

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-infarcted 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, heart transplantation being 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 protein administration presents serious limitations due to their 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 proteins 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 cytokines 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 cytokines secreted by stem cells (SC), capable of responding to the host environment, opens up a possible solution to that drawback. Moreover, MP possess many features that make them suitable for use 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), 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.

2.1 *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. (Warrington, USA). Collagen 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 provided by ATCC. Poly D-Lysine 1 mg/ml (PDL) was provided by Merck-Millipore (Darmstadt, Germany). rh Neuregulin-1b-iso (NRG) was provided by EuroBioSciences (Friesoythe, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfofenyl)-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 μ g of NRG, 5 mg of HSA and 5 μ l of PEG 400 dissolved in 200 μ l 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 \times g, 10 min). NRG-releasing particle scaffolds were lyophilized for 48h without cryoprotective agents (Virtis Genesis 12 EL, Gardines, NY). The conditions of freeze drying were -50°C to +15°C 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 μ L 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 4°C with primary antibody goat IgG-NRG-1 β -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% CO₂/95% 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 MTT assay. 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 a Tukey post hoc correction when the values measured were normally distributed

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, the particle scaffold was overlaid with 0.5 $\mu\text{g}/\text{cm}^2$ of type 1 collagen and/or PDL [18]. 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 °C for 2 h. Coated particle scaffolds were washed 3 times with distilled sterile water and lyophilized for long term storage. For ADSC adhesion, coated MP were resuspended with complete α -MEM medium, and were ultrasounded and briefly vortexed prior to addition of $2,5 \times 10^5$ or 5×10^5 cells. The mixture was then gently flushed and plated in Costar® Ultra Low Cluster Flat Bottom Sterile Polystyrene Plate. Plates were incubated at 37 °C 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 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 dispersing media were sterilized by autoclaving prior to use. 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*) 2.5×10^5 ADSCs-0.75 mg of particle scaffold coated with $0.5 \mu\text{g}/\text{cm}^2$ of collagen in 100 μL of resuspension medium, (*Group 2*) 5×10^5 ADSCs-0.75 mg of particle scaffold coated with $0.5 \mu\text{g}/\text{cm}^2$ of collagen in 100 μL of resuspension medium, (*Group 3*) 5×10^5 ADSCs-0.75 mg of particle scaffold coated with $0.5 \mu\text{g}/\text{cm}^2$ of mixture of collagen and poly-D-lysine (1:1) in 100 μL of resuspension medium, (*Group 4*) 5×10^5 ADSCs-0.75 mg of NRG-releasing particle scaffold coated with $0.5 \mu\text{g}/\text{cm}^2$ of mixture of collagen and poly-D-lysine (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 zone 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- μ m-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 $20 \pm 5 \mu\text{m}$, with a range of 2 to $70 \mu\text{m}$. As can be seen in Fig. 1A, particle scaffolds size distribution confirms that TROMS allows the obtention of reproducible batches of MP of a desired particle size.

MP with a size of $20 \mu\text{m}$ 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 $20 \mu\text{m}$ 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 $20 \mu\text{m}$ particle scaffolds reaching encapsulation efficiency values of $92.58 \pm 3.84\%$, 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 to the encapsulation method and to the use of TROMS to prepare the multiple emulsion system. On the one hand, the water/oil/water evaporation technique has been described as resulting in extremely efficient loading of biodegradable microparticles with hydrophilic compounds [39, 40] and, on the other hand, higher encapsulation efficiencies were found using TROMS compared to conventional encapsulation techniques. For instance, for VEGF, an encapsulation efficiency of 83.8 % was achieved using TROMS [8]. In contrast, King *et al.* reported an entrapment efficiency of 16 % using the solid/single emulsion/ solvent extraction technique to prepare the microparticles [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 cytokine 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, cytokine therapy efficacy is generally hindered for the short plasma half live, gastrointestinal tract instability and low bioavailability of proteins. 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. 1B).

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 cytokine) 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 the biological activity of NRG 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 cytokines involved in cardiac regeneration. Interestingly, this is the first report of NRG-releasing scaffold for cardiac tissue engineering applications. The importance of multiple growth factor 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 cytokines 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 stromal vascular fraction [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 $2,5 \times 10^5$ or 5×10^5 ADSC to particle scaffolds coated with different concentrations of collagen and PDL was studied. The concentration that showed better adherence was $0.5 \mu\text{g}/\text{cm}^2$, 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.

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 μ m 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).

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

Finally, ADSC combined with empty or cytokine 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, hematoxylin and eosin 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). Hematoxylin and eosin 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.

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 still 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.

Taking together our *in vitro* ADSC adhesion studies and our present *in vivo* biocompatibility findings on infarcted rats, 5×10^5 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.

Conclusion

The data presented in this article offers 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:

This work was partially supported by FEUN (Fundación Empresa Universidad de Navarra). P. Díaz-Herráez is beneficiary of a predoctoral fellowship from the Association of Friends of the University of Navarra.

E. Garbayo's work was supported by the Spanish Ministry of Science and Innovation through a Juan de la Cierva Program (JCI-2011-10737).

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Figure captions

Figure 1: Nrg-releasing particle scaffold characterization. A) Representative particle size distribution measured by laser diffractometry 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). * $p < 0.05$, *** $p < 0.001$

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-infarct area. Scale bars: 50 μm .

Figure 3: Macroscopic view of the infarcted heart following ADSC combined with Nrg-releasing particle scaffold implantation. Seven days after LAD 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.

Figure 4: Histological evaluation of myocardial tissue reaction 14 days after ADSC combined with Nrg-releasing particle scaffold administration in hematoxylin-eosin 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).

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-infarct area that encompassed the infarct 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).

Graphical abstract. Results of this work demonstrated the potential value of ADSC cells combined with NRG delivery systems for cardiac tissue engineering. The two most relevant findings of this study were as follows: (1) ADSC were efficiently attached to NRG-releasing particles *in vitro* (A, B) and (2) the ADSC-cytokine delivery system injected in the infarcted heart (C) proved to be compatible with an intramyocardial administration in terms of injectability through a 23-gauge needle and tissue response (D, E). Taken together, this proof of concept study provides important evidences required for future effectiveness studies and for the translation of this approach to the context of cardiac regeneration.