based on its ability to promote cardiomyocyte proliferation.

Another issue is the preferable route of administration, which also depends on the GF in question. EPO, for instance, has almost always been SC administered in a single dose, being sufficient to promote cell mobilization. Conversely, VEGF and FGF-2 have been IV, IC and IM administered. In general, IM administration avoids adverse effects associated with systemic administration and allows a better dose control. To reduce the invasiveness of IM administration, novel commercially available technologies are now being applied, such as the transendocardial injection with MyoStar^{TM} injection system guided by NOGA$^{®}$. This method allows direct IM injection with a tissue penetration up to 10 mm and low risk of myocardial perforation or rupture (Kharlamov *et al*., 2012).

Finally, it can also be concluded that the suitable manner for making these proteins available at the target site, with a desired dosage and for a determined period of time, remains unclear. Proteins, due to their limited bioactivity, short half-life, pharmacokinetic properties and instability, require specialized delivery modalities. However, reports of heart-specific drug delivery vehicles are scarce. Thus, there is an unmet need for cardiac drug delivery technologies able to administer biopharmaceuticals. Drug delivery systems (DDS) could also play an important role in multiple GF therapy since they can be made of different materials and can incorporate two or more therapeutic proteins with different release profiles. Currently, these systems are at the preclinical stage of safety and efficacy evaluation (See section 4).

2.2 Cell therapy:

On the basis of preclinical data, different cell types have been explored to regenerate infarcted heart (Fig. 3) (reviewed in Pelacho *et al*., 2013). Myoblasts were one of the first cells found to differentiate towards cardiomyocytes. However, it has been demonstrated that myoblasts act in a paracrine manner and that they are not able to generate new cardiac cells. Bone marrow-derived stem cells (BMSC) have been the most widely adult stem cells used for cardiac repair. BMSC include hematopoietic stem cells, mesenchymal stem cells (MSC) and endothelial progenitor stem cell

subpopulations. Adipose-derived stem cells (ADSC), which are easily isolated by liposuction, and umbilical cord blood-derived mesenchymal stem cells, are also showing great promise for use in cardiac repair. Contrary to classical conceptions, a population of cardiac progenitor stem cells (CSC) has been found in the heart, indicating an intrinsic regenerative potential of this organ. These cells appear in clusters that can be isolated and differentiated *in vitro* towards cardiomyocytes and vascular cells. Fetal cardiomyocytes and embryonic stem cells (ESC) are also an attractive cell source due to their totipotency. However, they present important concerns that limit their use, such as availability, immunogenicity, teratogenic potential and ethical issues due to their origin. An alternative to ESC are induced pluripotent stem cells (iPS). iPS are pluripotent cells obtained from adult cell reprogramming. iPS derived from rodent cells were obtained for the first time in 2006 (Takahashi and Yamanaka, 2006). Next, in 2007, human iPS were derived from human fibroblast by the transduction of several transcription factors (Oct3/4, SOX2, Nanog, Lin28) (Takahashi *et al.*, 2007). Improvements in iPS obtention protocol have been performed in recent years in order to avoid integrating virus use. However, the technology to create iPS is relatively new and it is still not clear whether these cells are safe for transplantation.

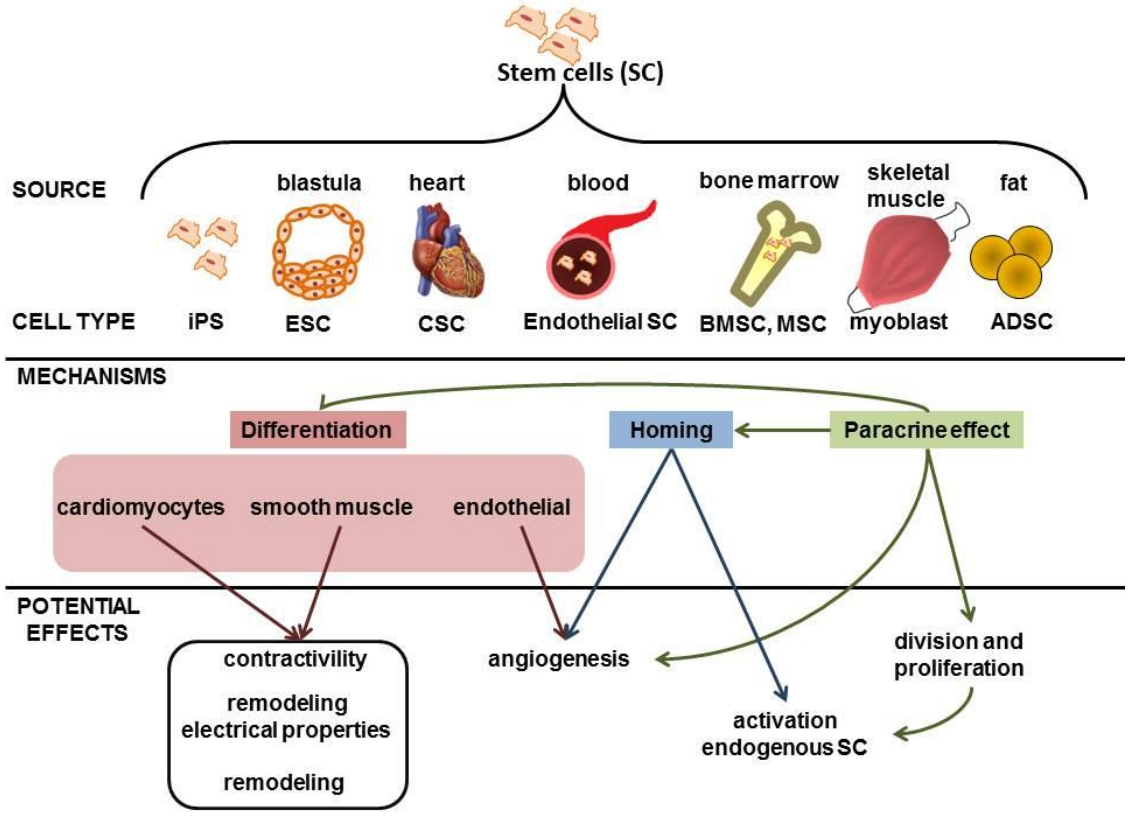

Figure 3: Schematic representations of the main stem cell sources used for myocardial infarction treatment showing the mechanisms and the potential effects in heart regeneration.

2.2.1 Clinical trials with cells:

Although many preclinical studies using different cell sources have been performed, only two cell types, BMSC and myoblasts, have been tested in CTs.

The first CT employing cell therapy for MI regeneration was the Strauer *et al.* trial in 2002. It enrolled 20 patients that received conventional therapy after MI, 10 of whom received autologous BMSCs in addition 3 months later, patients in the BMSC group showed significant infarct region reduction along with the improvement of the infarction wall movement velocity, stroke volume index, left ventricular end-systolic volume and contractility and myocardial perfusion of the infarcted region (Strauer *et al*., 2002). Trials performed later on showed different results. Some of them, such as TOPCARE-AMI, BOOST, REPAIR-AMI and FINCELL were successful in improving disease progression. On the other hand, LEUVEN-AMI, ASTAMI and HEBE trials did not confirm the previous positive results. In order to conclude about the effect of BMSC transplantation in acute MI, a meta-analysis was performed by Martin-Rendon *et al*. in 2008, concluding that BMSC transplantation was not only safe, but also effective in improving the LVEF and reducing the scar size (Martin-Rendon *et al*., 2008).

On the other hand, the first studies using myoblasts were published 10 years ago (Table 2). In 2005 a phase I CT demonstrated their safety and feasibility for cardiac regeneration. Moreover, potential functional benefits were described, with an improvement in the ejection fraction even 2 years after implantation. However, the first randomized placebo-controlled trial (MAGIC study) did not show any functional improvement or benefit in the electrocardiography. In fact, patients receiving cell treatment showed a higher number of arrhythmic events (Menasche *et al*., 2008). CAuSMIC study included 23 patients with MI and heart failure (Dib *et al*., 2009a).

Unlike the results of the previous study, these patients did not suffer arrhythmias, and showed improvement on the New York Heart Association and Minnesota Living Heart Failure Questionnaires, which are recognized to be representative of the heart failure impact and patients' quality of life.

Table 2: Current clinical trials using cell therapy for the treatment of myocardial infarction.

YEAR	TRIAL	CELLS	ADM. ROUTE	N	REF
2003	-	SkM	CABG	10	Menasche *et al*., 2003
			Tec	5	Smits *et al*., 2003
2004				12	Ince *et al*., 2004
			Tep	10	Siminiak *et al*., 2004
				20	Chachques *et al*., 2004
2005				30	Dib *et al*., 2005
	POZNAN		TC	9	Siminiak *et al*., 2005
2006	-		Tep	26	Gavira *et al*., 2006
			TEc	10	Biagini *et al*., 2006
2008	MAGIC		Tep	97	Menasche *et al*., 2008
2009	CAuSMIC		TEc	23	Dib *et al*., 2009b
2011	SEISMIC			40	Duckers *et al*., 2011
2002	-	BMC	IC	20	Strauer *et al*., 2002
2003	TOPCARE-AMI	BMC/CSC	IC	30	Britten *et al*., 2003
2006		BMC/CSC	IC	75	Assmus *et al*., 2006
2004	-	BMC	IC	33	Fernandez-Avilés *et al*., 2004
			Tec	20	Perin *et al*., 2004
2005			IC	26	Erbs *et al*., 2005
			dMI	20	Patel *et al*., 2005
2004	BOOST		IC	60	Wollert *et al*., 2004
2006	LEUVEN-AMI			66	Janssens *et al*., 2006
	-		dMI	20	Hendrikx *et al*., 2006
			IC	60	Meyer *et al*., 2006
			dMI	36	Mocini *et al*., 2006
			TEc	27	Fuchs *et al*., 2006
			IC	10	Briguori *et al*., 2006
	ASTAMI			97	Lunde *et al*., 2006 and Beitnes *et al*., 2009
2007	REPAIR-AMI			204	Schachinger *et al*., 2006
2006	-			66	Meluzin *et al*., 2006
2007				10	de la Fuente *et al*., 2007
	PROTECT-CAD		TEc	28	Tse *et al*., 2007
	-		dMI	40	Stamm *et al*., 2007
2008				36	Zhao *et al*., 2008
			Tep	63	Ang *et al*., 2008
	FINCELL		IC	77	Huikuri *et al*., 2008
2009	-		dMI	50	Akar *et al*., 2009
			IC	50	van Ramshorts *et al*., 2009
				67	Herbots *et al*., 2009

				60	Plewka *et al*., 2009
	REGENT			120	Tendera *et al*., 2009
	MYSTAR		IM/IC	60	Gyongyosi *et al*., 2009
2010	STAR-Heart		IC	391	Strauer *et al*., 2010
	-			40	Traverse *et al*., 2011
	HEBE			200	Hirsch *et al*., 2011
2004	-	MSC	IC	69	Chen *et al*., 2004
2009			IV	53	Hare *et al*., 2009
2009			EV	20	Dib *et al*., 2009 B
2010			dMI	30	Viswanathan *et al*., 2010
-		ADSC	IM/IV	estimated 10	ClinicalTrials.gov identifier NCT01502514 (recruiting)
	APOLLO		IC	estimated 48	ClinicalTrials.gov identifier NCT00442806 (ongoing)
	ADVANCE			estimated 216	ClinicalTrials.gov identifier NCT01216995 (recruiting)
	ATHENA			estimated 45	ClinicalTrials.gov identifier NCT01556022 (recruiting)
	PRECISE		IM	estimated 36	ClinicalTrials.gov identifier NCT00426868 (ongoing)
	-		IC	estimated 6	ClinicalTrials.gov identifier NCT01709279 (recruiting)
2004	MAGIC-cell	G-CSF + PBSC	IC	27	ClinicalTrials.gov identifier Kang *et al*., 2004.
2013	ALCADIA	CPC + bFGF	IM	estimated 6	ClinicalTrials.gov identifier NCT00981006 (ongoing)

The phase IIa study SEISMIC, which also employed myoblasts, concluded that this therapy was safe. However, no improvement in the heart's functional activity was observed (Duckers *et al*., 2011). In the same way, patients included in the MARVEL-1 study underwent sustained ventricular tachycardia without significant improvement in functional capacity, or in the Minnesota Living Heart Failure Questionnaire (Povsic *et al*., 2011).

Therefore, myoblast therapy is feasible and safe but its beneficial effects are still not clear. Larger studies with long-term follow-up are therefore needed.

2.2.2 Lessons from clinical trials with cells:

When cell therapy was suggested as a possible strategy for treating heart ischemic patients, it was hypothesized that cells would be able to engraft and differentiate, contributing to the cellular repopulation of the infarcted area. However, it

was soon observed that the benefits were principally due to the paracrine effect of the injected cells (Gnecchi *et al*., 2008; Reinecke *et al*., 2008). Cell survival rates in the tissue were very low, partly due to cell escape through capillaries and the stressful environment that the infarcted tissue entails for the cells. Therefore, one of the main lessons from cell CTs is the need to increase cell survival rates. Other lessons concluded from these studies were the need to establish which type of cell is more appropriate for a given application and the best trial end point determination. Other procedural aspects that must be revised are related to cell processing for obtaining higher quality cell populations, as well as cell dosing, timing and delivery route optimization. All these issues have recently been reviewed elsewhere (Menasche *et al*., 2011).

In summary, additional strategies are needed to achieve cardiac regeneration. Improvement of the existing approaches will depend on drug discovery and on the development of new technologies to effectively deliver these compounds.

3 Drug delivery systems to address unmet medical needs in cardiovascular diseases

As noted above, the development of new technologies that enable effective drug delivery to the heart would optimize cardiovascular treatment and would address some of the limitations of current therapies. DDS were developed to improve drug therapeutic properties and to render them more safe, effective, and reliable. In general terms, incorporating a medicine into a DDS can significantly improve its performance. The major advantages of these systems could be summarized as delivery of drugs at a constant rate, drug protection, drug control pharmacokinetics, minimization of possible side effects, better efficacy and enhanced patient compliance (Verma and Garg, 2001). Further goals in drug delivery are to target the drug at particular organs or cells in the body, or to overcome certain tissue or cellular barriers (Langer and Peppas, 2003). Delivery systems can be designed with different mechanical and physical properties, and they can be biodegradable or non-biodegradable, depending upon the nature of the polymer or the material used for their preparation.

DDS can be used either for local or for systemic delivery. Most strategies for local cardiac therapy have used direct myocardial injection, intrapericardial delivery or coronary injection, using epicardial surgery or a catheter-based endocardial approach (Revised in Rolfes *et al*., 2012). However, which is the safest and most effective of

these delivery strategies is currently unknown.

In the context of heart diseases, DDS could improve the therapeutic properties of standard pharmacological treatments and reduce unwanted side-effects. Drug delivery for interventional treatments after MI is one area of great importance under investigation. Drug eluting metallic stents are an example of DDS developed for localized drug delivery to a specific location and to minimize restenosis associated with bare metal stents (Langer and Peppas, 2003). Various approaches using metal stents delivering paclitaxel (Heldman *et al*., 2001), sirolimus (Oberhoff *et al*., 2002) and other drugs have been well developed and tested showing remarkable results in keeping blood vessels open (Morice *et al*., 2002). But safety concerns have led to improvements in conventional DES with the use of more biocompatible and biodegradable polymers (Onuba *et al*., 2011). Regarding their clinical use, only the Absorb™ (Abbot Laboratories) biodegradable DES has been approved for its use in peripheral disease in Europe. However, this device does not have the Food and Drug Administration (FDA) approval yet. Nowadays several CTs using this DES, manufactured with the biodegradable polymer poly-L-lactic acid (PLLA), are ongoing (Table 3). Lately, cytokine-eluting stents have been proposed to stimulate arteriogenesis in the peripheral circulation of the rabbit (Grundmann *et al*., 2007). This intra-arterial delivery platform combines the advantages of therapeutic proteins and DES. With continuing advances in chemical engineering and material sciences, greater progress in this DES application is expected in the future.

Table 3: Current clinical trials using bioresorbable drug eluting stents for the treatment of myocardial infarction.

ClinicalTrials.gov IDENTIFIER	**TRIAL NAME**	**DRUG**	**ESTIMATED N**	**SITUATION**
NCT01711931	EVERBIOII	EVEROLIMUS	240	Recruiting
NCT01583608	ABSORB		180	Ongoing
NCT01023789	ABSORB EXTEND		1000	Recruiting
NCT01425281	ABSORBII		501	Recruiting
NCT01308346	ABSORB PHYSIOLOGY		36	Recruiting
NCT01751906	ABSORB RCT		2000	Recruiting
NCT00856856	ABSORB B		101	Ongoing

But, the most important potential applications of DDS in the cardiovascular field are without any doubt the development of novel protein and cell delivery systems for cardiac repair. From a therapeutic perspective, proteins offer the advantage of specific mechanisms of action and high potency. However, as pointed out in the preceding sections, one important limitation is the difficulty of administering them efficiently to treat cardiac tissue diseases. The major obstacles, as previously mentioned, are their short half-lives, their low stability and their immunogenicity. The use of DDS might overcome the limitations associated with protein administration, and would improve its potential and efficacy. Notably, such strategies have the potential to become viable therapeutic protein products (Pisal *et al*., 2010). On the other hand, regarding cell therapy, DDS could provide a supportive scaffold for cells to enhance their engraftment and survival in the heart. DDS can be designed to direct cell organization, growth and differentiation in the process of forming functional tissue by providing physical, mechanical and chemical cues (Pelacho *et al*., 2013). They can also reduce acute cell loss after cell transplantation due to the wash out from the infarcted myocardium (Dai *et al*., 2009; Segers and Lee, 2011). Natural polymers like collagen, gelatin, and alginate have inherent peptide sequences that can be easily recognized by the cell-surface receptors, and are therefore suitable biomaterials for cell adhesion.

The following are some of the most common vehicles explored so far for cardiac protein and cell delivery (Fig. 4) (revised in Formiga *et al*., 2012):

Liposomes: Sphere-shaped vesicles that consist of one or more phospholipid bilayers (Akbarzadeh *et al*., 2013). Due to their size and hydrophobic and hydrophilic characteristics they are extensively used as carriers for numerous molecules (Fig. 4.A).

Hydrogels: Three-dimensional polymer networks swollen by aqueous solvent, which is the major component of the gel system (Silva *et al*., 2009) (Fig. 4.B). They can carry diverse molecules and are very versatile systems. For instance, they can swell in aqueous medium, they can be pH and temperature sensitive or/and be sensitive towards other stimuli.

Micro and nanoparticles: Solid particles in the nanometer (nanoparticles (NP)) or micrometer (microparticles (MP)) size range (Ravi Kumar *et al*., 2000) (Fig. 4.C). They can be prepared with many different materials and polymers, and they have been extensively used for protein delivery (Tan *et al*., 2010; Mundargi *et al*., 2008; Almeida and Souto, 2007).

Self-assembling peptide nanofibers (NF): Well defined scaffolds made of up to 99% water and amenable to incorporate a variety of bioactive cues. They are peptide repeats that have both hydrophilic and hydrophobic components and alternating charges, allowing them to undergo self-assembly in physiological solutions (Sy *et al.*, 2010) obtaining systems that slowly degrade, with low immunogenicity, and which are able to release in a sustained pattern (Fig. 4.D).

Polymer scaffolds: Three-dimensional matrices with network architecture that can be manufactured in different forms. They are useful to incorporate and release therapeutic proteins (Chung and Park, 2007) (Fig. 4.E).

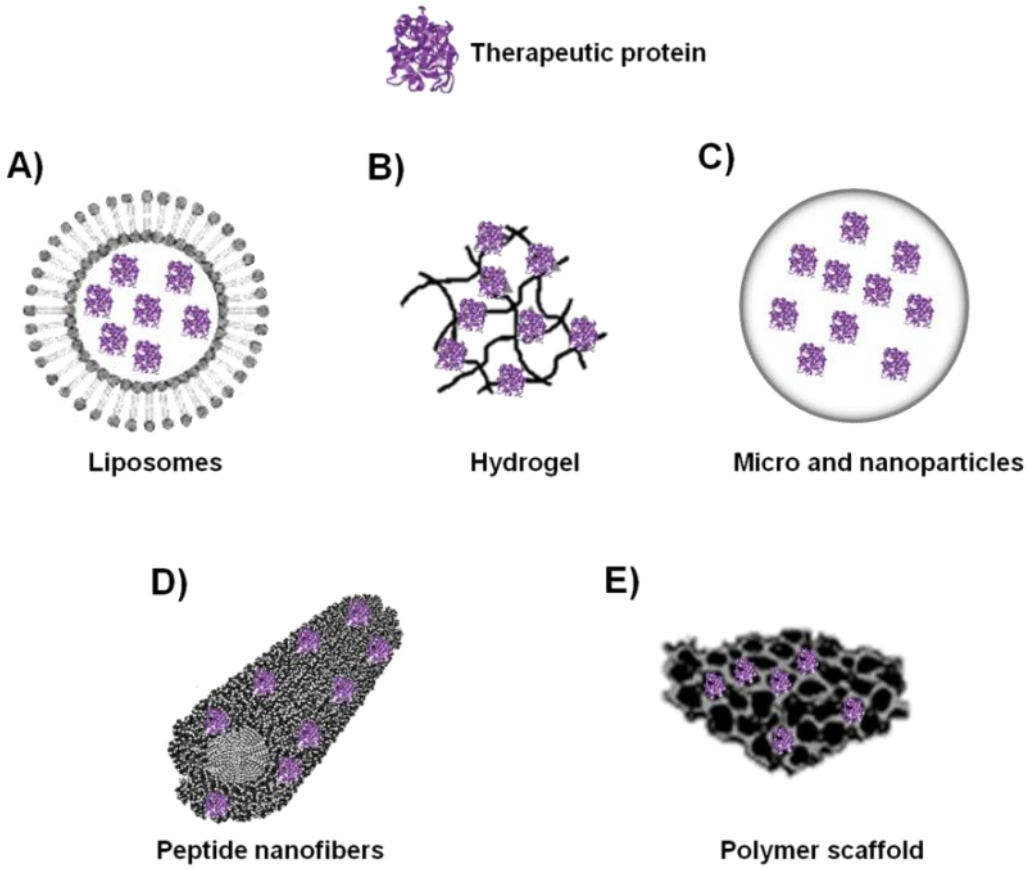

Figure 4: Schematic representation of the most common vehicles explored for cardiac drug delivery of proteins and cells with therapeutic potential.

Examples of DDS used for therapeutic protein and cell delivery in a cardiac context will be discussed in deeper detail in the next section.

Finally, a major focus of interest is targeted DDS development (Scott *et al.*, 2008). Targeted therapeutics can be delivered systemically at lower doses and could be used to increase drug concentration to the myocardium. Drug targeting would be possible for all the above mentioned DDS by coupling site-specific ligands like antibodies or receptors.

The search for appropriate biomarkers significantly and differentially up-regulated in diseased cardiac tissue is another unmet demand necessary to develop targeted drug delivery technologies with less toxic effects (Scott *et al*., 2008).

4 Current data on cardiac drug delivery systems and future trends

As mentioned in the previous sections, over recent years, research to combine protein and cell therapies with DDS has increased in order to minimize or eliminate possible drawbacks. Here studies from the last 10 years have been reviewed to show how these delivery systems have the ability to improve heart infarction therapeutics.

4.1 Drug delivery systems for growth factor delivery:

Currently, there are three main ways to incorporate GFs into DDS: immobilization, encapsulation or embedding. All these strategies have been applied to design an effective therapy for cardiac repair.

4.1.1 Drug delivery systems with VEGF:

Zhang *et al*. used a system based on a collagen-binding domain able to bind VEGF. The collagen-binding domain-VEGF formed was incorporated into a collagen membrane which, in a rat acute MI model, produced scar size reduction and cardiac function improvement. The beneficial effect observed is possibly due to high local VEGF concentration and prolongation of the protein's biological effect (Zhang *et al.*, 2009). Similarly, Miyagi *et al.* covalently immobilized VEGF, at two different concentrations, in collagen patches and tested them in a rat MI model. Although VEGF immobilization rate within the cardiac patch was low, positive results were obtained. VEGF-patched hearts were significantly thicker than control ones, which correlated with an increase in neovascularization which was more significant in the high dose patch-treated group (Miyagi *et al.*, 2011).

Immunoliposomes have also been found to deliver VEGF, as the attachment of specific immunogens can facilitate liposome targeting at the infarcted heart. In the study of Scott *et al.*, liposomes were conjugated to anti-P-selectin, one of the major molecules responsible for leukocyte enrolment in inflammation. Immunoliposomes were administered via tail vein and were able to reach the heart infarction area. The immunoliposome-treated group showed a significant improvement in cardiac function

compared to controls, showing a moderate left ventricle wall loss and vasculature improvement 4 weeks after DDS administration (Scott *et al.*, 2009).

Synthetic polymers have been studied for VEGF encapsulation. Among them, poly lactic co-glycolic acid (PLGA), a biocompatible and biodegradable polymer approved for human use by the FDA, is a widely used biodegradable polymer for delivery of protein drugs. Our group prepared PLGA MP encapsulating VEGF and examined its potential in a rat MI model. One month after implantation, VEGF MP produced a significant increase in angiogenesis and arteriogenesis, correlating with a positive remodeling of the heart. A significantly greater left ventricular wall thickness was observed when compared to free-VEGF group (Formiga *et al.*, 2010). Similar results were obtained in the study by Simón-Yarza *et al*. employing VEGF-PEG-PLGA MP (Simón-Yarza *et al*., 2013).

Another attractive approach for delivering VEGF to the heart is to embed the protein into hydrogels. Wu *et al.* prepared an aliphatic polyester hydrogel that allows localized, sustained VEGF release. Their *in vivo* study, performed in a rat MI model, showed that this hydrogel attenuated the adverse cardiac remodeling and caused ventricular function improvement by increasing blood vessel formation and by preserving the scar thickness (Wu *et al.*, 2011).

Self-assembling peptide NFs are a different strategy to embed VEGF. Guo *et al.* and Lin *et al*. tested them in rat and pig MI models, respectively. Fiber administration not only showed an improvement in angiogenesis, arteriogenesis and cardiac performance, but also transformed the injection site microenvironment into one capable of recruiting endogenous myofibroblasts, which helped to achieve an effective revascularization (Guo *et al.*, 2012; Lin *et al.*, 2012).

4.1.2 Drug delivery systems with FGF-2:

Fujita *et al.* studied the efficacy of a chitosan hydrogel encapsulating FGF-2 in a rabbit chronic MI model. The hydrogel was able to retain biologically active FGF-2 and to sustainably release it until the *in vivo* complete biodegradation of the system, 4 weeks after injection. A substantial angiogenesis induction and collateral circulation in the ischemic myocardium was reported (Fujita *et al.*, 2005). Similar results were obtained by Wang *et al.* who directly injected FGF-2 chitosan hydrogel into the infarcted

myocardium border, producing infarction size reduction, cardiac function improvement, collagen deposition reduction and an increase in arteriole density 4 weeks after administration (Wang *et al.*, 2010).

Sakakibara *et al.* used FGF-2 incorporated in gelatin microspheres to evaluate their distribution in the rat heart, using different administration methods. They also investigated their efficacy in pigs after MI. In the efficacy study, the mean infarct size was not significantly different between groups. However, the group treated with FGF-2-microspheres showed cardiac function improvement associated with higher angiogenic and vascular density rates (Sakakibara *et al.*, 2003). In a different approach, Shao *et al.* studied the effects of the IM injection of FGF-2 incorporated in gelatin hydrogels on neoangiogenesis in a rat MI model. FGF-2-hydrogel produced neoangiogenesis stimulation, and also decreased cardiomyocyte apoptosis in the infarct border zone, infarction wall thinning reduction, left ventricular remodeling attenuation and consequently, cardiac function improvement (Shao *et al.*, 2006).

More recently, FGF-2 was delivered using a pH- and temperature-responsive acrylic polymer hydrogel. This system allowed local FGF-2 retention in the heart apex, with minimal diffusion. Not only was the system beneficial effect demonstrated, which increased microvessel density, regional blood flow and improved cardiovascular function, but it was also shown that the hydrogel produced some benefit on its own. The acrylic polymer hydrogel was able to increase left ventricular thickness and improve cardiac function in the absence of exogenous GF delivery, although at a lower rate with respect to the one that contained FGF-2 (Garbern *et al.*, 2011).

4.1.3 Drug delivery systems with other growth factors:

In the study by Davis *et al.*, IGF-1 was entrapped in peptide NFs resulting in systolic function improvement and ventricular dilation reduction in a rat MI model (Davis *et al.*, 2006). Another study entrapping IGF-1 in peptide NFs revealed that IGF-1-NF treatment reduced the infarct size, improved the ventricular function and favored cardiomyocyte regeneration and coronary vessel formation, showing better outcomes than free IGF-1 in a rat MI model (Padin-Iruegas *et al.*, 2009).

The studies by Hsieh *et al*. also employed peptide NFs for PDGF administration. The NFs were injected in a rat MI model, and it was observed that the system remained at the targeted site 14 days post injection. Animals treated with PDGF-NFs significantly

improved fractional shortening compared with controls. Of particular importance was the point that the improvement of fractional shortening was maintained only in those animals treated with the highest GF dose, implying dose-dependent cardioprotection. Interestingly, these researchers found that cardiac function improvement after PDGF-NF injection may not result from improvement of blood supply directly, but from cardiomyocyte apoptosis prevention and myocardial function preservation. The system also improved hemodynamic parameters and cardiac performance 4 months after PDGF-NF injection (Hsieh *et al.*, 2006a and 2006b).

PIGF was encapsulated into chitosan-alginate NP and administered in a rat MI model. LVEF measurement showed that PIGF-NP beneficial effect had a delay in time, but was more sustained than after free PIGF administration. This suggests that chitosan-alginate NP provide a protective sustained-release mechanism to PIGF. These researchers also analyzed scar area, angiogenesis and arteriogenesis, detecting statistical differences from the control groups (Binsalamah *et al.*, 2011).

More recently, Purcell *et al*. applied a hyaluronic acid hydrogel containing SDF to a MI mice model. The system was not designed to favor regeneration of the infarcted heart on its own, but indirectly due to the capacity to chemoattract BMSC. SDF-hydrogel increased circulating BMSC number, but further studies are needed to elucidate post MI remodeling using this system (Purcell *et al*., 2012).

4.2 Drug delivery systems for multiple growth factor delivery:

Although DDSs with single GF therapy have shown promising results, normal vasculature was not completely achieved. For instance, it has been demonstrated that VEGF delivery may lead to immature and leaky vasculature with poor function (Yancopoulos *et al.*, 2000). This could be due to the requirement of more than one GF, as in physiological neovascularization mechanisms in which several GFs are implicated. Thus, the combination of more than one therapeutic protein has produced great interest.

An example of DDS for multiple GF delivery is the work of Hao *et al.* They evaluated the angiogenic effect of sequential VEGF and PDGF release from an alginate hydrogel in MI rats. The hydrogel was almost degraded *in vivo* after 4 weeks. At this time point, an increase in capillary density was observed both in the VEGF and VEGF-PDGF hydrogels, while PDGF hydrogel did not modify it. However, the capillary density was

higher in the group treated with VEGF-PDGF hydrogel than VEGF hydrogel. The authors suggested that PDGF could potentiate VEGF action (Hao *et al.*, 2007). In a similar way, the alginate hydrogel prepared by Ruvinov *et al*. sequentially delivered IGF-1 and HGF in a rat MI model, based on the different binding affinity of both GFs to alginate. A pronounced beneficial effect in the infarcted area was observed in the cytokine treated group compared with the control. The system preserved from fibrosis, scar thickness, attenuated infarct expansion and also increased angiogenesis and mature blood vessel formation 4 weeks after its administration (Ruvinov *et al.*, 2011).

More recently, a PEG based protease-degradable hydrogel combining VEGF with HGF demonstrated that dual factor release from a bioactive hydrogel was feasible and had the capacity to significantly improve the cardiac function in a ischemia/reperfusion rat model (Salimath *et al.*, 2012).

Gelatin microspheres have also been used for multiple GF delivery. For instance, this system was employed for VEGF and IGF-1 administration in a rat MI model by Cittadini *et al.* IGFs anti-apoptotic and anti-remodeling actions were boosted by VEGF neoangiogenic effect. Animals treated with microspheres containing both GFs showed remarkably better effects on infarct size and left ventricular volume reductions, heart function improvement, vascularization enhancement and apoptosis and inflammation reduction when compared with single GF microsphere administration (Cittadini *et al.*, 2011).

Kim *et al*. incorporated PDGF and FGF into self-assembling peptide NFs. The system mimicked extracellular matrix porosity and gross structure, which allows cells to reside, migrate and/or differentiate within the fibers. In the rat MI model, animals treated with the system containing both GFs almost recovered cardiac function. This effect correlated with a decrease in cardiomyocyte apoptosis, capillary and arterial density recovery, with stable vessel formation, higher reduction in the infarction size and improvement in wall thickness. Both GFs were detected 1 month after administration, a much longer period of time than after free GF administration (Kim *et al.*, 2011).

Recently our group examined whether the administration of MP containing NRG1 and FGF1 in a rat MI model promoted cardiac regeneration (Formiga *et al*., 2013). 3 months after treatment, cardiac function improvement was observed in rats treated with FGF1-MP, NRG1-MP or FGF1/NRG1-MP in comparison with the control group. Positive cardiac remodeling with smaller infarct size, a lower degree of fibrosis and induction of tissue revascularization was also noticed. Cardiomyocyte proliferation and progenitor

cell recruitment were also detected. Based on NRG1 and FGF1 putative activities, we hypothesized that a combination therapy involving administration of both cytokines would be more beneficial than each individually; however, we did not observe a consistent synergistic effect *in vitro* or *in vivo*. This important observation should be considered when designing new studies involving combination therapies.

4.3 Drug delivery systems for cell therapy:

Combining DDS and cell therapy generates great interest as it is expected to increase cell engraftment and survival after administration. To date, there are two ways to incorporate cells to DDS: cells can be encapsulated or adhered to the DDS surface.

4.3.1 Drug delivery systems with myoblasts:

The first study combining cell therapy with DDS in an animal MI model was performed by Christman *et al.* who seeded skeletal myoblasts in a fibrin glue scaffold. The group treated with myoblasts combined with fibrin glue scaffold presented higher myoblast density within the infarct area and smaller infarct scar size when compared to fibrin glue, cells and PBS groups 5 weeks after administration (Christman *et al*., 2004). Skeletal myoblasts were also seeded and cultured on a biodegradable collagen and Matrigel™ hydrogel by Giraud *et al*. These authors tested the system's effectiveness in a rat MI model. 4 weeks post-implantation they observed that the majority of the cells were washed out from the heart. However, systolic function and neovascularization were improved, presumably due to the cell paracrine effects (Giraud *et al*., 2008). This group also adhere skeletal myoblasts to polyurethane scaffolds. When implanted in a rat MI model, a delay in functional impairment was observed both in the free myoblasts group and in the scaffold group. Nevertheless, it must be noted that DDS use, prolonged the beneficial effect on global heart function. In fact, scaffolds showed vascularization within them, suggesting that they were able to facilitate good nutrient and oxygen cell supply, improving cell viability. However, after 1 year these authors observed that neither of the therapies prevented progression toward heart failure, so the system failed to produce a long-term effect (Giraud et al., 2010).

In another study, Blumenthal *et al*. used polyurethane scaffolds seeded with myoblasts, which were previously genetically modified to augment their paracrine activity in order

to enhance their possible beneficial effect in a rat MI model. After 6 weeks, an angiogenic effect and infarct size reduction were observed, probably due to VEGF, HGF and SDF released from the cells (Blumenthal *et al.*, 2010).

More recently, von Wattenwyl *et al*. seeded skeletal myoblasts overexpressing VEGF on polyurethane scaffolds, and implanted them epicardially in MI rats. The treatment enhanced angiogenesis, although infarction size was not reduced and cardiac function was not improved (von Wattenwyl *et al.*, 2012).

4.3.2 Drug delivery systems with embryonic stem cells:

Regarding ESC, they were seeded in Matrigel™ and studied in a rat MI model. Interestingly, animals treated with ESC-Matrigel™ showed greater improvements in cardiac function and cardiac remodeling after 2 weeks (Kofidis *et al*., 2004). 1 year later, the same group seeded ESCs in a collagen type I matrix and they observed the formation of a stable IM graft into the surrounding infarcted area without distorting myocardial geometry in a rat MI model. This construct was able to prevent wall thinning (Kofidis *et al.*, 2005). The same cell type was also used by the group of Ke *et al*., who grafted them on poly-glycolic acid scaffolds. 8 weeks after administration in a mice MI model, the system improved left ventricular function and reduced scar size. Interestingly, cells presented a higher survival ratio when attached to the scaffold than when freely administered (Ke *et al*., 2005).

In another study, ESCs were seeded onto porous fibrin scaffolds and were injected in the peri-infarct region of rat and swine MI models. First, in the rat MI model, cells were detected 1 month post-injection and a significant improvement in cardiac function was found. In the swine MI model, 4 weeks after injection, infarct size was significantly smaller in the ESC fibrin scaffold group than in controls. Left ventricular contractile function was also improved, as well as angiogenesis processes (Xiong *et al*., 2011).
More recently, a study demonstrated that ESC engraftment via fibrin-based patches represented a promising therapeutic approach to achieve efficient cell implantation in a rat MI model. Authors observed an improvement in global and regional cardiac function (Vallee *et al.*, 2012).

4.3.3 Drug delivery systems with mesenchymal stem cells:

In the last 3 years numerous studies including MSC in DDS have been performed with encouraging results. In a pioneering study MSC were embedded by Simpson *et al*. in collagen patches and epicardially applied in a rat MI model. 1 week after implantation, MSC collagen patchtreated rats showed progenitor cell engraftment increase in all heart regions, but more specifically in the epicardium. At 4 weeks, a significant improvement in full myocardial remodeling and cardiac function was observed. The authors concluded that a marked increase in α-smooth muscle actine positive cell number in patch-treated animals suggests that myofibroblast recruitment and differentiation was promoted (Simpson *et al*., 2007). The same group also performed a study with collagen patches to compare the effect of human MSC with human ESC-derived mesenchymal cells, in a rat MI model. Both cell types incorporated in the patch allowed similar cardiac function and angiogenic response (Simpson *et al*., 2012).

Similar results were obtained by Dai *et al*. in a rat MI model after seeding MSC onto a collagen matrix. They reported an increase in cell retention and survival. A reduction in the relocation of transplanted cells to non-infarcted areas was observed, possibly due to cell adhesion to the matrix interfering with the washout from the infarcted area (Dai *et al.*, 2009).

Jin *et al.* adhered MSC to poly-(lactid-co-Ɛ-caprolactone) (PLCL) scaffolds, an elastic and biodegradable polymer with good cell interaction. The study, performed in a rat MI model, did not show statistical differences in the LVEF compared with the free MSC group. Both groups, MSC-PLCL and MSC, had reduced infarction size compared to the saline group (29% and 18% respectively). Although in the infarction size there were no statistical differences, the expression of cardiac markers (MHC, α-actin, troponin-T) and GATA-4 was significantly greater in the MSC-PLCL scaffold group with respect to free MSC, indicating that the scaffolds favor higher cardiac differentiation (Jin *et al.*, 2009).

In another study, MSCs were encapsulated in RGD-alginate microbeads. The *in vitro* study demonstrated good cell growth and a satisfactory survival rate. The *in vivo* study in a rat acute MI model showed that after 10 weeks in MSC-microspheres and non-loaded microspheres groups there was a significant improvement in the cardiac function and arteriole formation enhancement when compared to free MSC and control groups

(Yu *et al.*, 2010). In the same year Lin *et al*. combined MSCs, with self-assembling peptide NFs. After 28 days of implanting the system in a pig MI model, capillary density increase was observed, accompanied by an increase in cell engraftment and survival. The ability of peptide NFs to provide a suitable microenvironment for MSC adherence and the maintenance of their ability to perform normal cellular function might be responsible for the positive effect. These effects allowed a higher MSC differentiation ratio in endothelial and smooth muscle cells, although not in cardiomyocytes. The study also demonstrated a synergistic effect between NFs and the cells. It is known that MSC significantly increase systolic function, whereas self-assembling peptide NFs increase diastolic function, so as expected their combination improved both functions (Lin *et al.,* 2010). Similar results were obtained by Cui *et al*. who seeded MSCs on other self-assembling peptide NF and proved their efficacy in a rat MI model. After 4 weeks, infarction size reduction and cardiac function improvement were observed in animals treated with MSC-NF when compared to free MSCs (Cui *et al*., 2010).

More recently, MSCs were seeded on collagen type I scaffolds and administered in a rat MI model. Histological examination showed that patches were well integrated in the tissue. After 1 month, global cardiac function and infarction size were improved in the MSC-scaffold group when compared with untreated ones. The MSC-scaffold also produced an increase in angiogenesis when compared to controls (Maureira *et al*., 2012).

Another interesting study was accomplished by Le Visage *et al*. who studied in a rat MI model, MSC incorporation into a polysaccharide-based porous scaffold. After 2 months, engraftment was almost 3 times higher in the MSC-scaffold group than when the cells were endocardially administered. Left ventricular fractional shortening was improved in the MSC-scaffold group when compared to the rest of the groups, possibly due to a paracrine cell effect (Le Visage *et al*., 2012).

4.3.4 Drug delivery systems with bone-marrow derived stem cells:

In 2005, Ryu *et al*. combined BMSCs with a fibrin matrix. This matrix facilitated cell survival until its complete degradation (8 weeks) in a rat MI model. This prolonged cell survival, allowed better heart vascularization, producing a significant increase in microvessel density with larger average of internal diameter, when compared with the free BMSC group (Ryu *et al.*, 2005).

2 years later, BMSCs were seeded in a biodegradable poly-glycolide-co-caprolactone (PGCL) scaffold and tested in a rat MI model by Piao *et al*. After 4 weeks, both BMSC-scaffolds and non-seeded scaffolds showed mechanical properties against progressive left ventricle dilation, suggesting that PGCL acted as a mechanical barrier. However, only BMSC-scaffolds showed an effective neovascularization induction. Interestingly, a portion of the BMSCs seeded on scaffolds exhibited cardiomyocyte differentiation markers (Piao *et al*., 2007).

More recently, BMSC were incorporated into self-assembling peptide NFs by Guo *et al.* and studied in a rat MI model. In one of the animal groups, peptide NF was combined with RGD, showing that the BMSC-RGD-NF group had collagen deposition decrease and higher heart function improvement when compared with free BMSC and BMSC-NF. BMSC-RGD-NF also allowed mature muscle fiber formation and gap junctions with the myocardium. This higher beneficial effect when the NFs were combined with RGD was attributed to RGD's ability to give the cells a temporary three-dimensional NF microenvironment, which favors survival and cardiomyogenic differentiation, having a key role in improving stem cell transplantation efficiency (Guo *et al.*, 2010).

4.3.5 Drug delivery systems with cardiac progenitor stem cells:

The combination of CSC with DDS has not yet been extensively studied. Tokunaga *et al.* analyzed the regenerative properties of different cell populations adhered to Puramatrix™ complex, a self-assembling nanopeptide, in a mouse MI model. The cell populations studied were CSC, BMSC, skeletal myoblasts and ADSC. They observed that the matrix with CSC produced the highest improvement in capillary density, cardiac remodeling and dysfunction prevention, all of which was possibly due to its angiogenic and anti-apoptotic effects. One of the mechanisms by which CSCs produced this benefit was by VEGF secretion, highlighting the importance of the paracrine effect. The other cell types combined with the matrix produced a benefit in the infarcted area, but it was not as pronounced as with the CSC. Despite the promising results obtained, the system still requires further improvements as it was shown that most of the cells were washed out from the treatment area in the first 24 hours (Tokunaga *et al.*, 2010).

4.3.6 Drug delivery systems with adipose-derived stem cells:

In 2010, Danoviz *et al*. evaluated the effects of free ADSCs or ADSCs seeded either into collagen or fibrin scaffolds on cardiac performance in a rat MI model. ADSC on either scaffold, regardless of its composition, retained significantly more cells than the control group 24 hours and 4 weeks after its administration. A significant improvement in cardiac function and cardiac structure was observed 4 weeks post-treatment in the ADSC-scaffold groups with respect to controls (Danoviz *et al*., 2010). In the same year, Zhang *et al*. isolated rat ADSCs and loaded them onto fibrin glue scaffolds, which were then injected in a rat MI model. After 4 weeks, the ADSC-scaffold group showed higher cell retention, significant arteriole density increase and cardiac function improvement compared to free ADSC or to non-loaded scaffold groups. Moreover, animals treated with ADSC-scaffold showed infarct size reduction and higher left ventricular thickness (Zhang *et al*., 2010).

More recently, Araña *et al.* incorporated ADSC into collagen patches with a different cross-linking degree. *In vitro,* ADSC adhered homogenously and showed a similar proliferation ratio in the different collagen patches. However, when collagen patches were tested *in vivo*, only the non-cross-linked one was able to have a complete, long-lasting adhesion 1 month after its injection in a chronic rat MI model. 1 week after ADSC-non-crosslinked collagen patch administration 25.3% of the transplanted cells were detected, whereas no cells were found in animals receiving free ADSC (Araña *et al*., 2013). Efficacy studies are now needed to confirm collagen patch benefit in cardiac repair.

4.4 Tissue engineering

As stated above, the combination of more than one GF is required for mature vessel formation and global cardiac regeneration. This multiple factor effect can be achieved by combining DDS with cells in order to take advantage of its paracrine effect. Moreover, if GF are incorporated into DDS they could promote tissue regeneration and/or potentiate cell regenerative role by increasing cell engraftment, proliferation and survival. The combination of GFs, cells and biomaterials is what is known as tissue engineering. At present, only a few studies have assessed the efficacy of tissue engineering strategies for MI treatment. However, preliminary but very promising results have been obtained.

In the study by Fukuhara *et al*., the authors adhered BMSC into a bFGF loaded polyglycolic acid scaffold impregnated with collagen type I hydrogel and studied them in a rat MI model. Interestingly, the bFGF-BMSC-scaffold group obtained the highest density vessel formation and the best cardiac function, leading to a better improvement with respect to the BMSC-scaffold group, 4 weeks post-treatment. It was thus demonstrated that a further response was obtained when bFGF was present (Fukuhara *et al*., 2005).

More recently, Kang *et al.* used a porous collagen scaffold to incorporate VEGF and FGF in combination with MSC, which was tested in a rat MI model. GFs-MSC-scaffold treated group showed higher angiogenic effect and better cardiac function compared to the MSC-scaffold group. This higher effect could be related to a higher cell survival in the GF-scaffold (Kang *et al*., 2012).

Penna *et al.* encapsulated VEGF in PLGA MP, which were then coated with fibronectin for MSC adhesion, aiming to obtain a good candidate to produce more global heart regeneration. *In vitro* results showed that the system was able to enhance cell proliferation and survival, but further studies are required (Penna *et al*., 2013). Díaz-Herráez *et al.* used PLGA MP for NRG encapsulation. The particles were next coated with collagen and/or poly-D-lysine for ADSC adhesion and studied *in vivo* in a rat MI model. After 2 weeks the systems were well integrated in the peri-infarcted area, indicating that they were biocompatible. This work, although preliminary, has yielded favorable results that require further effectiveness studies (Díaz-Herráez *et al*., 2013).

Finally, there is an ongoing phase I trial named ALCADIA in which human CSC IM injected are being combined with a gelatin hydrogel sheet incorporating bFGF for its controlled release (ClinicalTrials.gov identifier NCT00981006).

5 Drug delivery systems in heart regeneration: limitations and future perspectives:

As it has been discussed in the previous section, numerous studies involving DDS have been designed to tackle MI. Different materials have been used, from materials naturally present in the heart tissue to synthetic acrylic polymers, as shown in table 4. At the moment no evidence has been reported demonstrating the supremacy of one of

these. All DDS allow local delivery. Whereas particles, hydrogels and liposomes can be administered by transendocardial injection, patches and scaffolds need to be attached to the pericardium, so a more invasive administration technique is required. On the other hand, these systems have been proven to be able to contribute more efficiently to the heart mechanical properties.

Table 4: Biomaterials under investigation for drug delivery of proteins and cells in the myocardial infarction context.

MATERIAL	DDS	PROTEINS	CELLS
Chitosan	NP	**PIGF** (Binsalamah *et al*., 2011)	
	Hydrogel	**FGF** (Fujita *et al*., 2005; Wang *et al*., 2010)	
Alginate	NP	**PIGF** (Binsalamah *et al*., 2011)	
	Hydrogel	**VEGF+PDGF** (Hao *et al*., 2007) **IGF+HGF** (Ruvinov *et al*., 2011)	**MSC** (Yu *et al*., 2010)
Hyaluronic acid	Hydrogel	**SDF** (Purcell *et al*., 2012)	
Collagen/ Gelatin	Hydrogel	**FGF** (Sakakibara *et al*., 2003; Fukuhara *et al*., 2005; Shao *et al*., 2006)	**BMSC** (Fukuhara *et al*., 2005) **ESC** (Kofidis *et al*., 2005) **Myoblasts** (Giraud *et al*., 2008)
	Scaffold	**VEGF** (Zhang *et al*., 2009; Miyagi *et al*., 2011) **VEGF+FGF** (Kang et al., 2012)	**MSC** (Simpson *et al*., 2007; Dai *et al*., 2009; Kang *et al*., 2012 ; Simpson *et al*., 2012 ; Maureira *et al*., 2012) **ADSC** (Danoviz *et al*., 2012 ; Araña *et al*., 2013)
	Microsphere	**VEGF+IGF** (Cittadini *et al*., 2011)	
Fibrin	Scaffold		**ESC** (Xiong *et al*., 2011 ; Vallee *et al*., 2012) **ADSC** (Danoviz *et al*., 2010 ; Zhang *et al*., 2010)
	Hydrogel		**Myoblasts** (Christman *et al*., 2004) **BMSC** (Ryu *et al*., 2005) **MSC** (Lisi *et al*., 2012)

PLGA	MP	**VEGF** (Formiga *et al*., 2010; Simón-Yarza *et al*., 2013; Penna *et al*., 2013) **FGF+NRG** (Formiga *et al*., 2013) **NRG** (Díaz-Herráez *et al*., 2013)	**ADSC** (Díaz-Herráez *et al*., 2013) **MSC** (Penna *et al*., 2013)
Peptide nanofibers	Hydrogel	**VEGF** (Guo *et al*., 2012; Lin *et al*., 2012) **PDGF** (Hsieh *et al*., 2006 A and B) **IGF** (Davis *et al*., 2006; Padin-Iruelas *et al*., 2009) **PDGF+FGF** (Kim *et al*., 2011)	**MSC** (Lin *et al*., 2010 ; Cui *et al*., 2010) **BMSC** (Guo *et al*., 2010) **CSC, BMSC, myoblasts, ADSC** (Tokunaga *et al*., 2010)
Acrylic polymers	Hydrogel	**FGF** (Garben *et al*., 2011)	
	Scaffold		**Myoblast** (Giraud *et al*., 2010; Blumenthal *et al*., 2010 ; von Wattenwyl *et al*., 2012)

DDS for delivering GFs and/or cells are showing improvements in infarcted heart regeneration. Nevertheless, there are still many limitations that must be overcome (Fig. 5 summarizes most of them) and further studies are required in this area.

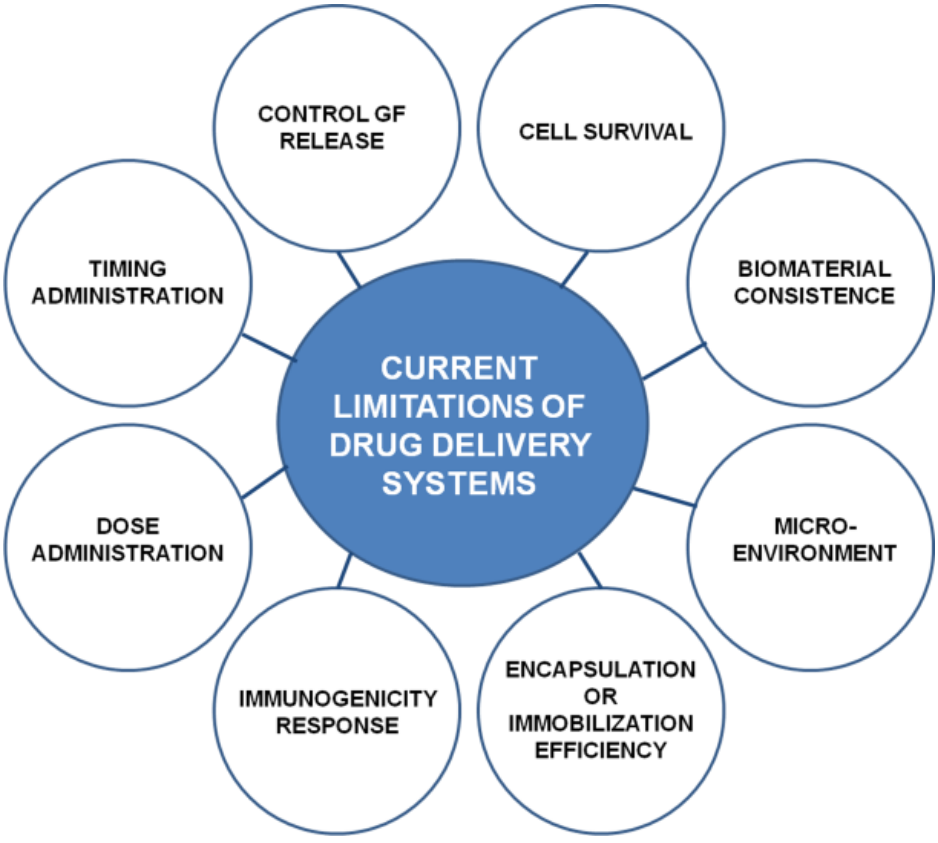

Figure 5: Current limitations of drug delivery systems used for growth factor and/or cell administration in cardiac regeneration.

For instance, one challenge is to obtain systems that can tightly control GF release. Many of the DDS mentioned in the previous section showed a high initial rate of GF delivery, which is known as a burst effect. A strategy to obtain a more sustained release could be to control GF affinity with the biomaterial. For example, as many GFs possess a high affinity with heparin, some *in vitro* and *in vivo* studies have incorporated this molecule in the formulation obtaining a delay in GF release (Cai *et al*., 2005; Huang *et al*., 2007). Controlling biomaterial porosity and/or biodegradation rate could be another way to control GF release. Alginates, for instance, typically present low and uncontrolled degradation. However, partial alginate oxidation or alginate combination with polymers that possess different molecular weights can provide controlled degradation kinetics, allowing better control of incorporated factor release (Hao *et al*., 2007).

Cell survival in the tissue has increased with DDS use. However, cell survival rates need to be ameliorated. In this sense, a better interaction between cells and biomaterials could help for this purpose. As previously mentioned, natural polymers can be easily recognized by the cell-surface receptors. On the other hand, synthetic polymers, as they are hydrophobic and lack cell-recognition moieties, are preferably used in combination with natural polymers or small peptide sequences in order to promote cell-biomaterial interactions (Ravichandran *et al*., 2012). Another way to increase cell interactions with synthetic polymers is by covering their surfaces with biomimetic substances, such as collagen (Lu *et al*., 2007, Hao *et al*., 2008, Qu *et al*., 2009, Díaz-Herráez *et al*., 2013), fibronectin (Garbayo *et al.*, 2011), poly-D-lysine (Lu *et al*., 2006, Lin *et al*., 2009, Díaz-Herráez *et al*., 2013), laminine (Jung *et al*., 2012), and tenascine (Sahoo *et al*., 2010, Chen *et al*., 2012), among others.

Biomaterial consistence is another aspect under investigation. Most of the biomaterials used until now have unmatched mechanical properties with the infarcted myocardium. For example, they are significantly softer than the human cardiac muscle at the end of diastole (Ravichandran *et al.*, 2012). However, this point has generated some controversy between authors since softer biomaterials are more flexible, facilitating normal heart contraction, and harder ones could hinder this function. Thus biomaterials with strength similar to normal human cardiac muscle seem to be the most appropriate.

Furthermore, biomaterials have to improve the generation of a suitable microenvironment that facilitates cell survival and engraftment and new vessel

formation. It has been seen that some hydrogels are formed really fast on the implantation area forming a consistent structure that does not allow the correct oxygen and nutrient entrance inside the system and the surrounding area, while scaffolds with an adequate porosity facilitates it. Self-assembling NFs have also been shown to be able to create NF microenvironments that can promote vascular cell recruitment and cell survival (Davis *et al*., 2005).

Encapsulation efficiency or immobilization rate are other important aspects that have to be optimized since GFs are high cost molecules. Currently, different manufacturing processes and the affinity between GFs and biomaterial are being studied to reduce protein loss during the production process and consequently minimizing global treatment cost. In addition, by increasing the percentage of GF that is encapsulated or immobilized, the quantity of biomaterial administered can be reduced, lowering the possible immunogenic response. Another way to reduce immunogenicity is by increasing surface hydrophilicity with the incorporation of, for example, PEG chains (Simon-Yarza *et al*., 2013).

Dose and timing of administration and the choice of the optimal GFs or cells are also under study. At present, VEGF and BMSC are the GF and cells that are receiving most attention, but further research is required to find out more about the real ischemic heart requirements. A deeper knowledge of the processes implicated in cardiac disorders and cardiac repair will help to establish which GFs are most adequate for each situation and at which time and dose they should be administered.

In conclusion, combining DDS with GF and/or cell therapy can be crucial in increasing the beneficial results in MI regeneration. Regarding these, it is expected that in the next 10-20 years, these therapies will constitute more than half of the new drugs introduced in the market (Tarun *et al.*, 2011). At the moment, more preclinical studies with consistent results are required, in order to proceed to CT. Tomorrow's drugs will definitely be more exciting in terms of the development of delivery systems because, as we have mentioned, these biopharmaceuticals present drug delivery challenges.

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HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

The use of polymeric devices, able to release biological molecules implicated in self-healing and mechanically increase stem cell retention, may potentiate stem cell activity for cardiac repair after myocardial infarction.

OBJECTIVES

The general objective of this thesis is to develop novel tissue engineering strategies based on the combination of polymeric devices, growth factors and stem cells to improve cardiac regeneration after myocardial infarction.

The following partial objectives have been established in order to achieve the general objective:

1. To develop and characterize novel strategies to improve stem cell homing and engraftment based on neuregulin (NRG) releasing microparticles (MP) combined with adipose-derived stem cells (ADSC): ADSC-NRG-MP, and to study their *in vivo* biocompatibility with the infarcted cardiac tissue.

2. To assess the therapeutic efficacy of ADSC-NRG-MP in an acute rat myocardial infarction model.

3. To design, develop and characterize injectable Dextran-Hyaluronic acid hydrogel embedding ADSC and NRG-MP for cardiac tissue engineering.

CHAPTER 1

ADIPOSE-DERIVED STEM CELLS COMBINED WITH NEUREGULIN-1 DELIVERY SYSTEMS FOR HEART TISSUE ENGINEERING

Chapter 1:

Adipose-derived stem cells combined with Neuregulin-1 delivery systems for heart tissue engineering

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Abstract:

Myocardial infarction (MI) is the leading cause of death worldwide and extensive research has therefore been performed to find a cure. Neuregulin-1 (NRG) is a growth factor involved in cardiac repair after MI. We previously described how biocompatible and biodegradable microparticles, which are able to release NRG in a sustained manner, represent a valuable approach to avoid problems related to the short half-life after systemic administration of proteins. The effectiveness of this strategy could be improved by combining NRG with several cytokines involved in cardiac regeneration. The present study investigates the potential feasibility of using NRG-releasing particle scaffold combined with adipose-derived stem cells (ADSC) as a multiple growth factor delivery-based tissue engineering strategy for implantation in the infarcted myocardium. NRG-releasing particle scaffolds with a suitable size for intramyocardial implantation were prepared by TROMS. Next, ADSC were adhered to particle scaffolds and their potential for heart administration was assessed in a MI rat model. NRG was successfully encapsulated reaching encapsulation efficiencies of 92.58 ± 3.84 %. NRG maintained its biological activity after the microencapsulation process. ADSC cells adhered efficiently to particle scaffolds within a few hours. The ADSC-cytokine delivery system developed proved to be compatible with intramyocardial administration in terms of injectability through a 23-gauge needle and tissue response. Interestingly, ADSC-scaffolds were present in the peri-infarted tissue two weeks after implantation. This proof of concept study provides important evidence required for future effectiveness studies and for the translation of this approach.

Keywords: Particle scaffold, PLGA Microparticles, ADSC, NRG-1, Myocardial infarction, Cardiac repair

1 Introduction:

Cardiovascular diseases cause more than 17 million deaths each year according to the latest report of the World Health Organization (available in http://www.who.int/cardiovascular_diseases), constituting the greatest health risk in western countries [1]. Despite the advances in pharmacological treatment, a major improvement able to repair the massive loss of cardiomyocytes after a myocardial infarction (MI) has not yet been reached, being heart transplantation the only real option for severe cases. Due to this situation new approaches have been explored in the last few years [2-5]. One of these strategies is the use of growth factors (GF). GF are thought to benefit the damaged heart through direct effects in the myocardium and by stimulating and mobilizing progenitor cells [6]. However, GF administration presents serious limitations due to the short *in vivo* half-life, physical and chemical instability, and the low oral bioavailability of these macromolecules [7]. The use of drug delivery systems (DDS) that encapsulate GF might overcome these drawbacks. Microparticles (MP), one of these DDS, could protect GF from degradation and ensure sustained release among time [8]. Recently, our group explored new therapeutic strategies for MI treatment, based on the use of polymeric MP that release different GF involved in cardiac angiogenesis and neovascularization [8-11]. Neuregulin-1 (NRG) deserves special attention in heart regeneration because it is involved in cardiac repair after MI [12]. This protein plays a crucial role in the adult cardiovascular system by inducing sarcomere membrane organization and integrity [13], cell survival [14, 15] and angiogenesis [16]. We recently proved that NRG-releasing MP promoted cardiac repair and improved cardiac performance [11]. NRG-releasing MP effectiveness could be improved by combining this protein with several other GFs involved in cardiac regeneration. This could be achieved by preparing a polymer-based GF delivery system that allows the release of multiple factors [6]. However, to date GF delivery systems have not demonstrated the ability to deliver cocktail of factors with distinct kinetics [17]. This aspect, besides the limitation that GF dose and timing are crucial for helping regeneration, makes it difficult to co-administer different GFs [6, 7]. The combination of NRG-releasing MP with GFs secreted by stem cells (SC), capable of responding to the host environment, opens up a possible solution to that drawback. Moreover, MPs possess many features that make them suitable to be used as cardiac scaffolds. In

particular, they are biodegradable, biocompatible, non-toxic and, importantly, they can provide structural support for cell survival and differentiation [18-24].

Among the different SC sources, adipose-derived stem cells (ADSC) have shown promising results in cardiac repair [25-28]. They are good candidates for cell therapy studies because of their easy isolation from the stromal vascular fraction [29-32] and their extensive differentiation potential. In addition, ADSCs are able to secrete angiogenic and/or anti-apoptotic factors [33], such as granulocyte-macrophage colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β) [31].

For all of these reasons, the primary purpose of this work was to investigate the potential feasibility of NRG-releasing particle scaffold combined with ADSC as a multi GF delivery-based tissue engineering strategy for the ischemic heart. To this end, NRG-releasing delivery system was prepared using Total Recirculation One-Machine System (TROMS), a technique based on the multiple emulsion solvent evaporation method which is suitable for the encapsulation of labile molecules like cytokines and GFs [8, 34]. We primarily investigated the physical characteristics of the particle scaffold such as morphology or size. Then, NRG-releasing particle scaffolds were combined with ADSC and flow properties such as dispersability and injectability of the ADSC particle scaffold suspension were analyzed to avoid complications during their administration. The myocardial response to ADSC combined with NRG-releasing particle scaffold was finally evaluated using a MI rat model to ensure safety and biocompatibility requirements.

2 Material and Methods:

2.1 Materials

Poly(lactic-co-glycolic acid) (PLGA) with monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (Mw: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; Mw: 400), human serum albumin (HAS), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), carboxymethyl-cellulose, mannitol, polysorbate 80, sodium azide and rhodamine B isothiocyanate were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain).

Poly(vinylalcohol) (PVA) 88% hydrolyzed (Mw: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Collagen type I of rat tail 3 mg/mL, Minimum Essential Medium Alpha (α-MEM) Medium, 0.05% Trypsin-EDTA, Heat inactivated Fetal Bovine Serum (FBS), Phosphate Buffered Saline pH 7.2 (PBS) and Dulbecco´s Modified Eagle Medium (DMEM) were provided by Gibco-Invitrogen (Carlsbad, CA, USA). ADSC cells were obtained from inguinal adipose tissue of male Sprague-Dawley transgenic rats. H9c2 cells were provided by ATCC. Poly-D-Lysine 1 mg/ml (PDL) was provided by Merck-Millipore (Darmstadt, Germany). rh Neuregulin-1b-iso was provided by EuroBioSciences (Friesoythe, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, USA). Goat polyclonal anti-human NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Preparation of NRG-releasing particle scaffold

NRG-releasing PLGA particle scaffolds were prepared by the emulsion solvent evaporation method using TROMS as previously described [11] with minor modifications. In order to obtain batches with the defined particle size the following TROMS parameters were adjusted: pumping flow, recirculation times to obtain both W_1/O and $W_1/O/W_2$ emulsions, and inner diameters of the needles used to prepare the emulsions. Briefly, the organic phase (O) composed of 100 mg of PLGA dissolved in 4 ml of a dichloromethane/acetone mixture (ratio 3:1) was injected into the inner aqueous phase (W_1) containing 200 μg of NRG, 5 mg of HSA and 5 μl of PEG 400 dissolved in 200 μl of phosphate-buffered saline (PBS pH 7.9). Next, the inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow through a needle. After this homogenization step, the W_1/O emulsion was injected into the outer aqueous phase (W_2) composed of 20 ml of a 0.5% w/v PVA solution. The turbulent injection through a second needle resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. Particle scaffolds were washed three times with ultrapure water by consecutive centrifugations at 4 °C (20,000×g, 10 min). NRG-releasing particle scaffolds were lyophilized for 48 h without cryoprotective agents (Virtis Genesis 12 EL, Gardines,

NY). The conditions of freeze drier were -50 ºC to +15 ºC over 2 days. After complete lyophilization, the vials were sealed under vacuum and stored at -20 °C until use. Unloaded particle scaffolds were prepared in the same manner without adding NRG. For fluorescence-labeled formulation, rhodamine B isothiocyanate (0.5 mg/mL) was added to the inner aqueous phase and particle scaffolds were prepared as described.

2.3 NRG-releasing PLGA particle scaffold characterization

2.3.1. Particle size analysis

The mean particle size and size distribution were examined by laser diffractometry using a Mastersizer® (Malvern Instruments, Malvern, UK). Particle scaffolds were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers.

2.3.2. Drug content

The amount of NRG encapsulated in the particle scaffold was determined by dissolving 0.5 mg of lyophilized loaded particles in 25 µL of DMSO, and was quantified using western blot. After electrophoresis and transference, the membranes were blocked with 5% nonfat dried milk in TBS plus 0.05% Tween 20 for 2 h, then incubated overnight at 4ºC with primary antibody goat IgG-NRG-1β-IGGF2 (sc-1793) 1:50. After several washes the membranes incubated with antigoat IG-HRP (sc-2020) 1:2000 secondary antibody for 2 h. Immunoreactive bands were, after several washes, visualized using LumiLight plus western blotting substrate (Roche Diagnostics, Mannheim, Germany). The quantifications were determined by ImageQuant RT ECL. Sample values were quantified using a blotting standard curve with known amounts of NRG.

2.3.3. In vitro bioactivity assay

The bioactivity of NRG released from particle scaffolds was evaluated *in vitro* by determining the proliferative capacity of H9c2 cells after NRG treatment. H9c2 cells obtained from embryonic BD1X rat heart tissue were cultured in DMEM medium supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin at 37 °C under 5% CO_2/95% air [35-37]. Cells were subcultured when 60% confluency was achieved. In order to quantify cell proliferation after NRG stimulation, cells were

seeded in 96-well tissue culture plates at a density of 2×10^3 cells/well. After 24 h, medium was removed and the cells were incubated with 150 ng/mL of NRG released from particle scaffolds over 24 h, which had previously been quantified by western blot, with 150 ng/mL of free NRG or medium alone as control. Culture medium supplementation was modified for these experiments by reducing the FBS to 5% in the culture medium. Treatments were removed every day, and fresh treatment was added to the cells. After three days of treatments, the number of viable cells was determined by MTS assay. Results were statistically analyzed with GraphPad Prism 5, employing the ANOVA and Tukey tests.

2.4 Isolation and culture of ADSC cells

ADSC cells were obtained by *in vitro* culture of the stromal vascular fraction (SVF) isolated from inguinal adipose tissue of male Sprague-Dawley transgenical rats that expressed the green fluorescent protein (GFP), as previously described [38]. ADSC cells were cultured in α-MEM medium supplemented with 10% FBS, 1 ng/mL bFGF and 1% penicillin/streptomycin. Cells were subcultured when 80% confluence was reached.

2.5 Adhesion of ADSC cells to particle scaffold

To favor cell attachment to the MP surface, particle scaffolds were overlaid with 0.5 μg/cm^2 of type I collagen and/or PDL. Particle scaffold coating was performed in 15 mL falcon tubes. Scaffolds were re-suspended in DPBS and the mixture was sonicated until the particles were completely dispersed in the liquid. Then, coating solutions were added to the falcon tube and mixed with the particles under rotation at 37 ºC for 2 h. Coated particle scaffolds were washed 3 times with distilled sterile water and lyophilized for long term storage [18]. For ADSC adhesion, coated MP were resuspended with complete α-MEM medium, and were ultrasounded and briefly vortexed prior to addition of 2,5 X 10^5 or 5 X 10^5 cells. The mixture was then gently flushed and plated in Costar® Ultra Low Cluster Flat Bottom Sterile Polystyrene Plate. Plates were incubated at 37 ºC for 4 h. At different times cells were observed to study the evolution of the adhesion.

2.6 Determination of dispersability and injectability of ADSC adhering to particle scaffold

Particles with adhered ADSC cells were dispersed in two autoclave sterile resuspension medium consisting of: (1) 0.1% (w/v) carboxymethyl-cellulose, 0.8% (w/v) polysorbate 80 and 0.8% (w/v) mannitol in PBS, pH 7.4 and (2) 0.4% (w/v) carboxymethyl-cellulose, 3.2% (w/v) polysorbate 80 and 3.2% (w/v) mannitol in PBS, pH 7.4. The suspension injectability was assessed by the ability of the particles combined with ADSC to pass through different needles (23, 24, 25, 27 and 29 gauge (G) needles).

2.7 In vivo studies using chronic myocardial infarction model

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC.

2.7.1 Induction of myocardial infarction

A total of 26 female Sprague-Dawley rats (Harlan-IBERICA, Spain) underwent permanent occlusion of the left anterior descending coronary artery, as previously described [11]. Briefly, rats were anesthetized with 4% isoflurane in an induction chamber and supported with a mechanical ventilator. Prior to surgery, animals received analgesic drug ketoprofen 5 mg/Kg subcutaneously. The rats were then intubated and 1.5–2% isoflurane was maintained for continuous anesthesia. The heart was accessed through a left thoracotomy through the fourth intercostal space, and the left anterior descending coronary artery was permanently occluded 2–3 mm distal from its origin. The chest was then closed in layers and rats allowed to recover on a heating pad.

2.7.2 Intramyocardial implantation

Seven days post-myocardial infarction, rats were placed into the following groups: (*Group 1*) 2.5 x 10^5 ADSCs-0.75 mg of particle scaffold coated with 0.5 µg/cm^2 of collagen in 100 µL of resuspension medium, (*Group 2*) 5 x 10^5 ADSCs-0.75 mg of particle scaffold coated with 0.5 µg/cm^2 of collagen in 100 µL of resuspension medium, (*Group 3*) 5 x 10^5 ADSCs-0.75 mg of particle scaffold coated with 0.5 µg/cm^2 of mixture of collagen and PDL (1:1) in 100 µL of resuspension medium, (*Group 4*) 5

x 10^5 ADSCs-0.75 mg of NRG-releasing particle scaffold coated with 0.5 µg/cm^2 of mixture of collagen and PDL (1:1) in 100 µL of resuspension medium and (*Group 5*) 100 µL of resuspension medium. Two animals in groups 1, 2 and 3 were injected with rhodamine B fluorescent particle scaffolds to visualize particles by fluorescent microscopy. All the treatments were injected in 4 sites of the border surrounding the infarct zone using a 23 G needle. After treatment injection, the chest was closed and rats were allowed to recover on a heating pad.

2.7.3 Histological assessment of myocardial tissue after treatment implantation

Two weeks after treatment implantation, animals were sacrificed and hearts were collected for histology. After being harvested, the hearts were perfused-fixed in 4% paraformaldehyde at 4 °C, and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4 °C, embedded in paraffin and 5-µm-sections were cut. Hematoxylin–eosin (HE) staining was performed to visualize tissue structure and to study tissue retention of implanted treatment.

2.7.4 Verification of NRG-releasing particle scaffold retention in the infarcted tissue and ADSC cell fate

Fluorescence microscopy was used to evaluate tissue retention of rhodamine particle scaffold and the fate of the ADSCs cells.

3 Results and Discussion:

3.1 Characterization of NRG-releasing particle scaffold prepared by TROMS

3.1.1 Particle size

NRG-releasing particle scaffolds were successfully prepared by $W_1/O/W_2$ emulsion/extraction method using TROMS technology. The mean particle size measured by laser diffractometry was distributed around a mean size of 20 ± 5 µm. As can be seen in Fig. 1.A, particle scaffolds size distribution confirms that TROMS allows the obtention of reproducible batches of MP of a desired particle size.

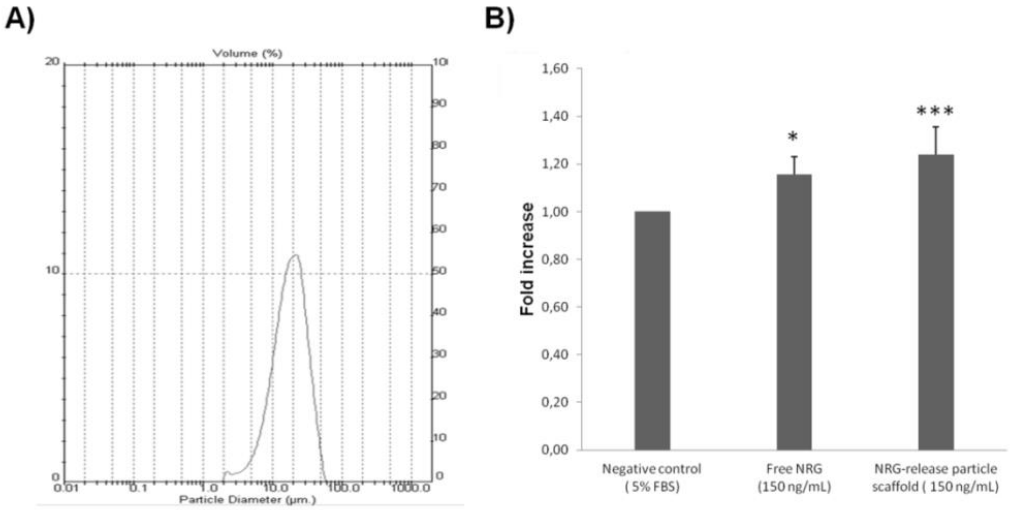

Figure 1: NRG-releasing particle scaffold characterization. A) Representative particle size distribution measured by laser difractometry of NRG-releasing particle scaffold prepared by TROMS. B) H9c2 proliferation induced by NRG stimulation (free or released from particle scaffold at 150 mg/mL) (*y axis* represents fold increase *vs* negative control). *p< 0.05, ***p< 0.001

MP with a size of 20 µm were selected as scaffolds for ADSCs. The particle size was chosen on the basis of previous research by our group in cardiac regeneration [8, 9, 11], where particles with different size were prepared and characterized to select the most suitable size for intramyocardial administration of GF [9]. In the present paper a slight modification in the particle size was made considering a balance between having sufficient surface for cell adhesion and not causing damage to the heart. The best device settings to obtain 20 µm particles were: 20 mL/min as pumping flow, 90 s and 60 s as recirculation times for obtaining homogeneous W_1/O and $W_1/O/W_2$ emulsions respectively and 0.17 mm and 0.5 mm as needles inner diameter of needles also for respectively W_1/O and $W_1/O/W_2$ emulsion formation.

3.1.2 Encapsulation efficiencies

NRG was efficiently encapsulated in 20 µm particle scaffolds reaching encapsulation efficiency values of 92.58 ± 3.84%, which corresponds to a final loading of 1851.50 ± 38 ng of NRG per mg of polymer. This amount of loaded NRG is suitable for *in vivo* studies [11]. Modifications in the preparation process resulted in 25% higher encapsulation efficiencies than those prepared before. The high NRG entrapment achieved could be related with the encapsulation method and with the use of TROMS to prepare the multiple emulsion system. On the one hand, the water/oil/water evaporation technique has been described to result in extremely efficient loading of biodegradable

microparticles with water soluble compounds [39, 40] and, on the other hand, higher encapsulation efficiencies were found using TROMS compare to conventional encapsulation techniques. For instance, an encapsulation efficiency of 83.8% was achieved for VEGF using TROMS [8]. In contrast, King *et al*. reported an entrapment efficiency of 16% of VEGF in PLGA-MP, employing the solid/single emulsion/ solvent extraction technique [41].

NRG is a GF involved in cardiac repair after MI that deserves special attention in heart regeneration [12]. Multiple *in vivo* studies have established the therapeutic potential of this GF after MI. NRG administration after myocardial injury improved systolic function, reduced infarct size and attenuated myocardial hypertrophy in small and large animal models of MI [12]. Its beneficial effects might be due to myocyte protection from death stimuli and through repair of dysfunctional cardiac myocytes [12]. Several clinical trials to evaluate the effect of NRG in humans are ongoing. However, GF therapy efficacy is generally hindered for the short plasma half live, gastrointestinal tract instability and their low bioavailability. Thus, the development of a DDS able to release NRG in a sustained manner would improve its potential and efficacy.

3.1.3 *In vitro* bioactivity assay

The bioactivity of encapsulated NRG released from the particle scaffolds was evaluated *in vitro* by determining its capacity to induce H9c2 proliferation (Fig. 1.B). The daily addition of NRG released from particle scaffolds (150 ng/mL) induced a statistically significant 1.24 fold increase in the proliferation of H9c2 in comparison with control (culture medium without GF) after 3-day treatment. The increase was similar to that observed when H9c2 cells were cultured with the daily addition of free NRG for 3 days at doses of 150 ng/mL indicating that NRG biological activity was maintained after encapsulation and release from particle scaffolds prepared by TROMS.

3.2 Adhesion of ADSC to NRG-releasing particle scaffold

The development of a DDS able to protect NRG from degradation and to ensure its sustained release throughout time would reinforce NRG efficacy in cardiac repair as it was mentioned before in this section. Moreover, in the present work we move one step forward combining NRG DDS with ADSC able to secrete multiple GFs involved in cardiac regeneration. Interestingly, this is the first report of NRG-releasing scaffold for

cardiac tissue engineering applications. The importance of multiple GF action in cardiac tissue regeneration has been extensively described [19, 21, 42]. In this regard, the possibility of increasing the beneficial effect obtained with NRG releasing particles combining this DDS with several other GFs secreted by SC would open up new possibilities in heart regeneration. Among SC, ADSC are particularly suitable for cell therapy because of their easy isolation from the SVF [29-32], their extensive differentiation potential and the secretion of several angiogenic and anti-apoptotic factors that activate the revascularization process and the positive remodeling of the heart [31, 33]. Although it is known that ADSC exert their positive effect via paracrine secretion, the beneficial factors remain partly unidentified. Moreover, it is possible that multiple factors might be functioning synergistically [43-45]. For that reason, ADSC transplantation for their paracrine effects still represents a reasonable strategy. In addition, SC are able to sense and respond to changes in the host environment modifying its paracrine secretion. Moreover, until now, NRG secretion by ADSC has not been described, meaning that the association of these two strategies might have complementary effects on cardiac tissue repair.

In the present study, the adhesion of 2,5 X 10^5 or 5 X 10^5 ADSC to particle scaffolds coated with different concentrations of collagen and PDL was studied. The concentration that showed better adherence was 0.5 $\mu g/cm^2$, either with the collagen or the mixture of collagen and PDL (1:1). The administration of these amounts of cells induced an improvement in the cardiac function when administered in combination with collagen-based carrier sheets after MI [26]. On the other hand, the dose of NRG administered with that quantity of MP also promoted cardiac repair and improved cardiac performance.

Both cell densities adhered efficiently to all particle scaffolds assayed. Observations of cell adhesion over time indicated that collagen coated particle scaffolds required 4 h for total cell attachment, while particle scaffolds coated with collagen and PDL attached to the cells after 2 h (Fig. 2). Differences in cell adhesion time might be due to collagen and PDL net charge at pH value of the culture medium used for adhesion (pH 7.2). Thus, collagen net charge at pH 7.2 is negative due to its isoelectric point of 5.5, hindering cell adhesion. On the other hand, PDL with an isoelectric point of 12.9, has a net charge positive at pH 7.2 that favors the adhesion of the cells. As in the coating and adherence processes a certain amount of encapsulated NRG is released over time, the less time required for ADSC NRG-particle scaffold preparation, the less amount of

NRG would be lost during the manufacture process.

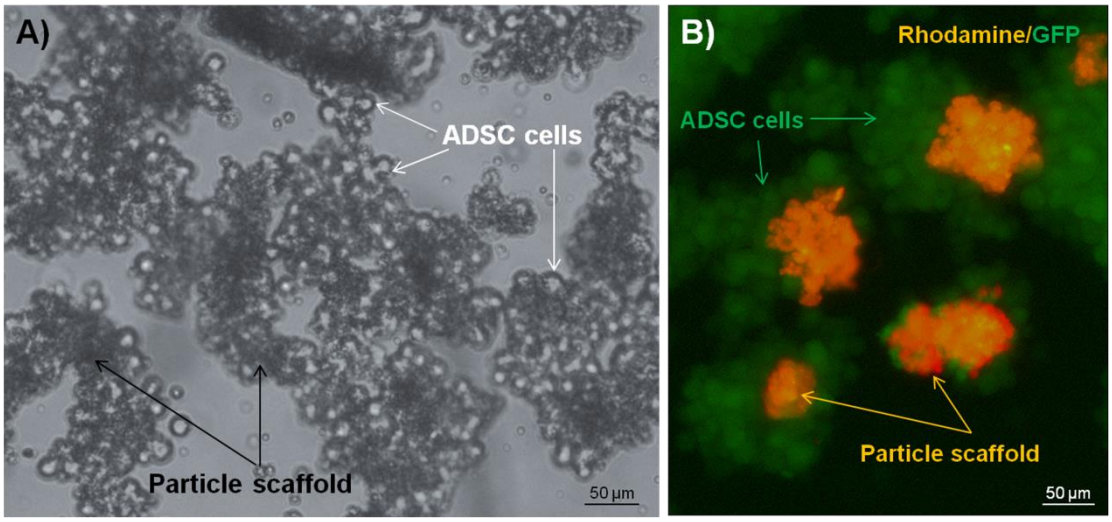

Figure 2: Representative images showing (A) bright field and (B) fluorescence images of ADSC combined with NRG-releasing particle scaffolds shortly before intramyocardial implantation in the peri-infart area. Scale bars: 50 µm.

The use of biodegradable microparticles as injectable scaffolds for tissue engineering applications was first proposed by Montero-Menei C. N. and colleagues [46, 47]. After that, several authors investigated their potential to improve cell survival and differentiation [18-20, 22-24, 48]. The differentiation of ADSC towards a cardiac lineage is, however, not the main objective of the present research. ADSC cells were combined with NRG delivery systems for their beneficial paracrine effect on endogenous cells. Thus, a multidrug delivery system able to respond to the surrounding tissue would be obtained.

3.3 Dispersability and injectability of NRG-releasing particle scaffold combined with ADSC

The injection of ADSC-particle scaffolds in the infarcted heart required the selection of a good dispersing medium that allows the cell-particle scaffold suspension to pass through a needle without needle blockage or sedimentation. A cell-particle scaffold suspension with good rheological properties will ensure dose uniformity and safety requirements during the local myocardial injection of the treatments. Two dispersing media containing different percentages of DMEM, carboxymethyl-cellulose, polysorbate 80 and mannitol were investigated in the present study. These excipients are

included in the Handbook of Pharmaceutical Excipients [49] and are frequently used in commercial formulations. DMEM is a solution commonly used for drug/cell injection into the infarcted heart [50, 51], carboxymethyl-cellulose is a wetting and biocompatible agent that prevent particle aggregation and makes their injection through a thin needle and polysorbate 80 and mannitol has been previously used to suspend PLGA microparticles prior to intracerebral implantation [34]. The ADSC particle scaffold suspension showed the best flow properties in the dispersing medium containing 0.4% (w/v) carboxymethyl-cellulose, 3.2% (w/v) polysorbate 80 and 3.2% (w/v) mannitol in PBS, pH 7.4. No toxicity or cell detachment was observed when using this medium.

Prior to injection in the infarcted myocardium, the injectability of the cell-particle scaffold suspension was analyzed. To this end, ADSCs adhered to 20 µm particle scaffolds were delivered through needles with different diameter (23, 24, 25, 27 and 29 G). ADSC particle scaffolds were only able to go through the 23G needle without blocking or sedimentation, and carrying ADSC cells adhered on the surface. This needle was therefore used for intramyocardial administration (Fig. 3).

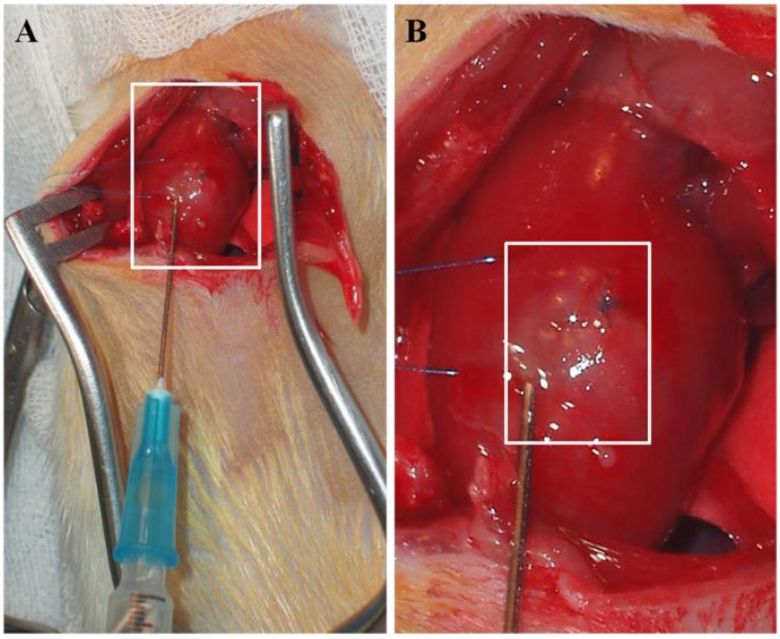

Figure 3: Macroscopic view of the infarcted heart following ADSC combined with NRG-releasing particle scaffold implantation. Seven days after left anterior descending coronary artery occlusion, ADSC combined with NRG-releasing particle scaffolds were injected into the peri-infarct zone through a 1 mL insulin syringe with a 23-G needle while the heart was beating. Note the presence of the ADSC-scaffold in the beating heart demonstrating that ADSC-scaffolds were not washed out from the infarcted myocardium.

3.4 Histological evaluation of myocardial tissue after the injection of NRG-releasing particle scaffold combined with ADSC

Finally, ADSC combined with empty or GF delivery systems coated with collagen or collagen:PDL were intramyocardially injected in the infarcted beating heart (Fig. 3). Two weeks later, animals were sacrificed to further evaluate myocardial tissue reaction and the non-toxic properties of the implanted scaffold *in vivo*. Upon implantation, HE staining revealed that ADSC particle scaffolds were well tolerated by the infarcted myocardium and they seem to integrate well within the host tissue (Fig. 4 C,D). Heart response after ADSC-scaffold injection was the typical reaction observed following mechanical trauma and exposure to a foreign body. ADSC-scaffolds (Fig. 4 C,D) did not induce inflammatory reactions when compared to resuspension medium injection (Fig. 4 A,B). HE staining did not evidence noticeable differences in terms of biocompatibility and local tolerance between groups. Moreover, the tissue adjacent to the implanted treatments maintained its physiological characteristics and no adverse cellular reactions were observed.

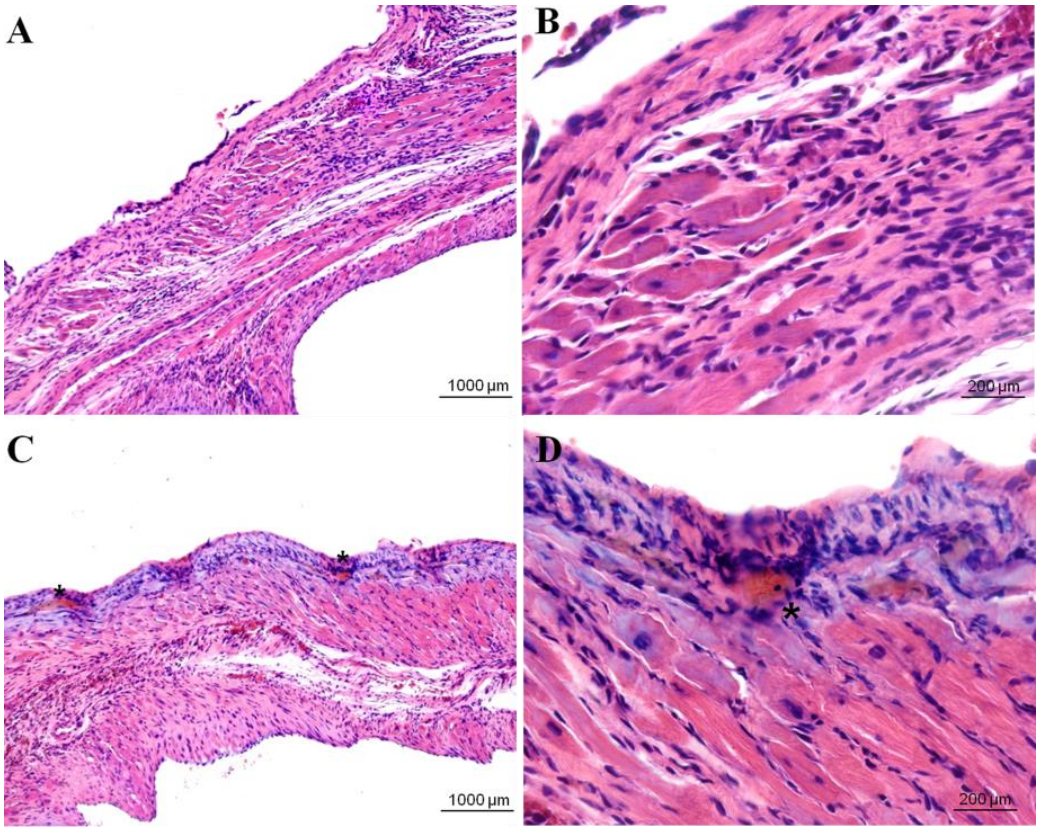

Figure 4: Histological evaluation of myocardial tissue reaction 14 days after ADSC combined with NRG-releasing particle scaffold administration in HE stained sections. The administration of ADSC combined with NRG-releasing particle scaffold was well tolerated by the tissue and no differences in tissue inflammation were found between the administration of medium (A, B) or ADSC combined with NRG-releasing particle scaffold (C, D). At higher magnifications, the ADSC-scaffold (indicated by asterisk) were much more clearly visualized (D). Scale bars: 1000 µm (A, C) and 200 µm (B, D).

3.5 Confirmation of NRG-releasing particle scaffold retention in the infarcted heart and ADSC cell fate

Fluorescent and brightfield microscopy showed that two weeks after intramyocardial implantation, particle scaffolds appeared grouped at the implantation site independently of the coating used to attach ADSC (Fig. 4 C,D and 5 A,B). At day 14, particle scaffolds were not totally biodegraded and a significant quantity of them were detectable. No differences in terms of scaffold degradation were observed among the various groups during the two-week implantation period. As can be seen in Figure 4 C,D and 5 A,B, counterstaining of nuclei revealed that particle scaffolds were always surrounded by cells suggesting that ADSC remained attached to the particle scaffold.

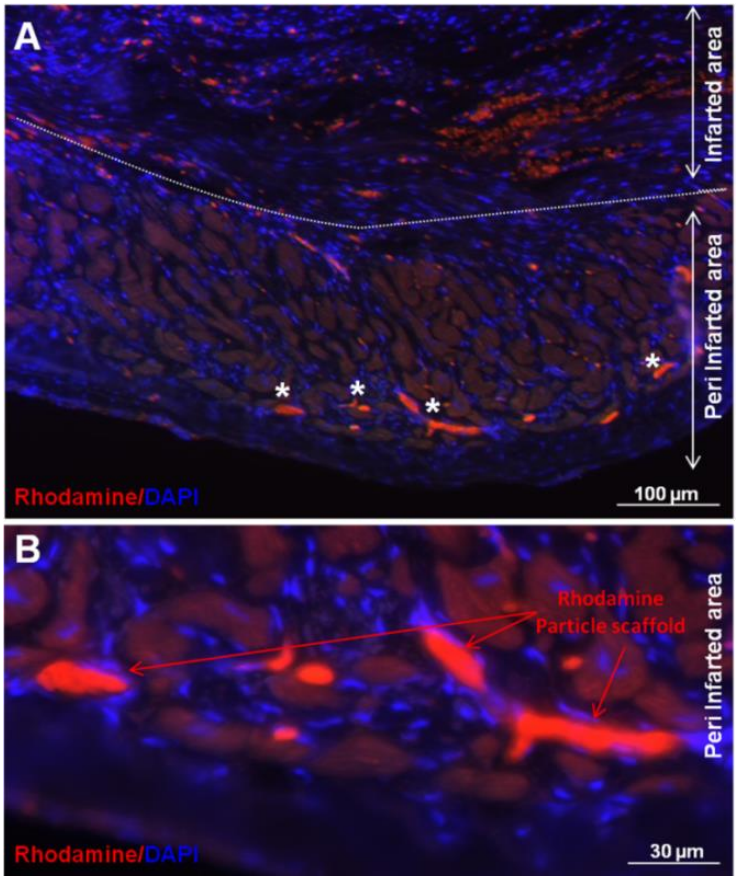

Figure 5: ADSC-scaffold visualization in the heart tissue. ADSC combined with NRG-releasing particle scaffold (indicated by asterisk) were clearly visualized in the peri-infart area that encompassed the infart zone on day 14 after implantation by fluorescence microscopy. Nuclear staining was performed with DAPI (blue). Scale bars: 100 µm (A) and 30 µm (B).

Taking together our *in vitro* ADSC adhesion studies and our present *in vivo* biocompatibility findings on infarcted rats, 5 X 10^5 ADSC cells combined with NRG-releasing particle scaffolds coated with collagen and PDL will be selected to further assess the therapeutic potential of this strategy in cardiac regeneration.

4 Conclusion:

The data presented is this article offer valuable evidence of the feasibility of using NRG-releasing particle scaffolds combined with ADSC as a multi GF delivery-based tissue engineering strategy to treat the ischemic heart. Future studies will be focused on the response produced by the treatment, to demonstrate whether the combination of ADSC with NRG and using particle scaffolds as support is helpful in the regeneration of the infarcted heart.

Acknowledgments

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CHAPTER 2

TRANSPLANTATION OF ADIPOSE-DERIVED STEM CELLS COMBINED WITH NEUREGULIN-MICROPARTICLES PROMOTES EFFICIENT CARDIAC REPAIR IN A RAT MYOCARDIAL INFARCTION MODEL

Transplantation of Adipose-Derived Stem Cells combined with Neuregulin-Microparticles promotes efficient cardiac repair in a rat myocardial infarction model

Chapter 2:

TRANSPLANTATION OF ADIPOSE-DERIVED STEM CELLS COMBINED WITH NEUREGULIN-MICROPARTICLES PROMOTES EFFICIENT CARDIAC REPAIR IN A RAT MYOCARDIAL INFARCTION MODEL

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Submitted

Abstract

Tissue engineering (TE) is a promising strategy to promote heart regeneration after myocardial infarction (MI). In a previous study, we developed a system that combined adipose-derived stem cells (ADSC) with microparticles (MP) loaded with neuregulin (NRG), named ADSC-NRG-MP, which was biocompatible with the infarcted heart tissue. In the present paper we investigate its beneficial effect on a rat MI model. MPs coated with a 1:1 mixture of 0.5 μg/cm^2 of collagen and poly-D-lysine (PDL) were able to adhere 500,000 cells in 60 minutes. In the *in vivo* study, an increase in cell engraftment was induced when ADSCs were adhered to the MPs, and was detectable up to three months after administration. Better tissue repair was observed in the animals treated with ADSC-MP and ADSC-NRG-MP, which presented thicker left ventricles. Moreover, the presence of NRG in the system promoted a more complete regeneration, since a smaller infarct size was observed. Regarding vasculogenesis, all the groups stimulated vessel formation when compared to the control group. However, it was seen that only when the ADSC were administered adhered to the MP, they were incorporated to newly formed vessels. Also, the ADSC-NRG-MP group proved to be the most favorable system since it promoted more formation of capillaries. Finally, the presence of MPs and ADSC favored the shift of macrophage expression from a pro-inflammatory to a regenerative cardiac response. Collectively these findings suggest that the combination of ADSC, NRG and MP favored a synergy for inducing a greater and more complete improvement in heart regeneration.

Keywords: Tissue engineering, Cardiac repair, Microparticles, Stem cells, Growth factor

1 Introduction:

Tissue engineering (TE) is a promising strategy for the regeneration of damaged tissues. The combination of stem cells (SC) and growth factors (GF) with a biomaterial scaffold [1] has been demonstrated to protect GF from fast degradation [2] and to provide a three-dimensional support that favors cell engraftment and survival [3]. Moreover, the combination of GF and SC increases the possibility of improving the activation of different pathways to promote tissue repair [4,5]. All this considered, TE seems to be a promising therapy in heart damage [6–9]. Within the cardiovascular diseases, myocardial infarction (MI) is the most frequent, causing thousands of deaths per year worldwide according to the World Health Organization [10]. After a MI, the heart is dramatically damaged and, to date, there is no treatment available to repair or reduce the massive loss of cardiomyocytes. Accumulative research evidence indicates beneficial effects of adipose-derived stem cells (ADSC) for treating MI in both animal models and humans, even though low cell survival and engraftment have been observed [11–13]. ADSC are easily isolated from adipose tissue, grow fast in culture media and share common properties with bone marrow stem cells in terms of multipotency and immunoregulatory properties [14–17]. ADSC implantation participates in the repair of the damaged cardiac muscle by inducing angiogenesis, mainly due to the paracrine effect of ADSC in the infarcted area [18,19]. These adult SCs are able to secrete various GFs, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), among others [6,20]. As the secretion of GFs by cells is regulated by tissue microenvironment signals, the concentrations of secreted GF are in the physiological range and can be adapted according to the requirements of the different stages of heart healing [21]. Although some studies have already demonstrated the efficacy of ADSC in cardiac regeneration [11,22], the possibility of combining these with GFs different from those secreted by those cells, such as neuregulin-1 (NRG), may induce a better regenerative response. NRG is a GF that plays a crucial role in the adult cardiovascular system [23] by inducing sarcomere membrane organization and integrity, cell survival and angiogenesis [24–27]. Our group has recently demonstrated that microparticles (MP) allow controlled delivery of therapeutic GFs like NRG in the MI region, accompanied by a significant improvement in cardiac function, in both rat and pig models of MI [28,29]. We have also demonstrated that NRG-MP combined with ADSC,

known as ADSC-NRG-MP, is totally biocompatible with infarcted rat hearts [30].

In the present study, we sought to improve ADSC survival and tissue repair using NRG-MP after implantation into the injured myocardium of a rat MI model. The potential reparative activity of ADSC, with or without NRG-MP, was first investigated. We further determined SC survival and cardiac differentiation. Finally, the interactions between MP and ADSC with the macrophages of the innate immune system were examined to determine whether a shift to regenerative macrophages was induced. Collectively, the results obtained indicate that the use of NRG-MP combined with ADSC led to increased SC engraftment, thus improving treatment efficacy and providing a rationale for the future application of this technique in clinical studies.

2 Material and Methods

2.1 Materials

Poly(lactic-co-glycolic acid) (PLGA) with a monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (Mw: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; Mw: 400), human serum albumin (HAS), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), carboxymethyl-cellulose, mannitol, polysorbate 80, sodium azide, sigmacote (SL2), monoclonal anti-actin α-smooth muscle-Cy3 (C6198) were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane was obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinylalcohol) (PVA) 88% hydrolyzed (Mw: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Collagen type I of rat tail 3mg/mL, Minimum Essential Medium Alpha (α-MEM) Medium, 0.05% Trypsin-EDTA, Heat inactivated Fetal Bovine Serum (FBS), Phosphate Buffered Saline pH 7.2 (PBS) and Dulbecco´s Modified Eagle Medium (DMEM) were provided by Gibco-Invitrogen (Carlsbad, CA, USA). ADSC cells were obtained from inguinal adipose tissue of male Sprague-Dawley transgenic rats. H9c2 cells were obtained from ATCC. Poly-D-Lysine (PDL) 1 mg/mL was obtained from Merck-Millipore (Darmstadt, Germany) and rh Neuregulin-1b-iso by EuroBioSciences (Friesoythe, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, USA). Goat polyclonal anti-human NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz,

CA, USA). Rabbit polyclonal GFP antibody (ab290) and anticardiac troponin T Ab [284(19c7)] (ab19615), monoclonal rabbit anti-CCR7 (ab3227) were supplied by Abcam (Cambridge, UK). Alexa Fluor goat anti-rabbit 488 (A11008), Alexa Fluor goat anti-mouse 594 (A11032), DAPI nucleic acid stain (D1306) were supplied by Molecular Probes-Invitrogen (Carlsbad, CA, USA). Mouse anti-CD163 (MCA342R) was provided by AbD Serotec. Goat serum (X0907) was provided by Dako. Donkey anti-rabbit FITC (711-096-152) provided by Jackson Immuno Research. Rabbit anti-caveolin-1 (3238) provided by cell signaling. Alexa Fluor 647 mouse anti-human Ki-67 (558615) provided by BS Pharmigen.

2.2 Preparation of NRG-releasing particles

NRG-releasing PLGA particles were prepared by multiple emulsion solvent evaporation method using total recirculation one-machine system (TROMS) as previously described [30]. Briefly, the organic phase (O) composed of 100 mg of PLGA dissolved in 4 mL of a dichloromethane/acetone mixture (ratio 3:1) was injected into the inner aqueous phase (W_1) containing 200 µg of NRG, 5 mg of HSA, and 5 µL of PEG 400 dissolved in 200 µL of PBS. Next, the inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow through a needle. After this homogenization step, the W_1/O emulsion was injected into the outer aqueous phase (W_2) composed of 20 mL of a 0.5% w/v PVA solution. The turbulent injection through a second needle resulted in the formation of a multiple emulsion (W_1/O/W_2), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. MPs were washed three times with ultrapure water by consecutive centrifugations at 4 ºC (20,000 g, 10 min). Unloaded MPs were formulated without adding NRG. TROMs parameters were adjusted for the preparation of particles of 20 µm in diameter [30].

2.3. Characterization of NRG particles

Particle size and size distribution were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, Malvern, UK). Particles were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers. Encapsulation efficiency was analyzed by western-blot assay, as described elsewhere [30]. The bioactivity of MP-released proteins

was evaluated *in vitro* by determining H9c2 proliferative capacity following GF treatment by MTS assay as previously described [30].

2.4. Particle surface modification

In order to favor cell attachment to the particle surface, MPs were overlaid with 0.1 or 0.5 μg/cm^2 of type 1 collagen and/or PDL (1:1). Particle coating was performed in 15 mL sygmacoted falcon tubes. MPs were re-dispersed in acidified PBS (pH 5.7) and the mixture was sonicated until the particles were completely dispersed in the solution. Then coating molecules were added to the falcon tube and mixed with the particles under rotation at room temperature for 60 minutes. Coated particles were washed with distilled sterile water and lyophilized for long term storage without cryoprotectant. Particle surface charge was determined by zeta potential measurement (Zeta Plus Potential Analyzer, Brookhaven Instruments Corp., New York, USA) and scanning electron microscope (SEM, Philips XL 30 ESEM-FEG) images were taken in order to observe the differences between coated and uncoated MP.

2.5. Isolation and culture of ADSCs

ADSCs were obtained by *in vitro* culture of the stromal vascular fraction (SVF) isolated from inguinal adipose tissue of 5 male Sprague–Dawley transgenic rats that expressed the green fluorescent protein (GFP). Cell isolation was performed as previously described [31]. ADSCs were cultured in α-MEM medium supplemented with 10% FBS, 1 ng/mL bFGF and 1% penicillin/streptomycin. Cells were sub-cultured when 80% confluence was reached. The percentage of ADSCs that expressed GFP was assessed by flow cytometry.

2.6. Adhesion of ADSC to the particles

For ADSC adhesion, 1 mg of coated MP was re-dispersed with complete α-MEM medium, and was then ultrasounded and quickly vortexed prior to addition of 5X10^5 cells. The mixture was then gently flushed and plated in Costar® Ultra Low Cluster Flat Bottom Sterile Polystyrene Plate. Plates were incubated at 37 ºC. The evolution of the adhesion of the cells to the particles was observed by bright field microscopy, within different time points (0, 10, 30, 60 and 90 minutes).

2.7. In vivo studies using chronic myocardial infarction model

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC.

Female Sprague-Dawley rats (Harlan-IBERICA, Spain) underwent permanent occlusion of the left anterior descending coronary artery, as previously described [28]. Among the animals (n=38), only those with a left ventricular ejection fraction (LVEF) between 40-50% (n=31) at day 2 post-MI were included in the study. LVEF was obtained by echocardiographic measurements with Vevo 770 ultrasound (Visualsonics, Toronto, Canada). One week post-MI, rats were divided into four groups, and the chests were reopened. The groups were: 1) $5X10^5$ ADSC (n=6), 2) $5X10^5$ ADSC adhered to one milligram of non-loaded MP (ADSC-MP; n=7), 3) $5X10^5$ ADSC adhered to one milligram of loaded NRG-MP (ADSC-NRG-MP; 1294.06 ng NRG; n=10) and 4) control resuspension medium (n=8). Treatments were dispersed in 80 µL of injection medium previously described [30] and implanted with a 23-gauge needle into 2 regions surrounding the border of the infarct. At one week (n=10) and three months (n=21) post-injection the animals were sacrificed.

2.8. Morphometric and histological studies

After harvesting, the hearts were perfused-fixed in 4% paraformaldehyde at 4 ºC and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4 ºC, embedded in paraffin and 5-µm-sections were cut. Hematoxylin-eosin (HE) staining was performed to visualize tissue structure and to locate the tract of the injection and the treatments.

Cell fate was assessed by immunofluorescence assay against GFP with the animals sacrificed one week and three months post-treatment. Moreover, at one week, GFP-ADSC co-localization with the MPs was studied by evaluating contiguous slices with immunofluorescence against GFP and HE stain, respectively. Proliferation of the ADSC was assessed by double immunofluorescence against GFP and Ki-67 in the animals sacrificed one week after treatment. Besides, ADSC differentiation was studied in animals that had received treatment for three months by double immunofluorescence against GFP and smooth muscle actin (α-SMA) or troponin (cTnT). The differential

immunological response to the treatments was evaluated one week after implantation by immunofluorescence against CCR7 (M1) and CD163 (M2) macrophages. Furthermore, heart tissue remodeling and revascularization were investigated. Quantification of infarction size and left ventricle (LV) thickness was performed by analyzing Sirius red stains. To evaluate these, 2.5X images were obtained to observe the complete area of the hearts, and quantification was performed by Fiji software. To appraise the revascularization effect produced between the different treatments, immunofluorescence was performed against α-SMA for the vessels and caveoline for the capillaries.

2.9. Statistical analysis

Statistics were calculated with Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). The differences among treatment groups were assessed by Anova, with Tukey post hoc correction, when the values measured were normally distributed.

3. Results

1.1. Characterization of NRG particles

Particles prepared by TROMS had a mean particle size of 20 ± 5 µm. Encapsulation efficiency was found to be 65 ± 2%, which corresponds to a final loading of 1294.06 ng of NRG per mg of polymer. The bioactivity of the NRG released from the MPs was assessed by induction of H9c2 cell proliferation, and we observed that NRG remained bioactive after the encapsulation process, as had been the case in previous studies [30].

1.2. Particle surface modification

In the study, two different molecules were used to favor cellular adhesion to the MP, PDL and collagen type I. Different concentrations were analyzed: 0.1 and 0.5 µg/cm^2 of PDL, collagen or a mixture 1:1 of both. Zeta potential was measured to analyze the changes on surface charge, to estimate the adherence of the cells to the MP. The values obtained are summarized in figure 1.A. Particles coated with PDL showed a positive charge with an increased magnitude corresponding to an increase in concentration (0.1 µg/cm^2 resulted in 5.92 ± 1.67 mV and 0.5 µg/cm^2 was 30.87 ± 0.72 mV). Collagen also increased zeta potential value with respect to the uncoated MPs, but it remained negative and the values were not concentration-dependent (0.1 µg/cm^2 was -4.19 ± 1.58

mV and 0.5 µg/cm^2 was -5.56 ± 3.58 mV). The 0.5 µg/cm^2 collagen:PDL coating produced a zeta potential close to neutrality (1.16 ± 1.52 mV). The SEM images showed that the incorporation of biomimetic substances in the particles induced a change in the appearance of the surface, becoming foamier with respect the uncoated particles, as can be seen in figure 1.B. Also, no changes in the structure of the MP were observed and the biomimetic substances were homogenously distributed through the surface of all the particles.

A)

Coating	-	Collagen		PDL		Collagen:PDL
[µg/cm^2]	-	0,1	0,5	0,1	0,5	0,5
Z Potential (mV)	-19,92 ± 2,49	-4,19 ± 1,58	-5,56 ±3,58	5,92 ± 1,67	30,87 ± 0,72	1,16 ± 1,52

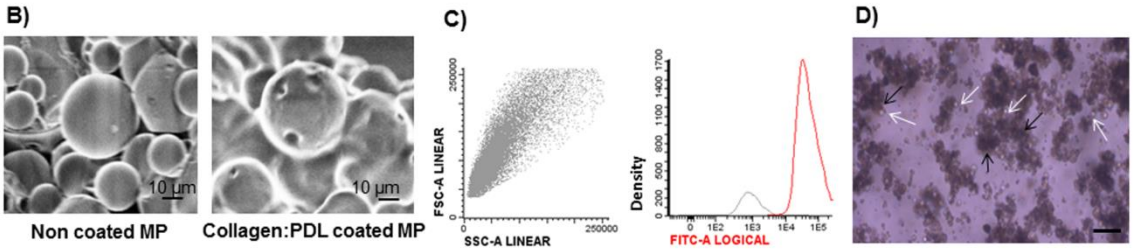

Figure 1: Characterization of the ADSC-NRG-MP. A) Zeta potential values of the MPs with different coatings (results shown as mean ± SD); B) SEM images of the MPs uncoated and coated; C) Representative cytometry analysis of the amount of GFP within the ADSC population; D) Image of 60 minutes attachment of the 500,000 ADSCs to one milligram of the 0.5 µg/cm^2 collagen:PDL 1:1 coated MPs (White arrows: ADSC, Black arrows: MP, Scale bar: 50 µm).

1.3. Isolation and culture of ADSCs

ADSC were obtained from transgenic rats that expressed GFP in order to track ADSC cells once injected in the tissue. The cytometry analysis showed that 80.09 ± 9.82% of the ADSC expressed GFP. A representative cytometry analysis can be seen in figure 1.C.

1.4. Adhesion of ADSC to the particles

Adhesion of the cells to MP with the different coatings and at different time points was examined using 500,000 cells. Complete adhesion was only observed after 60 minutes in the MP coated with 0.5 µg/cm^2 of the 1:1 collagen:PDL mixture (see figure 1.D), while the MP coated either with PDL or collagen alone, independently of the

concentration, required longer times for the total adhesion of the cells, and in some cases was more than 90 minutes (images not shown).

1.5. Morphometric and histological studies

1.5.1. Cell fate *in vivo*

Cell fate was analyzed one week and three months after transplantation by immunofluorescence against GFP. One week post-treatment, ADSCs were evidenced in the heart tissue in all the treatment groups. Remarkably, the amount of cells detected at this time was greater when the ADSCs were attached to the MPs (ADSC-MP: 53.76 % and ADSC-NRG-MP: 66.78 %) (representative images in figure 2). It was then demonstrated that the cells co-localized with the MPs when compared with H/E consecutive stains (image not shown), indicating that ADSCs remained in the injection track without diffusing through the tissue. Interestingly, three months after treatment, some GFP-ADSC were also detected, but only in the animal groups treated with the particle-scaffolds (ADSC-MP and ADSC-NRG-MP) (see figure 2).

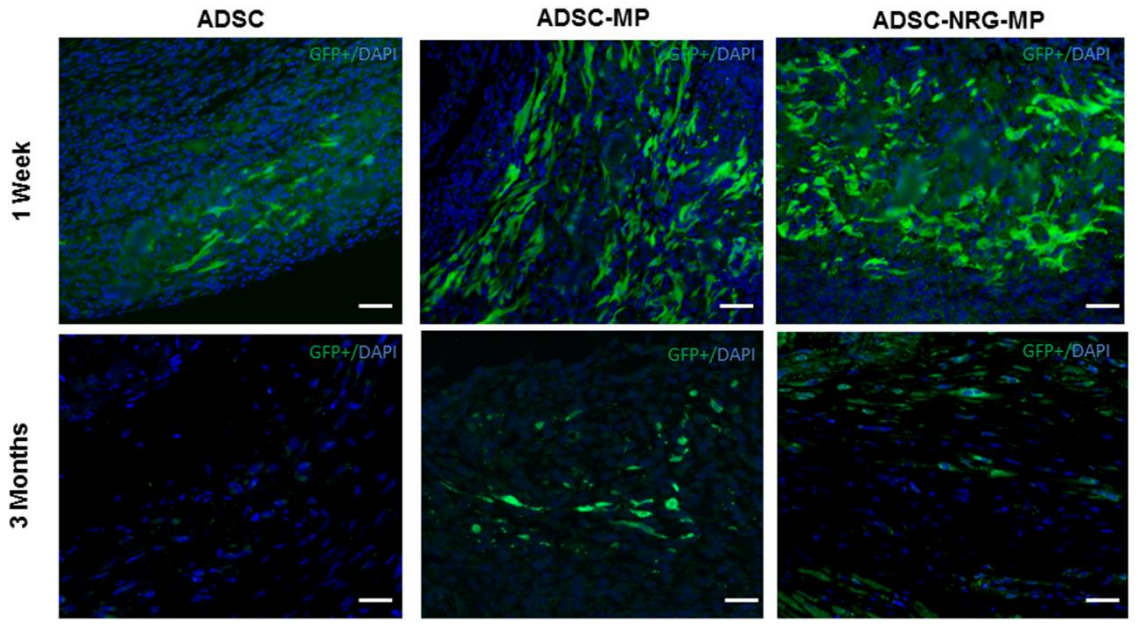

Figure 2**:** Representative images of the GFP-ADSCs of the different treatment groups at short (one week) and long (three months) term (Scale bar: 10 µm).

Although the injected ADSC did not proliferate, there was observed proliferation in the area surrounding the track of injection, as positive Ki-67 cells were detected (figure

3.A). This proliferation was evidenced in all the treatment groups, one week after the injection.

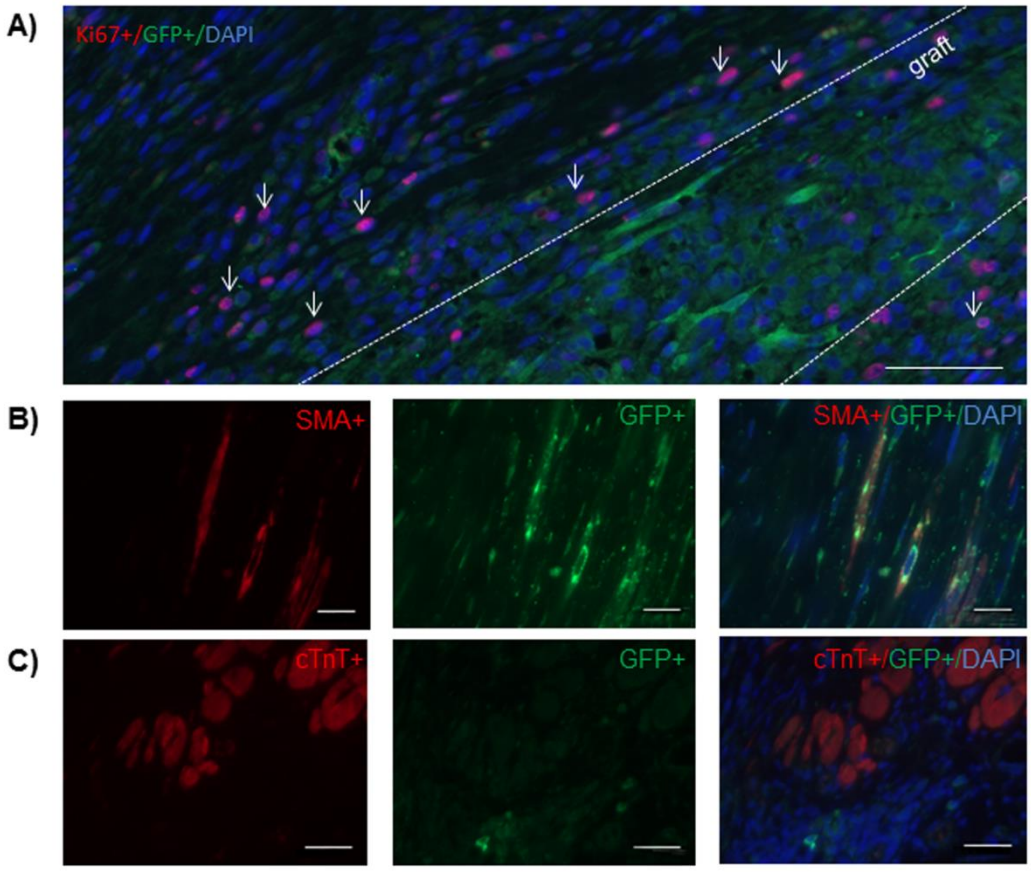

Figure 3: A) Ki-67/GFP immunostaining were Ki-67 positives cells (arrow) have been detected closed to the GFP-ADSCs (lines) indicating the induction of proliferation (Scale bar: 25 µm), B) Representative image of GFP-ADSC co-stained with α-SMA three months after treatment (Scale bar: 25 µm), C) Representative image of GFP-ADSC which not co-stained with cTnT three months after treatment (Scale bar: 50 µm).

Finally, the expression of α-SMA and cTnT were analyzed to investigate the differentiation of the ADSC to smooth and cardiac muscle respectively. Three months after treatment we found that some of the ADSC GFP^+ cells were positively stained for α-SMA. Moreover, some GFP^+/SMA^+ cells were incorporated into newly formed vessels (figure 3.B). However, no cardiomyocytes differentiated from ADSC were found (figure 3.C). It therefore appears that grafted cells differentiate into blood vessels but not to cardiomyocytes.

1.5.2. Macrophage response to the implanted tissue engineering strategy

Macrophage phenotype was evaluated to assess the activation state of implant-associated macrophages. A statistically significant higher ratio of M2 ($CD163^+$):M1 ($CCR7^+$) macrophages were observed in the surrounding tissue of ADSC (1.20 ± 0.06), ADSC-MP (0.87 ± 0.04) and ADSC-NRG-MP (1.00 ± 0.07) treated animals when compared with the control group (0.20 ± 0.02) 1 week after graft (see figure 4). This increased CD163 expression suggests a shift toward an anti-inflammatory pro-healing phenotype.

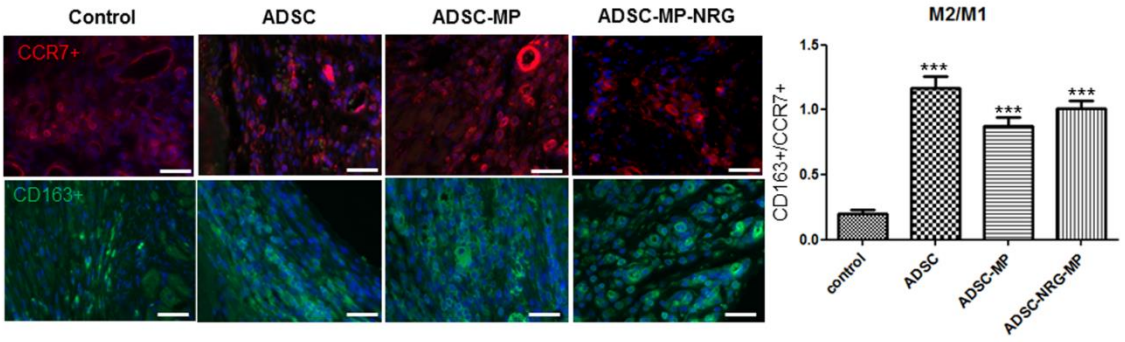

Figure 4: Representative immunostaining images of M1 (CCR7) and M2 (CD163) of the different treatments (Scale bar: 25 µm) and graphic representing the ratio of macrophages M2:M1 one week post-treatment. Results shown as mean ± SEM (***P<0.001 vs. Control).

1.5.3. Cardiac tissue repair

Different parameters were studied in order to assess cardiac tissue regeneration *in vivo* in the rat MI model. Animals sacrificed three months after treatment had a reduction of the infarct size (ADSC: 7.96 ± 0.73%, ADSC-MP: 8.13 ± 1.09%, ADSC-NRG-MP: 6.84 ± 0.57%) when compared with the control group (10.73 ± 0.92%). This reduction was only statistically significant in the ADSC-NRG-MP group (P<0.001) (see figure 5.A and C). The LV thickness was significantly increased in the groups treated with ADSC-MP (0.85 ± 0.03 mm) and ADSC-NRG-MP (0.89 ± 0.02 mm) when compared to control (0.66 ± 0.01 mm) and ADSC (0.73 ± 0.02 mm) groups (P<0.001) (see figure 5.B).

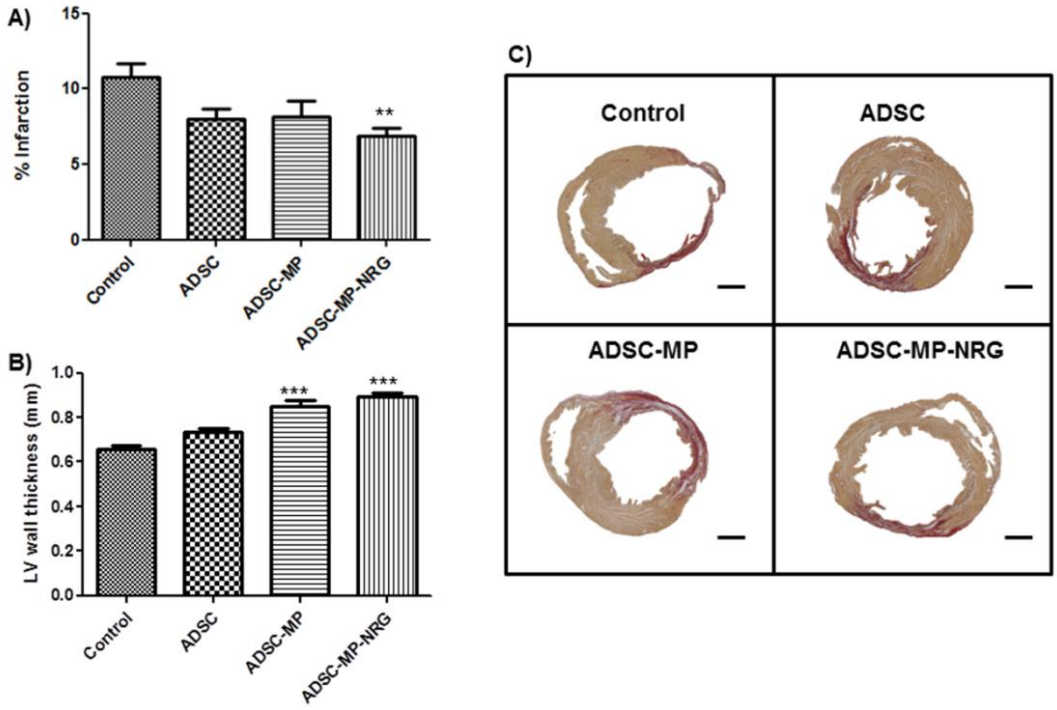

Figure 5: Sirius red staining three months after injection of the treatmens. A) Quantification of infarcted size; B) Left ventricle wall thickness of the different treatments; C) Representative images of the infarcted hearts with the different treatments (Scale bar: 1mm). Results are shown as mean ± SEM (**P<0.01 and ***P<0.001 vs. Control).

We also evaluated the effect of the treatments in vasculogenesis. To do this, we evaluated the number of arterioles (SMA^+ vessels) and capillaries (small caliber caveolin vessels). Concerning the density of SMA^+ vessels, a significantly greater number of vessels was observed in all the treatment groups (ADSC: 217.20 ± 10.20 mm^2, ADSC-MP: 225.37 ± 10.32 mm^2, ADSC-NRG-MP: 203.45 ± 7.87 mm^2) (P<0.001) when compared to the control (129.57 ± 6.68 mm^2) (see figure 6.A). Meanwhile, significantly more capillaries were found in the animals treated with ADSC-MP (738.34 ± 22.09 mm^2) (P<0.5) and ADSC-NRG-MP (738.62 ± 22.09 mm^2) (P<0.01) when compared to the control group (612.06 ± 27.95 mm^2), whereas ADSC did not yield similar results (685.96 ± 32.46 mm^2) (see figure 6.B).

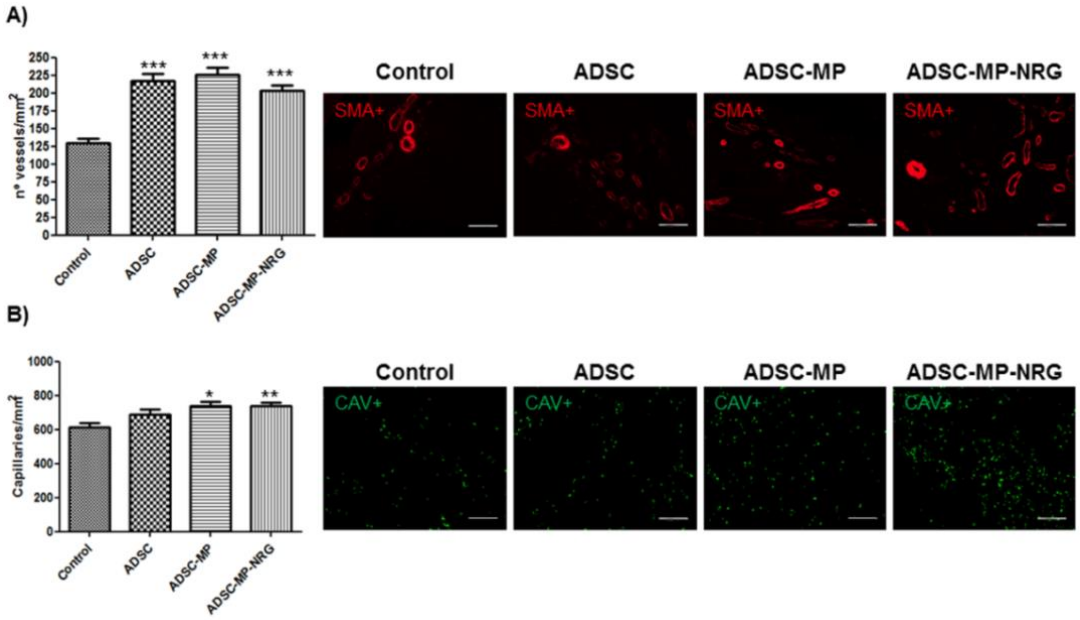

Figure 6: Representative images and quantification of A) α-SMA vessel density (arterioles) and B) caveolin (CAV) in infarcted and peri-infarcted zones three months after injection of the treatments. Results are shown as mean ± SEM (*P<0.05, **P<0.01, ***P<0.001 vs Control).

4 Discussion

Cardiac tissue bioengineering has opened up new possibilities for heart regeneration after a MI. To date, most of the strategies developed for myocardial regeneration have been focused on the combination of either proteins or cells with different biomaterial scaffolds. However, at present few studies have investigated the effect of the three elements administered in combination [32,33]. As different pathways for regeneration can be reached, this strategy might favor greater regeneration of the damaged tissue when compared with each element administered alone. We report here for the first time an approach that combines ADSC, NRG and PLGA MPs showing more efficient cardiac regeneration than when these are administered individually. Our data demonstrate that the combination of ADSC with NRG-MPs enhanced ADSC survival once in the tissue. This might account for the higher cardiac remodeling detected *in vivo* in the rat MI model.

Previous studies on cardiac TE were performed using hydrogels or sheet scaffolds typically made of natural biomaterials such as alginate, collagen, chitosan and hyaluronic acid, which brought about improvements in cardiac performance [34–38]. However, the administration of sheet scaffolds or hydrogels may be problematic [39]. On the one hand, sheet scaffolds require an invasive administration procedure, as the

chest has to be opened to adhere the system directly to the heart. On the other hand, hydrogels might have certain limitations. First, they usually present fast gelation times that can block the administration device. Second, in some cases, the procedure for inducing the gelation, such as ultra-violet light, may also require chest opening and can cause damage to the cells surrounding the treatment area [40,41]. Conversely, MPs of a size safe to be intramyocardially administered can be easily delivered to target areas in the heart through minimally invasive approaches, using the NOGA guided catheter [29], which should facilitate the transference for future clinical application.

Interestingly, we employed MPs made of PLGA, a synthetic polymer that has received Food and Drug Administration (FDA) approval for clinical application in TE. Although the use of MPs is more common for protein than for cell delivery [42], in the present study we move a step further employing the MPs as cell carriers. MPs have the limitations that they can only incorporate one or two GFs as most at the same time and it is difficult to release each factor with different kinetics [28,43,44]. For this reason the combination of GF-MPs with cells such as ADSC, with a high paracrine activity [31], is a promising alternative. We have previously demonstrated that MPs are good candidates as heart regenerative devices, having a mean particle size which is safe for intramyocardial administration, enough surface to favor cell adhesion [30] and a final NRG loading suitable for *in vivo* studies [28]. In order to favor the attachment of the cells, the biomimetic substances PDL [45] and collagen type I [8], which have previously shown positive cell adhesive capacities [30], were incorporated to improve PLGA MPs-cellular interactions [46,47].

One of the goals of the particles developed was to tackle the low proportion of grafted cells that survive in the infarcted heart after transplantation, which is the most important drawback of myocardial cell therapy [6]. It has been previously demonstrated that free ADSCs administration promote vasculogenesis and reduction of infarct size even with low cell retention [31,48,49]. This beneficial effect was higher when cells were combined with a polymeric device [11,12]. However, although a certain improvement in cell survival was observed, the longest the cells were detected in the heart was one month [50–53]. In our study, MPs were able to improve cell survival both in the short (one week) and in the long (three months) term, indicating that MPs are good carriers to improve ADSCs retention in the tissue and to enable localized delivery of cells without

mechanical washout. We next wanted to know whether ADSC remained at the injection site over a long period of time or migrated toward other areas. The comparison of the GFP immunofluorescence stain with the consecutive HE stain slice allowed us to confirm the co-localization of the cells with the MPs within the injection site, meaning that cell attachment to the MPs was strong and prolonged in time, and ruling out the possible migration of the grafted cells to other areas. This aspect has also been observed when employing other systems [11,13,54,55] and in opposition to free cell administration [56–58].

Going a step further, we investigated whether ADSCs were able to proliferate after being injected in the cardiac tissue. As MI induces a high loss of cardiomyocytes, it is important to repopulate the infarcted area. Although ADSCs did not proliferate, the cells surrounding the treatment area did show a certain proliferation, indicating that a putative trophic effect could be induced either by the transplanted cells and/or by NRG released from the MPs, as previously reported [28,31]. Nevertheless, although injected ADSC did not proliferate, they did expresses smooth muscle cell markers three months after administration, as previously described by others [31,59–61]. Our results showed that certain GFP-ADSC co-stained with α-SMA, but not with cTnT, in both ADSC-MP and ADSC-NRG-MP treated groups, demonstrating that ADSCs contribute principally to blood vessel formation, providing blood flow to the tissue [13,54].

The characteristics of the tissue-engineered implant determine the host response to the treatment by interacting positively or negatively with the immune system. In this way, biomaterials and ADSC may modulate wound healing and may induce a shift in the local macrophage phenotype that may be associated with better tissue recovery [17,62,63]. It has been demonstrated that both biomaterials and ADSC can prompt M1 macrophages, which correspond to classically-activated pro-inflammatory macrophages, towards M2 macrophages associated with regulatory and homeostatic functions [17,64]. The present study demonstrates that our cardiac tissue-engineered implant can modulate the immune response by inducing a shift in macrophage phenotype toward the M2 state in the short term as previously seen [65]. This increase of macrophage M2 expression has been shown to induce positive LV remodeling of MI animal models with both biomaterials [66] and cells [67].

We next studied the capacity of the system to induce heart regeneration. Smaller infarct size and thicker LV were detected in the ADSC-NRG-MP group, indicating that the TE strategy induced globally a greater, more complete improvement. According to these results, an increase in the vasculogenesis of the infarcted area, both in arterioles and capillaries was observed, indicating that tissue remodeling is closely associated with the growth of the vascular network. Indeed, the majority of the studies with ADSC that reported cardiac function improvement displayed neoangiogenesis in the ischemic tissue [11,31,68], which is also consistent with ADSC ability to differentiate into endothelial cells and to secrete GFs as VEGF [20]. Moreover, tissue revascularization may also be helpful for improving cell survival and proliferation [20,69]. In short, MPs were able to improve cell survival, and hence consistent higher efficacy was observed, resulting in a better outlook.

5 Conclusion

This study describes the long-term positive effects that can be achieved when ADSC are combined with a drug delivery system as support. Longer cell survival was observed since cells were detectable in the tissue after three months of treatment. It was also observed that both cells and biomaterials favored the shift of the macrophage expression, inducing an increase in the ratio M2:M1, which favored the regeneration of the heart. This aspect was observed as there were improvements in the histological analysis. In the groups in which the cells were attached to the MPs, the treatments were able to promote an increase in capillary density, reducing tissue remodeling and favoring the differentiation of the surviving cells into new vessels. Moreover, when the MPs were loaded with NRG, a more complete regeneration was observed with respect to the non-loaded MPs, as some of the parameters studied, such as infarct size and capillary density, were greatly improved when using ADSC-NRG-MP. Taking all these factors into account, the ADSC-NRG-MP has been shown to be an effective treatment for heart regeneration, since a longer and stronger response was observed. Nevertheless, further studies in large animal models must be performed in order to assess if this is a worthy candidate to proceed to clinical trials.

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CHAPTER 3

INJECTABLE DEXTRAN-HYALURONIC ACID HYDROGELS EMBEDDING NEUREGULIN-LOADED MICROPARTICLES AND ADIPOSE-DERIVED STEM CELLS AS A STRATEGY FOR CARDIAC TISSUE ENGINEERING

Chapter 3:

Injectable Dextran-Hyaluronic acid hydrogels embedding Neuregulin-loaded microparticles and Adipose-Derived Stem Cells as a strategy for cardiac tissue engineering

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Abstract

To date, hydrogels are the only biomaterial-based engineering strategy that has reached clinical trials for heart tissue repair. Hydrogels were used in this study as cell carriers for therapeutic adipose-derived stem cell (ADSC) administration and to improve the delivery of GFs like neuregulin (NRG), both of which are promising cell and protein sources in the field of cardiac regeneration. The controlled delivery of the GF from hydrogels, still present some challenges. A controlled release may be achieved by embedding GF loaded microparticles (MP) in hydrogels. The objective of this study was therefore to prepare and characterize injectable hydrogel with different ratios of dextran (Dex) and hyaluronic acid (HA) containing ADSC and different amounts of NRG loaded MP, in order to identify a system suitable for heart regeneration. The 50:50 Dex:HA hydrogel proved to be the most favorable, as it had proper gelation time (320 seconds), good injectability through a 29G needle and an elastic modulus value of 5.7 ± 1.21 kPa suitable for heart regeneration. Cells and MPs were homogenously distributed through the hydrogel. Scanning electron microscopy showed that hydrogels had an open network structure and a pore size of 30 μm that allowed cell survival. In fact, more than 95% of the entrapped ADSC cells survived at least 14 days after encapsulation in the hydrogel. Finally, the hydrogel required 25 days for total *in vitro* degradation. Further experiments are mandatory in order to assess the biocompatibility and efficacy of the system once in the infarcted heart.

Key words: Hydrogel, Microparticles, Tissue engineering, Stem cells, Growth factors

Injectable Dextran-Hyaluronic acid hydrogels embedding Neuregulin-loaded microparticles and Adipose-Derived Stem Cells as a strategy for cardiac tissue engineering

1. Introduction

There is a growing need to develop improved tissue engineering (TE) strategies combining growth factors (GF), cells and biomaterials to enhance heart repair and regeneration [1]. Regarding biomaterial-based strategies, many studies have been performed in the field of heart TE [2–4], but only hydrogels, microparticles (MP) and nanofibers have been tested in relevant large animal models of myocardial infarction (MI) [5–7]. Even more importantly, only hydrogels have reached clinical trials with Algisyl-LVR [8]. Thus, hydrogels have been shown to be one of the most promising candidates for cardiac TE. This could be due to their unique compositional and structural similarities to the natural extracellular matrix (ECM) which favors the survival and engraftment of cell such as the adipose-derived stem cells (ADSC) which have heart regenerative properties [9–12]. The enzymatic crosslinking of polysaccharide tyramine hyaluronic acid (HA) and dextran (Dex) using horseradish peroxidase (HRP) and hydrogen peroxide has shown a versatile method towards injectable hydrogels applicable in TE [13–15]. However, the potential of enzymatically cross-linkable hydrogels of natural polymer conjugates for cardiac repair has never been explored. HA hydrogels have previously been employed in heart TE [16–19]. Moreover, HA is a biomaterial that mediates wound repair, cell proliferation and differentiation, promotes angiogenesis and suppresses fibrous tissue formation, among other effects [20–22]. Solutions of HA are generally viscous, hydrogels prepared from HA can retain considerable amounts of water and is a biocompatible and biodegradable polymer [21,23]. Nevertheless, it is known that HA degrades very fast once injected in the heart [16]. To decrease the degradation time of HA type hydrogels, a strategy that has shown to be applicable is the co-crosslinking of HA and Dex. Dex is a biomaterial included in the Food and Drug Administration (FDA) list of Generally Recognized As Safe (GRAS) products (available on http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=scogslisting&id=101 last update 10/31/2006). The application of Dex in heart repair has not been investigated up to now, requiring further studies in heart TE [24,25].

Hydrogels have also been widely investigated for GF encapsulation [26,27]. However, GF release, and consequently GF degradation, remains fast [15]. To retard GF release from hydrogels a possible solution may be the microencapsulation of those GFs in MPs,

and the incorporation of the MPs into the hydrogels. MPs have the capacity to protect GFs from degradation and ensure sustained release over time [28]. Our group has previously shown that poly(lactic-co-glycolic acid) (PLGA) MPs may improve the efficacy of protein therapy in pre-clinical studies, protecting GFs against degradation and allowing controlled, localized release for up to three months [28–32]. In the present study, neuregulin (NRG) was microencapsulated in MPs, based on recent results obtained by our group that demonstrated beneficial effects in tissue remodeling and cardiac function after treatment with NRG-loaded MPs in both rat and pig models of MI [6,31].

The aim of this study was to prepare a novel strategy for heart TE based on enzymatic cross-linkable hydrogels of Dex-TA and HA-TA conjugates mixed in different ratios the delivery of ADSCs and NRG-MPs. We first studied the hydrogel gelation time and its rheological parameters to determine if these hydrogels were suitable for use in cardiac repair. We further used confocal and scanning electron microscopy (SEM) to study the structure of the hydrogel and the distribution of both MPs and cells. Next, a swelling/degradation assay was performed to elucidate the possible behavior of the hydrogel once in the tissue. Finally, the survival of the ADSCs in the hydrogels combined with the NRG-MP was studied to assess the cytocompatibility of the system. Collectively, the results obtained indicate that the use of a 10% w/v Dex-TA:HA-TA hydrogel (50:50) combined with 1 mg of NRG-MP and embedding 500,000 ADSC seems to be a promising strategy for heart tissue repair.

2. Material and Methods

2.1. Material

PLGA with monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (Mw: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; Mw: 400), human serum albumin (HAS), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), dextran (Mw 15000-30000), tyramine (TA), hydrogen peroxide (H_2O_2), hyaluronidase (HAse, w300 U/mg) and horseradish peroxidase (HRP, type VI, 300 purpurogallin U/mg solid) were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinylalcohol) (PVA) 88% hydrolyzed (Mw: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Minimum Essential

Medium Alpha (α-MEM) Medium, 0.05% Trypsin-EDTA, heat inactivated Fetal Bovine Serum (FBS), Phosphate Buffered Saline pH 7.2 (PBS), Dulbecco´s Modified Eagle Medium (DMEM), PrestoBlue® Cell viability Reagent and Live/Dead® Viability/Cytotoxicity Kit were provided by Gibco-Invitrogen (Carlsbad, CA, USA). ADSC cells were obtained from inguinal adipose tissue of male Sprague-Dawley transgenic rats. H9c2 cells were provided by ATCC. rh Neuregulin-1b-iso was provided by EuroBioSciences (Friesoythe, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, USA). Goat polyclonal anti-human NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sodium hyaluronate (15–30 kg/mol, laboratory grade) was purchased from CPN Shop.

2.2. Methods

2.2.1. Formulation of microparticles containing NRG

NRG PLGA MPs were prepared by the emulsion solvent evaporation method using total recirculation one machine system (TROMS) as previously described [28]. Briefly, the organic phase (O) composed of 50 mg of PLGA dissolved in 4 ml of a dichloromethane/acetone mixture (ratio 3:1) was injected into the inner aqueous phase (W_1) containing 50 μg of NRG, 5 mg of HSA and 5 μl of PEG 400 dissolved in 200 μl of PBS. Next, the inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow through a needle. After this homogenization step, the W_1/O emulsion was injected into the outer aqueous phase (W_2) composed of 20 ml of a 0.5% w/v PVA solution. The turbulent injection through a second needle resulted in the formation of a multiple emulsion (W_1/O/W_2), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. MPs were washed three times with ultrapure water by consecutive centrifugations at 4 °C (20,000×g, 10 min). NRG-MPs were lyophilized for 48 h without cryoprotective agents (Virtis Genesis 12 EL, Gardines, NY). After complete lyophilization, the vials were sealed under vacuum and stored at -20 °C until use. Unloaded and rhodamine MPs were prepared in the same manner without adding NRG and stored at 4 ºC.

2.2.2. Microparticles characterization

The mean particle size and size distribution were examined by laser diffractometry using a Mastersizer® (Malvern Instruments, Malvern, UK). MPs were dispersed in ultrapure water and analyzed under continuous stirring. The average particle size was expressed as the volume mean diameter in micrometers.

The amount of NRG encapsulated in the MPs was determined by dissolving 0.5 mg of lyophilized loaded particles in 25 µL of DMSO followed by quantification using western blot assay as previously described [30]. The bioactivity of NRG released from MPs was evaluated *in vitro* by determining the proliferative capacity of H9c2 cells after NRG treatment as previously described [30].

2.2.3. Isolation and culture of ADSC cells

ADSCs were obtained by *in vitro* culture of the stromal vascular fraction (SVF) isolated from inguinal adipose tissue of male Sprague-Dawley transgenic rats that expressed the green fluorescent protein (GFP), as previously described [33]. ADSC cells were cultured in α-MEM medium supplemented with 10% FBS, 1 ng/mL bFGF and 1% penicillin/streptomycin. Cells were subcultured when 80% confluence was reached.

2.2.4. Hydrogels formulation and incorporation of microparticles and ADSCs

Dex–TA and HA-TA conjugates were prepared as reported previously [34]. The degree of substitution (DS), denoted as the number of TA units per 100 anhydroglucose rings, of Dex-TA and HA-TA for all the experiments were 15 and 2.5, respectively. Hydrogel samples were prepared at room temperature. Solution of Dex-TA and HA-TA 12.5% concentration in PBS, at different ratios (Dex-TA:HA-TA: 100:0, 50:50, 30:70, 10:90 and 0:100) were employed to prepare the hydrogels. Different amounts of MPs (0, 0.2, 0.4, 0.8 and 1 mg MP/ 100 µL of hydrogel) were added to the Dex-TA:HA-TA solution. Freshly prepared solutions of H_2O_2 (0.3% stock solution) and HRP (150 U/mL) in PBS were added to give a final concentration of 10% and the mixture was gently vortexed. Different concentrations of HRP and H_2O_2 were studied in order to produce a hydrogel with a suitable gelation time for myocardial injection. Meanwhile, hydrogels containing ADSCs, were prepared under sterile conditions by mixing a 50:50 Dex-TA:HA-TA solution in presence or absence of loaded or un-loaded MPs, with the

cells under suspension and with a freshly prepared mixture of HRP and H_2O_2. Solutions of the polymers were prepared using medium, while HRP and H_2O_2 stock solutions were prepared in PBS. Cell seeding density in the gels was $5x10^5$ ADSC/150 µL of hydrogel solution. Samples were incubated at 37 ºC and 5% CO_2, and the medium was replaced every 2 days. The final volume of the hydrogels, 150 µL, was selected according to previous studies [35] and considering future biocompatibility and effectiveness studies in a rat MI model.

2.2.5. Hydrogels characterization

2.2.5.1. Gelation time

Hydrogel gelation time was determined using the vial tilting method. No flow upon inverting the vial was regarded at the gel state.

2.2.5.2. Rheology

Rheological experiments were carried out with a MCR 301 rheometer (Anton Paar) using a parallel plate (25 mm diameter, 0º) configuration and at 20 ºC in the oscillatory mode. The evolution of the storage or elastic (G´) moduli was recorded as a function of time. A frequency of 1 Hz and a strain of 0.1% were applied in order to maintain the linear viscoelastic regimen.

2.2.5.3. Hydrogel structure study

The structure of the hydrogel was studied by two techniques, SEM and confocal microscopy. SEM analysis also gave information about the pore size and the porosity of the hydrogel. Samples for SEM were prepared by freezing the gels in liquid nitrogen for 1 minute, followed by freeze-drying for 24 hours to remove all the water from the hydrogels. The samples were next analyzed with a Philips XL 30 ESEM-FEG SEM operating at a voltage of 10 kV. Samples were gold sputtered (Carringdon) before SEM analysis. Hydrogel samples containing ADSC were fixed with formalin followed by sequential dehydration and critical point drying. Samples were gold sputtered and analyzed with SEM.

Hydrogels containing GFP-ADSCs and rhodamine-MPs were examined by confocal microscopy.

2.2.5.4. Swelling and degradation assay

Hydrogels (150 µL) with different ratios of Dex-TA and HA-TA and with different amounts of MPs were prepared as described above and weighted.

Subsequently, hydrogels were incubated at 37 ºC in 1 mL of PBS solution containing 20 U/mL of hyaluronidase enzyme. Samples were weighted at different time points: 6 h, 24 h, 4, 7, 11, 14, 18, 21, 25 and 30 days. The remaining gel (%) was calculated from the original gel weight after preparation. The buffer was replaced every 2 days and the experiments were performed in triplicate.

2.2.5.5. Hydrogel injectability

The injectability of Dex-TA:HA-TA solution with the MPs embedded was assessed by the ability of the hydrogel solution to pass through needles with different inner diameters (23, 25, 27 and 29 gauge (G) needles). The viscosity of the hydrogel solution was measured by MCR 301 rheometer, in order to study the correlation with the flow through the needles.

2.2.6. Cell viability and metabolic activity within the hydrogel

Viability study of ADSCs encapsulated in hydrogels was performed with PrestoBlue and Live/Dead assays. The PrestoBlue assay was performed at days 1, 3, 7 and 14 to study metabolic activity of the cells within the different treatment groups. The treatments studied were hydrogels: 1) with ADSCs, 2) with MPs and ADSCs and 3) with NRG-MPs and ADSCs. The metabolic values were obtained using 10 µL PrestoBlue for each 100 µL of total volume. 500 µL were added to each sample, incubated for 30 min and measured. Afterwards those same samples were submitted to the Live/Dead assay. The samples were stained with calcein AM/ethidium homodimer using Live/Dead assay Kit, according to the kit protocol and were visualized using confocal microscopy: living cells emit green fluoresce and the nuclei of dead cells are red.

2.2.7. Statistical analysis

Statistics were calculated with Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). The differences among treatment groups were assessed by Anova, with Tukey post hoc correction, when the values measured were normally distributed. A correlation study was also performed when statistical differences between groups were not detected.

3. Results

3.1. Microparticles characterization

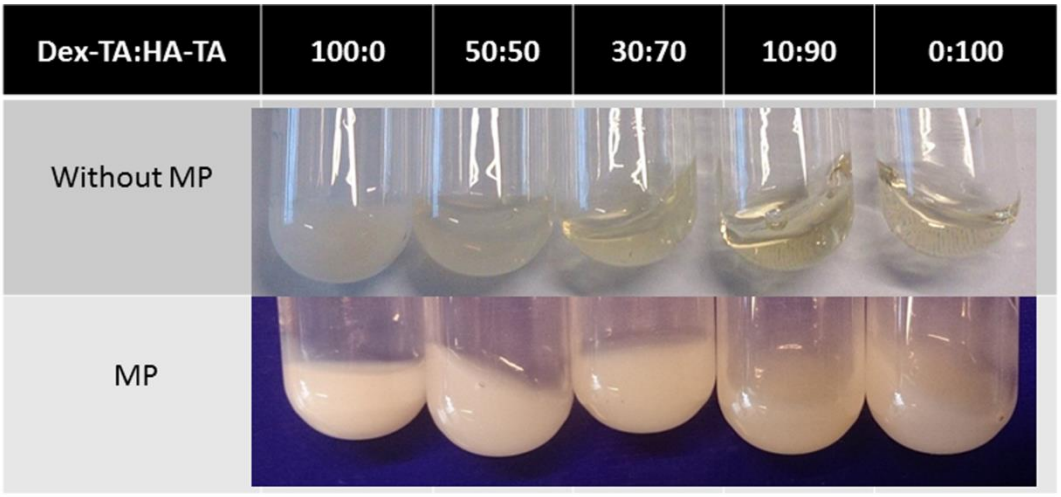
The MPs incorporated into the hydrogel were prepared by TROMs, a system suitable for GF encapsulation [29–31,36]. TROMS avoids high temperatures that can degrade the GFs, and allows higher encapsulation efficiency when compared to other microencapsulation methods [37]. The MPs had an average size of 5 ± 2 μm. NRG encapsulation efficiency was 77.81%, which correspond to a final loading of 778.1 ng of NRG per mg of polymer. The bioactivity of the NRG released from the MPs was assessed by induction of H9c2 cell proliferation NRG remained bioactive after the encapsulation process, as it had been the case in previous studies (data not shown) [30].

3.2. Hydrogel characterization

3.2.1. Gelation time:

Hydrogels (10% w/v) with different ratios of Dex-TA:HA-TA were prepared. Macroscopic differences were detected among the hydrogels (fig. 1.A). The increase in the amount of HA-TA favored the formation of more transparent hydrogels with slightly yellowish color. Meanwhile, no differences were detected in the aspect of the hydrogels that contained MPs as all presented an opaque white color. As figure 1.B shows, hydrogel gelation time was reduced when the amount of HA-TA was increased in the hydrogel. Gelation time varied from 49 seconds in 100:0 Dex-TA:HA-TA hydrogels, to 8.5 seconds in 0:100 Dex-TA:HA-TA hydrogels. The increase in the amount of MPs amount did not affect the gelation time. As all the Dex-TA:HA-TA hydrogels showed a very fast gelation time that occurred in less than 60 seconds, changes in the formulation were tested to obtain hydrogels suitable for being injected into the heart through clinically available cardiac injection catheters [5,6]. The best results were obtained when incorporating 2% of HRP (150 U/mL) and 18% of H_2O_2 (0.3%) to the hydrogels solutions, obtaining gelation times of 320 seconds with the 50:50 Dex-TA:HA-TA hydrogel, without affecting other parameters. The longer gelation times were due to the interaction of the excess of H_2O_2 with HRP, as previously assessed [34]. Also, it has previously been reported that these hydrogels and with similar concentrations of H_2O_2 are biocompatible and non-toxic [38].

A)



B)

Ratio Dex-TA:HA-TA	Gelation Time (seconds ± SD)	0 mg MP	0.2 mg MP	0.4 mg MP	0.8 mg MP	1 mg MP
	100:0	37.3 ± 6.8	49.3 ± 1.2	35.5 ± 2.1	34.3 ± 1.2	41.5 ± 2.1
	50:50	18.7 ± 1.2	23.7 ± 0.6	20.5 ± 3.5	19.0 ± 1.0	23.5 ± 0.7
	30:70	13.0 ± 0.0	13.0 ± 0.0	14.0 ± 1.4	12.0 ± 1.7	17.5 ± 0.7
	10:90	11.3 ± 1.5	9.0 ± 0.0	10.0 ± 0.0	10.0 ± 1.7	11.5 ± 0.7
	0:100	11.0 ± 1.7	9.0 ± 1.0	9.0 ± 0.0	8.7 ± 0.6	8.5 ± 0.7

Figure 1: A) Representative images of the different hydrogels with and without MPs; B) Gelation times of the hydrogels with different ratios of Dex-TA:HA-TA and with different amounts of MPs (gelation time measured in second ± SD).

3.2.2. Rheology:

The rheological parameters of the different hydrogels are summarized in figure 2.A. All the G´ values were smaller than 10 kPa and varied from 8.7 ± 1.2 to 3.4 ± 0.2 kPa. Then it was studied if either the amount of HA-TA or MP affect G´ values. Regarding the amount of HA-TA, it was observed a positive correlation between the increasing amounts of HA-TA and the increasing values of G´ (50:50 Dex-TA:HA-TA hydrogel vs 30:70 R = 0.58; p = 0.3 and vs 10:90 R = 0.36; p = 0.56), except for the hydrogel composed of 100% HA (fig. 2.B). Meanwhile, as figure 2.C illustrates, the presence of MPs slightly increased the G´ with respect to the hydrogels without MPs (0 mg MP: 4.88 ± 1.25, 0.2 mg: 6.06 ± 1.91, 0.4 mg: 6.22 ± 1.50, 0.8 mg: 5.86 ± 1.77 and 1 mg: 5.76 ± 1.60 kPa), but no statistical differences were detected within the different amounts of MPs incorporated to the hydrogel.

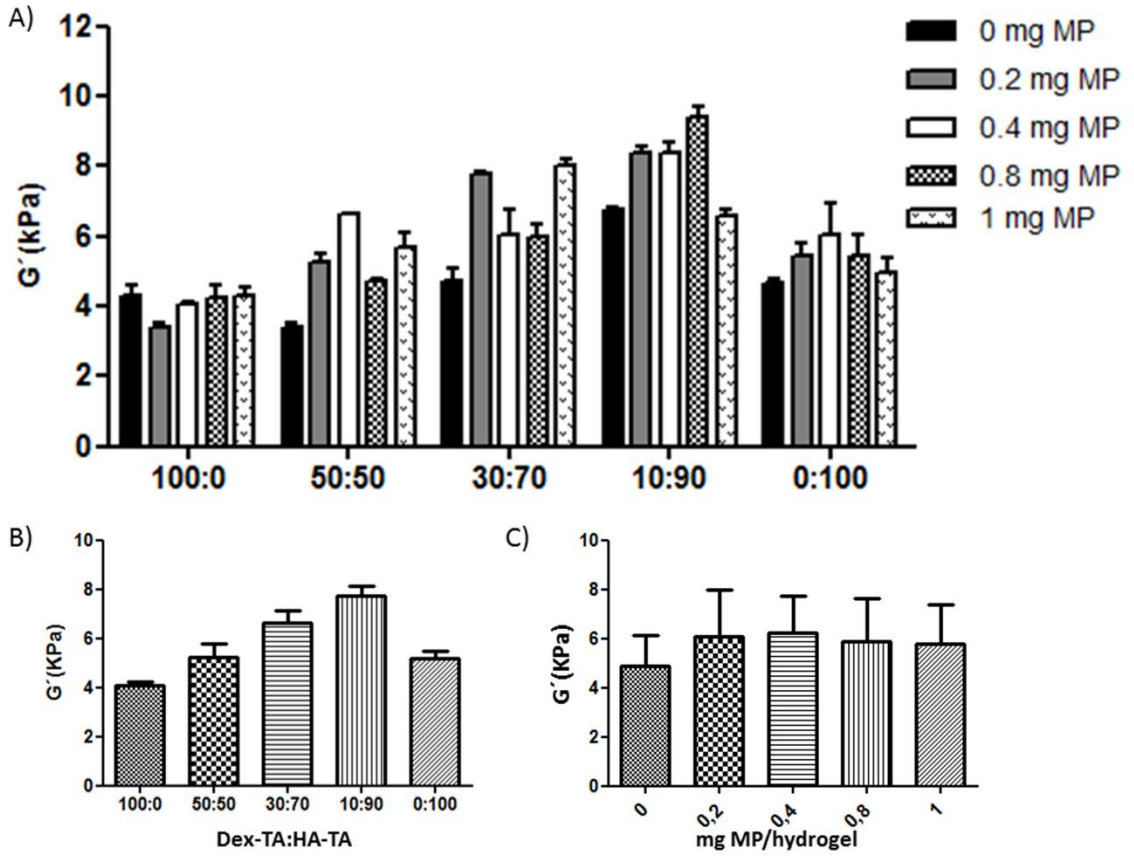


Figure 2: A) Measurements of the rheological parameters of elastic modulus (G´) of the different hydrogels, B) Rheology taking into account the composition of the hydrogel, C) Rheology taking into account the amount of MPs incorporated to the hydrogel. Results shown as mean ± SD.

3.2.3. Hydrogel structure:

Figure 3 shows representative images of the different hydrogels by SEM, with and without MPs. No modifications on the surface structure of the hydrogel due to the incorporation of MPs were observed. All HA-TA hydrogels had a more open network structure (with pores of 30 µm) than Dex-TA hydrogels (with pore size of 17 µm). Interestingly, all the hydrogels that contained HA-TA maintained the same structure independently of the amount of HA-TA incorporated into the hydrogel. A closer view of the hydrogels with MPs can be seen in figure 4.A where it can be observed that size of MPs is smaller than the pore mesh size of the 10% w/v 50:50 Dex-TA:HA-TA hydrogel. On the other hand, figure 4.B shows the presence of ADSCs on the hydrogel surface.

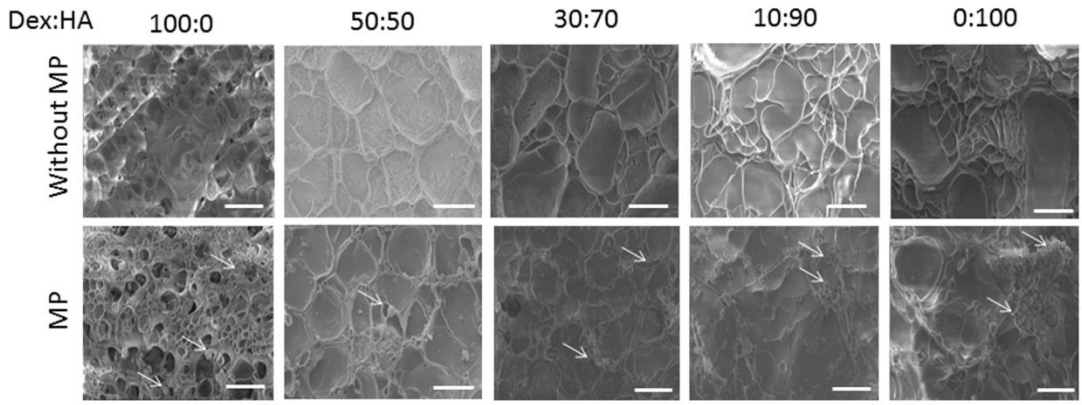


Figure 3: Representative SEM images of the surface of the different hydrogels with and without MPs (Scale bar: 20 µm, MPs are shown by arrow).

Hydrogels embedding rhodamine-MPs and GFP-ADSCs were prepared and studied by confocal microscopy to observe the distribution of both MPs and cells inside the hydrogel. Stack images were taken observing that MPs and ADSCs were homogenously distributed through all the hydrogell, rather than on the surface (fig. 4C).

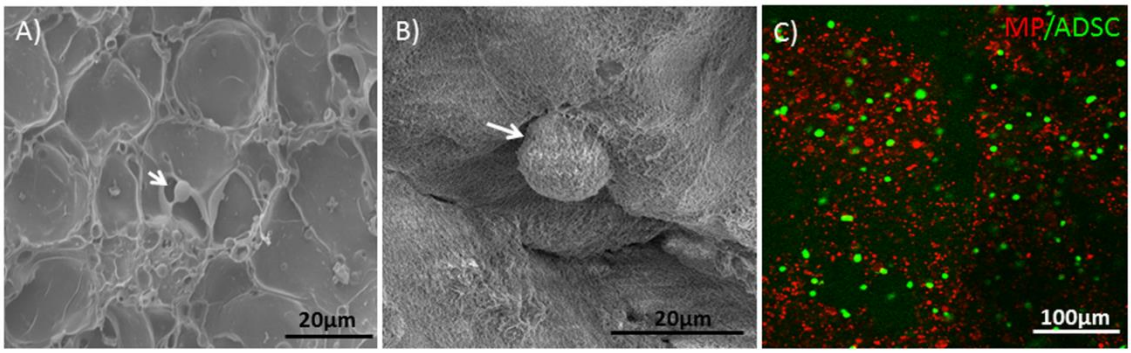


Figure 4: A) Representative SEM image of the 50:50 hydrogel with MPs (MPs are shown by arrow) , B) Representative SEM image of the 50:50 hydrogel with ADSC (Cell is shown by arrow), C) Representative confocal image of the 50:50 hydrogel with rhodamine-MPs and GFP-ADSC.

3.2.4. Swelling and degradation assay:

During the swelling phase (first 24 hours), an increase in the weight of HA hydrogels was observed (0:100 Dex-TA:HA-TA hydrogel: 60%, 10:90 Dex-TA:HA-TA hydrogel: 30%, 30:70 Dex-TA:HA-TA hydrogel: 17%) (Table 1). This is probably due to water retention in the hydrogel during the swelling phase. Once the swelling phase was over, hydrogels started their degradation. The degradation rate was a fundamental parameter to determine which of the hydrogels has more potential for cardiac application. Hydrogels composed of 0:100 and 10:90 Dex-TA:HA-TA were completely degraded in less than 7 days. As this is relatively fast, these hydrogels were discarded.

Meanwhile, the 100:0 Dex-TA:HA-TA hydrogel show no modifications in its weight during the 30 days of the experiment. When comparing the hydrogels prepared with or without MPs, there were no statistical differences in the swelling or degradation rate. The 100:0 Dex-TA:HA-TA hydrogel showed differences in weight according to whether the MPs were incorporated or not, although those differences were not statistically significant. But as 100:0 Dex-TA:HA-TA has a very low degradation rate and as previously mentioned, did not possessed an open structure, which may affect cell survival as previously reported [15], this was also discarded. Finally, Dex-TA:HA-TA 30:70 and Dex-TA:HA-TA 50:50 hydrogel degradation took 21 and 25 days respectively, which are acceptable degradation rates for further *in vivo* studies. For this reason, as 10% w/v hydrogel 50:50 Dex-TA:HA-TA had a longer degradation rate and has been more deeply studied [13–15], this hydrogel was selected for further experiments. Meanwhile, as MPs showed no negative effect on the 50:50 hydrogel, the amount of MP selected was 1 mg, having NRG concentrations suitable for *in vivo* effectivity [31].

Table 1: Degradation assay table with the percentage of weight of the different hydrogels over time. Results shown as mean ± SD.

% Weight ± SD	Dex:HA hydrogel with out MP					Dex:HA hydrogel with 1 mg MP				
Days	100:0	50:50	30:70	10:90	0:100	100:0 MP	50:50 MP	30:70 MP	10:90 MP	0:100 MP
0	103.0±3.8	107.2±6.5	103.0±2.7	102.8±3.1	109.2±8.0	97.8±4.2	104.2±4.4	104.6±4.1	103.6±3.6	101.6±2.5
0,25	95.5±2.9	104.0±5.3	121.1±7.9	137.7±5.3	158.8±36.2	95.3±2.9	113.0±7.4	134.6±9.2	140.0±3.9	172.0±7.5
1	100.7±2.3	87.5±10.8	114.5±3.2	129.9±16.1	169.1±18.4	89.0±5.2	103.7±8.3	120.0±3.4	136.0±2.4	160.9±17.6
4	97.9±6.5	66.3±14.0	83.9±11.3	104.5±25.4	1.0±1.7	87.6±17.7	65.1±9.0	79.4±22.0	133.8±39.4	8.9±7.1
7	92.5±10.2	38.7±5.7	52.3±12.3	0.7±0.7	-	79.6±20.3	40.0±0.7	44.4±19.8	12.4±15.5	-
11	87.2±10.8	28.7±5.4	16.3±1.7	-	-	62.7±21.5	34.3±6.7	29.7±5.4	-	-
14	94.1±11.6	23.8±7.1	12.7±0.9	-	-	82.3±1.7	26.3±1.4	13.3±3.8	-	-
18	94.2±11.3	11.2±8.4	2.0±2.8	-	-	79.2±3.2	17.0±3.1	6.5±1.4	-	-
21	91.0±12.7	7.6±4.0	0.5±0.8	-	-	77.8±0.1	8.9±2.1	1.1±1.1	-	-
25	93.6±13.8	3.2±2.1	-	-	-	75.3±1.9	6.5±1.8	-	-	-
30	93.6±13.8	-	-	-	-	75.3±1.9	-	-	-	-

3.2.5. Injectability:

Needles with different diameters were tested in order to assess the injectability of the system. All the needles tested allowed the passage of the 10% w/v 50:50 hydrogel through them, even when the MPs and cells were incorporated in the hydrogel, as no resistance to the flow of the syringe was observed. In order to confirm this capacity the viscosity of the solution formed by the hydrogel previous to the addition of the HRP and H_2O_2 was also studied, showing that 50:50 Dex-TA:HA-TA hydrogel has a viscosity

value similar to water (1.04 10^{-3} Pa/s).

3.2.6. Cell survival

Cell viability was assessed using two assays, the PrestoBlue and the Live/Dead assay. Regarding the PrestoBlue assay, similar cell metabolic activity was observed among the 3 hydrogel groups (figure 5.A). Meanwhile, the live/dead staining was conducted at the different time points studied, demonstrating that 95% of the cells encapsulated in the hydrogel matrix remain alive, independently of the treatment and the time measurement (see figure 5.B). Green fluorescence designates live cells, whereas red fluorescence indicates dead cells.

A)

	1 day	3 days	7 days	14 days
Hydrogel + ADSC	0.34 ± 0.01	0.55 ± 0.02	0.53 ± 0.01	0.57 ± 0.01
Hydrogel + ADSC + MP	0.34 ± 0.01	0.56 ± 0.04	0.056 ± 0.03	0.56 ± 0.02
Hydrogel + ADSC + NRG-MP	0.35 ± 0.01	0.58 ± 0.03	0.58 ± 0.03	0.57 ± 0.02

B)

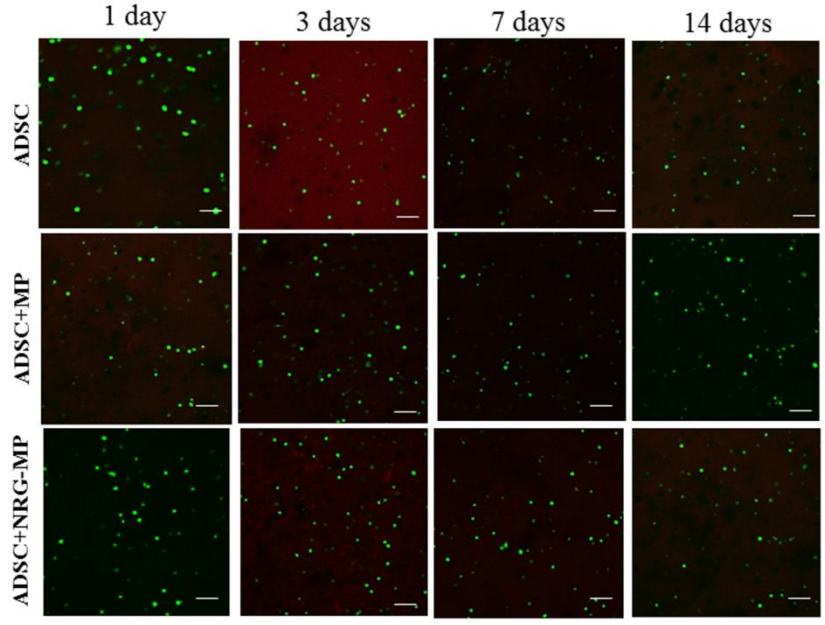


Figure 5: A) Absorbance signals produced by PrestoBlue assay, indicating the metabolic activity of the cells embedded in the hydrogel (Results shown as absorbance mean ± SD), B) Representative images of the live/dead assay of the ADSCs with the different treatments in the hydrogel over time (Scale bar: 50 µm, Green: living cells, Red: dead cells).

4. Discussion

So far, injectable hydrogels appear to be one of the most promising biomaterial-based strategies for cardiac regeneration [39]. They can be used as cell carriers for

therapeutic ADSC administration and to improve the delivery of GFs [17,40]. Interestingly, worthwhile regenerative improvements have been reported following the co-delivery of a combination of GFs, cells and biomaterials compared with each treatment on its own [30,41]. However, certain aspects are still challenging, like the controlled delivery of the GF from the hydrogels [15]. More controlled release could be achieved from MPs embedded in hydrogels. In the present study, we have for the first time designed Dex-TA:HA-TA hydrogels embedding NRG-MPs and ADSCs for heart repair. One of the most attracting features of Dex-TA:HA-TA biomaterial for cardiac application is that it does not need an external stimulus for gelation, as is the case with photo-polymerizable hydrogels [42], which would facilitate clinical translation. Gelation is achieved in situ upon mixing of the polymer conjugates with the enzyme and low non-toxic concentration of the initiator H_2O_2 during the injection.

Hydrogels in TE must meet a number of design criteria to mimic the ECM and consequently promote new tissue formation. Some of those parameters are gelation time, injectability, stiffness, porosity, induce vascularization, cytotoxicity and swelling/degradation ratio [39]. Several compositions of Dex-TA:HA-TA hydrogels with different physicochemical characteristics were therefore prepared to select the most suitable hydrogel for heart application in the diseased heart. The possible effect of MPs and ADSCs on hydrogels properties were also taken into account. In order to design hydrogels suitable for biomedical application, gelation time is a key parameter, as a fast gelation time may not translate to catheter delivery [7]. A gelation that is not too short is preferable to ensure homogeneous distribution of MPs and cells and for successful treatment localization at the site of injection [43]. Nevertheless, gelation that is too slow could favor MP and cell sedimentation in the hydrogel bottom, inducing a much slower NRG release and increasing cell death due to lack of nutrient/excretion exchange [14]. Within the prepared hydrogels, the 10% w/v Dex-TA:HA-TA (50:50) hydrogel had a gelation time of 320 seconds, a proper gelation time for administration with current catheter technology. Related to the gelation time, another important parameter that has to be considered when designing materials for clinical application is the injectability and the delivery method of the material. Hydrogels that can pass through a fine-gauge needle (~27G) meets the requirement to be injectable. Such hydrogels are capable of being administered safely into the heart in a minimally invasive manner [44] using catheter systems such as NOGA [7,45]. The 10% w/v 50:50 Dex-TA:HA-TA hydrogel

embedding 1 mg of MP was able to go through the different needles tested, even the 29G needle, being amenable to current cardiac catheters and indicating their suitability for future *in vivo* studies.

Furthermore, materials designed to be injected into the heart to act as a temporary matrix for transplanted cells should have sufficient stiffness as well as elasticity to lope the cyclic heart contraction and dilatation [44]. Substrate stiffness also influences cell morphology and function [46]. All the hydrogels developed, independently of the ratio Dex-TA:HA-TA or the amount of MP incorporated, were suitable for use in heart tissue regeneration [35], with G´ values smaller than 10 kPa. It has been previously demonstrated that hydrogels with 10 kPa stiffness induce functional maturation of neonatal rat ventricular cardiomyocytes and optimal sarcomere structure [47]. Stiffer substrates have reported faster cell proliferation, however, this is a disadvantage for heart TE. A stiffer substrate, leads to higher cell density and as a result, increase competition amongst the cells for limiting the substrate attachment sites as well as oxygen and nutrients, decreasing the cell viability [46].

Not only matrix stiffness affects cell migration, but also pore size determines cell migration [45]. Cellular infiltration is an important component of cardiac repair as it allows regeneration of the damaged MI region [45]. Other authors reported that hydrogels must be highly porous with an open interconnected geometry, to allow a large surface area [39], encouraging cell ingrowth, uniform cell distribution and to assist matrix neovascularization [48]. Inaddition all cells must be within 200 μm of the blood supply in order to provide adequate mass transfer of nutrients and oxygen. Thus, pore interconnectivity is also of critical importance [44,49]. If pores are too small, pore blocking by the cells would occur, inhibiting cellular penetration, ECM production and neovascularization of the inner areas of the hydrogel [39]. Madden *et al.* demonstrated that maximal vascularization was achieved in rats after cardiac implantation of acellular poly (2-hydroxyethy methacrylate-co-methacrylic acid) hydrogels with a pore diameter between 30-40 μm [50]. Our hydrogels had a pore diameter in the range of 30 μm, with the exception of the 100:0 Dex-TA:HA-TA that showed a pore diameter of 17 μm, which indicates an open network structure. This open network structure favors nutrient and detritus exchange to aid cell survival. Furthermore it facilitates the diffusion to the surrounding tissue of the GFs released either from the MPs or ADSCs. The favorable pore geometry of our hydrogels was subsequently shown in Live/Dead experiments: 95% of the ADSCs embedded in the gels remained alive during the 14 days

experiments. This is in agreement with a previous work, which showed that endothelial-like cells derived from mesenchymal stromal cells interact well with Dex-TA grafted on HA-TA enhancing proliferation, migration, and/or matrix remodeling [15]. In addition, metabolically active cells are expected to interact more intensively with the surrounding matrix [15]. The ability to sustain metabolic activity of the cells might be a key factor in successful clinical application of a biomaterial for TE purposes. These aspects also suggest that the gelation process does not compromise cell viability and that sufficient mass transport of nutrients and oxygen to the cells inside the gel matrix takes places, as observed in previous works [13].

Finally, the swelling/degradation ratio is another important design criteria to be considered when designing cardiac scaffolds in order to ensure mechanical support to the left ventricle for an appropriate amount of time [39]. In addition, hydrogels have to degrade at a rate that allow both cellular infiltration and tissue repair, as it is essential to provide an adequate extracellular milieu for transplanted cells, and consequently increasing their survival [35,44,45]. In the hydrogels developed, a weight increase was first observed, and this increase was higher and faster when the amount of HA-TA increased. This is due to the charged HA-TA conjugates which have increased water attraction [13]. Consequently, HA-TA hydrogels degraded faster. Meanwhile, as Dex-TA induced an increase in the stability of the hydrogel [13], a longer degradation was observed when the amount of Dex-TA was increased. It is remarkable that neither the swelling nor degradation rates of the Dex-TA:HA-TA and HA-TA hydrogels, were modified by the presence of the MPs, indicating that MPs do not affect hydrogel degradation rates. Interestingly, the 100:0 Dex-TA:HA-TA hydrogel, did not show swelling phase and nearly maintained a constant weight during the degradation assay. This may indicate that this hydrogel has a much more rigid structure and a slow degradation.

5. Conclusion

We were able to prepare a promising cardiac TE system combining Dex-TA:HA-TA hydrogels, NRG-MP and ADSC to be injected into damaged heart tissue. With this work, the 50:50 Dex-TA:HA-TA hydrogel embedding 1 mg of NRG-MP and 500,000 ADSC in 150 µL, showed the best properties for heart tissue repair, is established as a promising candidate for future studies. This injectable hydrogel demonstrated good

mechanical properties (5.7 ± 1.2 kPa), proper gelation time (320 s), adequate pore size (30 μm) to favor ADSCs survival, prolonged degradation rate to give support either to the left ventricle and to ADSCs and NRG-MPs (25 days) and good injectability through 29G needles. Nevertheless, further experiments are mandatory in order to assess the biocompatibility and efficacy of the system once in the infarcted heart.

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Injectable Dextran-Hyaluronic acid hydrogels embedding Neuregulin-loaded microparticles and Adipose-Derived Stem Cells as a strategy for cardiac tissue engineering

GENERAL DISCUSION

Cardiovascular diseases (CVDs) are the leading cause of death worldwide [1]. Among them, myocardial infarction (MI) is the main CVD, causing 7.4 million deaths each year and accounting for 13.2% of the global deaths (World Health Organization fact sheet: http://www.who.int/mediacentre/factsheets/fs310/en/). It is therefore fundamental to investigate new therapies to improve the outcome of patients who already suffer from these conditions. However, at present, neither pharmacological nor surgical treatments for MI have resulted in heart regeneration, as they are only useful for mitigating symptoms. As described in the **Introduction** to this thesis, various strategies including cell and growth factor (GF) therapies, have been extensively explored in order to favor heart regeneration. However, the results obtained with these therapies remain controversial due to the issues observed when they were tested in clinical trials (**Introduction** and [2]). Briefly, on the one hand, cell therapy requires an improvement in engraftment after transplantation. On the other hand, GFs have to be protected from the harsh environment due to their low stability in order to remain active for long periods of time [3,4]. For these reasons, many investigations have proposed biomaterial-based devices as a possible solution to overcome cell and protein delivery issues (**Introduction** and [2]). In most of those studies, biomaterials have shown potential to improve heart regeneration either on their own [5–7], or combined with cell [8–11] or GF therapies [12–16]. However, many aspects still need to be solved in order to obtain a system able to fully regenerate the heart. Recently, better regenerative improvements have been obtained when using combinations of cells, GF and polymeric devices, in what is known as the tissue engineering (TE) strategy [12,17–22].

In this thesis, and with the aim to develop more effective regenerative therapies for MI, two different TE strategies were investigated. The first strategy was the use of poly(lactic co-glycolic acid) (PLGA) microparticles (MPs) containing neuregulin-1 (NRG) as support for attaching adipose-derived stem cells (ADSC), in a system named ADSC-NRG-MP (**Chapters 1** and **2**). The second strategy, included in **chapter 3**, was the combination of two different polymeric devices, MPs and hydrogels. The MPs were loaded with NRG and were embedded together with the ADSCs in hydrogels composed of different ratios of dextran (Dex) and hyaluronic acid (HA). Both strategies were developed in order to increase cell survival and to activate different pathways to favor heart regeneration.

Many questions can be highlighted concerning the strategies that we have developed:

why were MPs and hydrogels used as polymeric devices? Why the ADSC as cell source? Why NRG as GF? And more importantly, is the combination of all the elements more effective than each one on its own? Answering the first question, many studies have been performed in the field of heart TE [4,20,23], but from all the delivery systems investigated, only hydrogels, MP and nanofibers have been tested in relevant large animal models of MI [24–26]. Additionally, only hydrogels have reached clinical trials [27]. Regarding MPs, our group has extensive experience in the elaboration of PLGA MPs for heart regeneration [28–32]. In these studies, prolonged efficacy of GF therapy was demonstrated [32,31]. Meanwhile, hydrogels have been shown to be one of the most promising candidates for TE, due to their unique compositional and structural similarities to the extracellular matrix (ECM), which favors cell survival and engraftment [33–35]. Another key aspect in favor of MPs and hydrogels is that they can be administrated by minimally invasive routes using guided catheter systems such as NOGA [25,36].

The selection of the biomaterials used to prepare the scaffolds (MPs and hydrogels) was another fundamental point of this thesis. The MPs were made of PLGA. This biomaterial has attracted significant interest in drug delivery due to its favorable properties such as good biocompatibility, biodegradability, low immunogenicity and low toxicity [37]. Also, the US Food and Drug Administration (FDA) has granted the approval of PLGA for human use [38]. On the other hand, hydrogels were prepared with HA and Dex. HA is an immunoneutral polysaccharide that is ubiquitous in the human body and is crucial for many cellular an tissue functions [39], which has facilitated its use in the last three decades [40]. Another interesting aspect of HA is that it is rapidly turned over in the body by hyaluronidase, with a tissue half-life ranging from hours to days [41]. This aspect has been seen to be favorable for heart tissue regeneration as it can be helpful in reducing the stresses in the heart wall that are induced after a MI [6,42,43]. On the other hand, Dex is a commercially available bacterial-derived polysaccharide [44]. Dex hydrogels have been previously investigated for drug delivery applications [45]. This biomaterial is highly hydrophilic and biocompatible. Its degradation products can be excreted through the kidney as long as the molecular weight of the original Dex components are below the filtration threshold of the kidney [44]. Nevertheless, this was the first time Dex-TA:HA-TA hydrogels have been employed for heart tissue repair.

Regarding the biomaterial manufacture method, MPs were prepared by TROMs, a

system that has previously been demonstrated to be suitable to encapsulate GFs [28,31]. TROMS avoids high temperatures that can degrade the GF, and allows higher encapsulation efficiency when compared to conventional microencapsulation methods [46]. Within hydrogels, the most frequent elaboration methods are chemical and physical crosslinking. But both methods have limitations that have led them to be ruled out as good systems for the elaboration of cardiac hydrogels. For instance, both methods require external activation for inducing gelation, such as ultra-violet light [47], and the opening of the chest is mandatory to direct the light directly onto the injected area of the heart. This increases the risk of adverse consequences, such as possible damage to the already injured tissue [48,49]. For these reasons, hydrogels employed in this research were prepared by enzymatic crosslinking. Enzymatic crosslinking is a method that is biologically compatible, with a high degree of substrate specificity that potentially avoids secondary reactions [50,51]. The only limitation of enzymatic crosslinking is that gelation time is very fast and occurs in a period of seconds. Thus, in order to allow catheter administration, the concentration of the reaction inducers has to be tightly adjusted to obtain longer gelation times.

Regarding the cell source, although different cell sources have shown potential for heart regeneration (**Introduction** and [2]), the use of ADSCs was based not only on their easy isolation, with a non-invasive system and in large amount [52,53], but also because they have been shown to have the ability to regenerate the heart [54–60]. Several preclinical studies and a certain number of clinical trials have tested the potential of ADSCs for regenerating the heart ([55,57–59,61,62] and ClinicalTrials.gov NCT01502514, NCT00442806, NCT01216995, NCT01556022, NCT01974128, NCT01449032). From those studies, the conclusions that can be drawn are that the improvements in heart regeneration are rather minor, as cell survival remained low. In this regard, the combination of cell therapy with polymeric devices has opened up new possibilities to improve cell survival.

Classically, stem cell therapy aimed to repopulate the damaged tissue with the transplanted cells once differentiated [53,63]. However, cell differentiation seems to be insufficient on its own to produce all the beneficial effects that have been observed after cell transplantation in the infarcted heart. That is the reason why the theory of a paracrine effect induced by the grafted cells has gained ground [64,65]. The paracrine effect has demonstrated that cells are able to secrete different GFs that can induce a

favorable response to the environment of damaged tissue, to help its regeneration. In previous studies, it has been demonstrated that ADSCs are able to secrete different GFs, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), among others [60,66]. Thus, the paracrine effect can help to build a system for tissue regeneration in which we can apply multiple GF therapy in a much easier and more effective way. Stem cells would secrete the concentration and the GFs proper for each precise moment of the damaged tissue, favoring the activation of different pathways to produce heart regeneration. In this case, the combination of cells and GFs would not be justified. But, as ADSCs are not able to produce all the GFs that can be involved in heart regeneration, the combination of cells with GFs different from the ones that they can release might be helpful. In the present work we selected NRG as GF to be combined with ADSCs, as among all the GFs that these cells are able to secrete, no published study includes NRG [60,66]. Moreover, previous research by our group [31] showed that NRG is a good regenerative GF for MI. NRG not only induced an improvement in the infarct size, fibrosis, vasculogenesis and cardiac function, but also favored a significant increase in cardiomyocyte proliferation and in the recruitment of cardiac progenitor cells in preclinical models of MI [25,32].

Against this background on the field, we worked on the elaboration of two TE strategies for cardiac repair, ADSC-NRG-MP and Dex-TA:HA-TA hydrogels embedding NRG-MP and ADSC.

Firstly, we investigated the potential feasibility of NRG-releasing MP combined with ADSC, the ADSC-NRG-MP system, as a multi GF delivery-based tissue engineering strategy for the ischemic heart (**chapters 1** and **2**). We were able to prepare PLGA MPs of 20 µm which efficiently encapsulate NRG, maintaining its bioactivity. As was described in **chapter 1**, the size of the particles was a key parameter, as on one hand MPs need to have enough surfaces to be able to attach the ADSCs, and on the other hand their size must be adequate so as to not induce further damage to the injected tissue. Based on the work of Formiga *et al.* [31], we estimated that 20 µm would be suitable to be injected in the heart, an aspect that was confirmed in this thesis (**chapters 1** and **2)**. From the results of the characterization and biocompatibility study (**chapter 1**), we demonstrated that the ADSC-NRG-MP system was able to favor the attachment of at least $5x10^5$ ADSCs to 1 mg of MP coated with the mixture 0.5 µg/cm^2 of collagen and poly-D-lysine (PDL) 1:1. Within the different coatings, it was the 0.5 µg/cm^2 of collagen and PDL 1:1 coating that favored faster attachment of the cells to their surface.

This coating only required 60 minutes, while the others required at least 90 minutes for complete cell adhesion (**chapter 2**). Also, the 0.5 μg/cm^2 collagen:PDL coated MPs proved to be faster in the attachment of the cells with respect to other coatings of MPs, which required at least 3 hours for complete adhesion [56]. In **chapter 1**, cells were detected surrounding the MPs in the infarcted myocardium two weeks after administration in a rat MI model. Another issue was the injectability of the system, because a 23G needle was mandatory for the administration of the ADSC-NRG-MP. Collectively, the results presented in **chapter 1** offered valuable evidence of the feasibility of using the system which was shown to be a promising treatment without inflammatory negative response caused either by the size/composition of the ADSC-NRG-MP system or by the needle diameter.

Next, we investigated the efficacy of ADSC-NRG-MP in a rat MI model (**chapter 2**). The ADSC-NRG-MP system was shown to be able to improve cell survival once in the tissue. Furthermore, ADSCs were detectable 3 months after their administration when they were adhered to the MPs. Previous work in the field of heart regeneration has already tried to increase ADSC survival by using polymeric devices [67–70]. Although improved cell survival and more complete regeneration of the MI heart were observed when compared to injection of ADSCs alone, no study was able to detect the ADSCs in the infarcted myocardium 3 months after their administration [67–70]. Next**,** it was observed that ADSCs expressed smooth muscle actine (α-SMA), a protein found within the walls of blood vessels. More importantly, some of those cells were inserted into newly formed vessels (**chapter 2**). Another aspect studied was cell proliferation, although no proliferation of the transplanted ADSC was observed, we did observe that cells surrounding the treatment area did proliferate. This suggests that the treatment was able to promote cardiac cell proliferation and indicates that a putative trophic effect could be induced either by the transplanted cells and/or by NRG released from MPs, as previously reported [32,59]. Further, it has been shown that cells and biomaterials favored the shift of the local macrophage expression from M1 macrophage (pro-inflammatory) to M2 (regulatory and homeostatic functions), inducing an increase in the ratio M2:M1, which favored heart regeneration [71–73]. Finally, regarding the regenerative effect of the ADSC-NRG-MP, it was seen that the ADSC-NRG-MP group showed the smaller infarct size and thicker left ventricle (LV) when compared to the other treatments (ADSC and ADSC-MP groups), indicating that the TE strategy

induced a greater effect. According to these smaller infarct size and thicker LV, an increase in vasculogenesis of the infarcted area was favored, both in arterioles and capillaries, which indicates that tissue remodeling is associated with vascular network growth. Moreover, tissue revascularization may also be helpful for improving cell survival and proliferation. In short, MPs were able to improve cell survival, and hence consistent higher efficacy was observed, resulting in a better outlook.

Taking a global overview of the results obtained, we saw that the ADSC-NRG-MP group was the treatment that induced the more pronounced regeneration of the infarcted heart. This allowed us to conclude that the use of the three elements of the TE strategy, and moreover, using ADSCs as cell source, NRG as GF and MP as polymeric device, are crucial to obtain a favorable response in the damaged tissue. For this reason, in the future ADSC-NRG-MPs will be administered in a more relevant animal model.

Finally, in the last part of this work (**chapter 3**) a new TE strategy was developed. Up to now only hydrogels have reached clinical trials for heart repair [27], and seem to be a good strategy to favor cell survival as they mimic the ECM [33–35]. Although GFs have also been embedded in hydrogels [14,48], their release remains fast [74]. To retard GF release from hydrogels, a possible solution may be the microencapsulation of those GFs in MPs, and the incorporation of the MPs into the hydrogels. Another aspect that attracts the attention of these hydrogels is their composition, as HA has previously been shown to induce heart regeneration [5,75]. But as HA degrades too fast, it was combined with Dex, as this biomaterial induces an increase on the stability of the hydrogel [76]. In this way it is expected that the improvement will not only be induced by the cells and the GFs, but also due to the polymer. Taking all this together, we developed and characterized hydrogels with different ratios of Dex-TA:HA-TA and different amounts of MP. All the hydrogels studied, independently of Dex-TA:HA-TA ratios and the amount of MP, had elastic modulus suitable for heart administration [77,78]. The next parameter studied was hydrogel pore size, a parameter that affects cell migration [79]. Some authors have reported that hydrogels must be highly porous with an open interconnected geometry [80,81]. Scanning electron microscopy (SEM) images showed that the Dex-TA:HA-TA hydrogels had an adequate mesh pore size, of 30 μm, which would allow cell survival and possible vascularization. As it is important to ensure mechanical support to the LV for the appropriate amount of time, the swelling/degradation assay was performed [81]. In the hydrogels developed, an increase

in their weight was firstly observed, being higher and faster when the amount of HA was increased [76], consequently having a much faster degradation. Regarding injectability, the developed hydrogels were able to pass through a fine-gauge needle (~27G), like catheter systems [36,79,82]. Finally, the cytotoxicity study showed that, independently of the presence of MPs or NRG-MPs, cells were metabolically active. Moreover 95% of them remained alive during the 14 days of the experiments. Although the elaboration of this system seems to be more complex, the beneficial effects expected would warrant a further survival of the cells, a further protection of the GF and also the beneficial effect from the HA. In summary, the hydrogel that showed the best properties for heart tissue repair was the 50:50 Dex-TA:HA-TA embedding 1 mg of NRG-MP and 500,000 ADSC, as it has good mechanical properties (5.7 ± 1.2 kPa), proper gelation time (320s), adequate pore size (30 µm), prolonged degradation rate (25 days), injectable through 29G needles and favoring ADSC survival.

To conclude, in the present thesis two candidates for heart repair after a MI were developed. On the one hand, the ADSC-NRG-MP demonstrated to increase long term cell survival and to induce a synergic effect between ADSC and NRG that favors cardiac regeneration. The next step would be the scale up of the ADSC-NRG-MP system and efficacy studies in a large animal model of MI. On the other hand, we were able to prepare a 50:50 Dex-TA:HA-TA hydrogels embedding ADSC and NRG-MP, but a release and biocompatibility study would be mandatory prior to an efficacy study to stablish wether it is a good candidate for regenerating the heart after a MI.

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CONCLUSIONS

The studies included in this work allow us to conclude:

1. PLGA microparticles (MP) prepared by TROMS technology presented a size of 20 µm. This technique was capable to efficiently microencapsulate neuregulin-1 (NRG) and its bioactivity was not affected during the manufacturing process, as demonstrated by a proliferation assay using the H9c2 cardiomyocytic cell line.

2. The coating of 1 mg of MP with 0.5 µg/cm^2 of a mixture 1:1 of collagen:poly-D-lysine allowed the fast and efficient adherence of 500,000 adipose-derived stem cells (ADSC), requiring only 60 minutes of incubation for the formation of the system denominated ADSC-NRG-MP.

3. The ADSC-NRG-MP showed to be compatible with the infarcted tissue in terms of size, injectability through 23G needles and tissue response two weeks after their administration in a rat myocardial infarction model.

4. The adhesion of the ADSC cells to the MP favored an increase of their survival in the tissue, either at short (one week) or long term (three months), inducing a higher and more sustained therapeutic effect.

5. Ki-67$^+$ cells were detected in the proximity of the injection area, indicating that the factors released either from the MP and the ADSC might favor the proliferation of cardiac cells.

6. The histological analysis showed that three months after the administration of the ADSC-MP and ADSC-NRG-MP, some ADSC cells expressed the endothelial smooth muscle actine (SMA$^+$) marker. Furthermore, some of those ADSC-SMA$^+$ cells were inserted inside vessels, showing their capacity to favor vasculogenesis.

7. The results obtained in the rat myocardial infarction model, showed that the use of ADSC-NRG-MP favored a better regeneration of the cardiac tissue than the ADSC or ADSC-MP. The transplantation of ADSCs adhered to the NRG-MP induced long term significant improvement of the infarct size, improvement in the mechanical behavior of the heart and a higher formation of new vessels, either of capillaries and arteriols.

8. The presence of biomaterials and ADSCs favored a shift in the expression from an inflammatory macrophage phenotype to a regenerative phenotype. One week after the administration of the treatments, it was observed an increase in the M2:M1 macrophage ratio, what could accelerate cardiac tissue regeneration.

9. Hydrogels composed of tyramined dextran and hyaluronic acid were capable to efficiently embed ADSCs and NRG-MP. Both, ADSCs and MP were homogenously distributed through the hydrogel.

10. The hydrogel composed of 50% dextran and 50% hyaluronic acid, embedding 1 mg of MP and 500,000 ADSCs, showed to be the hydrogel with better properties for cardiac tissue. This hydrogel proved to possess optimal mechanical properties to be administered in the heart. Furthermore, as the hydrogel has a prolonged degradation it would give support to the ventricle as well as to the ADSC and NRG-MP.

CONCLUSIONES

Los estudios realizados en este trabajo permiten concluir:

1. Las micropartículas (MP) de PLGA preparadas por la técnica TROMS presentaron un tamaño de 20 µm. Dicha técnica fue capaz de encapsular neuregulina (NRG) de manera eficiente y su bioactividad no se vio afectada durante el proceso de elaboración tal y como se ha demostrado en un ensayo de proliferación de la línea celular cardiomiocítica H9c2.

2. Se observó que el recubrimiento de 1 mg de MP con 0,5 µg/cm^2 de la mezcla 1:1 de colágeno:poli-D-lisina favorecía una rápida y eficiente adhesión de 500.000 células madre derivadas de tejido adiposo (ADSC), requiriendo únicamente 60 minutos de incubación para formar los complejos denominados ADSC-NRG-MP.

3. Las ADSC-NRG-MP mostraron ser compatibles con el tejido infartado en cuanto a tamaño, inyectabilidad con agujas de 23G y respuesta tisular dos semanas tras su administración en un modelo de infarto de miocardio en rata.

4. El trasplante de las células ADSC adheridas a las micropartículas favoreció una mayor supervivencia de las mismas en el tejido, tanto a corto (una semana) como a largo plazo (tres meses), ejerciendo así un mayor y más prolongado efecto terapéutico.

5. Se detectaron células Ki-67$^+$ en la proximidad de las zonas de inyección lo que indica que los factores de crecimiento liberados tanto por las MP como por las ADSC podrían favorecer la proliferación de células cardiacas.

6. Los análisis histológicos demostraron que tres meses después de la administración de los complejos ADSC-MP y ADSC-NRG-MP, algunas células ADSC expresaban el marcador endotelial actina de músculo liso (SMA$^+$). Además, algunas células ADSC-SMA$^+$ se encontraban incluidas dentro de vasos, mostrando su capacidad de favorecer la vasculogénesis.

7. Los resultados obtenidos en el modelo de infarto de miocardio en rata demostraron que el uso de las ADSC-NRG-MP mejoraba notablemente la regeneración del tejido cardiaco que las ADSC o las ADSC-MP. El trasplante de ADSCs en un soporte de NRG-MP indujo, a largo plazo, una mejora significativa del tamaño de infarto, mejora en el comportamiento mecánico del corazón y una mayor formación de nuevos vasos sanguíneos, tanto de capilares como de arteriolas.

8. La presencia de biomateriales y ADSCs favoreció un cambio del fenotipo de los macrófagos de uno inflamatorio hacia un fenotipo regenerador. Una semana después de la administración de los tratamientos se observó un aumento del ratio macrófagos M2:M1 lo que podría acelerar la regeneración del tejido cardiaco.

9. Los hidrogeles de dextrano y ácido hialurónico tiraminados fueron capaces de embeber en su interior ADSCs y NRG-MP, de manera eficiente. Tanto las ADSCs como las MP se distribuyeron de manera homogénea por todo el hidrogel.

10. El hidrogel compuesto por 50% dextrano y 50% ácido hialurónico, embebiendo 1 mg de MP y 500.000 ADSC, mostró ser el que mejores propiedades presentaba para su uso en tejido cardiaco. Dicho hidrogel se caracteriza por poseer propiedades mecánicas óptimas para ser administrado en el corazón. Así mismo, la degradación lenta del hidrogel favorecería su función de soporte tanto al ventrículo como a las ADSC y NRG-MP.

ANNEX I

CONTROLLED DELIVERY OF FIBROBLAST GROWTH FACTOR-1 AND NEUREGULIN-1 FROM BIODEGRADABLE MICROPARTICLES PROMOTES CARDIAC REPAIR IN A RAT MYOCARDIAL INFARCTION MODEL THROUGH ACTIVATION OF ENDOGENOUS REGENERATION.

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Controlled delivery of fibroblast growth factor-1 and neuregulin-1 from biodegradable microparticles promotes cardiac repair in a rat myocardial infarction model through activation of endogenous regeneration

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ABSTRACT

Acidic fibroblast growth factor (FGF1) and neuregulin-1 (NRG1) are growth factors involved in cardiac development and regeneration. Microparticles (MPs) mediate cytokine sustained release, and can be utilized to overcome issues related to the limited therapeutic protein stability during systemic administration. We sought to examine whether the administration of microparticles (MPs) containing FGF1 and NRG1 could promote cardiac regeneration in a myocardial infarction (MI) rat model. We investigated the possible underlying mechanisms contributing to the beneficial effects of this therapy, especially those linked to endogenous regeneration. FGF1- and NRG1-loaded MPs were prepared using a multiple emulsion solvent evaporation technique. Seventy-three female Sprague–Dawley rats underwent permanent left anterior descending coronary artery occlusion, and MPs were intramyocardially injected in the peri-infarcted zone four days later. Cardiac function, heart tissue remodeling, revascularization, apoptosis, cardiomyocyte proliferation, and stem cell homing were evaluated one week and three months after treatment. MPs were shown to efficiently encapsulate FGF1 and NRG1, releasing the bioactive proteins in a sustained manner. Three months after treatment, a statistically significant improvement in cardiac function was detected in rats treated with growth factor-loaded MPs (FGF1, NRG1, or FGF1/NRG1). The therapy led to inhibition of cardiac remodeling with smaller infarct size, a lower fibrosis degree and induction of tissue revascularization. Cardiomyocyte proliferation and progenitor cell recruitment were detected. Our data support the therapeutic benefit of NRG1 and FGF1 when combined with protein delivery systems for cardiac regeneration. This approach could be scaled up for use in pre-clinical and clinical studies.

1. Introduction

Ischemic heart disease is the leading cause of morbidity and mortality worldwide [1]. Thus, there has been great interest in novel therapeutic options, such as gene (reviewed in [2]) and stem cell therapy (reviewed in [3]), or even direct administration of pro-angiogenic cytokines [4]. In the case of growth factor-based therapy, although preclinical studies and initial clinical trials had suggested beneficial effects [5,6], double-blinded clinical trials with large cohorts of patients failed to validate the efficacy [7–9]. These negative findings may have resulted from issues related to growth factor selection, monotherapy instead of combinatorial therapy, and/or timing of growth factor delivery. Moreover, the therapeutic benefit of directly administered growth factors can be limited by the short circulating half-life and high instability of these proteins after injection. In this context, new strategies involving injectable biocompatible and biodegradable microparticles (MPs), which mediate sustained release of cytokines, might offer valuable approaches for overcoming these limitations [10].

Poly(lactic-*co*-glycolic acid) (PLGA) is a biopolymer that is FDA-approved for use as a drug delivery platform due to its excellent biocompatibility, high safety profile, and suitable biodegradation [11]. PLGA MPs were already shown to be useful for growth factor delivery [12,13]. Moreover, we demonstrated the efficacy of treating infarcted hearts with PLGA MPs loaded with vascular endothelial growth factor (VEGF), which induced neovascularization and reduced cardiac remodeling after myocardial infarction (MI) in rats [14]. Indeed, many

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pre-clinical and clinical studies aimed at repairing an infarcted heart tissue have explored pro-angiogenic cytokine administration as a means to promote tissue revascularization.

In addition to mediators of angiogenesis, the list of potential therapeutics for cardiac regeneration has continued to grow, and the use of factors involved in cardiac development, stem cell homing, cardiac differentiation/proliferation, or direct cardioprotection could lead to novel approaches for repairing a damaged heart (reviewed in [4]). In this regard, in vitro studies have shown that adult cardiomyocytes do not proliferate under resting conditions, but may divide in response to extracellular mitogens, such as periostin [15], acidic fibroblast growth factor (FGF1) [16], and neuregulin-1 (NRG1) [17]. These findings have supported a new paradigm, which suggests that the heart might be capable of repair and regrowth in response to extracellular mitogens. Consistent with this idea, it is known that FGF1 regulates cardiac remodeling by exerting a protective and proliferative effect after MI [18,19]. On the other hand, neuregulins play crucial roles in the adult cardiovascular system by inducing structural organization of sarcomeres, cell integrity, cell–cell adhesion [20], cell survival [21,22] and angiogenesis [23]. In fact, several studies using animal models of heart failure have demonstrated the therapeutic benefits of neuregulins, which improved cardiac performance, attenuated disease markers, and prolonged animal survival [24,25]. Furthermore, phase I and II clinical trials for chronic heart failure in humans confirmed the favorable effects mediated by neuregulins [26,27], highlighting the therapeutic potential of these growth factors in cardiac repair.

In this study, we have examined the efficacy of novel MP-based delivery of NRG1 and FGF1 in a rat model of MI. Notably, the use of MPs prevented issues related to growth factor stability, facilitating sustained treatment in the damaged tissues. As a result, we observed significant improvement in cardiac function upon MP-mediated delivery of these factors to infarcted hearts. Finally, we investigated the underlying mechanisms contributing to this positive effect, especially those linked to endogenous regenerative capacity.

2. Materials and methods

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC. An expanded Methods section is available in the Supplementary Material.

2.1. Materials

Recombinant human FGF1 and NRG1 were supplied from ImmunoTools GmbH (Friesoythe, Germany). PLGA with a monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA), bovine serum albumin (BSA), dimethylsulfoxide (DMSO) and sodium azide were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Química S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Murine HL-1 cardiomyocyte-cell line (kindly donated by Dr. Claycomb, Louisiana State University Medical Center, USA) was used in the in vitro assays. Claycomb medium was provided by SAFC Biosciences (Lenexa, KS, USA) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, USA). Rabbit polyclonal anti-human FGF-1 antibody (ab9588) was supplied by Abcam (Cambridge, UK) and horseradish-peroxidase conjugated donkey anti-rabbit (NA934V) were purchased from GE Healthcare. Goat polyclonal anti-human NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™ anti-rat IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Monoclonal anti-alpha smooth muscle actin-Cy3 (C6198) was provided by Sigma (St. Louis, MO, USA), anti-caveolin-1 and rat anti mouse CD45 (550539) from BD Pharmingen (Heidelberg, Germany). Rabbit polyclonal anti-human c-Kit antibody (A4502) was supplied from Dako (Carpinteria, CA, USA), monoclonal anti-human Ki-67 antibody (RM9106) was purchased from Thermo Fisher Scientific (Fremont, CA, USA) and mouse monoclonal cardiac troponin I antibody (ab19615) was obtained from Abcam (Cambridge, UK). DAPI nucleic acid stain was supplied from Molecular Probes-Invitrogen (Carlsbad, CA, USA) and TOPRO-3 was from Molecular Probes.

2.2. Preparation and characterization of MPs containing FGF1 and NRG1

FGF1- and NRG1-loaded PLGA MPs were prepared through a solvent extraction/evaporation method using the Total Recirculation One Machine System (TROMS) [14]. Particle size and size distribution were measured by laser diffractometry. Cytokine encapsulation efficiency and in vitro release from MPs was quantified by western blot. The bioactivity of MP-released proteins was evaluated in vitro by determining HL-1 cardiomyocyte proliferative capacity following growth factor treatment.

2.3. MI model and intramyocardial administration of MPs

Seventy-three female Sprague–Dawley rats underwent permanent left anterior descending coronary artery occlusion to induce MI. Among the surviving animals ($n = 57$), only those with a left ventricular ejection fraction (LVEF) below 50% ($n = 46$) at 2 days post-MI were included in the study. Four days post-MI, rats were divided into four groups, and the chests were reopened. Two milligrams of FGF1-loaded MPs (FGF1-MP; 1740 ng of FGF1), NRG1-loaded MPs (NRG1-MP; 1300 ng of NRG1), a mixture of MPs loaded with FGF1 and NRG1 (FGF1/NRG1-MP; loaded with the same doses), or control non-loaded MPs (NL-MP) were injected with a 29-gauge needle into four regions surrounding the border of the infarct. At 1 week ($n = 6$ rats) and 3 months ($n = 40$ rats) post-injection the animals were sacrificed.

2.4. Morphometric and histological studies

The heart function was assessed 3 months post-treatment. In addition, heart tissue remodeling, revascularization, cardiac proliferation, and endogenous stem cells were investigated. All results obtained from the growth factor-treated groups were compared to the NL-MP-injected control group.

2.5. Statistical analysis

Results are expressed as mean ± SEM. Statistics were calculated using Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). P values <0.05 were considered significant.

3. Results

3.1. FGF1 and NRG1 induce adult cardiomyocyte proliferation and survival in vitro

The effect of FGF1 and/or NRG1 on adult cardiomyocyte proliferation and apoptosis was studied in vitro. The treatment of HL-1 cardiomyocytes with different doses of FGF1 or NRG1 (alone or in combination) led to a statistically significant increase in cell proliferation (Fig. 1A) ($P < 0.01$). HL-1 cell apoptosis could be induced by hypoxia and serum deprivation, and addition of both FGF1 and NRG1 resulted in a statistically significant decrease in the apoptotic phenotype (Fig. 1B and C).

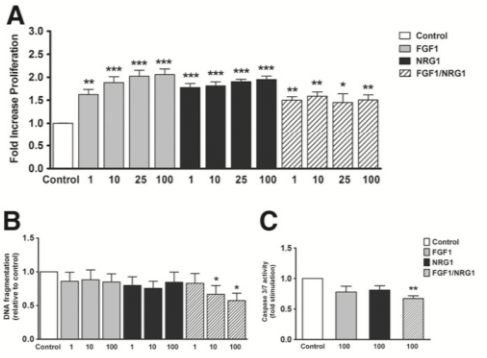

Fig. 1. FGF1 and NRG1 effects on cardiomyocyte proliferation and apoptosis in vitro. (A) HL-1 cardiomyocyte proliferation following treatment with free FGF1 or free NRG1 (1, 10, 25, and 100 ng/ml) administered alone or in combination (FGF1/NRG1). (B,C) HL-1 cell apoptosis in the presence of FGF1, NRG1, or FGF1/NRG1 was measured by ELISA detection of histone-associated DNA fragmentation (B) or detection of caspase-3/7 activity (C). Data are expressed as mean ± SEM from three independent experiments (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control).

3.2. Formulation and characterization of FGF1- and NRG1-loaded MPs

MPs prepared by TROMS had a spherical shape with an average size of 5.1 ± 1.4 μm (Fig. 2A and B). Encapsulation efficiency was found to be 87.4 ± 2.3% for FGF1 and 65.5 ± 5.1% for NRG1, which corresponded to final loading of 874.1 ± 23.4 ng of FGF1 and 655.3 ± 50.1 ng of NRG1 per mg of polymer. In vitro growth factor release profiles revealed an initial burst effect, which indicated that both factors displayed very similar release rates from day 7 to day 28, with 65% of NRG1 and almost 70% of FGF1 being released within 28 days (Fig. 2C). Also, we assessed the bioactivity of the MP-released cytokines by induction of HL-1 proliferation. NRG1 and FGF1, either released from the particles or as free cytokines, induced 1.5–1.7 fold increase in HL-1 proliferation in comparison with controls (NL-MP or no cytokine), indicating that both cytokines retained their biological activity after encapsulation into PLGA MPs (Fig. 2D).

3.3. Treatment with FGF1- and NRG1-loaded MPs improved cardiac function in a rat model of acute MI

Next, we investigated the effect of the growth factor-loaded MPs on cardiac repair using a rat model of acute MI. Four days after inducing MI, rats were treated with FGF1-MP, NRG1-MP, FGF1/NRG1-MP, or control (NL-MP). Three months after transplant, we observed significant improvement in LVEF of animals treated with FGF1-MP and NRG1-MP compared to those injected with NL-MP (Table 1). Moreover, absolute changes in LVEF (3 months post-infarction LVEF − baseline infarction LVEF) were significantly greater in the rats treated with FGF1-MP (15.0 ± 4.9%, $P < 0.05$), NRG1-MP (18.0 ± 5.7%, $P < 0.05$) or FGF1/NRG1-MP (13.0 ± 1.9%, $P < 0.05$) when compared with the NL-MP group (1.1 ± 3.6%) and similar among the three growth factor-loaded MP treatments (P = NS). Left ventricular end-systolic and end-diastolic diameters and volumes were higher in the NL-MP group, consistent with left ventricular (LV) chamber dilatation and progression of myocardial dysfunction (see Table 1; LVEDV, LVESV, LVEDD, and LVESD). In contrast, a statistically significant improvement in LVEDV and LVESV as well as LV mass was observed in FGF1-MP, NRG1-MP, and FGF1/NRG1-MP treated animals in comparison with NL-MP, indicating a beneficial effect on heart remodeling (Table 1).

Three months following transplantation, the infarct size was significantly reduced in animals treated with growth factor loaded MPs in comparison with NL-MP (NL-MP: 16.8 ± 2.8%; FGF1-MP: 11.9 ± 3.8%, $P < 0.01$; NRG1-MP: 12.3 ± 3.6%, $P < 0.01$; FGF1/NRG1-MP: 11.7 ± 3.8%, $P < 0.01$) (Fig. 3A), but no differences were detected among the growth factor treated rats. Similarly, fibrosis (collagen deposition) was significantly reduced in animals treated with FGF1-MP, NRG1-MP, or FGF1/NRG1-MP when compared with the NL-MP group (Fig. 3B), whereas LV thickness was significantly increased in the animals treated with FGF1-MP (2.06 ± 0.18 mm, $P < 0.05$), NRG1-MP (1.67 ± 0.07 mm, $P = 0.05$), and FGF1/NRG1-MP (1.93 ± 0.14 mm, $P < 0.05$) compared with control (NL-MP: 1.55 ± 0.14 mm).

Next, we evaluated the effect of growth factor loaded MPs on the number of arterioles/arteries (alpha smooth muscle actin [α-SMA]-coated vessels) and capillaries (small caliber caveolin-1$^+$ vessels). The density of α-SMA$^+$ vessels was significantly greater in animals treated with FGF1-MP and/or NRG1-MP (Fig. 4A). In addition, we observed a significant increase in the area of α-SMA$^+$ vessels after administration of any of the cytokine loaded MPs in comparison with the NL-MP (FGF1-MP: 5519 ± 448 μm^2; NRG1-MP: 8489 ± 803 μm^2; FGF1/NRG1-MP: 8064 ± 925 μm^2; NL-MP: 3972 ± 779 μm^2; $P < 0.05$).

Also, significantly more capillaries were found in the infarcted and peri-infarcted zones of animals treated with NRG1-MP or FGF1/NRG1-MP in comparison with NL-MP, whereas FGF1-MP treatment alone did not yield a similar effect (Fig. 4B). Notably, we observed a negative correlation between LVEF and the degree of fibrosis ($R = -0.599$; $p = 0.002$), while there was a positive correlation of LVEF with vasculogenesis (the area occupied by α-SMA-coated vessels) ($R = 0.591$; $p = 0.002$) (Table 2).

3.4. Apoptosis of cardiomyocytes is attenuated by growth factor-loaded MPs

Three months after treatment, there was a similar percentage of TUNEL-positive, apoptotic cardiomyocytes detected in the peri-infarcted zones of hearts treated with FGF1-MP, NRG1-MP, or control (NL-MP: 2.8 ± 0.9%; FGF1-MP: 1.5 ± 0.2%, P = NS; NRG1-MP: 1.8 ± 0.6%; P = NS); however, there was a clear trend in cardiomyocyte protection observed when the combination of growth factors was used (NL-MP: 2.8 ± 0.9% vs. FGF1/NRG1-MP: 1.1 ± 0.3%; $P = 0.08$). Therefore, consistent with the in vitro experiments (Fig. 1B and C), these findings suggest that dual treatment with NRG1 and FGF1 has a protective effect.

3.5. Growth factor-loaded MPs promote cardiomyocyte proliferation

In vivo, NRG1 and FGF1 were reported to induce proliferation of cardiomyocytes.[19,24] Thus, to determine whether growth factor loaded MPs could have a similar effect, expression of Ki67 was examined in myocyte-specific enhancer factor 2c (MEF2c)$^+$ and cardiac troponin-T (cTnT)$^+$ cardiomyocytes. We observed a significant increase in the number of Ki67$^+$ cardiomyocytes in the infarcted and peri-infarcted zones following treatment with NRG1-MP compared with NL-MP at 1 week (Fig. 5A) and 3 months (Fig. 5B) post-implantation (1.5- and 3.4-fold increases in Ki67$^+$ cardiomyocytes/mm^2 at 1 week and 3 months, respectively).

3.6. NRG1-MP induced c-Kit$^+$/CD45$^-$ progenitor cell recruitment during early myocardial repair

Finally, we investigated the effect of growth factor-loaded MPs on cardiac progenitor cell recruitment by measuring the number of c-Kit$^+$ cells in ischemic myocardium. Although the majority of c-Kit$^+$ cells in the peri-infarct and infarct zones were CD45$^-$ one week after treatment, c-Kit$^+$/CD45$^+$ cells represented the largest c-Kit$^+$ population at 3 months (Fig. 6). Notably, NRG1-MP injection into the ischemic myocardium resulted in a significant 10-fold increase in c-Kit$^+$/CD45$^-$ progenitor cell recruitment compared to the control

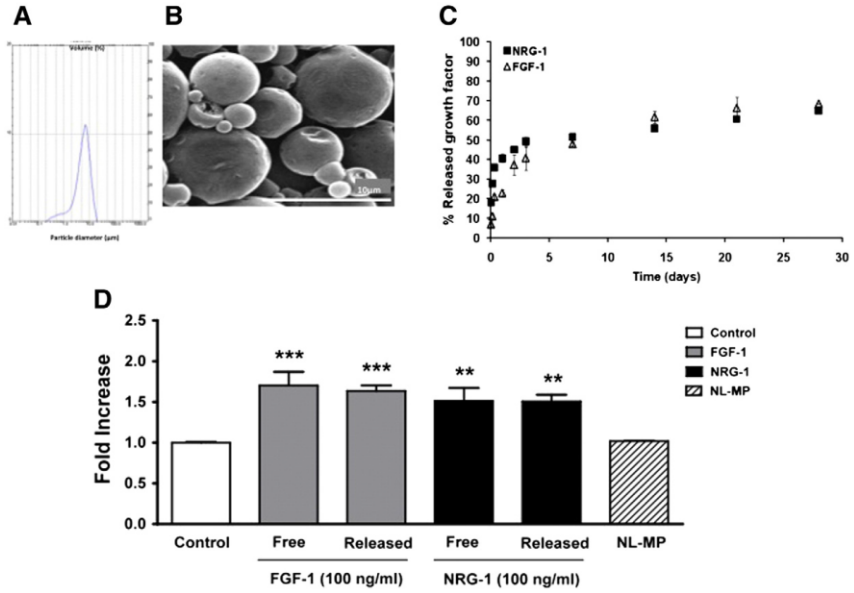

Fig. 2. Characterization of MPs loaded with FGF1 and NRG1. (A) Particle size distribution and (B) scanning electron microscopy images of MPs loaded with FGF1. (C) FGF1 and NRG1 in vitro release from MPs. (D) HL-1 cells were cultured in the presence of either MP-released or free FGF1 and NRG1 (100 ng/ml) and cell proliferation was assessed at 72 h using an MTS assay. Data are expressed as mean ± SEM from three independent experiments (**$P < 0.01$ and ***$P < 0.001$).

group during the early phase of myocardial repair (1 week after MP injection) (NL-MP: 4.88 ± 1.14 c-Kit$^+$/CD45$^-$ cells/mm^2; NRG1-MP: 48.40 ± 3.73 c-Kit$^+$/CD45$^-$ cells/mm^2, $P < 0.001$), but not at 3 months post injection (Fig. 6). In fact, the number of c-Kit$^+$/CD45$^-$ cells detected at 3 months was much lower than at 1 week. These results suggested a transient recruitment of cardiac progenitors shortly after injury. Moreover, when c-Kit$^+$/CD45$^+$ cells were quantified 1 week and 3 months after injection, we observed no significant differences between the groups that received growth factor-loaded MPs and NL-MP.

Table 1
Cardiac function data by echocardiography.

	NL-MP	FGF1-MP	NRG1-MP	FGF1/NRG1-MP
LVEF				
Baseline	38.0 ± 2.6	29.2 ± 2.9	30.1 ± 4.1	38.4 ± 4.7
Day 90	39.1 ± 3.0	44.2 ± 4.0*	48.1 ± 3.1*	51.4 ± 4.9**
FS				
Baseline	14.4 ± 1.9	11.9 ± 1.3	12.4 ± 2.0	16.2 ± 2.2
Day 90	16.8 ± 1.5	19.4 ± 2.1*	21.4 ± 1.7*	23.0 ± 2.8**
LV mass				
Baseline	1.35 ± 0.04	1.48 ± 0.07	1.63 ± 0.16	1.46 ± 0.09
Day 90	1.78 ± 0.07**	1.39 ± 0.07	1.41 ± 0.06	1.44 ± 0.06
LVEDV				
Baseline	1.151 ± 0.119	1.364 ± 0.098	1.690 ± 0.219	1.454 ± 0.190
Day 90	1.916 ± 0.194**	1.225 ± 0.170	1.154 ± 0.088*	1.177 ± 0.110
LVESV				
Baseline	0.756 ± 0.083	0.963 ± 0.073	1.159 ± 0.153	0.912 ± 0.134
Day 90	1.186 ± 0.158*	0.721 ± 0.131	0.614 ± 0.058*	0.585 ± 0.072**
LVEDD				
Baseline	0.800 ± 0.031	0.855 ± 0.023	0.912 ± 0.053	0.868 ± 0.043
Day 90	0.965 ± 0.037**	0.810 ± 0.045	0.788 ± 0.039	0.809 ± 0.028
LVESD				
Baseline	0.685 ± 0.030	0.753 ± 0.022	0.796 ± 0.044	0.729 ± 0.624
Day 90	0.804 ± 0.039*	0.658 ± 0.049	0.642 ± 0.021*	0.624 ± 0.030*

LVEF: left ventricular ejection fraction (%); FS: fractional shortening (%); LV mass: left ventricular mass (g); LVEDV: left ventricular end-diastolic volume (ml); LVESV: left ventricular end-systolic volume (ml); LVEDD: left ventricular end-diastolic diameter (cm); LVESD: left ventricular end-systolic diameter (cm). Values (mean ± SEM); *$P < 0.05$, **$P < 0.01$, vs. NL-MP.

4. Discussion

Protein therapies have failed to show consistent benefits in clinical trials of ischemic cardiovascular diseases [4,28]. Therefore, efforts aimed at understanding the factors limiting these strategies are fundamental for the successful development of future protein-based approaches. Our findings have suggested that PLGA MPs loaded with NRG1 and FGF1 can be used to improve cardiac function after acute MI by inducing an increase in vasculogenesis, inhibiting cardiac remodeling, and recruiting c-Kit$^+$ cardiac progenitors. Thus, the combination of this clinically applicable platform for drug delivery along with proteins involved in cardiac biology may represent a new therapeutic approach, which overcomes some of the classical drawbacks of protein therapy.

A number of studies have investigated systems that allow controlled delivery of therapeutic proteins, such as hydrogels, peptide nanofibers, liposomes, nanoparticles, and MPs. These strategies have been used mainly for the delivery of VEGF [14,29–31], FGF1 [19] and FGF2 [32,33]. Although hydrogels represent an appealing class of delivery vehicles, technical difficulties related to injection of the gelatin hydrogel into the thin ventricular wall of infarcted rat hearts have been reported [34]. Moreover, while liposomes have been shown to accumulate experimentally in areas of MI [30,35,36], their clinical application has been hindered because they are unstable and readily interact with high-density lipoproteins in blood. Also, self-assembling nanofiber scaffolds

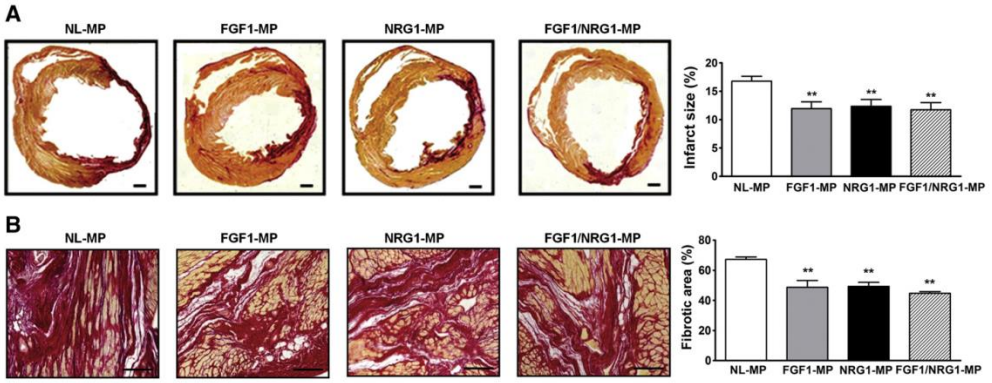

Fig. 3. Cardiac remodeling was inhibited by treatment with growth factor-loaded MPs. Representative images and quantification of infarct size (% of left ventricular [LV] infarcted area vs. total LV area) (A) and fibrosis (B) as measured by Sirius Red staining 3 months after injection of growth factor-loaded MPs or NL-MP. Results are shown as mean ± SEM (**$P < 0.01$ vs. NL-MP). Scale bars: 1 mm (A), 200 μm (B).

coated with VEGF were recently shown to improve cardiac function in rat and pig models of MI by inducing arteriogenesis and recruitment of endogenous myofibroblasts and cardiomyocyte-like cells [37]. In contrast to some of these systems, PLGA MPs are advantageous because they allow local drug delivery over an extended period of time. In fact, we have shown that these MPs persist in the heart tissue for at least 90 days following implantation, favoring long-term growth factor therapy [38]. The importance of this sustained release is supported by the fact that previous studies analyzing intravenous injection of free NRG1 did not observe improvements in infarct size [22], supporting the benefit of using PLGA MPs for delivery. Furthermore, while MPs were implanted by direct injection in this study, the size of the particles employed should allow for delivery through a less invasive procedure, such as using a NOGA-guided catheter. Thus, future studies validating this system in larger models might pave the way for future clinical application of this strategy, which should be relatively straightforward since PLGA has already been approved for clinical use.

NRG1 regulates cardiovascular homeostasis during development and adulthood by stimulating recruitment and proliferation of cardiac progenitors and cardiomyocytes as well as inducing angiogenesis, vasculogenesis, and cardiac remodeling [24]. On the other hand, FGF1 is a potent cardiac mitogen capable of reducing damage-induced cardiac scarring [19]. Based on the putative activities of these two growth factors, we hypothesized that a combination therapy involving administration of both cytokines would be more beneficial than each individually; however, we did not observe a consistent synergistic effect in vitro or in vivo. Instead, we observed that the effect of both cytokines combined was lower than either alone. Thus, as both proteins may utilize similar signaling pathways [15,17], it is possible that they compete, limiting their combined effect. Indeed, this important observation should be considered when designing new studies involving combination therapies. Interestingly, even though the cytokines showed an additive effect in preventing cardiomyocyte apoptosis in vitro, this finding did not translate in vivo.

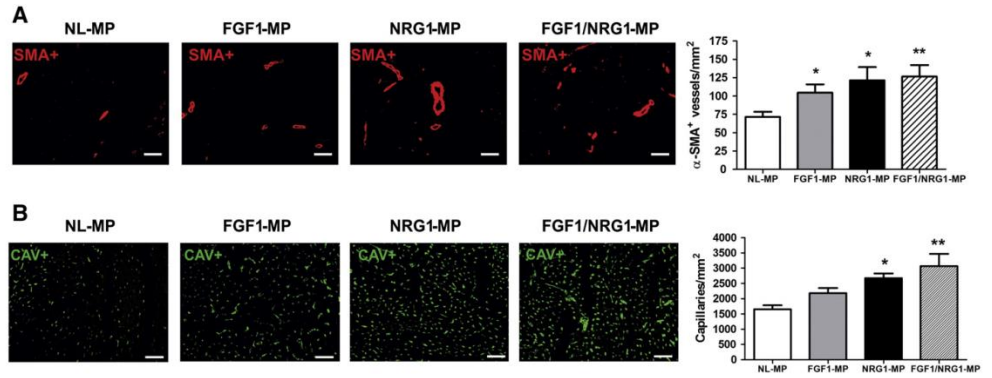

Fig. 4. FGF1 and NRG1 released from MPs in the ischemic myocardium exerted arteriogenic and angiogenic effects. Representative images and quantification of α-SMA$^+$ vessel density (arteriogenesis) (A) and caveolin-1$^+$ vessels (capillary staining) (B) in infarcted and peri-infarcted zones 3 months after injection of growth factor-loaded MPs or NL-MP. Results are shown as mean ± SEM (*$P < 0.05$ and **$P < 0.01$ vs. NL-MP control group). Scale bars: 50 μm.

Table 2
Pearson correlation analysis between functional echocardiographic parameters and histological results of growth factor loaded MP-treated hearts.

	Fibrosis	Vessel area	Apoptosis
Ejection fraction	−0.599(p = 0.002)**	0.591(p = 0.002)**	−0.364(p = 0.081)
LVESV	0.543(p = 0.006)**	−0.428(p = 0.037)*	0.517(p = 0.010)**

Data are shown as correlation coefficient (significance). LVESV: left ventricular end-systolic volume (ml).
*$P < 0.05$, **$P < 0.01$.

Further elucidation of the mechanisms by which different growth factors contribute to cardiac repair should help to refine protein-based therapies, as cytokines with different functional mechanisms may act synergistically. Interestingly, when tissue revascularization was assessed, a significant increase in arteriolar/arteries density and capillaries were found after treatment in each of the groups treated with growth factor-loaded MPs. These effects were only significant in those groups treated with NRG1, suggesting that at least in vivo, NRG1 has a greater vasculogenic effect in comparison with FGF1. While various populations of cardiac progenitor cells have been described, one of the most commonly employed cardiac progenitor markers is c-Kit, which identifies cells that participate in endogenous cardiac repair following MI [39]. Indeed, we identified a dramatic 10-fold increase in the number of c-Kit$^+$/CD45$^-$ progenitor cells 7 days after injection of NRG1-loaded MPs, which coincided with the time when the most drug was released from MPs. Thus, this finding suggests a potential role for NRG1 in cardiac progenitor recruitment. Moreover, it has been reported that c-Kit$^+$ progenitor cells can present a vascular phenotype, which might suggest that they participate in de novo vessel formation [40]. Nevertheless, we did not detect the recruitment of specific progenitors with a vascular phenotype.

In addition to cardiac progenitors, recent studies have suggested that adult cardiomyocytes can contribute directly to cardiac regeneration through either proliferation or dedifferentiation processes [41,42]. Thus, targeting cardiomyocyte proliferation could also represent a useful therapeutic approach. Although our study was not designed to specifically investigate cardiomyocyte proliferation, we observed increased Ki67$^+$ in cardiomyocytes 1 week and 3 months after injection of growth factor-loaded MPs, suggesting that a sustained release of NRG1 and FGF1 may increase the in vivo proliferative potential of myocytes. As observed in the case of c-Kit$^+$ progenitors, the effect on myocyte proliferation was negligible at 3 months but was 100-fold higher 1 week after injection.

5. Conclusions

Taken together, our present findings demonstrate the therapeutic efficacy of combining sustained protein delivery platforms with growth factors for achieving cardiac regeneration. Specifically, we have successfully delivered FGF1 and NRG1 to ischemic myocardium using a slow-release polymer. This strategy significantly contributed to global myocardial function during post-MI remodeling by promoting angiogenesis and arteriogenesis, inducing cardiac proliferation, and eliciting stem cell recruitment. Further validation of this therapeutic approach in large preclinical models could pave the way for implementation of this strategy in patients with ischemic heart disease.

Acknowledgments

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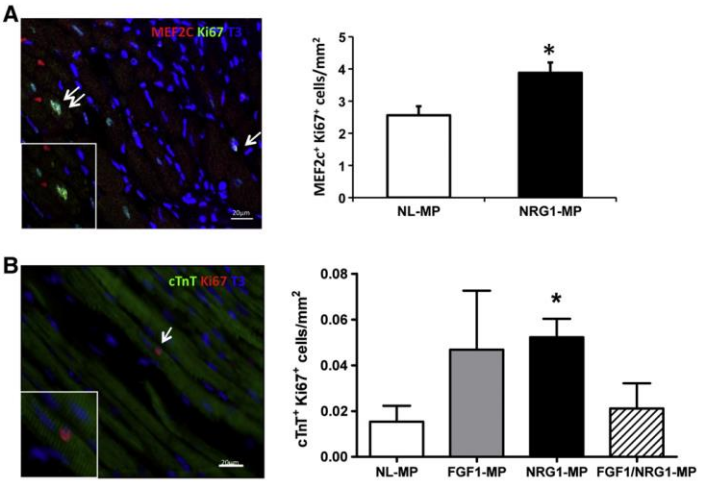

Fig. 5. Growth factor-loaded MPs induced cardiomyocyte proliferation. Proliferating cardiomyocytes were measured 1 week (MEF2c$^+$/Ki67$^+$) after NRG1-MP or NL-MP injection (A) or 3 months (cTnT$^+$/Ki67$^+$) after FGF1-MP, NRG1-M, FGF1/NRG1-MP or NL-MP injection (B). Representative images and quantification are shown. Data are represented as mean ± SEM (*$P < 0.05$ and **$P < 0.01$ vs. NL-MP control group). Scale bars: 20 μm.

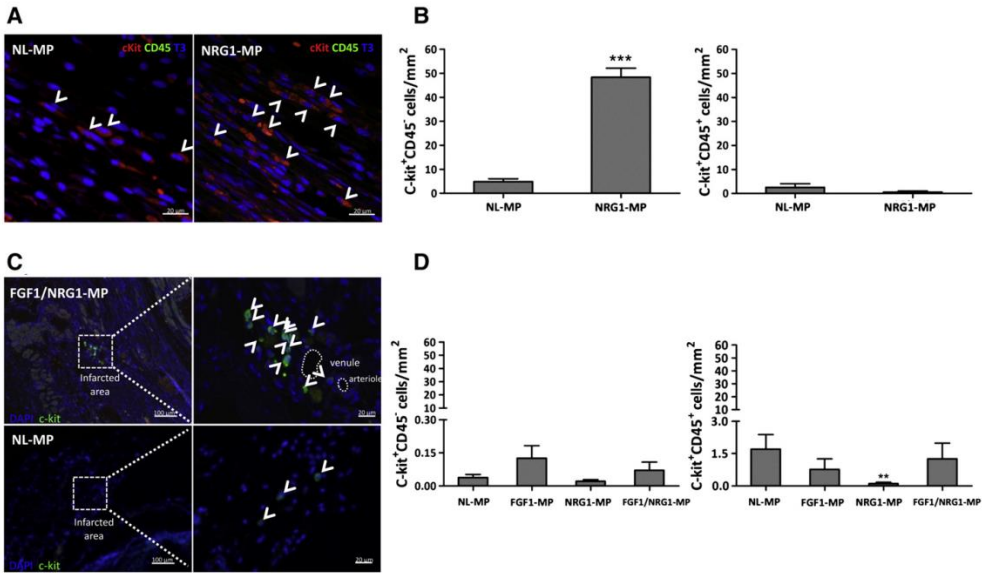

Fig. 6. NRG1-MP treatment induced cardiac progenitor cell recruitment. Cardiac progenitor cell (c-Kit$^+$/CD45$^+$ and c-Kit$^+$/CD45$^-$) recruitment was assessed 1 week after injection of NRG1-MP or NL-MP (A,B) and 3 months after injection of NRG1-MP, FGF1-MP, NRG1/FGF1-MP or NL-MP (C,D). Representative immunofluorescence images (A,C) and quantitation (B,D) are shown. Data are represented as mean ± SEM (*$P < 0.05$ and **$P < 0.01$ vs. NL-MP control group).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.10.034.

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ANNEX II

BIODEGRADATION AND HEART RETENTION OF POLYMERIC MICROPARTICLES IN A RAT MODEL OF MYOCARDIAL ISCHEMIA

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Research paper

Biodegradation and heart retention of polymeric microparticles in a rat model of myocardial ischemia

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ABSTRACT

Poly-lactide-*co*-glycolide (PLGA) microparticles emerged as one of the most promising strategies to achieve site-specific drug delivery. Although these microparticles have been demonstrated to be effective in several wound healing models, their potential in cardiac regeneration has not yet been fully assessed. The present work sought to explore PLGA microparticles as cardiac drug delivery systems. PLGA microparticles were prepared by Total Recirculation One-Machine System (TROMS) after the formation of a multiple emulsion. Microparticles of different size were prepared and characterized to select the most suitable size for intramyocardial administration. Next, the potential of PLGA microparticles for administration in the heart was assessed in a MI rat model. Particle biodegradation over time and myocardial tissue reaction were studied by routine staining and confocal microscopy. Results showed that microparticles with a diameter of 5 μm were the most compatible with intramyocardial administration in terms of injectability through a 29-gauge needle and tissue response. Particles were present in the heart tissue for up to 3 months post-implantation and no particle migration toward other solid organs was observed, demonstrating good myocardial retention. CD68 immunolabeling revealed 31%, 47% and below 4% microparticle uptake by macrophages 1 week, 1 month, and 3 months after injection, respectively ($P < 0.001$). Taken together, these findings support the feasibility of the developed PLGA microparticles as vehicles for delivering growth factors in the infarcted myocardium.

1. Introduction

Myocardial infarction (MI) is a great threat to life in developed countries, and so, research efforts are being focused on the development of new therapies. Therapeutic angiogenesis induced by exogenous administration of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) has been considered a promising strategy to treat patients with MI. However, although many preclinical studies have reported beneficial effects of angiogenic growth factor administration after MI, neither VEGF nor FGF have demonstrated efficacy in double-blinded clinical trials [1,2]. These disappointing results were attributed, at least partially, to the high intrinsic instability of the protein when systemically administered and the short half-life during which growth factors retain their biologic activity *in vivo*.

Current methods for growth factor delivery require administration of high protein concentration and repeated injections, which may result in abnormal vessel formation and unwanted side effects such as hypotension [3,4]. Targeted delivery of angiogenic proteins into the ischemic heart could therefore be useful. Delivery strategies that provide sustained local release of growth factors would not only control protein concentration, but could also minimize systemic exposure. A number of approaches have been designed to deliver growth factors in the heart in a controlled fashion. These include hydrogels, peptide nanofibers, liposomes, nano- and microparticles mainly for delivery of VEGF [5–9], FGF-1 [10], and FGF-2 [11,12]. While each delivery platform has both merits and drawbacks in the controlled delivery of angiogenic growth factors, there are few reports about the feasibility of these approaches via the intramyocardial route in relation to injectability, local retention and tissue response.

Polymeric microparticles encapsulating protein drugs offer the possibility of controlling the release of macromolecules over extended time periods [13]. Copolymers of lactic and glycolic acids (PLGAs) have been studied most commonly for this purpose because of their proven safety record and established use in marketed products for controlled delivery of several peptide drugs [14,15]. Nevertheless, PLGA microparticles have not been thoroughly investigated as a feasible delivery system for growth factors into the myocardium.

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In this study, the compatibility of PLGA microparticles with intramyocardial administration was evaluated in a rat model of myocardial ischemia. To this end, PLGA microparticles were prepared using Total Recirculation One-Machine System (TROMS), a technique based on the multiple emulsion solvent evaporation method which is suitable for the encapsulation of labile molecules like proteins [7,16]. Physical characteristics of the microparticles such as morphology, size or surface charge were primarily investigated. Furthermore, flow properties such as dispersability and injectability of microparticle suspension were analyzed to avoid complications during their administration. The *in vivo* biodegradation of the particles in the infarcted tissue and the evaluation of the myocardial responses to PLGA microparticles were finally evaluated using a rat model of MI to ensure safety and biocompatibility requirements.

2. Materials and methods

2.1. Materials

PLGA with a monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA) and rhodamine B isothiocyanate were provided by Sigma–Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc. (Warrington, PA, USA). Dulbecco's modified eagle medium (DMEM) was provided from Gibco-Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-rat CD68 antibody (MCA341R) was purchased from Serotec (Oxford, UK). Alexa Fluor 488 goat anti-mouse IgG antibody was provided by from Molecular Probes (Eugene, OR, USA).

2.2. Microparticle preparation

PLGA microparticles were obtained after the preparation of a multiple emulsion by solvent evaporation method using the Total Recirculation One-Machine System (TROMS) [7,16,17]. Briefly, the organic phase (O) composed of 50 mg of PLGA dissolved in 2 ml of a dichloromethane/acetone mixture (ratio 3:1) was injected into the inner aqueous phase (W_1) containing 5 mg of HSA and 5 μl of PEG 400 dissolved in 200 μl of phosphate-buffered saline (PBS pH 7.9). Next, the previously formed inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow through a needle with an inner diameter of 0.17 mm. After this homogenization step, the W_1/O emulsion was injected into the outer aqueous phase (W_2) composed of 20 ml of a 0.5%$_{w/v}$ PVA solution. The turbulent injection through a second needle resulted in the formation of a multiple emulsion (W_1/O/W_2), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. Microparticles were washed three times with ultrapure water by consecutive centrifugation at 4 °C (20,000g, 10 min). Finally, the particles were resuspended in 1 ml of ultrapure water, frozen at −80 °C, lyophilized (Genesis 12EL, Virtis) and stored at 4 °C. In order to obtain batches with different particle sizes, the following TROMS parameters were adjusted during microparticle preparation: pumping flow, recirculation times to form W_1/O and W_1/O/W_2 emulsions, and the inner diameter of the needle used to prepare the W_1/O/W_2 emulsion. A needle with an inner diameter of 0.17 mm was used to form the primary W_1/O emulsion of all microparticle batches.

For fluorescence-labeled microparticle formulation, rhodamine B isothiocyanate (0.5 mg/mL) was added to the inner aqueous phase and microparticles were prepared as described.

2.3. Size, surface charge analysis and morphological observation of PLGA microparticles

Particle size and size distribution of the microparticles were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, UK). The average particle size was expressed as the volume mean diameter in micrometers and samples were measured in triplicate.

For rhodamine-labeled microparticles, particle size was estimated using the software imaging system Cell° connected to the camera fluorescence microscopy system CH40 (Olympus GmbH, Münster, Germany). The morphology of the particles was characterized by scanning electron microscopy (SEM). Briefly, the lyophilized microparticles were mounted on carbon conductive disks attached to aluminum stubs. Samples were then coated with gold to a 16-mm thickness (Emitech K550 equipment). Microparticles were randomly scanned using SEM (Zeiss DSM 940A, Germany) and photomicrographs were taken.

Particle surface charge was determined by zeta potential measurement (Zeta Plus Potential Analyzer, Brookhaven Instruments Corp., New York, USA). A dilute suspension (0.5 mg/ml) of microparticles was prepared in 1 mM KCl (pH = 7.6) and the zeta potential measurements were performed after 10 cycles in the high precision mode.

Rhodamine-labeled microparticles were imaged at high-power by fluorescence microscopy. Microparticles were resuspended in water, mounted on a microscope slide, and visualized using a camera microscopy system (Olympus CH40).

2.4. Determination of microparticle dispersability and injectability

Prior to *in vivo* studies, microparticle dispersability was tested in three different resuspension media: PBS, DMEM and DMEM supplemented with a surfactant mixture composed of 0.1%$_{w/v}$ carboxymethyl cellulose, 0.8%$_{w/v}$ polysorbate 80 and 0.8%$_{w/v}$ mannitol in PBS, pH 7.4 (DMEM-S). Microparticle suspension injectability was assessed by its ability to pass through a 29-gauge needle, since these needles are used for heart injection [11]. Particle concentration and particle size were evaluated as injectability parameters in order to define the optimal microparticle formulation for heart injection.

2.5. Quantification of residual PVA content

The residual PVA associated with microparticles was determined by a colorimetric method [18]. Two milligrams of dry microparticles were hydrolyzed with 2 ml of 0.5 M NaOH for 15 min at 60 °C. The solution was then neutralized with 900 μl of 1 N HCl, and the volume was adjusted to 5 ml with distilled water. Next, 3 ml of a 0.65 M solution of boric acid, 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M) and 1.5 ml of distilled water were added. These conditions allowed the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule. After 15 min of incubation, the absorbance was measured at 690 nm using an Agilent 8453 UV–visible spectrophotometer (Agilent technologies, Palo Alto, CA, USA). A standard plot of PVA was prepared under identical conditions and measurements were performed in triplicate.

2.6. In vivo studies using PLGA microparticles

2.6.1. Induction of myocardial infarction

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 2010/63/EU. Animal experiments were carried out using a rat model of

cardiac acute ischemia-reperfusion. Rats were initially anesthetized with 4% isoflurane in an induction chamber. Prior to surgery, animals received analgesic drug ketoprofen 5 mg/kg subcutaneously, fentanyl 0.15 mg/kg and heparin 0.1 mg/kg both administered by intraperitoneal route. The rats were then intubated and ventilated at 90 cycles/min (1.5–2% isoflurane was maintained for continuous anesthesia). A left thoracotomy through the fourth intercostal space was performed, and the left anterior descending (LAD) coronary artery was occluded 2–3 mm distal from its origin for 1 h and then reopened. The chest was then closed in layers and rats allowed to recover on a heating pad.

2.6.2. Intramyocardial administration of microparticles

Four days after LAD coronary artery occlusion, animals were assigned to receive microparticles of different sizes (2, 5, 14 and 30 μm) or medium alone. Microparticle suspensions (2 mg/100 μl) were injected with a 29-gauge needle into four regions of the border zone surrounding the infarct while the heart was beating. A total of 22 animals were used in the *in vivo* experiments. The chest was closed and rats were allowed to recover on a heated pad.

2.6.3. Histological assessment of myocardial tissue after microparticle administration

Animals were sacrificed at different times after microparticle injection and their hearts were collected for histology. After being harvested, the hearts were perfused-fixed in 4% paraformaldehyde at 4 °C and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4 °C, embedded in paraffin and cut 5-μm-sections. Hematoxylin–eosin (HE) staining was carried out to localize the microparticles and to visualize tissue structure. Samples from control zone (non-injected tissue), right ventricle, and other organs (kidney, liver and spleen) were also analyzed.

2.6.4. In vivo biodegradation, tissue retention and phagocytic uptake of microparticles

A group of infarcted animals ($n = 8$) was injected 4 days after LAD coronary artery occlusion with 5 μm-sized fluorescence-labeled microparticles and sacrificed 8, 30 and 90 days later. Rhodamine B was used as a fluorescent marker to localize the injected microparticles by confocal microscopy in the heart tissue. After the hearts were frozen in OTC compound, frozen sections were prepared. In order to assess the phagocytic uptake of microparticles after their intramyocardial delivery, rat macrophage staining was carried out. CD68 immunofluorescence was performed as follows: slides were dried for 15 min at room temperature. Next, tissue sections were hydrated by passing through a graded ethanol series for 2 min each from absolute ethanol, 96%, 80%, and 70% followed by washing in running tap water and subsequently in distilled water. Prior to blocking with 5% bovine serum albumin (BSA) for 30 min, sections were rinsed with Tris Buffered Saline (TBS) plus 0.05% Tween 20 (TBST). Labeling with primary antibody was performed using a mouse anti-rat CD68 antibody (diluted 1:100 in TBS) by incubating at 4 °C overnight. After three consecutive washings with TBST (5 min each), fluorescent Alexa Fluor 488 goat anti-mouse IgG secondary antibody (1:100 dilution) was applied to sections for 1 h in the dark followed by nucleus staining with TOPRO-3 (diluted 1:50 in PBS-glycerol). For confocal microscopy, a LSM 510 META (Carl Zeiss, Minneapolis, USA) microscope was used. Digital images at 40× were analyzed in order to quantify the cardiac phagocytic uptake of injected microparticles. The extent of phagocytosis was expressed in terms of microparticle phagocytosis index determined as the ratio between the number of rhodamine-loaded microparticles internalized in CD68-positive macrophages and the total number of microparticles detected in each section. Eight serial sections of each rat were analyzed.

2.6.5. Statistical analysis

Data are presented as mean ± S.D. Statistics was calculated with Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). The differences among microparticles batches or groups of animals were first evaluated using the Kruskal–Wallis Test, followed by Mann–Whitney *U*-test when values followed a nonparametric distribution. The differences among the batches of microparticles or the groups of animals were assessed by ANOVA with a Tukey's post hoc correction when the measured values were normally distributed. Shapiro–Wilk test was used to justify the use of a parametric test. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. PLGA microparticles prepared by TROMS

A wide range of formulation methods have been used for encapsulating drugs into PLGA microparticles. These include solvent extraction [19], phase separation [20], spray drying [21], solid encapsulation [22], static mixer extrusion [23], and expansion in a supercritical fluid [24]. But the most frequently utilized method for the entrapment of fragile molecules is the water/oil/water (W/O/W) multiple emulsion solvent evaporation method [25]. Based on this method, TROMS has the advantage, over the conventional solvent evaporation techniques, of encapsulating compounds without the need for aggressive techniques or heating during the emulsification process. Thus, the method is especially useful for the encapsulation of fragile molecules such as growth factors. Previously, we successfully encapsulated VEGF and GDNF into PLGA-MP using TROMS, which maintained their biological activities [7,16,26]. In fact, higher encapsulation efficiencies were found when using TROMS. For instance, VEGF encapsulation efficiency up to 83% was achieved by TROMS [7]. In contrast, Golub et al. reported an entrapment efficiency of 5.3% of VEGF in PLGA-MP, employing a modification of the double emulsion method [27]. In turn, King et al. proposed a combination of the multiple emulsion technique and the atomization-freeze process into a unique solid-encapsulation/single-emulsion/solvent extraction method, but the entrapment efficiency of VEGF into PLGA microparticles using this manufacturing strategy was not improved, achieving a 16% entrapment efficiency [22]. Furthermore, another significant benefit of TROMS in view of its industrial application is the consistent production of very homogeneous batches of microparticles allowing for an easy scale-up of the manufacturing process.

In this study, TROMS-produced microparticles in the size range of 2–30 μm varying apparatus conditions during microparticle production. Particle size remained unchanged after lyophilization. The needle diameter for $W_1/O/W_2$ emulsion formation, pumping flow and recirculation times of both W_1/O and $W_1/O/W_2$ emulsions yielded batches with different particle sizes (Table 1). The inner diameter of the needles is a critical factor determining the final size of microparticles prepared by TROMS [17]. Microparticles with a diameter around 30 μm (batch 1) were obtained using the largest needle diameter to form the multiple emulsion (0.50 mm). A significant reduction in particle size was observed with needle diameters of 0.25 mm and 0.12 mm (batches 2 and 3, respectively, compared with batch 1, $P < 0.01$). Table 1 also shows the influence of recirculation times of both W_1/O and $W_1/O/W_2$ emulsions on the final size of microparticles, which was strongly dependent on the recirculation time of the primary W_1/O emulsion. A reduction in 1 min on the recirculation time of this emulsion increased significantly the particle size from 4.1 μm (batch 4) to 14.7 μm (batch 5) ($P < 0.01$). The pumping flow also played a key role in the final size of the microparticles, whereas increasing flows led to more turbulent regimes to form both primary and multiple emulsions. Conse-

Table 1
Influence of TROMS conditions on the final particle size.

Batch no.	Pumping flow (ml/min)	Needle diameter[a] (mm)	Recirculation times (min)		Mean size (μm)
			W_1/O	$W_1/O/W_2$	
1	25	0.50	3	4	30.1 ± 2.4
2	25	0.25	3	4	20.4 ± 1.8*
3	25	0.12	3	4	21.4 ± 1.4*
4	30	0.17	3	6	4.1 ± 0.7
5	30	0.17	2	6	14.7 ± 1.6#
6	30	0.17	2	4	19.8 ± 2.6
7	30	0.17	3	4	5.1 ± 1.4
8	35	0.17	3	4	3.3 ± 0.9
9	50	0.17	3	4	2.0 ± 0.8†

[a] Corresponding to the conditions for $W_1/O/W_2$ emulsion formation. A needle with diameter of 0.17 mm was employed in W_1/O emulsion formation for all batches.
* $P < 0.01$ vs. Batch 1.
# $P < 0.01$ vs. Batch 4.
† $P < 0.05$ vs. Batch 7.

quently, smaller microparticles were formed under higher homogenization energies supplied by more vigorous flows (batches 7 and 9, $P < 0.05$).

Colloidal stability was analyzed by measuring the zeta potential of PLGA microparticle surface. Particles were negatively charged (around −30 mV at pH 7.6) and no significant differences in zeta potential values were observed among all TROMS-produced microparticles batches. The morphology of the microparticles was examined by fluorescence microscopy (Fig. 1A). Microparticles appeared spherical in shape. Red fluorescence was distributed in the polymer matrix, indicating good rhodamine encapsulation. SEM visualization of the microparticles confirmed these findings, revealing a spherical shape with a smooth surface and few small pores in some particles (Fig. 1B). Physical characteristics of microparticles such as morphology, size or surface charge were investigated as well as quality controls for batch-to-batch consistency. Concerning the residual PVA content, the percentage of PVA recovered in the microparticles ranged from 1.1% to 1.6% depending on the formulations. These values are several times lower than 13%$_{w/w}$ PVA content previously reported [28]. The differences could be explained by the polarity of organic solvent that was used by Zambaux et al. [28] compared to that used in our study (dichloromethane vs. dichloromethane–acetone). Probably, the co-solvent system composed by a dichloromethane–acetone mixture led to less PVA partitioned into the polymeric phase, resulting in the lower deposition of PVA on the surface of TROMS-produced microparticles. Residual PVA differences could also be attributed to other formulation parameters such as PVA concentration in the external aqueous phase and PVA molecular weight.

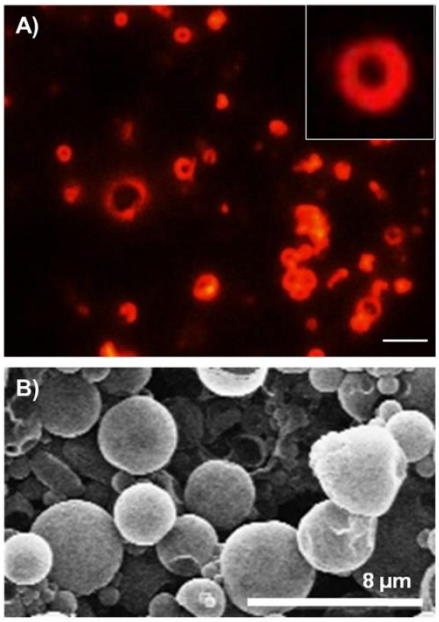

Fig. 1. (A) Fluorescence image of representative 5 μm rhodamine-loaded microparticles. Scale bars: 10 μm. (B) Scanning electron microscopy of representative 5 μm PLGA microparticles prepared using TROMS. Scale bars: 8 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Dispersability and injectability of microparticle suspension

Injectable microparticles encapsulating therapeutic agents require that the microparticles be well dispersed in the media used to deliver the agent. Therefore, good flow properties of the microparticles are necessary to ensure dose uniformity and safety requirements. In the present work, dispersability and injectability of TROMS-produced microparticles were assessed to select an adequate injectable PLGA microparticle suspension for local myocardial injection. These flow properties describe the ability of the microparticle suspension to pass easily through a needle. Dispersability of freeze-dried microparticles was tested in three different saline solutions: PBS, DMEM and DMEM-S. PBS and DMEM are solutions commonly employed to inject drugs/cells into the infarcted heart [29,30] and the carboxymethyl cellulose solution containing polysorbate 80 and mannitol has been previously used to suspend PLGA microparticles prior to intracerebral implantation [26]. Carboxymethyl cellulose is a wetting and biocompatible agent that prevents particle aggregation and makes easier their injection through a thin needle. Furthermore, carboxymethyl cellulose solution is a viscosity building agent commonly used in the formulation of oral and injectable pharmaceutical suspensions [31,32]. Microparticles were better dispersed in DMEM-S, probably due to the surfactant mixture added to medium, which increased DMEM viscosity and reduced particle aggregation and sedimentation. Thus, DMEM supplemented with carboxymethylcellulose,

polysorbate 80 and mannitol was selected as injection medium for animal experiments. Microparticle suspension in the range of 2–5 mg/100 µl was found to be both dispersible and injectable. Particle batches of 14 µm and 30 µm exhibited some resistance to resuspension in DMEM-S compared to smaller ones, probably due to sedimentation of particles with a diameter higher than 14 µm. Concerning microparticles' ability to pass through a 29-gauge needle, a typical needle size for heart injection [11], moderate levels of sedimentation could also explain the resistance observed when 30 µm-sized particles passed through the needle. In contrast, microparticles of 2 µm and 5 µm were flowable. They were easily injected through a 29-gauge needle.

3.3. Histological evaluation of myocardial tissue after microparticle injection

Microparticle batches of 2, 5, 14 and 30 µm were tested according to their compatibility with an intramyocardial injection. Microparticles were intramyocardially injected in the infarcted beating heart (Fig. 2). As mentioned above, large microparticles did not exhibit suitable resuspension in the injection medium. Indeed, blockages in the 29-gauge needle were detected during the administration of 30 µm-sized particles in the myocardium. Probably, the presence of aggregates obstructed the flow through the needle, which limited the injection of large microparticles in the rat heart. Despite the obstruction, a residual amount of 30 µm-sized particles reached the infarcted area. HE staining showed a more consistent accumulation of inflammation-mediated cells (IMCs) after injection of large microparticles (30 µm-injected particles, Fig. 3C and D) than in smaller ones (2 µm-injected particles, Fig. 3A and B). In fact, it has been described that large microparticles (75 µm) caused extensive myocardial necrosis in a porcine model [33]. On the other hand, there were fewer IMCs in the surrounding areas of 2 µm-sized particles, which did not induce severe responses. However, these 2 µm diameter particles exhibited a low retention in the heart, probably due to local phagocytic activity (results not shown). Taken together, these observations demonstrated that particles with an intermediate diameter could be adequate for heart injection. Consequently, 5 µm-sized particles were selected for long-term tissue retention and response

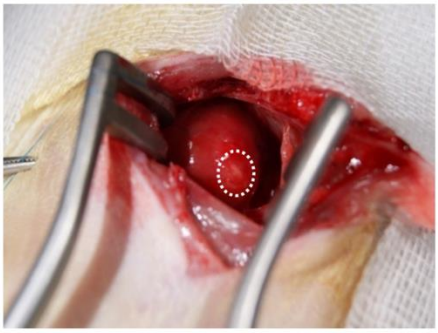

Fig. 2. Macroscopic view of the heart following 5 µm-sized PLGA microparticle implantation. Four days after LAD coronary artery occlusion, rats were anesthetized with isoflurane and ventilated. The chest was held opened by a retractor. Microparticles were injected into the infarct zone through a 29G needle while the heart was beating. Note the presence of the microparticles in the beating heart demonstrating that microparticles were not rapidly washed out from the infarcted myocardium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

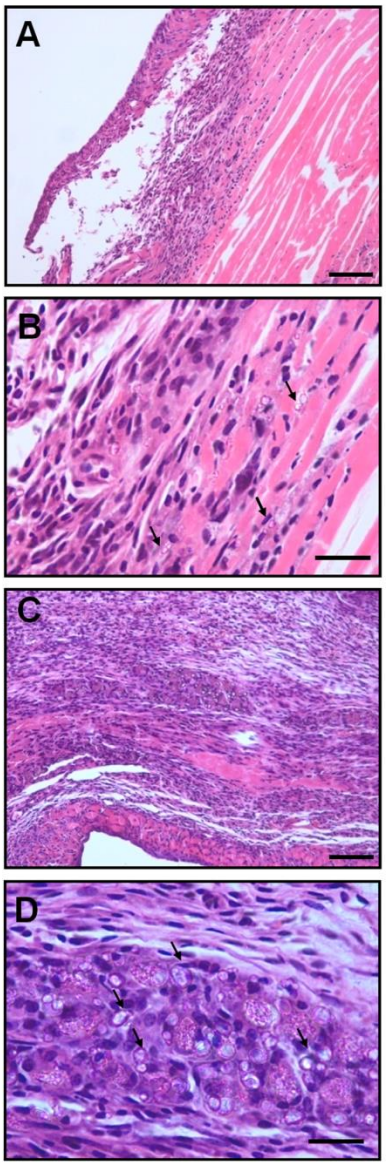

Fig. 3. Histological evaluation of myocardial tissue reaction 4 days after microparticle administration in hematoxylin–eosin stained sections. Microparticles with a diameter of 2 µm (A and B) and 30 µm (C and D) are clearly visualized at high magnification (B and D indicated by arrows). Scale bars: 20 µm (A and C) and 100 µm (B and D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

studies. Interestingly, 5 μm-sized particles did not induce inflammatory reactions when compared to the injection of resuspension medium. The response of the heart was the typical reaction observed following mechanical trauma and exposure to a foreign body. Slightly inflamed areas provoked by the needle during the myocardial administration of DMEM-S or suspension of microparticles with 5 μm diameter were observed. This finding correlates with the report that 7 μm resin particles encapsulating FGF-2 did not cause myocardial damage [34]. However, the clinical application of these microparticles is limited because the resin material is non-biodegradable, unlike PLGA which degrades generating monomeric acids (lactic and glycolic acids) that are consequently eliminated from the body as carbon dioxide and water [35]. Importantly, no myocardial hemorrhage was observed in our HE sections. Additionally, no signs of physiological disturbances such as fibrillation upon microparticles injection and no adverse cellular reactions in the tissue adjacent to the implanted microparticles were observed.

3.4. Biodegradation, tissue retention and phagocytic uptake of microparticles in the infarcted heart

Confocal microscopy and HE staining were carried out to study biodegradation of 5 μm-sized particles over time. The results are illustrated in Fig. 4. Fluorescent microparticles were always grouped at the implantation site and they remained at the site of injection during the entire 90-day experiment. At the beginning of the study, microparticles showed a spherical shape but they underwent progressive changes in size and morphology over time. Between the first and second week, there was a decrease in the microsphere size that continued to diminish, at a lower rate, over time. Most of the microparticles lost their spherical shape and showed a reduction in their size 2 months after implantation. Importantly, 3 months after implantation, microparticles were not totally biodegraded and a significant amount of them were still detectable (Fig. 4D and E).

Some studies have demonstrated a rapid loss of intramyocardially implanted cells or drugs which is biologically caused by the contracting myocardium [36,37]. In the present paper, we have demonstrated that microparticles were not rapidly washed out from the infarcted myocardium (Fig. 2). Immediately after microparticle administration, a persistent blanching surrounding the injection point and a change of tissue color from dark pink to light pink after polymer injection was observed. There was no microparticle loss or leakage during the intramyocardial implantation, indicating a localized retention of the microparticle suspension in the epicardial zone. Confocal microscopy and HE staining was performed to evaluate the temporal retention of fluorescence-labeled PLGA microparticles in the heart tissue. Fluorescent microparticles were found in the injection sites up to 3 months post-implantation (Fig. 4). Whereas Sy et al. reported retention of 20 μm-sized poly(cyclohexane-1,4-diyl acetone dimethylene ketal) (PCADK) microparticles for up to 10 days in the myocardium [38], and another study carried out by our group showed retention of 5 μm-sized PLGA microparticles encapsulating VEGF for at least 30 days [7], the results of the present study showed retention of 5 μm-sized PLGA particles for up to 90 days. These tissue retention results indicate the capacity of PLGA microparticles to remain in the myocardium for a prolonged period of time, a requirement for sustained growth factor treatment. Correlating with histological observations of HE stained sections, no fluorescent signal of rhodamine-loaded microparticles was observed in other tissues such as kidney, liver and spleen, indicating no migration of the microparticles toward solid organs. This is an important feature of PLGA microparticles for local delivery of therapeutics into myocardium, preventing systemic side effects of the loaded drugs.

While there are reports that have described the phagocytic uptake of PLGA microparticles in macrophage cultures [39,40], there is no detailed *in vivo* study on the macrophage-mediated phagocytosis of PLGA microparticles in the heart tissue. As the macrophage is a primary responder cell involved in the regulation of post-MI wound healing, eliminating apoptotic/necrotic myocytes and other debris [41], phagocytic activity of cardiac macrophages upon injected microparticles was further assessed. Quantification of phagocytic uptake of rhodamine-loaded microparticles was carried out by detection of CD68 macrophages. The extent of phagocytosis measured as the ratio between rhodamine-loaded microparticles internalized into CD68-positive macrophages and the total number of microparticles detected in each section was assessed in three groups of animals: rats sacrificed 1 week, 1 month, and 3 months

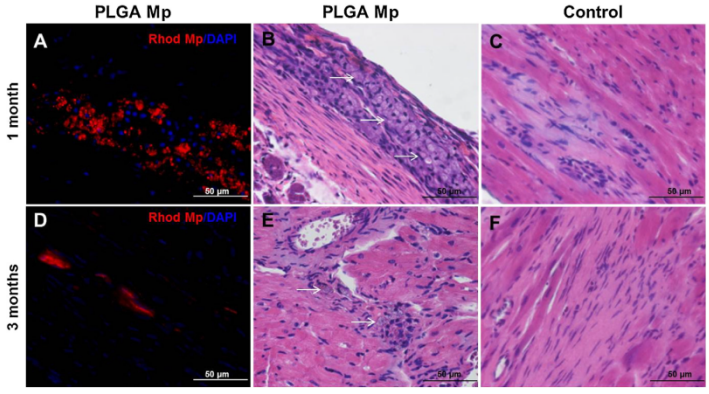

Fig. 4. Biodegradation and tissue retention of 5 μm-sized fluorescent microparticles intramyocardially implanted in the infarcted heart 4 days after LAD coronary artery occlusion. Representative images of confocal microscopy (A and D) and hematoxylin eosin staining (B, C, E, F) of the peri-infarted area 1 and 3 months after PLGA microparticle (A, B, D, E) or resuspension media (control) (C and F) injection. The administration of microparticles was well tolerated by the tissue and no differences on tissue inflammation were found between the administration of medium or microparticles. Note that microparticles were still present in the peri-infarted area 3 months after the injection. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

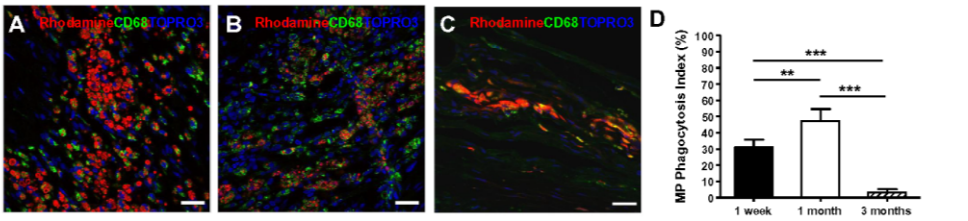

Fig. 5. *In vivo* phagocytic uptake of 5 μm-sized PLGA microparticles. Representative pictures of CD68 (green) macrophage immunofluorescence of heart sections 1 week (A), 1 month (B) and 3 months (C) after intramyocardial administration of microparticles labeled with rhodamine (red). Nuclear staining was performed with TOPRO-3 (blue). Scale bars: 20 μm. Quantification of microparticle phagocytosis was determined as the ratio between rhodamine-loaded microparticles internalized into CD68-positive macrophage and the total number of microparticles detected in each section (D). ***$P < 0.001$, **$P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

after intramyocardial administration of fluorescent microparticles. CD68 immunolabeling revealed a microparticle uptake of 31% 1 week after microparticle injection (Fig. 5A and D). An increase in the phagocytic activity of macrophages upon microparticles was detected 1 month after drug administration, with a microparticle phagocytosis index of 47% (Fig. 5B and D). In contrast, a very low microparticle uptake (below 4%) was found 3 months after implantation (Fig. 5C and D).

In rodent models of MI, within the first hours to 1 day, there are robust up-regulations of intramyocardial cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These cytokines mediate the acute remodeling process in the infarcted myocardium, which includes modulation of cardiac macrophages and phagocytosis [42]. After the initial increase of gene expression in the infarcted region, the cytokines normally begin to decrease toward baseline after 1 week [43]. Therefore, a decrease in the phagocytic uptake of microparticles 1 month after injection must be expected, because of decreased phagocytic activity of cardiac macrophages during the chronic remodeling post-MI. However, a larger number of microparticles internalized into CD68-positive macrophage were quantified in injected animals after 1 month compared with 1 week. One potential explanation is that microparticles maintained their spherical shape and diameter around 5 μm, presenting some resistance to phagocytosis 1 week after injection, a very short period for polymer degradation. In contrast, 1 month after their injection, particles originally 5 μm in size were observed as smaller ones (<2 μm) due to higher polymer degradation. Consequently, these small particles in the heart tissue were more susceptible to phagocytosis, whereas particle size around 1 μm is suitable for efficient uptake by macrophages [44]. Three months after microparticle injection, microparticles were visible in heart tissue. However, a low colocalization degree of microparticles with CD68-positive macrophage was observed. It is possible that macrophage response decreases considerably after this period, owing to very low levels of intramyocardial cytokines which modulate macrophage activation. At this time, damage due to the injection is repaired and the inflammatory response is ended.

On the other hand, in addition to particle size, other formulation parameters could affect the phagocytic uptake of microparticles. For example, particle hydrophobicity decreases with the amount of residual PVA associated with microparticles, reducing their recognition by macrophages [18]. We used a 0.5% PVA solution as stabilizer, which prevents microparticle aggregation during solvent removal. Using this PVA concentration, microparticles with a minimal content of residual PVA were obtained. Moreover, microparticles presented a high negative charge, which is associated with a stable colloid nature. An increase in PVA concentration used for microparticle formulation would result in the increase in the residual PVA content. However, as PVA is a potentially toxic non-biodegradable polymer, its administration should be minimized as much as possible [45]. Therefore, changes in formulation parameters must be rationally performed to alter microparticle hydrophobicity, with the aim of controlling its phagocytic uptake during the first month after implantation.

4. Conclusions

In this study, a PLGA microparticle formulation was developed that was compatible with intramyocardial injection in terms of particle size, injectability and tissue response. In addition, these particles exhibited the capacity to remain in the myocardium for up to 3 months. Concerning *in vivo* phagocytic uptake of microparticles, a moderate level of macrophage-mediated phagocytosis of PLGA microparticles was observed in the heart tissue. In particular, this result helps us to understand better the heart tissue response to a polymeric delivery system in the context of biomaterial research for cardiac regeneration.

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ANNEX III

HEART REGENERATION AFTER MYOCARDIAL INFARCTION USING SYNTHETIC BIOMATERIALS

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Review

Heart regeneration after myocardial infarction using synthetic biomaterials

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ABSTRACT

Myocardial infarction causes almost 7.3 million deaths each year worldwide. However, current treatments are more palliative than curative. Presently, cell and protein therapies are considered the most promising alternative treatments. Clinical trials performed until now have demonstrated that these therapies are limited by protein short half-life and by low transplanted cell survival rate, prompting the development of novel cell and protein delivery systems able to overcome such limitations. In this review we discuss the advances made in the last 10 years in the emerging field of cardiac repair using biomaterial-based delivery systems with focus on the progress made on preclinical *in vivo* studies. Then, we focus in cardiac tissue engineering approaches, and how the incorporation of both cells and proteins together into biomaterials has opened new horizons in the myocardial infarction treatment. Finally, the ongoing challenges and the perspectives for future work in cardiac tissue engineering will also be discussed.

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1. Introduction

1.1. Myocardial infarction and current treatments

Myocardial infarction (MI) remains a leading cause of morbidity and mortality worldwide, being responsible for nearly 7.3 million deaths each year. Moreover, as the World Health Organization highlighted in the last "Global Atlas on cardiovascular disease prevention and control" report [1], the number of deaths is expected to increase within the next decades due to the rising prevalence of the key risk factors for this pathology, such as behavioral and metabolic factors.

MI is principally caused by the occlusion of a coronary artery due to atherosclerotic and thrombotic processes, with the consequent reduction of the blood flow to the heart muscle. That loss of blood supply to the myocardium induces functional and morphological consequences. First, ischemic conditions lead to cardiomyocyte (CMC) death by necrotic or apoptotic processes, generating an infarcted area and causing a defect in contractile function. As a consequence, progressive and negative left ventricle (LV) remodeling and scar tissue formation take place [2]. These changes affect the ventricular chamber geometry, leading to the emergence of a larger, thinner and more spherical heart shape. Although the collagen-rich scar provides a rapid solution that avoids total LV wall disintegration, progression of the MI event often culminates in total heart failure and death [2]. A schematic representation of MI development with the principal steps is shown in Fig. 1.

Death from MI could be prevented by accurate early-stage diagnosis and proper subsequent proper treatment [3]. The more quickly the blood flow is restored, the better the outlook. Current therapies include surgical procedures such as coronary bypass, balloon angioplasty, stents and heart transplant as a last option [4]. Surgical interventions are generally combined/complemented with pharmacological treatments in order to improve patient outcomes [5]. However, although conventional interventions are useful in mitigating MI symptoms [6,7], they cannot repair the infarcted tissue, and so cardiac dysfunction remains an issue [8]. In view of the fact that current treatments are not able to regenerate the cardiac tissue and that the heart has shown limited post-natal cardiomyogenesis [9], patients who survive a MI might face serious functional limitations for the rest of their lives, which leads to secondary complications that impair their quality of life and place a major annual economic burden on the country [10].

1.2. New therapeutic strategies under investigation for myocardial infarction

As already stated above, conventional treatments are not enough to deal with functional and economic complications derived from MI and many aspects of the treatment for this pathology remain challenging. Therefore, in recent decades there has been a great research effort aimed at finding new alternative therapies for MI, focusing on myocardial regeneration. In these investigations, angiogenesis, CMC proliferation and recruitment of stem cells (SCs) to enhance endogenous healing of the heart have played an essential role, since they are considered to be key factors for adequate post-ischemic repair [11,12]. The advent of new molecular and cellular targets together with advances in genomics, proteomics and other biotechnologies have led to the discovery of novel pharmaceutical compounds with the potential to definitively change MI treatment. This emerging class of substances includes biological agents, genes, siRNAs, small molecules such as growth factors (GFs) and other therapeutics [13]. Among them, the ones that have shown the best results so far are cells and proteins [14,15]. In fact, exciting preclinical studies carried out to evaluate regenerative therapies for MI have prompted the initiation of clinical trials based on administration of SCs or GFs to the heart, as shown in the sections below.

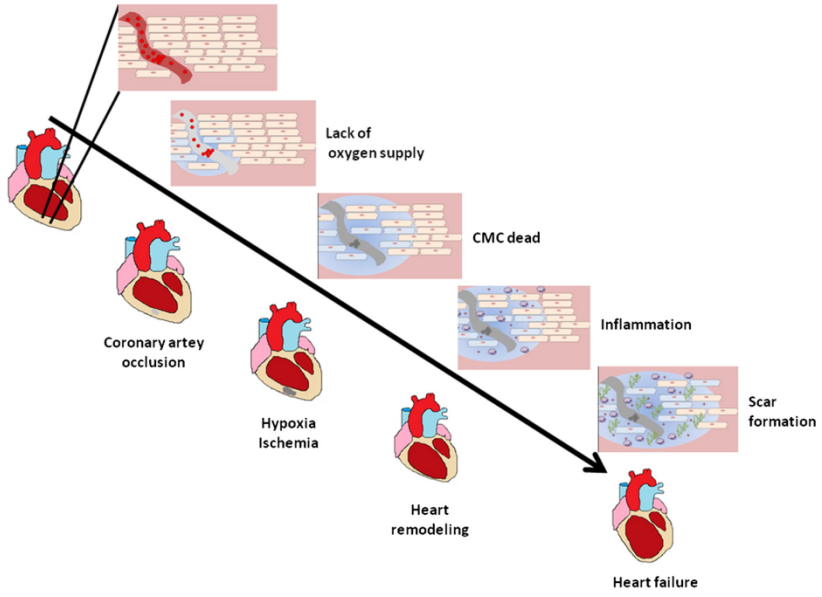

Fig. 1. Schematic representation of MI development including the principal steps. After a coronary artery occlusion, the heart gradually loses its function and suffers a negative remodeling that ends in total heart failure. The black arrow indicates the progression of the pathology.

1.2.1. Cell therapies for MI in clinical trials

Cell therapy relies on the administration of living cells for therapeutic purposes. Focusing on myocardial regeneration, it requires the administration of multipotent cells able to differentiate into the main cardiac cell lineages myocytes, vascular smooth muscle cells and endothelial cells [16] and to develop both CMCs and coronary vessels [17]. To date, the most popular cell candidates used to regenerate the damaged tissue include adipose derived stem cells (ADSCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), endothelial progenitor cells (EPCs), bone marrow derived stem cells (BMSCs), induced pluripotent stem cells (iPS), cardiac progenitor cells (CPCs) and induced cardiomyocytes (iCMs) [18]. However, although several SCs have been tested in *in vitro* and *in vivo* preclinical studies with promising results [19], not too many SCs have reached clinical trials. In fact, only BMSCs, myoblasts, CPCs and ADSCs have been employed in clinical trials yielding both encouraging and disappointing results [20]. The discrepancies in the results of different clinical trials using the same cell source have led researches to investigate the key aspects that determine the success of cell therapy. A careful analysis of the available data shows that negative results may be fundamentally due to the poor permanence of the injected cells inside the tissue [21,22], since SCs' therapeutic efficacy depend on their ability to survive in the hostile milieu of the damaged heart and to engraft within the myocardium [23]. Another critical point is the complexity of obtaining a reliable source of functional CMCs. Moreover, there are other aspects such as ideal cell type, source and dosing, route and time of delivery [24] and clinical trial design which should undergo further analysis to validate the safety and efficacy of cell therapy for MI [25]. In addition, cardiovascular regeneration may not be identical among individuals, and there should be an optimal cardiac regeneration therapy for each patient [26]. In summary, although the outcomes of clinical trials performed so far have displayed promising results, the overall beneficial effects of SCs therapies are still relatively modest. Moreover, the fundamental mechanisms of SC-mediated repair are largely unknown and controversial. Interestingly, the slight improvement observed after cell administration is frequently due to the paracrine effect of the cells rather than to their differentiation [19,27]. Thus, bioactive factor secretion may mediate the improvement in cardiac remodeling, function and metabolism. The latest research trend in SC therapy for cardiac healing identified exosomes secreted by SCs as crucial mediators of cell therapy-induced regeneration [28].

1.2.2. Protein therapies for MI in clinical trials

In addition to cell therapy, the administration of GFs able to promote cardiac repair holds great promise as a therapy capable of contributing to myocardial regeneration. GFs are administered next to the damaged tissue with the aim of favoring angiogenesis, chemotaxis, SC differentiation, CMC survival and proliferation, reduction of apoptosis and remodeling [5]. First, it was reported that during MI evolution the administration of therapeutic GFs could help to enhance the endogenous angiogenic process [29], thereby improving cardiac function and recovery. Interestingly, more recently it has been demonstrated that GF administration also has effects on stimulating progenitor cell recruitment to the heart and on inducing differentiation of SCs and existing CMCs [15]. In fact, the combination of all the three processes is mandatory for achieving the best possible heart regeneration. Therefore optimism about how protein-based approaches can be effective for cardiac regeneration and can avoid the fatal consequences of MI disorder has spread considerably in the last few years. Consequently, several GFs have been brought to clinical trials to test their therapeutic potential to regenerate the infarcted heart. This is the case of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), erythropoietin (EPO), hepatocyte growth factor (HGF), neuregulin (NRG), granulocyte-colony stimulating factor (G-CSF) and stromal cell-derived factor-1 (SDF-1) [5,30]. Again, as reported in clinical trials with cells, important but controversial results have been observed in clinical trials with GFs. By summarizing these results some interesting conclusions can be outlined. Firstly, the study designs vary considerably from one trial to another in terms of population, GF administered, route and dose of administration. Thus, a better definition of clinical trial requirements is needed in order to obtain more comparable results and conclusions. Secondly, a common drawback observed in all these trials is the low half-life of therapeutic proteins in the organism, which are rapidly degraded or removed from the site of injection. Therefore, the low efficacy and variable results reported so far might be attributed to this bioavailability issue. Thus, protein therapy needs to overcome those obstacles before it can attain clinical relevance.

1.2.3. Current challenges

Taking an overview of the aforementioned results, it can be gathered that SC and protein based therapies are potentially powerful strategies for treating MI. However, only partial improvements have been achieved, and more research is needed to optimize such therapies. The advantages and challenges of cell and protein therapies are illustrated in Fig. 2.

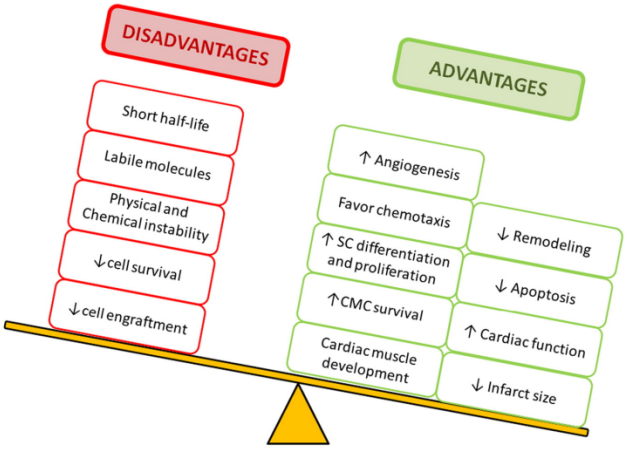

Fig. 2. Advantages and challenges of cell and protein therapies.

Principally, enhancement of cell engraftment, integration and coupling in the tissue must be at the center of efforts in cell therapy, whereas in protein therapy, improvements in bioactivity half-life and stability of therapeutic proteins are the principal points in where deeper investigation is mandatory. It is necessary to improve the efficacy of these novel strategies to reach their full potential. Importantly, specialized delivery modalities are highly recommended to achieve these goals.

2. Biomaterials to enhance cell and protein delivery to the heart

2.1. Biomaterials in cardiac repair

The development of new technologies that enable effective cardiac drug delivery would optimize cardiovascular treatment and would address the shortcomings of current and novel therapies. Biomaterials were developed to be used as medical devices for transplantation. However, the term biomaterial has evolved from simple implants to complex multifunctional interfaces with the body. The current definition proposed by the *European Society for Biomaterials* is a "material intended to interface with biological systems to evaluate, treat, augment or replace any tissue organ or function of the body" highlighting the role and importance of the material in influencing biological processes. Biomaterials should play a crucial role in the repair of the damaged heart. In a cardiac context, there are 4 ways in which biomaterials have shown to be useful (Fig. 3):

a) *The biomaterial by itself promotes cell migration or tissue regeneration.* In this case, biomaterials reproduce some aspects of the natural cardiac tissue environment and encourage tissue regrowth. Biomaterials are used for general cardiac reconstruction, vascular grafts, pediatric shunts, etc. Synthetic materials, metals, combinations of both and decellularized materials have been used for many years with significant success [31].
b) *The biomaterial is used to encapsulate cells acting as an immunoisolation barrier.* Encapsulation is one potential strategy to increase viable cell retention while facilitating paracrine effects. Synthetic biomaterials have evolved from polymers with no cell-recognition moieties to compounds mimicking the extracellular matrix, thus favoring cell–biomaterial interactions [32]. Nevertheless, they have not yet reached the cell viability and proliferation rates observed with natural biomaterials. Consequently, synthetic polymers are used in combination with natural compound or small peptide sequences in order to promote cell–biomaterial interactions for tissue regeneration [33].
c) *The biomaterial is used as a matrix to support cell growth and integration.* The biomaterial improves cell behavior due to the 3D environment as well as to the mechanical and signaling cues they provide to transplanted cells. These biomaterials are thus used as scaffolds. Several parameters must be taken into consideration in scaffold design to meet heart-specific requirements, such as shape, size and physical and mechanical properties [34].
d) *The biomaterial is used as a controlled release reservoir to locally deliver bioactive molecules.* Biomaterials can be used to prepare drug delivery systems (DDSs) that might provide protection from degradation to biomolecules (e.g. GFs, transcription factors, soluble paracrine factors) and a prolonged delivery. Thus, DDSs are able to decrease the amount of drug given to the patient, reducing serious side effects besides promoting cardiac repair. Each biomaterial, regarding its physico-chemical properties, provides a particular release profile. Thus, a specific biomaterial must be used for achieving the desired controlled release.

2.2. Current studies using biomaterial-based delivery systems in heart regeneration

Biomaterial-based delivery systems are essential in enhancing the therapeutic outcomes of cells and proteins in cardiac tissue engineering. The number of studies using biomaterials in combination with cell and protein therapies has therefore increased exponentially over the last decade. This section provides an overview of the advances made in the last 10 years in the field of cardiac repair using biomaterial-based

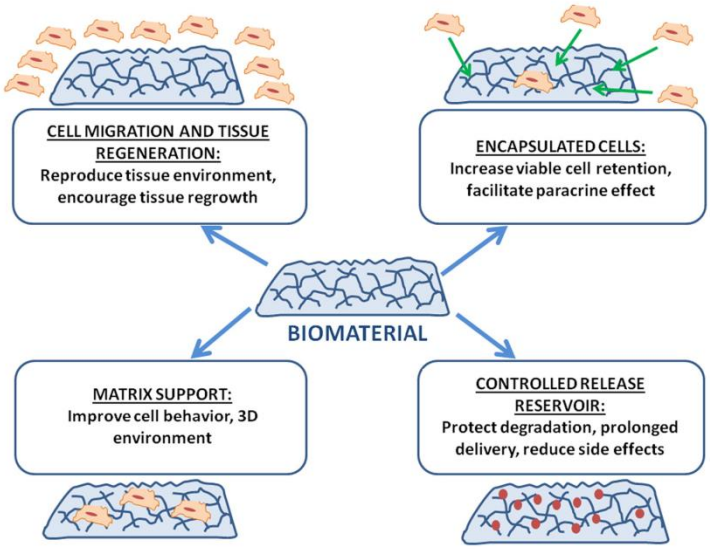

Fig. 3. Principal benefits of biomaterials in cell and protein therapies.

delivery systems with a focus on the progress made on preclinical *in vivo* studies done with synthetic biomaterials. We center on the use of synthetic biomaterials because they have been recently favored as cell and protein carriers. Nowadays there is a large list of synthetic biomaterials under investigation, including caprolactone, polyglycolic and polylactic acids, polyurethane and self-assembling peptides among others (Table 1). Each one has its own characteristics, but, in general all present important advantages over their natural counterparts. For instance, their physical, chemical, mechanical and biological properties can be modified, they can be produced uniformly in large quantities offering a multitude of possibilities. They also offer limited batch-to-batch variability. Concerning toxicity, nowadays synthetic biomaterials have reached similar safety levels to natural compounds, and their biocompatibility and biodegradability are well established in almost all cases (Table 1). A point that must be considered when designing biomaterial-based delivery systems for heart regeneration is the size of the DDS, since it could induce important side effects. On one hand, administration of "big" DDSs might favor tissue necrosis or hamper cardiac muscle contraction. On the other hand, very small DDSs could be rapidly phagocyted or cleared by the blood flow diffusing to other body organs. For instance, our preclinical studies using small (rat) and large (minipig) animal models of MI demonstrate that particles between 5 and 20 μm have the ideal size for intramyocardial injection. Smaller particles presented poor retention in the injected tissue, whereas injection of bigger particles resulted in damaged cardiac tissue [35–37].

2.2.1. Hydrogels

Injectable hydrogels are three-dimensional polymer networks extensively swollen in water (Fig. 4) [44], and represent a powerful delivery system for cardiac repair, since they are tri-dimensional networks that mimic the extracellular matrix and reproduce the natural environment and, in addition, they can be administered using non-invasive techniques like cardiac catheterization, thanks to their liquid-gel controllable nature [45]. In accordance with this potential, hydrogels have been developed using a long list of biomaterials. Nowadays synthetic materials have achieved high degrees of biodegradation and biocompatibility, like their natural counterparts. In fact, cardiac administration of synthetic hydrogels has proved to be effective in terms of promoting contractile phenotype smooth muscle tissue formation [46,47], preventing LV remodeling and scar expansion and improving cardiac function [48–51]. Interestingly, the timing of administration seems to be important because hydrogels assumed markedly different morphologies that determined heart remodeling. Thus, very early time points may not be beneficial, whereas hydrogel injection one week after the infarct event results in positive remodeling and cardiac function improvements [50].

2.2.1.1. Hydrogels in cell-based therapies. The use of hydrogels as cell carriers to repair the heart is a relatively new strategy. The resulting network that hydrogels form can reproduce specific biological functionality of the natural cardiac extracellular matrix and thus, seeded cells grow under conditions as similar as possible to those of the natural environment.

To examine the effect of cell delivery via hydrogels, Wall S.T. *et al.* developed a semi-interpenetrating hydrogel made of a crosslinked copolymer network of N-isopropylacrylamide and acrylic acid, interpenetrated with linear chains of polyacrylic acid with chemically tethered peptides combined with cell-surface integrin receptors for encouraging cellular attachment. The system was used as an assistive microenvironment for BMSC transplantation (2×10^5 cells per hydrogel) and tested in a mouse MI model. Six weeks after implantation, hydrogels were at the site of injection and GFP-BMSC could be detected. However, BMSC-hydrogel treatment did not show any improvement in ejection fraction (EF) and fractional shortening (FS) compared to non loaded hydrogels and free BMSC injection [52]. Concurrently, Li X.Y. *et al.* developed a crosslinked polymer hydrogel by mixing dextran-hydrophobic poly(e-caprolactone)-2-hydroxylethyl methacrylate chains with thermo-responsive poly(N-isopropylacrylamide) (NIPAM) chains. The biomimetic network was then combined with BMSC and placed onto the LV of rabbit MI model. A significant increase in cell engraftment 48 h after injection compared to free SC administration was observed. One month after treatment significant LV-EF preservation and attenuated LV dilatation accompanied by enhanced neovascular formation and prevented scar expansion were found in BMSC-hydrogel group compared to the rest of the groups [53].

Limited positive results have been reported with hydrogels prepared using only poly(ethylene-glycol) (PEG) [54]. However, several PEG copolymer hydrogels have shown benefits. For instance PEG has been combined with synthetic caprolactone by Wang T. *et al.* A triblock polymer was synthesized by mixing methoxy PEG and poly(caprolactone)-(dodecanedioic acid)–poly(caprolactone). BMSCs (2×10^7) were resuspended in α-cyclodextrin solution, which was intramyocardially co-injected with the hydrogel solution in a rabbit MI model. BMSC-hydrogels significantly enhanced cardiac function and increased both cell retention and vessel density around the infarct, preventing scar expansion compared with cells injected alone 4 weeks after treatment [55]. PEG has also been combined with natural materials to formulate hydrogels for cell transplantation. Naturally occurring biomaterials allow for the appropriate cell–matrix interactions, thus favoring cell engraftment [56]. The first example is the study by Habib M. *et al.* who PEGylated bovine fibrinogen to synthesize a biocompatible matrix where neonatal rat ventricular CMCs (3×10^6) were seeded. Irgacure 2959 photoinitiator was added to allow UV-light-activate in situ polymerization of the hydrogels after injection into the myocardium of rats suffering MI. Owing to the PEG additional crosslinkers, hydrogel

Table 1
Most employed synthetic biomaterials used to prepare cardiac DDSs and their principal advantages and disadvantages.

Synthetic biomaterial	Advantages	Disadvantages
Caprolactone and derivatives	Non-toxic, tissue compatible, mechanical properties, modifiable nature, pH sensitivity	Difficult to synthesize, slow biodegradation [38,39]
Polyglycolic and polylactic acids and derivatives	Well established biodegradation and biocompatibility, extended release rates	Acidic environment during degradation, bulk erosion [39]
Polyurethane	Biocompatible, mechanical properties	Biodegradable only when copolymerized with other polymers, no conductivity [39]
Self-assembling RAD 16 peptides	Self-assembly properties, bioreabsorbable, designed 3D microenvironment	Unknown toxicity and side effects [40,41]
Carbon nanotubes	Excellent mechanical and electrical properties	Strong hydrophobicity, physicochemical properties related toxicity, expensive [42]
Polyketals	Biodegradable, non-immunogenic, neutral degradation products, acid sensitivity, low cost	Rapid macrophage uptake and biodegradation, complex synthesis [43]

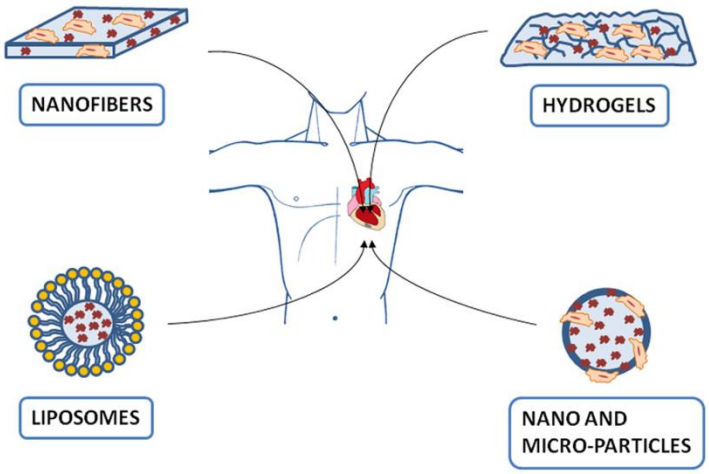

Fig. 4. Main types of drug delivery systems made of biomaterials that are being investigated in combination with proteins and/or cells for treating myocardial infarction.

permanence in the tissue varied from less than one month on the absence of additional PEG, to more than one month when additional PEG up to 2% was added. The combination of CMCs and hydrogels resulted in a favorable effect on cardiac remodeling with a significant increase in FS and functional outcomes 30 days after treatment administration. Higher anterior wall thickness was also detected in the CMC-hydrogel group when compared to controls, and transplanted CMCs were detected one month after administration inside the infarcted region [57]. In another approach Bearzi C. *et al.* synthesized a PEG–fibrinogen hydrogel using PEG–diacrylate as crosslinker and the photoinitiator Irgacure 2959 to control gelation. iPS (5×10^6) overexpressing placental growth factor (PlGF) and/or matrix metalloproteinase 9 (MMP9, protease involved in vascularization and engraftment processes) were combined with the hydrogels and tested in a mouse MI model. Animals that received iPS-hydrogel, regardless of whether cells over-expressed the therapeutic proteins or not, showed a significant increase in capillary density and cardiac function and a decrease in fibrotic and apoptotic indexes. Administration of SCs over-expressing both proteins resulted in better outcomes [58]. Positive results were also observed by Wang H. *et al.*, who combined PEG with fumarate. In this case, APS/TEMED solution was used for controlling hydrogel gelation. ESCs (1×10^6) were seeded and system efficacy was tested in a rat MI model. 24 h and 4 weeks after treatment a significant higher injected-cell population was detected in the infarcted tissue in ESC-hydrogel group compared to injection of cells alone. The heart area covered by transplanted cells was also significantly bigger when the hydrogel was used. Concerning cardiac parameters, echocardiography values, infarct size and arteriole/venule density were significantly improved in ESC-hydrogel group when compared to controls 4 weeks after treatment [59].

In addition to cardiac hydrogels based on PEG there is a growing interest on the development of hydrogels made from self-assembling peptides. Self-assembling is a process that is mediated by non-covalent interaction between molecules via ionic bonds, hydrogen bonding, hydrophobic interactions and van der Waal interactions [60]. Self-assembling peptides are normally 8–16 amino acids long and they are composed of alternating hydrophilic and hydrophobic residues that form a stable hydrogel of flexible nanofibers (NFs) upon exposure to physiological salt concentration or pH [61]. This is the case with the self-assembling polypeptide RADA16-II and its derivates. This polypeptide is able to spontaneously assemble into a stable three-dimensional NF scaffold that mimics natural extracellular matrix [62]. Davis M.E. *et al.* demonstrated that RADA16-II peptides rapidly gel when mixed with sterile sucrose solution. The resulting hydrogel created a microenvironment in the myocardium which promoted vascular cell recruitment and favored injected cell survival [63]. Then, Lin Y.D. *et al.* seeded BMSC (10^8) in such DDS and tested them in a pig MI model. BMSC-hydrogel injection resulted in significant higher improvement of cardiac function compared to other groups, and was accompanied by a significant increase in transplanted cell retention and capillary density in the peri-infarct area. Similar significant results were observed regarding the scar length fibrosis area, which were reduced in animals treated with seeded hydrogels compared to other groups [64]. More evidence about the use of RADA16 peptides for cardiac implantation was given by Cui X.J. *et al.* Here, MSCs (5×10^5) were seeded on the polypeptide hydrogel and injected in a rat MI model [65]. Injected cells underwent myogenic differentiation in the infarct and peri-infarct regions 4 weeks after administration. Smaller infarct size, higher capillary density and improved global cardiac function were observed, with significant differences between animals treated with MSC-RADA16-hydrogel and the rest of the groups [66]. These encouraging results led Guo H.D. *et al.* to attach an RGDSP cell-adhesion motif to RADA16 peptide to enhance cell survival and differentiation and thereby to improve SC efficacy. 5×10^6 BMSCs were seeded in these systems and final constructs were tested in a rat MI model. The system protected SCs from apoptosis and necrosis processes present in the ischemic myocardium. Moreover, MSC survival rate, cardiac function and collagen deposition were improved in animals treated with RGDSP-MSC-hydrogels with respect to MSC-hydrogels group 4 weeks after treatment [67]. Tokunaga M. *et al.* further demonstrated the efficacy of RADA16 peptide as cell carrier in a mice MI model. In this study, Puramatrix™, a commercial variant of RADA16 peptide, was used to create self-assembling hydrogel that underwent gelation in the presence of salts from the body. Authors seeded 2×10^4 BMSC, SM, ADSC or CPC in such systems and injected them onto the infarcted area of a mouse MI model. 2 weeks after treatment CPC-hydrogel significantly attenuated infarct size expansion, improved echocardiography parameters and favored neoangiogenesis. Cell apoptosis was reduced

when hydrogels were employed compared to free cell administration. These results suggest that CPCs are a promising cell source for preventing cardiac remodeling and dysfunction [68].

More recently, carbon nanotubes have been explored for cardiac delivery. They have good electrical conductivity and suitable and adaptable mechanical properties for cardiac application. A novel hydrogel made of carbon-nanotubes mixed with thermo-sensitive NIPAM was developed in order to enhance ADSC therapeutic efficacy. 2×10^6 ADSCs were seeded onto such systems and injected in a rat MI model. One week after treatment significant enhanced engraftment of seeding cells was detected when hydrogels were co-administered with the SCs in comparison to free ADSC administration. Moreover, LV-EF and FS, infarcted area and LV wall thickness were significantly improved in the ADSC-hydrogel group [69] providing evidence for the myocardial application of carbon nanotubes.

2.2.1.2. Hydrogels in protein-based therapies. The use of hydrogels for therapeutic protein delivery into the myocardium is quite recent. However, due to the liquid nature of the hydrogels, which facilitates cardiac administration, this DDS has rapidly attracted a great deal of attention.

Thermo-responsive hydrogels have been proved to favor positive remodeling and to improve cardiac function when combined with GF. For instance, a temperature-sensitive aliphatic polyester copolymer hydrogel made of PEG, N-hydroxysuccinimide and poly(δ-valerolactone) was conjugated with VEGF (40 ng) and intramyocardially injected in a rat MI model. No significant differences among groups were observed in any of the echocardiography parameters 7 days after treatment, but 35 days after treatment FS, EF, end-systolic elastance and end-systolic volume were significantly improved in the VEGF-hydrogel group compared to controls. Interestingly, conjugation of VEGF with the biomaterial prevented scar expansion and ventricular dilatation. Vascular density was significantly higher when VEGF was encapsulated inside the hydrogel [70]. Similar results were observed by Garbern J.C. *et al.* that entrapped biotinylated-FGF (5 μg) into a sharply pH–temperature-responsive injectable hydrogel system composed of a random terpolymer of NIPAM, propylacrylic acid and butyl acrylate. Hydrogels increased GF retention in the heart for 0–7 days when tested in a rat MI model. On the other hand, FS, regional myocardial blood flow, LV wall thickness and angiogenesis were significantly improved in the FGF-hydrogel group when compared to the other groups 28 days after treatment [71].

Apart from temperature sensitive hydrogels, other devices have been tested. For instance, Projahn D. *et al.* encapsulated Met-CCL5, a chemokine that inhibits neutrophil infiltration by competitive antagonism of CCL5 receptors, and SDF-1 in a star-shaped poly(ethylene oxide-stat-propylene oxide) and linear poly(glycidol) hydrogel. In this strategy, polymer chemical modification led to different GF release profiles. Thus, Met-CCL5 (0.5 μg) was mixed with fast degradable hydrogel and SDF-1 (3 μg) with slow degradable one in order to optimize GF retention in the myocardium and to adjust the GF release to heart necessities over time. One day after injecting the systems in a mouse MI model, high levels of Met-CCL5 were detected in mouse sera, but this trend was not maintained 4 weeks after hydrogel injection. On the other hand, levels of SDF-1 remained constant due to the slow release. Regarding heart recovery, EF was significantly higher 4 weeks after treatment when both GF-hydrogels were administered. Regarding neovascularization, apoptotic levels and infarcted area size, they were significantly improved when SDF-1 was administered, alone or in combination with the other GF, suggesting that there was accelerated wound healing in these groups [72].

In another study, PEG-based hydrogels were formulated by mixing this compound with maleimide macromers, and the systems were pre-functionalized with RGD adhesion peptide. HGF and/or VEGF (both at concentration of 1 μg per injected hydrogel) were incorporated into the matrices, which were tested in a rat MI model. The chemical-sensitive precursor hydrogel solution was crosslinked into a hydrogel by addition of a cysteine-flanked protease-degradable peptide sequence. Thus, before administration, crosslinked agent was added and final solution was injected into the ischemic zone. Only when both GF were coadministered a significant cardiac function improvement could be observed 21 days after treatment with respect to the non-treated group. Significant increase in both vessel density and fibrosis and in c-kit$^+$ cells were observed after HGF–VEGF-hydrogel treatment [73]. Nevertheless some controversial results were observed in larger animal models when other PEG based hydrogels were employed. This was the case of the study by Koudstaal S. *et al.*, who developed a pH-switchable supramolecular hydrogel with self-healing properties made of PEG and 2-ureido-4-pyrimidone (UPy). These systems were combined with insulin like growth factor 1 (IGF-1, 2 μg) and HGF (2 μg) and administered in a pig MI model. Although the dual GF-hydrogel combination resulted in improvements in LV end-systolic volumes, EF and formation of new capillaries in the infarct border zone one month after the injection procedure, no other benefits were detected. Interestingly, regarding CMC hypertrophy rate, although GF administration attenuated CMC degeneration, no significant differences were observed between the hydrogel group and the free GF administration group [74].

Another type of chemical-sensitive hydrogels was used by Wang T. *et al.* who employed their biocompatible PEG-based hydrogel solution, previously used as cell carrier (see [55]), as delivery system to administer EPO into rat MI model. In order to favor hydrogel formation, PEG solution and EPO solution dissolved with α-cyclodextrin were co-injected together. One month after treatment, animals that had received EPO dissolved in saline medium or incorporated into the hydrogel showed significant improvements in echocardiography parameters. However, significant infarct size reduction and apoptotic index, as well as increase in CD34$^+$ cell density and neovasculature formation were only detected in the EPO-hydrogel group indicating the benefits of this strategy [75].

Self-assembling peptides are nowadays attracting growing interest for protein delivery. For instance, RADA16-II peptide solution in combination with platelet-derived growth factor (PDGF, 4 and 8 μg) has been used to synthesize chemical depending hydrogels, which undergo gelation once peptide solution is mixed with sterile sucrose [76]. The highest dose system showed decreased CMC death and preserved systolic function 14 days after being injected in a rat MI model. Previous observations were correlated with a decrease in infarct size and induced PDGF receptor β expression and Akt phosphorylation in cardiomyocytes *in vivo* that indicated that CMCs were protected by endothelial cells through PDGF-pathway. The same group performed a long-term study in which intramyocardial delivery of PDGF by self-assembling peptide hydrogel led to an improvement in cardiac performance for at least 3 months [77]. In order to prolong and slow angiogenic factors release, Guo H.D. *et al.* constructed a novel self-assembling peptide by attaching the heparin-binding domain sequence LRKKLGKA to the self-assembling peptide RADA16 encapsulating VEGF (100 ng). In a rat MI model EF, FS, scar size, collagen deposition, cell survival and microvessel density were significantly improved in animals treated with the novel hydrogel compared to VEGF-RADA16 hydrogel (without LRKKLGKA sequence) 4 weeks after treatment [78]. Segers V.F. *et al.* also observed positive results in cardiac recovery using RADA16-II peptides. In this case, SDF-1 variant resistant to protease degradation was encapsulated into hydrogels and administered in a rat MI model. Treatment resulted in significant enhancements on SC recruitment, improved cardiac function and capillary density 28 days after administration [79]. Interestingly, when animals were treated with the SDF-1 resistant variant, a better heart recovery was observed compared to the normal SDF-1 treated group, although no significant differences were reported.

Encouraged by RADA16-II peptide result, Kim J.H. *et al.* optimized the therapy by combining PDGF and FGF-2 in the same hydrogels. The systems were injected in a rat MI model and dual GF loaded hydrogels showed the smaller CMC apoptosis rate compared to the other groups.

Infarct size and wall thickness followed similar significant trends 4 and 8 weeks after treatment. Interestingly, animals treated with PDGF–FGF-hydrogel showed similar vessel density to non-infarcted animals, suggesting an important angiogenic synergy between both GFs [80]. Dual GF delivery strategy for preserving cardiac function was also explored by Webber M.J. *et al.* in a mouse MI model, who loaded VEGF and FGF (10 ng of each GF per hydrogel) in heparin-binding-peptide-amphiphile hydrogels. VEGF–FGF-hydrogel treatment resulted in significantly improved LV contractility 30 days after administering the treatments [81].

Hydrogels made of semi-synthetic materials have also been explored as protein carriers for cardiac repair. He Y.Y. *et al.* used dextran in combination with hydrophobic poly (e-caprolactone)-2-hydroxylethyl methacrylate chain and thermo-responsive NIPAM forming thermosensitive hydrogels. 2.5 μg of high-mobility group box 1 (HMGB1, cytokine that attenuates cardiac remodeling after MI) were added per hydrogel, and then tested in a rat MI model. 24 h after treatment administration, cardiac SC proliferation and differentiation were found to be significantly higher in HMGB1-hydrogel group compared to the other groups. One month later, HMGB1-hydrogel treated animals showed the greatest increase in EF and the lowest collagen deposition, with significant differences from all other groups. Nevertheless both HMGB1-hydrogel and free HMBG1 significantly increased arterial density in the peri-infarcted area when compared to controls, but no significant differences were observed between these groups [82].

Hyaluronan is a natural polysaccharide which has been mixed with synthetic compounds to prepare hydrogels due to its excellent biocompatibility and biodegradability [83]. For instance, sodium hyaluronate was chemically modified with hydroxyethyl methacrylate to favor hydrolytic degradation, as in the work of MacArthur J.W. Jr. *et al.* A synthetic analog of SDF-1α was encapsulated at a concentration of 25 μg/50 μL and APS/TEMED was used for hydrogel gelation. These systems were injected intramyocardially in a rat MI model, where they proved to have significant benefits in improving echocardiography parameters such as EF, cardiac output and contractility when compared to controls. Loaded hydrogel also augmented capillary density. However, no significant differences were found between SDF-hydrogel and hydrogel groups regarding preservation of ventricular geometry and infarct size region, although both were significantly improved when compared to control groups [84].

2.2.2. Nanofibers

NFs are tridimensional, polymeric matrices with a network structure made of engineered fibers with diameters less than 500 nm (Fig. 4). To date several biomaterials have been tested as potential NFs for inducing cardiac repair after MI. For instance, in the work of Castellano D. *et al.*, collagen, poly(3-hydroxybutyrate), poly(e-caprolactone), poly-lactic acid and polyamide NFs were generated by electrospinning, being then transplanted into a rat MI model. Interestingly, poly(3-hydroxybutyrate) was the scaffold with the most beneficial reparative potential and positive remodeling capacity [85]. In another study polyester urethane urea NFs showed suitable mechanical properties and biocompatible characteristics, allowing cellular integration and endocardial endothelialization with minimal inflammation [86]. Thus, the evidences suggested that NFs result in positive outcomes for MI treatment.

2.2.2.1. Nanofibers in cell-based therapies. Since NFs are solid networks, cells can be entrapped within the polymeric matrix, augmenting their engraftment and survival. With this aim, Jin J. *et al.* combined MSCs with poly(lactide-co-ε-caprolactone) NFs. The systems, containing 1×10^6 MSCs per NF construct, were sutured onto the epicardial surface over the infarcted region of a rat MI model. Four weeks after treatment, echocardiography showed that SC administration, regardless the co-administration of NFs, resulted in LV dilation and improved EF compared with the control groups, and SCs survived and differentiated into cardiomyocytes. Only infarct area was significantly reduced in the MSC-NFs group compared to other groups [87]. More recently poly(ε-caprolactone) was mixed with gelatin to prepare NFs by electrospinning. 2×10^6 MSCs were seeded onto these hybrid scaffolds and transplanted into a rat MI model. Cells within the NFs were able to migrate towards the scar tissue, promoting new blood vessel formation at the infarct site. Consequently, 4 weeks after transplantation, the seeded NFs restricted the expansion of the LV wall, reduced the scar size and improved cardiac function significantly compared to the other groups [88]. Other similar co-polymers such as poly-glycolide-co-caprolactone (PGCL) and polyglycolic-acid (PGA) have been used as synthetic and biocompatible NFs for myocardial implantation. Piao H. *et al.* seeded 2×10^6 BMSC on PGCL-NFs and injected such systems into the epicardial surface in a rat MI model. Four weeks after implantation, BMSC-NF group showed higher but no statistically significant migration of BMSC into the epicardial region, as well as a greater differentiation rate towards cardiomyocytes. Induction of neovascularization, reduced fibrosis, positive remodeling and ameliorated LV function were detected in BMSC-NF treated group when compared to controls [89]. In the work of Ke Q. *et al.* PGA-NFs were combined with ESC (5×10^4), being then transplanted onto the surface of ischemic myocardium of infarcted mice. ESC-NF treatment not only improved blood pressure and ventricular function, but also had significantly higher survival rates compared to all other groups eight weeks after treatment [90].

More recently, polyurethane (PU) has drawn attention due to its softness, elastic and biodegradation characteristics. In addition PU allows CMC to grow in organized layers matching physical and mechanical properties of the native tissue [91,92]. Thus, Blumenthal B. *et al.* seeded SM (5×10^6 cells) on such systems but, interestingly, they previously transfected the SM with DNA of VEGF, HGF, SDF-1, or serine–threonine protein kinase (Akt1). Their final constructs resulted in GF-producing myoblast-seeded PU NFs. After being sutured at the epicardial zone of infarcted rats, SM-NFs were found to be accepted by the host with no inflammatory reaction detected after 6 weeks. This was correlated with enhanced angiogenesis when SMs were transfected with VEGF, HGF and Akt1, and with reduced infarction area when SM over-expressed SDF-1 and Akt1 or when SMs were untransfected [93]. Two years later, in 2012, a couple of interesting studies were performed in the same direction. On one hand, von Wattenwyl R. *et al.* used VEGF-overexpressing myoblasts (5×10^6) seeded on PU-NFs [94]. On the other hand, Poppe A. *et al.* transfected SM (5×10^6) with HGF and then seeded them on the same DDS [95]. Apart from stimulating endothelial cell motility and enhancing angiogenesis, intramyocardial HGF secretion after ischemic injury was associated with less severe ventricular enlargement and with an improved cardiac function [96]. In both cases, the seeded scaffolds were intramyocardially transplanted in infarcted rats, and six weeks later hemodynamic parameters and histological analysis were performed. The administration of HGF-overexpressing SM in PU-NFs resulted in an increased capillary density on the infarcted and peri-infarcted regions. Nevertheless, statistical analysis showed no significant changes in infarct size between groups. Regarding cardiac function, only the HGF overexpressing SM-NF treated group showed a significant improvement from baseline at the end of the study [94,95]. These results are in correlation with the study of Giraud M.N. *et al.*, who studied how myoblast-seeded PU-NFs could prevent cardiac dysfunction. Highly porous NFs with SM (5×10^6) were attached to the outer myocardial scar surface of MI rats. Only SM-NFs significantly prevented progression towards heart failure 9 months after treatment compared to the other groups, but this effect vanished 12 months after treatment. Interestingly, the systems were correctly incorporated into the cardiac tissue as new-formed vessels were formed inside the DDS [97].

2.2.2.2. Nanofibers in protein-based therapies. The use of NFs as protein delivery systems is a relatively new field and only a limited number of studies have explored their application in cardiac repair. For instance,

Wang Y. *et al.* formulated poly(lactic-co-glycolic acid) (PLGA) NFs loaded with FGF (15 μg). This system significantly enhanced neo-vascular formation, blood flow, FS and the number of proliferating cells 6 weeks after implantation in a mini-swine MI model [98]. In another approach, poly-vinyl-alcohol (PVA) was combined with dextran to form solid injectable NFs for the delivery of FGF (100 μg). These systems were tested in a large ovine MI model. FGF-NFs were sutured to animals' epicardium, showing a sustained release of FGF that strongly stimulated angiogenesis and increased wall thickness index in the infarcted myocardium 2 months after treatment. The NFs also significantly attenuated the increase in LV end-systolic diameter, but did not improve cardiac function [99]. Positive results were reported by Zhang G. *et al.*, who reported that PEGylated fibrin NFs loaded with SDF-1 (100 ng), when injected in a mouse MI model, significantly increased myocardial recruitment of c-kit$^+$ cells compared to controls two weeks after treatment. Enhanced stem cell homing was maintained at 28 days, when LV function was significantly improved in comparison with the controls [100]. More recently, our group prepared smooth polymeric NFs of stat-modified PLGA to deliver NRG to the heart. *In vivo* biocompatibility studies demonstrated that NFs were present in the heart 3 months after administration and a constructive tissue remodeling was observed indicating good incorporation into the organism [101]. In ongoing studies, the efficacy of this system is being evaluated.

2.2.3. Nano and micro-particles

DDSs based on nano and microparticles (NPs and MPs, respectively) have shown great potential to improve the treatment of many diseases, including cardiovascular disorders. They are solid particles in the nanometer or micrometer size range in which the active principle is dissolved, entrapped, encapsulated or adsorbed [102]. There is a long list of materials that can be used to prepare particles of a desired size. Depending on the raw materials employed, drug release profiles, particle degradation and location of the particles can be controlled. Generally, NPs and MPs suffer faster degradation processes than hydrogels or NFs. This higher biodegradability allows their total elimination from the biological tissues avoiding chronic inflammation responses. Together, these characteristics make particles one of the more versatile DDS on the market [103].

2.2.3.1. Nano/microparticles in cell-based therapies. Regarding strategies based on the use of SCs combined with NPs, covalent coupling, adsorption and internalization of NPs inside cells have been used [104]. It is important to note that NPs were not used for encapsulating or conveying SCs on their surface due to their relatively small size, but for augmenting their circulation time, targeting cells towards specific tissues, improving SC function *in vivo* [104], modifying cell behavior [105], delivering biomolecules and genes and for diagnostics and imaging methods [106].

On the other hand, MPs can be formulated to encapsulate [107,108] or to convey [37,109] cells on their surface. Nevertheless, although alginate and matrigel MPs have reported promising results [107,108], only few studies have been performed with MPs made of synthetic materials. In a recent study human amniotic fluid SCs (1×10^6) were encapsulated inside PLGA porous MPs of about 250 μm. The efficacy of these systems was tested in a rat MI model, showing that animals treated with SCs–MPs had a significantly increased capillary density and positive remodeling, which resulted in an improved cardiac function 4 weeks after treatment compared to other groups. SCs were clearly retained at the site of injection and were differentiated towards cardiomyogenic and angiogenic lineages [110]. In another study, Penna C. *et al.* demonstrated that PLGA-MP enhanced MSC survival and regeneration in the hostile environment of post-ischemic tissues [109].

2.2.3.2. Nano/microparticles in protein-based therapies. Encapsulation of proteins in NPs/MPs is one of the approaches that have been most extensively investigated to protect therapeutic molecules against *in vivo* degradation and to release drugs in a controlled manner [36,102]. Apart from their well-established efficiency as DDS, their surface modification possibilities [111] have given NPs and MPs an interesting therapeutic potential. In fact, active targeting [112] is a very common strategy that has given NPs a particular interest for intravenous administration [113], since they can pass through the microcirculation easily [114] and they are not very vulnerable to immune clearance [115,116], finally reaching heart tissue. Nevertheless, any targeted NP formulation has been clinically approved yet [111]. Regarding MPs, their relative large size makes their intravenous administration impossible without causing undesired side effects. Consequently, in the few *in vivo* studies developed so far, local delivery of NPs and MPs remains the most common way of administration. Along these lines, Sy J.C. *et al.* developed poly(cyclohexane-1,4-diyl acetone dimethylene ketal) MPs (polyketal-MPs) and PLGA-MPs of around 20 μm encapsulating SB239063 (0.5 mg), which were intramyocardially injected in a rat MI model. SB239063 molecule is an inhibitor of apoptotic protein p38, which is related to the progression of cardiac dysfunction. Both types of MPs allowed an *in vivo* sustained release of SB239063 at least for 7 days. Authors observed significant less fibrosis and improvements in cardiac function 21 days after treating MI rats with the SB239063-polyketal-MP group but, interestingly, no significant improvements with respect to controls were detected in the SB239063-PLGA-MP animals [117]. Based on these results, the same group also synthesized superoxide dismutase 1 (SOD1) polyketal-MPs (protein:polymer ratio of 0.05) (10 μm). SOD1 is a protein with antioxidant effects that has proved to favor infarct size reduction after a MI event [118]. When injected intramyocardially in a rat MI model, MPs were detected for up to 10 days in the myocardium. Superoxide levels were decreased in animals treated with SOD1-MPs when compared to controls, and the same significant trend was observed in CMC apoptosis ratios. Three days after treatment, improvements in FS were only observed when SOD1 and SB239063-MPs were co-injected, suggesting the need of multiple therapeutics dosage to combat the different phases of the disease [119]. In order to enhance NP uptake by CMC, SB239063-polyketal-NPs were covered with the sugar N-acetyl-D-glucosamine (GlcNAc) [120] and their efficacy was tested in a rat MI model. The number of apoptotic CMCs was significantly lower in the GlcNAc-SB239063-NP group compared to other groups 24 h after treatment. This result was confirmed by an uptake study, where GlcNAc-polyketal-NPs were clearly more captured by CMCs than non-coated NPs. Three days after treatment echocardiography analyses showed that only rats that received loaded-NPs had a significant reduction in infarct size/area-at-risk and an improved FS [121].

Due to their well-established *in vivo* biocompatibility, safety and FDA approval, polyesters like PLGA are widely used in cardiac tissue engineering. One interesting example is the study by Chang M.Y. *et al.*, where PLGA-NP of 60 nm, 200 nm and 1 μm were synthesized containing different concentrations of IGF-1. When intramyocardially injected in a MI mice model, 24 h after treatment IGF-1 was significantly more in IGF-1-NP treated group compared to free IGF-1 administration. IGF-NP treated animals also showed a significant reduction in infarct size and number of apoptotic CMCs and improved LV-EF 21 days after treatment compared to free IGF administration and non-loaded NPs. Finally, the authors reported that 60 nm NPs were most effective in binding IGF-1 and consequently preventing CMC apoptosis [122]. Our group has also examined the feasibility of using PLGA-MPs encapsulating therapeutic proteins to promote cardiac regeneration. First, Formiga F.R. *et al.* prepared PLGA-MPs (5 μm) containing VEGF (35 μg per 50 mg of MPs) by solvent extraction/evaporation method using TROMS technology. This technology based on double emulsion and solvent evaporation methods allowed them to encapsulate labile proteins without altering their natural properties and bioactivity [123]. The systems were administered via intramyocardial injection in a rat MI model. One month after treatment, PLGA-MPs were present in the myocardium, and significant increments in angiogenesis and arteriogenesis in the infarct and peri-

infarct areas of the injured hearts in the VEGF-MP group were detected in comparison to controls. The increased revascularization of the tissue translated into a beneficial effect in the remodeling processes, with a significantly greater thickness of the LV wall in the VEGF-MP treated animals in comparison to the rest of the groups [123]. In addition, Simón-Yarza T. *et al.* combined both angiogenic and antioxidant drugs to establish potential synergistic effects. With this aim, PLGA-MPs of 5 μm containing VEGF (50 μg per 50 mg of MP) and PLGA-NPs (150 nm) encapsulating Coenzyme Q10 (CQ10, 1.5 g per 3 g of NPs) were formulated. CQ10 is known due to its antioxidant and cardioprotective roles [124]. The efficacy of VEGF-MPs and CQ10-NPs was studied in a rat MI model, where MPs were intramyocardialy injected and NPs were administered orally. Separately, both treatments demonstrated significantly increased EF three months after administration when compared to the other groups. That was correlated with a highly significant increase in the number of capillaries in the infarct and peri-infarct areas. Interestingly, CQ10-NPs showed better outcomes than commercial CQ10, what was attributed to the ability of NPs to improve oral bioavailability and to the sustained release of the encapsulated CQ10. Unfortunately, combined treatment failed to offer synergy, and no EF improvements could be observed [125]. In another study, we successfully delivered FGF-1 and NRG-1 to the ischemic tissue using PLGA MPs (5 μm), administering a final amount of 1740 ng of FGF and/or 1300 ng of NRG in treated animals. Three months after treatment, global cardiac function, infarct size, fibrosis, revascularization and cardiac stem cell recruitment were significantly increased in GF-MP treated groups (FGF, NRG or FGF/NRG) when compared to controls [126] (Fig. 5). Our study is providing very useful data regarding the underlying mechanisms contributing to the beneficial effects of this therapy, especially those linked to endogenous regeneration, which might be very useful for the design of novel cardiac repair approaches. As a prerequisite for clinical application, we next determined the long-term therapeutic effectiveness and safety of this therapeutic strategy in a pre-clinical large animal model of myocardial infarction (mini-pigs) demonstrating that cytokine delivery MPs are able to restore cardiac function [35]. This technology could soon be translated to humans.

Finally, Oh K.S. *et al.* prepared semi-synthetic NPs made of a lecithin core and a Pluronic F-127 coating with a Capryol 90 hydrogel system in order to achieve a stable localization of VEGF-NPs at the ischemic area. In this case, VEGF (5 μg) was encapsulated inside the natural core of NPs and final systems (270 nm) were epicardially injected in a rat MI model. Although both VEGF-NP and VEGF-NP-hydrogel resulted in significantly improved capillary density, significantly higher cardiac function was observed in VEGF-NP-hydrogel group compared to all other groups [127].

2.2.4. Liposomes

The latest example of biomaterial-based DDSs reviewed here are liposomes. Liposomes are sphere-shaped vesicles consisting of one or more phospholipid bilayers (Fig. 4) [128]. The liposomal drug Doxil was the first lipid system to be used in clinical practice in 1999. Although nowadays they are used in other diseases [129,130], there are still no liposomal formulations approved for human use for the treatment of cardiovascular disease.

Concerning liposomes, active targeting has been deeply explored, and surface-attached targeting molecules have been employed for preparing liposomes used to target MI, constituting a promising approach for heart therapy [131]. Thus, immunoliposomes (ILs) presenting phosphatidylserine (PS) on their surface can be easily recognized by macrophages, which are relevantly concentrated in the cardiac tissue after MI due to the inflammatory process, providing specific accumulation of targeted ILs in the damaged heart. This strategy has been used by Harel-Adar T. *et al.* who synthesized PS-ILs. Firstly, the systems were intraperitoneally injected in rats, and 3 h later, the peritoneal cells were analyzed. The state of macrophages changed from pro-inflammatory to anti-inflammatory. That was translated in significantly higher levels of anti-inflammatory cytokines on the peritoneal lavage fluids in treated animals compared to controls, which confirmed the previous result. When this was translated to a rat MI model, the same protective trend was observed. Interestingly, PS-ILs induced cardiac macrophages to secrete anti-inflammatory cytokines 3 days after treatment, which is 1 day earlier than under normal conditions. The treatment also promoted angiogenesis, prevented ventricular dilatation and remodeling, and small scars were detected in comparison with control groups [132].

Concerning cell therapy and liposomes, no results have been published yet, so the following section will be focused on liposomes for protein delivery.

2.2.4.1. Liposomes in protein-based therapies. A large number of the physicochemical properties of liposomes [133] have been explored for active targeting of therapeutic drugs to myocardial ischemic regions [134]. PEGylation strategy is used to improve permanence-time and to reduce the opsonization process of systems administered in the blood [135,136], consequently increasing liposome therapeutic efficacy [137]. Regarding heart targeting, one adhesion molecule that is up-regulated on endothelium in response to ischemia and inflammation is P-selectin [138]. In the work of Scott R.C. *et al.*, PEGylated phosphatidylcholine/cholesterol liposomes were synthesized and incubated with IgG2a mouse antibody to rat P-selectin. VEGF (0.12 g/kg animal weight) was encapsulated in such systems and administered via tail vein immediately after induction of MI in rats. ILs were selectively accumulated in the myocardial infarct region [139], allowing targeted VEGF delivery to post-MI tissue, which resulted in significant increase of FS and improved systolic function. These functional improvements were associated with an increase in the number of vessels in the MI region of treated animals [140]. Similarly, Wang B. *et al.* developed anti-P-selectin conjugated ILs to target the delivery of VEGF to the heart, which significantly improved vascularization and cardiac function [141]. Using other approach,

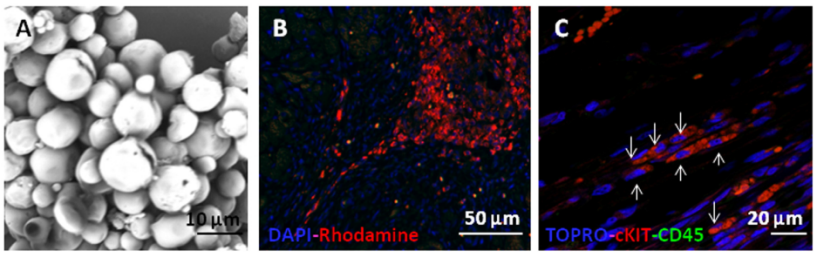

Fig. 5. NRG–PLGA MPs. A) Scanning electron microscopy of NRG–PLGA MPs. B) Tissue retention of fluorescent PLGA MPs 1 month after intramyocardial injection in a rat MI model. C) Cardiac progenitor cell recruitment 1 week after intramyocardial injection of NRG–PLGA MPs.

Yamada Y. *et al.* prepared Sialyl Lewis X molecule (SLX) ILs (100 nm) encapsulating EPO [142]. SLX is a carbohydrate present in the leucocyte membrane known for interacting with selectin cell-adhesion proteins and to play a vital role in cell-to-cell recognition processes [143]. SLX-EPO-ILs were intravenously administered in a rabbit MI model (2500 IU of EPO/kg body weight). Only ILs but no non-targeted liposomes were selectively accumulated at the border area of the infarcted myocardium, significantly increasing EPO levels in the heart 48 h after treatment. LV remodeling, EF, FS and reduction on MI size were significantly improved in the SLX-EPO-ILs group when compared to controls. Similar results were observed for the number of $CD31^+$ microvessels and for EPO receptor expression [142].

3. Emerging tissue engineering strategies for heart regeneration after myocardial infarction

The combination of cells or protein with biomaterials has proved to be effective in preclinical animal models of MI. In brief, regarding cell therapy, it has been possible to enhance cell viability and engraftment. Biomaterials have enabled cells to assemble into effective tissue substitutes that may restore cardiac functions and structure. Concerning protein therapy, the use of DDSs has allowed researchers to protect growth factors against *in vivo* degradation and to achieve a controlled release over time, favoring important processes during cardiac healing such as angiogenesis or SC differentiation towards cardiac lineages. Moreover, SCs can directly benefit from the action of therapeutic GFs. For instance, SCs depend on GFs for correct survival and differentiation (Fig. 6). In addition, SC paracrine secretions together with therapeutic GFs may achieve a better regenerative effect. Thus, some authors have investigated the combination of both cellular and protein therapies together with biomaterial-based delivery systems. This integrated approach, known as the tissue engineering triad, has attracted considerable attention over the past years (Fig. 6).

Since tissue engineering is a novel approach only a few studies have been published so far, although showing interesting results. In the first study in 2005, a bioengineered NF scaffold made of polyglycolic acid succeeded in incorporating BMSC (1×10^7) and FGF (0.2 μg). When such systems were transplanted in a rat MI model, cells were detected inside the scaffold 4 weeks after implantation, and NFs were absorbed by the host tissue indicating good incorporation into the organism. Global cardiac function and capillary density were significantly improved in BMSC–FGF-NFs treated animals when compared to BMSC or FGF loaded NF groups [144]. Similarly, our group investigated the feasibility of using NRG-releasing PLGA-MPs (20 μm diameter, 1.8 μg NRG/mg of MP) combined with ADSC (2.5×10^5 or 5×10^5) as a multiple growth factor delivery-based tissue engineering strategy for implantation in the infarcted myocardium [37]. ADSC–NRG-MPs proved to be compatible with intramyocardial injection in a rat MI model and systems were present in the peri-infarcted tissue 2 weeks after implantation [37]. Efficacy studies are currently being performed. Apart from those polymers, other synthetic materials have been employed for generating injectable DDSs. The group of Kraehenbuehl T.P. *et al.* formulated a three-dimensional metalloproteinase-sensitive PEG-based hydrogel, and used such systems to deliver thymosin β4 (Tβ4, 2.5 μg) in combination with ESCs (6.6×10^6) and smooth-muscle stem cells (3.3×10^6) in ischemic injuries of a rat MI model. Tβ4 protein activates the survival kinase Akt, protects cardiac muscle form death after ischemic damage and promotes angiogenesis, making it an interesting molecule for cardiac regeneration [145]. Thus, the cell seeded-Tβ4-hydrogels effectively preserved contractile performance 6 weeks after myocardial infarction and attenuated LV dilation compared to controls and to the Tβ4-hydrogel treated group [146]. Neovascularization and infarct size were also significantly improved in cell seeded-Tβ4-hydrogels and Tβ4-hydrogels groups compared to controls.

Concerning full synthetic biomaterials, self-assembling peptide RADA16-II has been used to create injectable hydrogels incorporating IGF (approximately 1 ng) in combination with CMCs (1×10^6) [147] or CPCs (1×10^5) [148] for cardiac repair. In both studies the administration of cell-seeded-IGF-hydrogels significantly improved the recovery of myocardial structure and function in rats one month after treatment. Apoptosis was also reduced regardless of the cell type, but a reduced infarct size and increased capillary density were only reported when CPCs were co-injected with IGF [148]. In any case, the presence of IGF resulted in a protective environment that favored SC proliferation. In other study using the same RADA16-II peptides, Dubois G. *et al.* compared the efficacy of skeletal myoblasts (SMs) and PDGF therapies to

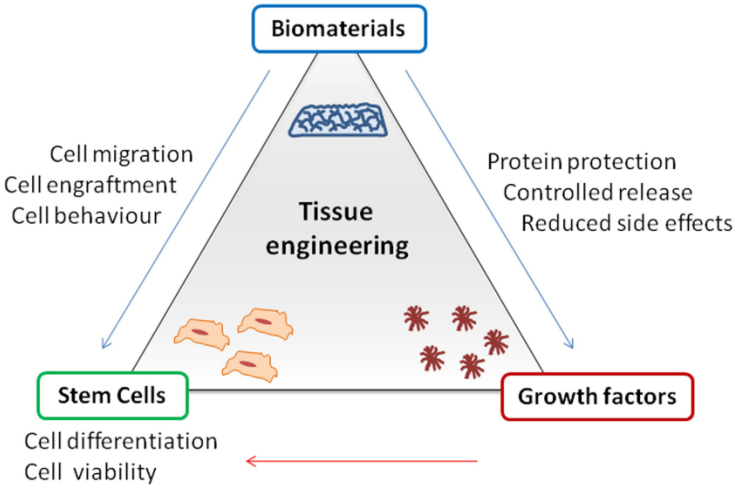

Fig. 6. Tissue engineering triad, with the benefits that each element (growth factors, SCs and biomaterials) gives to other element.

SM–PDGF tissue engineering in a rat MI model. Significantly greater angiogenesis was observed in all GF-treated groups compared to controls one month after treatment. However, this was not correlated with an improved cardiac function. In fact LV function was not improved in either of the treated groups compared to controls at the same time point. The lack of functional improvements observed *in vivo* was explained by an *in vitro* SM viability study. Authors concluded that specific tailoring of the biomaterial to the cell type is required for correct cell survival [149].

The combination of synthetic and natural biomaterials is common in tissue engineering, and relevant promising results have been obtained. For instance, semi-synthetic hydrogels made of PEGylated fibrin biomatrix efficiently bound HGF and entrapped BMSC (5×10^5). After administration in a mouse MI model, the systems allowed significant improvements in cell prevalence at the injection site for at least 4 weeks, compared to free cell administration. Interestingly, in BMSC–HGF-hydrogel treated animals, cell retention was accompanied by the lowest levels of apoptosis and the highest LV function recovery among all the groups, confirming that tissue engineering was more effective than protein or cell therapy alone [150].

In order to obtain a system inspired by tissue-specific niches able to mimic the real biological process of heart healing, several DDSs have been combined. Thus, in the work of Holladay C.A. *et al*. MSCs were seeded onto semi-synthetic hydrogels of collagen, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide and N-hydroxysuccinimide. 2 μg of interleukin-10 (i10), the most potent anti-inflammatory cytokine, was incorporated into dendrimer polyplexes and incubated with the hydrogels. Four weeks after treating MI rats with such systems, SC retention and FS were found to be significantly improved in animals which received MSC-i10-systems compared to the rest of the groups. Improved function was associated with increased infarcted wall thickness, decrease of cell death and a change in macrophage markers from mainly cytotoxic in the MSC-hydrogel group to mainly regulatory in MSC-i10-system treated group, confirming the success of tissue engineering over cell therapy [151]. Using another strategy, Miyagi Y. *et al.* combined both NFs and hydrogels. Thus, authors first synthesized a gelfoam/poly-(ε-caprolactone) NFs construct. Then, BMSC (1×10^6), stem cell factor and/or SDF (30 ng of each one) were incorporated in a polymeric temperature-sensitive hydrogel made of valerolactone and PEG. MI was induced in rats, and after two weeks NFs were transplanted covering the infarcted area. At the same time, hydrogels were injected next to the NFs. Four weeks after treatment, those animals treated with NFs and SC–GF-hydrogels showed a significant global cardiac function improvement compared to animals treated only with the NFs. Although NFs in combination with SC–GF-hydrogel treatment resulted in better heart recovery compared to controls, no significant differences were observed when compared to animals that had received NFs and SC-hydrogel or GF-hydrogel. Finally, neovascularization and wall thickness were enhanced in all treated animals compared to controls [152].

3.1. Challenges ahead

The above examples represent some of the ways in which tissue engineering strategies are being investigated to address cell and protein hurdles. Interestingly, several synthetic biomaterial-based DDS have been explored although no one has proved to be better than the others. In any case, if we want to reach clinical applications, new techniques for treating MI must not be more invasive than the existing cardiac procedures. Concerning this aspect, hydrogels, NPs, MPs, and liposomes achieve this goal, and can be administered by trans-endocardial injection or via catheters. On the other hand, NFs need to be attached to the pericardium, so a more invasive administration technique is required. However, it is highly desirable that biomaterials should provide satisfactory mechanical support to the infarcted heart, in order to favor functional recovery of the damaged organ [33]. In this sense, NFs have proved to be able to contribute more efficiently to the heart's mechanical properties than other DDS. However, given the intricate anisotropic mechanical behavior of myocardium, it is not easy to produce a biomaterial that responds to mechanical stresses in a way that is similar to the heart itself. In this regard, PU seems to be the most promising biomaterial [91,92]. Another recommendable characteristic for DDS in cardiac repair is the ability to mimic the natural heart microenvironment [153]. This way, biomaterials are used as an alternative to extracellular matrix, being NFs and hydrogels the DDS that reproduce natural conditions in the best way possible. Taking all of this into account, we can say that the search for the optimum biomaterial-based delivery system still continues and further research in this area is guaranteed.

We cannot forget that the mammalian heart is a complex organ composed of a heterogeneous cell population. Consequently, the potential of a long list of SCs and GFs for regenerating the infarcted heart tissue has been investigated so far. Tissue engineering has proved to be useful in regenerative medicine in terms of high viability and long-term engraftment of cells. In addition, cardiac repair and regeneration is favored by effective delivery of therapeutic GFs. Nonetheless, although all tissue engineering strategies regardless of the therapeutic agent employed have enhanced myocardial functional, the repair mechanisms remain unclear at the moment. It is still unknown whether the repair of the infarcted heart is caused by the functional activity of the cells or by structural changes brought by biomaterials or proteins. Therefore myocardial tissue engineering approaches have to be developed considering both cell and GF requirements of the heart for successful cardiac recovery. In addition, functional integration between the graft and the host tissue, in both electromechanical and vascular terms, still remains a major challenge that must be considered when designing new cardiac tissue engineering approaches [154]. The establishment of well-defined protocols and the optimization of the synergies between the different cells and GFs are required before clinical applications can be attained. In fact, tissue engineering is still at the development phase and the only clinical trial evaluating a tissue engineering strategy is the one called ALCADIA. In this ongoing trial CPCs and FGF are being combined in a gelatin hydrogel to treat ischemic cardiomyopathy (Clinicaltrials.gov identifier NCT00981006). Thus, all of these promising results should be considered preliminary, and further studies are needed to confirm the possible benefits of myocardial tissue engineering.

4. Conclusions and future prospects

New contributions to the advancement and optimization of classical treatments for MI have allowed a reduction in the number of death due to this pathology over recent decades. However, complications deriving from MI remain a big problem. Therefore, new strategies have been investigated to overcome such limitations, and the ones that have shown the most promising results so far are cell and protein therapies [14,15]. As this review has illustrated, both of these have encountered various challenges when tested in clinical trials, related to the low cell engraftment and the rapid degradation of therapeutic proteins once they are administered. Fortunately, it seems that nowadays we are close to reaching their full potential by combining them with biomaterials. Thus, this review has also demonstrated the relevance of biomaterials in the repair and regeneration of the damaged heart. Currently, synthetic hydrogels, NFs, NP, MP and liposomes are being investigated in depth in cardiac repair, in combination with cells and proteins. The capacities of these DDS to increase cell survival and engraftment, and to protect and control GF release are the main reasons for their success. However, the type of material, cell and GF sources, timing, dose and injection technique are still uncertain, and further investigation is mandatory in order to achieve the best patient outcomes. The current challenge is to establish a perfect combination of three components: biomaterials, cells and proteins. Tissue engineering is a rapidly evolving

discipline. In fact, it is expected that in the next 10–20 years, these therapies will account for more than half of the new drugs introduced on the market [155]. Great advances have been made in the last few years, although there are still several aspects to improve and current results should be considered preliminary. In the future, MI treatments will surely represent an amazing challenge in terms of biomaterials and delivery systems with the final goal of providing many benefits to MI patients.

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ANNEX IV

PLGA NANO- AND MICROPARTICLES FOR VEGF DELIVERY

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