Functional benefits of PLGA particulates carrying VEGF and CoQ10 in an animal of myocardial ischemia.

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Abstract

Myocardial ischemia (MI) remains one of the leading causes of death worldwide. Angiogenic therapy with the vascular endothelial growth factor (VEGF) is a promising strategy to overcome hypoxia and its consequences. However, from the clinical data it is clear that fulfillment of the potential of VEGF warrants a better delivery strategy. On the other hand, the compelling evidences of the role of oxidative stress in diseases like MI encourage the use of antioxidant agents. Coenzyme Q_{10} (CoQ_{10}) due to its role in the electron transport chain in the mitochondria seems to be a good candidate to manage MI but is associated with poor biopharmaceutical properties seeking better delivery approaches.

The female Sprague Dawley rats were induced MI and were followed up with VEGF microparticles intramyocardially and CoQ_{10} nanoparticles orally or their combination with appropriate controls. Cardiac function was assessed by measuring ejection fraction before and after three months of therapy.

Results demonstrate significant improvement in the ejection fraction after three months with both treatment forms individually; however the combination therapy failed to offer any synergism. In conclusion, VEGF microparticles and CoQ_{10} nanoparticles can be considered as promising strategies for managing MI.

Keywords: VEGF, $CoQ_{10,}$ protein delivery, angiogenesis, tissue engineering, myocardial ischemia.

Abbreviations:

CVD: Cardiovascular disease

VEGF: Vascular endothelial growth factor

CoQ_{10}: Coenzyme Q_{10}

DDS: Drug delivery system

MP: Microparticle

NP: Nanoparticle

TROMS: Total recirculation one machine system

PEG: Poly (ethylene glycol)

PLGA: Poly (lactic-co-glycolic) acid

PVA: Poly (vinyl alcohol)

DMSO: Dimethylsulfoxide

DCM: Dichloromethane

HUVEC: Human umbilical vein endothelial cell

EF: Ejection Fraction

MI: Myocardial ischemia

1. Introduction

Cardiovascular diseases (CVD) continue being the leading cause of death worldwide in spite of increasing efforts to improve its management. World Health Organization estimates that by 2030 almost 25 million of all global deaths will be due to CVD. Within this group of disorders coronary heart disease and stroke will be the main cause. Both, myocardial ischemia and strokes are principally caused by the accumulation of an atherosclerotic plaque in an artery. In the heart tissue ischemia is followed by a complex process involving cells (Tucka et al., 2012), growth factors (GFs) (Gullestad et al., 2012) and the extracellular matrix (Eckhouse and Spinale, 2012) that ends in the remodeling of the ventricle and heart failure.

To avoid consequences of ischemia in the heart the idea of growing new blood vessel for increased supply blood and better heart function has been proposed (Mitsos et al.,

2012). Vascular endothelial growth factor (VEGF) is endogenously released by cells after myocardial ischemia and its supplementation to help damaged tissue to efficiently complete vessel formation has been studied in depth (Wang et al., 2013, Formiga et al., 2012). However, one of the major drawbacks of the therapy with GFs is its short half-life, being good candidates to be incorporated into drug delivery systems that protect them from degradation while extending its performace by sustaining the release (Simon-Yarza et al., 2012).

Cardioprotection is more of a prophylactic measure to prevent or reduce myocardial damage and the use of antioxidants is gaining importance in this area (Burgoyne et al., 2012, Maksimenko and Vavaev, 2012). Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone, is a molecule that is responsible of the electron flow between complexes one and two with complex three in the electron transport chain (Sohal and Forster, 2007). It is well known because of its antioxidant role in several pathologies and it has been administered in coronary artery disease due to its cardioprotective role (Littarru et al., 2011). It is used as adjunctive therapy combined with different drugs and/or clinical interventions (Pepe et al., 2007; Fotino et al., 2012). However, CoQ_{10} is still seeking solutions to overcome poor oral bioavailability problems (Beg et al., 2010). Strategies to improve gastrointestinal absorption have been previously studied with proven success in several pathologies (Ankola et al., 2007; Ratnam et al., 2009).

The present study is an attempt to compare the efficacy of particulate forms of VEGF and CoQ_{10} and to investigate the possibilities of synergism of the respective combination in an animal model of MI.

2. Materials and methods

2.1 Chemicals

Human recombinant VEGF was from R&D Systems (Minneapolis, MN, USA) and CoQ_{10} was a gift from Tishcon Corp. (Westbury, NY). All chemicals were provided by Sigma-Aldrich (Barcelona, Spain), organic solvents by Panreac Quimica S. A. (Barcelona, Spain) except high-performance liquid chromatography-grade methanol, ethanol, and acetonitrile that were procured from J.T. Baker (now Avantor Performance materials, Phillipsburg, NJ). Polymers were purchased from Boehringer-Ingelheim (Ingelheim, Germany) and western blot reagents from BioRad, unless specified in the text. Rabbit polyclonal anti-human VEGF-A (clone A-20, sc-152) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™ anti-Rabbit IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Human umbilical venous endothelial cells (HUVECs) were obtained from umbilical cords from donors, after informed consent according to the guidelines of the Committee on the Use of Human Subjects in Research at the Clinic Universidad de Navarra. Components of cell culture media were purchased by ATCC-LGC. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was provided by Promega. Isofluorane employed for animal anaesthesia was IsoFlo® from Abbot Laboratories S.A. and sutures were from Ethicon (Johnson & Johnson, Brussels).

2.2 Preparation of Coenzyme Q_{10} encapsulated nanoparticles

CoQ_{10} encapsulated nanoparticles were prepared as reported previously in our laboratory (Ratnam et al., 2011) however with slight modifications that in the current study large scale batches were made instead of lab scale preparations (50 mg) The procedure in brief goes as follows: PLGA (Resomer RG 50:50 H; intrinsic viscosity 0.32-0.44 dl/g) (3 g) is dissolved in 150 ml of ethyl acetate under stirring and CoQ_{10} (1.5 g) was added to the polymer solution and stirring continued for one hour. In a separate bottle, PVA (PVA) (Mol. Wt. 30,000-70,000) (3 g) was dissolved in 300 ml of

distilled water over a period of one hour. The CoQ_{10} containing polymer solution (25ml x 6 lots) was emulsified into (50 ml x 6 lots) of PVA solution over 30 min stirring (1000 rpm). This emulsion is then homogenized for 30 min at 15000 rpm and added to 250 ml of distilled water and left for overnight stirring to ensure complete evaporation of ethyl acetate. The suspension was centrifuged at 14,000 g for 30 min and the pellet collected was then re-suspended in 4 ml of distilled water either by vortex or occasionally probe sonication. Particle size was measured using zeta sizer (Malvern Zata sizer, UK) before and after centrifugation. The suspension was divided into two equal parts (2 ml each) in a 5 ml vial and 5 % (w/v) trehalose was added and lyophilised using a bench top freeze dryer (Martin Christ, Germany) using the following condition 8 h freezing (-80 ºC and safety pressure 1.650); followed by main drying for 48 h (-50 ºC, 0.0035 mBar vacuum and safety pressure 1.650) and final drying of 12 h +20 ºC 0.0035 mBar vacuum and safety pressure 1.650). The freeze dried particles were characterized for particle size and CoQ_{10} entrapment using reported HPLC method in our laboratory (Ratnam et al., 2011). Nanoparticles without CoQ_{10} (NL-NPs) were prepared following similar procedure without adding CoQ_{10} to the preparation.

2.3 VEGF Microparticle preparation and characterization

VEGF microparticles (VEGF-MPs) were prepared by double emulsion solvent evaporation method, as previously described (Simon-Yarza et al., 2013). Briefly, VEGF (50 µg) was included in the aqueous solution. On the other hand, 25 mg PLGA Resomer® RG 503 and 25 mg poly [(d,l-lactide-co-glycolide)-co-PEG] diblock Resomer® RGP d 50105 were dissolved in a mixture of DCM and Acetone (3:1). By using TROMS® technology (Garbayo et al., 2008; Formiga et al., 2010; Simon-Yarza et al., 2013), organic phase was incorporated into the inner aqueous phase to form the first emulsion, that was then incorporated into the PVA 0.5 % external aqueous phase,

to form the multiple emulsion. Particles were then formed by solvent evaporation during 3 hours under constant stirring (300 rpm). Freeze-drying was employed to store particles until its use.

Non-loaded microparticles (NL-MPs) to be used in the control groups were prepared in the same way without VEGF.

Particle size was determined by laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). Sample preparation was done by dispersing 3 ml of particle suspension into the small volume dispersion unit. To do the measurements Mastersizer-s v2.19 software was used.

Microparticle (MP) residual PVA content was studied by a colorimetric assay based on the reaction occurring between two adjacent hydroxyl groups of PVA and iodine molecule leading to the formation of a colored complex (Joshi et al., 1979). Measurements were performed in triplicate.

To determine MP protein content 0.5 mg of loaded MPs were dissolved in 20 µl DMSO. Incubation with rabbit antihuman VEGF-A antibody (1:2000) lasted 2.5 h at RT. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (1 h, RT, 1:2000) was performed to detect antibody binding. Chemiluminescence detection was performed using LumiLight Plus western blotting substrate (Roche Diagnostics, Mannheim, Germany). The VEGF signal was quantified by densitometry using the Quantity One software (Bio-Rad Laboratories, Inc., Munich, Germany).

For the *in vitro* release studies, 1 mg of VEGF-MPs were introduced in 1.5 ml tubes and dispersed in 300 µl PBS pH 7.4 with 0.02 % (w/v) sodium azide, incorporated as a bacteriostatic agent. Samples were incubated under orbital shaking at 37 ºC. At different time points, tubes were centrifuged (20,000 g, 10 min) and 60 µl of supernatant were removed for its analysis and replaced with the same volume of fresh release medium.

VEGF concentration in the supernatants was measured by western blot, using the protocol described in the previous section. Release profile was expressed as cumulative release during a period of time. All measurements were done in triplicate.

2.4 Bioactivity of encapsulated VEGF

To confirm that protein bioactivity was not affected during the microencapsulation process a proliferation cell culture assay was done (Hervé et al., 2005). HUVECs were obtained from human umbilical cords. Tissue digestion was done with 0.1 % collagenase II (Jaffe et al., 1973). Afterwards, cells were expanded in F12K medium (ATCC 30-2004) supplemented with 30 μg/ml endothelial cell growth supplement (ECGS, BD Biosciences), 10 % fetal bovine serum, 1 % sodium heparin and 1 % penicillin/streptomycin.

For the proliferation study, cells were plated into 96-well culture plates (3×10^3 cells/well). 12 h after seeding, cells were treated with 10 ng/ml of non-encapsulated VEGF or released VEGF from the MPs. After 72 h incubation time under normal culture conditions proliferation in each group was measured using MTS assay.

2.5 *In vivo* experiments

The experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). 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 (40) weighing approximately 240-260 g were provided by Harlan-IBERICS (Barcelona, Spain) and housed in conventional animal quarters. Environment was controlled (12 h light/dark cycle at 21°C) and all animals received a standard diet and water *ad libitum*.

2.5.1 Animal model

Anesthesia was firstly induced in an induction chamber with 2 % Isoflurane. Then animals underwent tracheal intubation for ventilation that was maintained during the procedure (10-15 ml/kg, 80-90 respiratory rate). Anesthesia was maintained with 0.5 % isoflurane during the surgery. Before the heart operation, animals received analgesic drug ketoprofen 5 mg/Kg subcutaneously and fentanyl 0.15 mg/kg intraperitoneally. The surgical approach consisted of a left thoracotomy through the fourth intercostal space. After pericardium was opened, left anterior descending (LAD) coronary artery was blocked 2–3 mm distal from its origin with a ligature with an 7/0 polypropylene suture. The chest was closed in layers using resorbable suture Vicryl® 4/0 and rats were allowed to recover. Surgical procedure total time was 30 minutes. Only those rats with an EF equal or below 55% (as determined by echocardiography) 4 days after MI were included in the study, since it is established that an EF under 55 % is an indicator of heart damage. Animals were then divided into eight groups (n=5) as showed in Table 1.

2.5.2 Intramyocardial administration of the microparticles

One week after the LAD occlusion, MPs were intramyocardially administered using a 29 gauge needle in four points surrounding the ischemic area, evidenced by the scar formation. Intramyocardially treated groups consisted of 0.6 or 1.6 mg of VEGF-MPs (0.85 μg/mg). For its administration, particles were dispersed in a sterile buffered solution composed of 0.1 % (w/v) carboxymethylcellulose, 0.8 % (w/v) polysorbate 80 and 0.8 % (w/v) mannitol in PBS, pH 7.4. All animals received 80 μl total volume. After injection chest was closed and animals were allowed to recover.

Intramyocardial injection is becoming one of the main routes of administration of growth factors and cells in acute myocardial infarction preclinical and clinical trials. This administration path allows local delivery of the treatment with a better dose control

and avoiding adverse effects resulted from the dissemination of the growth factor through the organism. Despite being more invasive than other routes, for the large animal models and for the clinical application sophisticated devices have been constructed (Sherman et al. 2006). With this intramyocardial catheter-based delivery systems open chest surgery is no longer necessary, and intramyocardial injection has become a less invasive technique with excellent safety profiles in the clinical trials

2.5.3 Oral administration of the nanoparticles and commercial CoQ_{10}

Oral administration of the CoQ-NPs, NL-NPs or CoQ_{10} commercial formulation started 24 h after LAD occlusion and continued every three days during the first month and subsequently switched to weekly once until the end of the study. Animals treated with the nanoparticulate or with the commercial CoQ_{10} received a 100 mg/kg dose every three days throughout the first month and the 200 mg/kg dose once per week up to three months. Animals treated with NL-NPs received the equivalent amount of NPs. For oral administration, freeze dried particles were dispersed in distilled water using a sonicator prior administration that was performed with an oral rigid dosing cannula.

2.5.4 Ecochardiographical evaluation

The Echocardiographic analysis was performed using a Vevo770 high-resolution ultrasound system (Visualsonics, Toronto, Canada) as previously described (Benavides-Vallve C. et al., 2012)

The left ventricular (LV) systolic function of the heart was evaluated by measuring the EF, calculated using the Simpson's rule, from a parasternal long axis view and four parasternal short axis views at different levels of the LV. At the long axis view the left ventricle length was measured from the aortic annulus to the endocardial border at the apex level in both diastole and systole. At the parasternal short axis view, the

endocardium was traced at four different levels in both systole and diastole, to calculate the areas required to calculate the Simpson's value.

All measurements were performed offline using dedicated Vevo770 quantification software (Vevo 770 v. 3.0.0).

2.5.5 Histological studies

Three months after surgery animals were sacrificed and hearts were collected for subsequent histological analysis. Harvested hearts were fixed and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated and embedded in paraffin. Sections (5 μm) were cut from each slice.

To quantify the small caliber vessel density and area, anti-caveolin-1α antibody (diluted 1:50) was used as marker, and 2 peri-infarct and 2 intra-infarct images per section were analyzed. Secondary antibody was Alexa Fluor 488 goat conjugated anti-mouse IgG (diluted 1:100). Images were acquired using the Axio Cam MR3 video camera at 20× connected to the Zeiss Axio Imager M1 microscope equipped with epifluorescence optics. Digital images were analyzed using MatLab® software platform (Mathworks Inc., Natick, MA, USA).

3 Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test was used to analyze statistical significance. *P*-values corresponded to a two-tailed unpaired *t*-test for the group comparison. A $p < 0.05$ was considered statistically significant using Prism software (GraphPad software, San Diego, CA, USA).

4 Results and discussion

4.1 Preparation and characterization of Coenzyme Q_{10} encapsulated nanoparticles

The procedure was highly reproducible and we were able to produce large quantities of CoQ_{10} containing nanoparticles. The key variation we made from the small scale batch

preparation was the homogenization duration which was increased to 30 min from 15 min used for 50 mg batch size (Ratnam et al., 2011). The fresh CoQ_{10} containing particles were of 147±9 nm (fresh), however a slight increase in the size 156±9 nm was observed after centrifugation to separate the free CoQ_{10} and surfactant which is expected and this increase is due to aggregation upon centrifugation. However, freeze drying process did not affect the particle characteristics and remained same due to inclusion of cryoprotectant trehalose. The entrapment efficiency was found to be about 70% of initial CoQ_{10} entrapment (~35 mg CoQ10/100 mg polymer). CoQ_{10} entrapped PLGA nanoparticles were proven efficacious in diabetic and renal hypertensive models by preventing the free radicals, inflammation and lipid abnormalities (Ankola et al. 2007, Ratnam et al. 2008, Ratnam et al. 2011).

4.2 VEGF Microparticle preparation and characterization

The process led to uniform sized particles of about 4.89 μm with a VEGF content of 0.85 μg/mg equivalent to 85% EE. Our previous studies suggest the size of the particles is within the range for intramyocardial administration with a 29G syringe (Formiga et al., 2010). Residual PVA associated with the MP preparation was less than 1% of the initial amount used and this concentration should not cause any adverse reactions either by local injections or oral/systemic administration (DeMerlis and Schoneker, 2003; Baker et al., 2012). There are reports suggesting very high doses in rats (daily doses of 2,000-5,000 mg/kg) over a period of 90 days showed no adverse or toxicological effects (Kelly et al., 2003).

In vitro release profile showed a burst effect with 58 % of protein released within the first 4 h followed by a more sustained release until the end of the study (Figure 1). This burst effect could be a typical phenomenon of surface bound protein or the protein in the exterior layers of the particles releasing at much quicker rate than that is in the inner

cores. PEGylated particles exhibit a greater burst effect than non PEGylated owing to their increased hydrophilicity of the polymer. Once the superficial layers of the protein are depleted a more sustained release of protein is observed which is controlled by the diffusion as well as the amount of the protein remaining in the particles. *In vivo* release is expected to be slower due, in part, to the presence of tissue surrounding the particles, instead of an aqueous medium.

4.3 Bioactivity of encapsulated VEGF

HUVEC cell assay demonstrates the proliferative activity of VEGF-MPs and is as effective as respective free protein (Figure 2). This study further confirms the integrity of the protein during the encapsulation process by TROMS®.

4.4 *In vivo* experiments

Animals were divided into eight groups (n=5) and followed up to three months. Echocardiographic functional results have been analyzed grouped in three different efficacy studies to facilitate interpretation of obtained data.

4.4.1 CoQ_{10} loaded nanoparticle efficacy study

The CoQ-NPs improved the heart function significantly as indicated by increased EF rates when compared to the commercial CoQ_{10} which showed results comparable to that of the untreated group (Figure 3A). The improved performance of CoQ-NPs can be attributed to their ability to improve their peroral bioavailability and sustain the release of the encapsulated CoQ_{10} over a period of time allowing dose reduction while maintaining the activity. Even though the commercial formulation used in this study is reported to exhibit improved peroral bioavailability (Chopra, 2001), it is just not enough in the system under the current dosage regimen to elicit any positive response, may be a daily dosing would have been better with commercial form. Even though a precise mechanism of action of CoQ_{10} in MI is not known, in general it is attributed to its

antioxidant nature, ability to minimize inflammation, improve endothelium function (Dai et al., 2011; Lee et al., 2012). We have previously reported the efficacy of CoQ-NPs in other experimental models (Ankola et al., 2007; Ratnam et al., 2009) however a more systematic understanding is required on their therapeutic potential before much efforts are put in to prove the performance of these compounds that are in general high dose molecules and currently in use as supplements.

4.4.2 VEGF loaded microparticle efficacy study

We have studied two doses of VEGF to establish a dose response and the doses selected were based on the success from our previous study in a rat model of ischemic heart disease (Formiga et al., 2010). The animals receiving VEGF-MPs improved the heart function as indicated by the increase in EF however both low and high dose show same response, while the control group which received NL-MPs offered no protection (Figure 3B).

4.4.3 Combined therapy

Though the individual treatments of CoQ_{10}-NPs and VEGF-MPs resulted in significant improvement in the EF (Figure 3C) these two failed to offer synergism when administered together. Expected synergy between proangiogenic and antioxidant therapies mediated by VEGF and CoQ_{10} was not observed. Moreover, if we put together these results with those obtained with the VEGF-MPs alone it appears that CoQ_{10}-NPs counteract benefits of VEGF therapy (Figure 3D).

There is compelling evidence that CoQ10 is an independent predictor of mortality in chronic heart failure (Molyneux et al., 2008), while a recent study suggests the plasma concentrations of CoQ10 is not associated with lower risk of AMI (Naidoo et al., 2012). Unfortunately, molecules such as CoQ10 are used as supplements with no clear doses or plasma levels established to prevent/manage/treat any pathology. It is important at this

juncture that the inherent biopharmaceutical/physicochemical problems associated with such molecules are addressed by formulating them appropriately before we make substantial claims on their effectiveness or ineffectiveness (Kumar 2012).

CoQ_{10} role in the angiogenic process has not been studied in depth. There are some published works that explore CoQ_{10} in the tumor angiogenesis. It has been suggested an antiangiogenic effect of this molecule in the new vessel formation mediated by a mechanism in which endogenous VEGF serum levels are reduced (Premkumar et al., 2007, 2008). In our approach VEGF released from the particles in the damaged heart acts in cooperation with endogenous VEGF that is secreted by the cells after MI. An explanation to the results of this study will be that, on one side VEGF-MPs and CoQ-NPs will be exerting its proangiogenic and cardioprotective action respectively, and on the other CoQ-NPs will be also act reducing VEGF endogenous levels, thus limiting angiogenesis. This result emphasizes how important the co-administration of non-regulated actives can be in the effectiveness of the treatment of the patients. There is, therefore, a need to go into depth in the interactions of these micronutrients that need to be controlled when patients undergo treatment.

4.4.4 Histological studies

Animals receiving VEGF-MPs showed a statistically highly significant increase ($p<0.0001$) in the number of capillaries in the infarct and peri-infarct area when compared with the control group (Figure 4A). This difference was reduce in the group receiving VEGF-MPs and CoQ-NPs ($p=0.015$). This data are in accordance with the functional results that present the same standard, suggesting that CoQ-NPs are reducing the VEGF-MPs angiogenic effect. In relation with the vessel mean area we have proved that is significantly smaller in the VEGF-MPs & CoQ-NPs animal group ($p=0.02$), indicating that vessel development is more immature in this group (Figure 4B). Finally,

no differences in the total caveoline positive area were found between control group and VEGF-MPs group, but there were differences in the VEGF-MPs & CoQ-NPs group in which it was reduced (Figure 4C).

Taking all these data into account we can conclude that VEGF-MPs promotes vessel development, but its angiogenic effect is reduced when it is concurrently administered with CoQ-NPs, resulting in less functional benefit.

5 Conclusion

In this work polymeric nanoparticles containing antioxidant molecule ubiquinone and stealth microparticles containing VEGF were prepared by two different methods, both of them leading to high encapsulation efficiency. These drug delivery systems were applied in an animal model of MI by different administration routes. Treatments resulted in an improvement of the EF of the animals when administered separately, suggesting that they can be effective systems to treat this disease. In the case of VEGF-MPs an angiogenic process underlies this benefit, confirmed by the quantification of larger number of capillaries in the animal hearts. Treatment with the CoQ-NPs present several advantages based on its oral administration, more feasible and non-invasive for the patient, also with lower cost.

However, when both particles were administered concurrently no significant benefit was observed, suggesting that in the dose regime employed in this study, there is a counteraction between them. Histological analysis confirms that small vessel density and size is reduced in those animals included in this group. Finally, results in this study emphasize the need for extra care when using supplements with standard therapeutic interventions.

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7 Bibliography

Ankola, D.D., Viswanad, B., Bhardwaj, V., Ramarao, P., Kumar, M.N., 2007. Development of potent oral nanoparticulate formulation of coenzyme Q10 for treatment of hypertension: can the simple nutritional supplements be used as first line therapeutic agents for prophylaxis/therapy? Eur. J. Pharm. Biopharm. 67, 361-369.

Baker, M.I., Walsh, S.P., Schwartz, Z., Boyan, B.D., 2012. A review of polyvinyl alcohol and its uses in cartilage and orthopedic applications. J. Biomed. Mater. Res. B. Appl. Biomater. 100, 1451-1457.

Beg, S., Javed, S., Kohli, K., 2010. Bioavailability enhancement of coenzyme Q10: an extensive review of patents. Recent Pat. Drug Deliv. Formul. 4, 245-255.

Benavides-Vallve, C., Corbacho, D., Iglesias-Garcia, O., Pelacho, B., Albiasu, E., Castaño, S., Muñoz-Barrutia, A., Prosper, F., Ortiz-de-Solorzano, C., 2012. New strategies for echocardiographic evaluation of left ventricular function in a mouse model of long-term myocardial infarction. PLoS One. 7.

Burgoyne, J.R., Mongue-Din, H., Eaton, P., Shah, A.M., 2012. Redox signaling in cardiac physiology and pathology. Circ. Res. 111, 1091-1106.

Chopra, R.K., 2001. Coenzyme Q products exhibiting high dissolution qualities U.S. Patent Number 6,300,377.

Dai, Y.L., Luk, T.H., Yiu, K.H., Wang, M., Yip, P.M., Lee, S.W., Li, S.W., Tam, S., Fong, B., Lau, C.P., Siu, C.W., Tse, H.F., 2011. Reversal of mitochondrial dysfunction by coenzyme Q10 supplement improves endothelial function in patients with ischaemic left ventricular systolic dysfunction: a randomized controlled trial. Atherosclerosis. 216, 395-401.

DeMerlis, C.C., Schoneker, D.R., 2003. Review of the oral toxicity of polyvinyl alcohol (PVA). Food Chem. Toxicol. 41, 319–326.

Eckhouse, S.R., Spinale, F.G., 2012. Changes in the myocardial interstitium and contribution to the progression of heart failure. Heart. Fail. Clin. 8, 7-20.

Formiga, F.R., Pelacho, B., Garbayo, E., Abizanda, G., Gavira, J.J., Simon-Yarza, T., Mazo, M., Tamayo, E., Jauquicoa, C., Ortiz-de-Solorzano, C., Prósper, F., Blanco-Prieto, M.J., 2010. Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. J. Control. Release. 147, 30-37.

Formiga, F.R., Tamayo, E., Simón-Yarza, T., Pelacho, B., Prósper, F., Blanco-Prieto, M.J., 2012. Angiogenic therapy for cardiac repair based on protein delivery systems. Heart Fail. Rev. 17, 449-473

Fotino, A.D., Thompson-Paul, A.M., Bazzano, L.A., 2012. Effect of coenzyme Q10 supplementation on heart failure: a meta-analysis. Am. J. Clin. Nutr.

Garbayo, E., Ansorena, E., Lanciego, J.L., Aymerich, M.S., Blanco-Prieto, M.J., 2008. Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres. Eur. J. Pharm. Biopharm. 69, 844-851.

Gullestad, L., Ueland, T., Vinge, L.E., Finsen, A., Yndestad, A., Aukrust, P., 2012. Inflammatory cytokines in heart failure: mediators and markers. Cardiology. 122, 23-35.

Hervé, M.A., Buteau-Lozano, H., Mourah, S., Calvo, F., Perrot-Applanat, M., 2005. VEGF189 stimulates endothelial cells proliferation and migration in vitro and up-regulates the expression of Flk-1/KDR mRNA. Exp. Cell Res. 309, 24-31.

Jaffe, E.A., Nachman, R.L., Becker, C.G., Minick, C.R., 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745–2756.

Joshi, D.P., Lan-Chun-Fung, Y.L., Pritchard, J.G., 1979. Determination of poly(vinyl alcohol) via its complex with boric acid and iodine. Anal. Chim. Acta. 104, 153–160.

Kelly, C.M., DeMerlis, C.C., Schoneker, D.R., Borzelleca, J.F., 2003. Subchronic toxicity study in rats and genotoxicity tests with polyvinyl alcohol. Food Chem. Toxicol. 41, 719-727.

Kumar, M.N.V.R., 2012. Can efficient delivery systems leverage benefits of antioxidants leading to potential medicines? Drug Discov. Today. 17, 407-408.

Lee, B.J., Huang, Y.C., Chen, S.J., Lin, P.T., 2012. Effects of coenzyme Q10 supplementation on inflammatory markers (high-sensitivity C-reactive protein, interleukin-6, and homocysteine) in patients with coronary artery disease. Nutrition. 28, 767-772.

Littarru, G.P., Tiano, L., Belardinelli, R., Watts, G.F., 2011. Coenzyme Q(10) , endothelial function, and cardiovascular disease. Biofactors. 37, 366-373.

Maksimenko, A.V., Vavaev, A.V., 2012. Antioxidant enzymes as potential targets in cardioprotection and treatment of cardiovascular diseases. Enzyme antioxidants: the next stage of pharmacological counterwork to the oxidative stress. Heart Int. 7.

Mitsos, S., Katsanos, K., Koletsis, E., Kagadis, G.C., Anastasiou, N., Diamantopoulos, A., Karnabatidis, D., Dougenis, D., 2012. Therapeutic angiogenesis for myocardial ischemia revisited: basic biological concepts and focus on latest clinical trials. Angiogenesis. 15, 1-22.

Molyneux, S.L., Florkowski, C.M., George, P.M., Pilbrow, A.P. Frampton, C.M., Lever, M., Richards, A.M., 2008. Coenzyme Q10: an independent predictor of mortality in chronic heart failure. J. Am. Coll. Cardiol. 52, 1435-1441.

Naidoo, N., van Dam, R.M., Koh, W.P., Chen, C., Lee, Y.P., Yuan, J.M., Ong, C.N., 2012. Plasma vitamin E and coenzyme Q10 are not associated with a lower risk of acute myocardial infarction in Singapore Chinese adults. J. Nutr. 142, 1046-1052.

Pepe, S., Marasco, S.F., Haas, S.J., Sheeran, F.L., Krum, H., Rosenfeldt, F.L., 2007. Coenzyme Q10 in cardiovascular disease. Mitochondrion. 7, S154-167.

Premkumar, V.G., Yuvaraj, S., Vijayasarathy, K., Gangadaran, S.G., Sachdanandam, P., 2007. Serum cytokine levels of interleukin-1beta, -6, -8, tumour necrosis factor-alpha and vascular endothelial growth factor in breast cancer patients treated with tamoxifen and supplemented with co-enzyme Q(10), riboflavin and niacin. Basic Clin. Pharamcol. Toxicol. 100, 387-391.

Premkumar, V.G., Yuvaraj, S., Sathish, S., Santhi, P., Sachdanandam, P., 2008. Anti-angiogenic potential of CoenzymeQ10, riboflavin and niacin in breast cancer patients undergoing tamoxifen therapy. Vascul. Pharmacol. 48, 191-201.

Ratnam, D.V., Chandraiah, G., Sonaje, K., Viswanad, B., Bhardwaj, V., Ramarao, P., Kumar, M.N.V.R., 2008. A potential therapeutic strategy for diabetes and its complications in the form of co-encapsulated antioxidant nanoparticles (NanoCAPs) of ellagic acid and coenzyme Q10: Preparation and evaluation in streptozotocin induced diabetic rats. J. Biomed. Nanotechnol. 4, 33-43.

Ratnam, D.V., Chandraiah, G., Meena, A.K., Ramarao, P., Kumar, M.N., 2009. The co-encapsulated antioxidant nanoparticles of ellagic acid and coenzyme Q10 ameliorates hyperlipidemia in high fat diet fed rats. J Nanosci Nanotechnol. 9, 6741-6746.

Ratnam, D. V., Wadsworth, R. M., Kumar, M. N. V. R., 2011. Protective effects of nanoparticulate coenzyme Q10 and curcumin on inflammatory markers and lipid metabolism in streptozotocin-induced diabetic rats: a possible remedy to diabetic complications. Drug Deliv. and Transl. Res. 1, 448–455.

Sherman, W., Martens, T.P., Viles-Gonzalez, J.F., Siminiak, T., 2006. Catheter-based delivery of cells to the heart. Nat. Clin. Pract. Cardiovasc. Med. 3, S57-S64.

Simón-Yarza, T., Formiga, F.R., Tamayo, E., Pelacho, B., Prosper, F., Blanco-Prieto, M.J., 2012. Vascular endothelial growth factor-delivery systems for cardiac repair: an overview. Theranostics. 2, 541-552.

Simón-Yarza, T., Formiga, F.R., Tamayo, E., Pelacho, B., Prosper, F., Blanco-Prieto, M.J., 2013. PEGylated-PLGA microparticles containing VEGF for long term drug delivery. Int. J. Pharm. 440, 13-18.

Sohal, R.S., Forster, M.J., 2007. Coenzyme Q, oxidative stress and aging. Mitochondrion. 7, S103-111.

Tucka, J., Bennett, M., Littlewood, T., 2012. Cell death and survival signalling in the cardiovascular system. Front. Biosci. 17, 248-261.

Wang, B., Cheheltani, R., Rosano, J., Crabbe, D.L., Kiani, M.F. 2013. Targeted delivery of VEGF to treat myocardial infarction. Adv. Exp. Med. Biol. 765, 307-314.

Figure captions

Figure 1. *In vitro* release profile of VEGF-MPs in PBS-pH7.4 at 37 ºC.

Figure 2. Cell proliferation assay to assess the stability of encapsulated VEGF. Free VEGF and VEGF-MPs are equally googl in their cell proliferation ability and are significant when compared to untreated control (***$p<0.0001$)

Figure 3. Ejection fraction (cardiac function) observed in different groups: (A) CoQ-NPs (B) VEGF-MPs (C) VEGF-MPs and CoQ-NPs combination and (D) Summary of individual and combination VEGF treatments. EF was assessed by echocardiography before and 3 months after treatment and mean improvement±SD is represented.

Figure 4. The histological section were imaged and quantified for the analysis of (A) Vessel density (B) Average vessel diameter and (c) Total caveoline positive area. Small vessels were stained using anti-caveoline-1α antibody.

Table captions

Table 1. Animals were divided in eight different groups (n=5 / group). Three efficacy studies were performed to observe effects of CoQ-NPs, VEGF-MPs and combination of both.