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COMBINATION OF AN ADIPOSE PEDICLE WITH MYOCARDIAL MATRICES ENRICHED WITH MULTIFUNCTIONAL EXTRACELLULAR VEHICLES BY CARDIAC TISSUE ENGINEERING: PRECLINICAL AND CLINICAL STUDIES

Carolina Gálvez Montón
Fundació d'Investigació en Ciències de la Salut Germans Trias i Pujol

1. Project summary

The application of an autologous adipose flap (adipose graft transposition procedure, AGTP) obtained from pericardial surface is an efficient alternative to cardiomyoplasty to reduce infarct size and to improve cardiac function in the porcine model of acute myocardial infarction (MI). However, the benefits of AGTP in the chronic infarct model are only moderate, raising the need for improvements in modulation of host inflammatory responses as well as participation of the inherent myocardial regeneration mechanisms. Thus, a novel bioengineered 3D patch including a decellularized myocardial scaffold integrated by multifunctional extracellular vesicles (EV) may enhance the AGTP effects.

This project entailed two main objectives: first, to conduct a prospective, single-center, randomized, controlled phase I-II clinical trial to examine the safety and efficacy of AGTP intervention in patients with a chronic myocardial scar who were candidates for revascularization of other myocardial areas. The second goal was to isolate, characterize and functionally analyse (immunomodulation and chemoattractive properties) EV derived from cardiac adipose tissue-derived progenitor cells (cATDPCs), develop a decellularized myocardial scaffold enriched by EV and examine its regenerative capacity in a porcine model of MI.

2. Results

Phase I-II clinical trial for the safety and efficacy evaluation of AGTP for improving cardiac function in patients with transmural myocardial infarction

A prospective randomized clinical trial was performed involving 10 patients with chronic transmural MI (defined as >70% of infarcted wall thickness evaluated with gadolinium retention using cardiac magnetic resonance imaging) and who were candidates for cardiac revascularization (coronary by-pass) from another area distal to the scar. The candidates were divided into 2 groups: control (n = 5) and treatment (n = 5). In the control group, the patients underwent a bypass and the chronic infarction scar was not treated (according to standard clinical practice criteria), while in the treatment group, in addition to the bypass the chronic transmural infarction was covered with an autologous adipose vascularized pedicle of pericardial origin. After surgery, all patients

had a one-year follow-up with clinical visits (7, 90, and 365 days post-intervention), as well as undergoing 3 cardiac magnetic resonance imaging sessions (MRI before surgery, and on days 90, and 365 post-surgery) to evaluate cardiac function.
As a result, 1 out of the 10 candidates initially included in phase I clinical trial was excluded for presenting claustrophobia on MRI, initially assigned to the control group. All patients were male and no significant group differences were detected in the clinical variables, nor in the variables related to the severity of the pathology presented or to surgical risk. The same cardiovascular surgeon performed all surgeries and no adverse events were detected during surgeries. No differences were found in terms of safety, hospital admissions during the study follow-up, or the prevalence of arrhythmias, although the number of supraventricular ectopic arrhythmias was lower in the patients in the treatment group.

Relative changes in the percentage of necrotic tissue were detected from inclusion to 3 months of follow-up in the control and treated patients. However, no differences were observed between the study groups. Statistical analysis revealed a tendency towards a lower left ventricular systolic volume ($P = 0.09$) and lower necrosis ratio ($P = 0.06$) at 3 months of evolution in the treated patients. In addition, although not significant there was a reduction in extracellular space at 3 months follow-up between the treated and control group (-4.5% versus -1.1%, respectively; $P = 0.53$).

From the beginning of the study, at the different clinical evaluation points of the patients (7, 90 and 365 days), the values of highly sensitive NT pro-BNP and troponin T were analysed without significant differences being detected between the two study groups. There were no significant differences between the two study groups in terms of clinical benefit with respect to the different follow-up visits (7, 90 and 365 days). Importantly, one of the treated patients, who had the largest necrotic zone and the most dilated ventricles at the beginning of the study, had a remarkable benefit: necrotic mass of the left ventricle at baseline 33.2%, which decreased 4.4% at 3 months and 10.8% a year; left ventricular diastolic volume 315.1 mL and left ventricular systolic volume 218.5 mL at baseline when 31.9 mL and 23.8 mL decreased, at 3 months, and 55.2 mL, and 37.8 mL, at baseline. In addition, the Q waves (indicator of chronic ischemia) disappeared from the electrocardiogram at leads III and aVF at follow-up.

Isolation, characterization and functional analysis of extracellular vesicles (EVs) derived from cardiac adipose tissue-derived progenitor cells (cATDPCs), development of a decellularized myocardial matrix integrated by EV and analysis of their regenerative capacity in the swine model of myocardial infarction.

A stock of EV was produced from cATDPCs primary culture supernatants (n = 21) isolated by size exclusion chromatography. In order to test their immunomodulatory properties, the culture and stimulation of swine peripheral blood mononuclear cell cultures (PBMCs) was adapted to study the effect of the addition of cATDPC-EV in terms of modulation of proliferation and cytokine secretion by PBMCs. PBMCs were activated by PMA and ionomycin (Io) to simulate the pro-inflammatory environment characteristic of ischemic myocardium, obtaining a high proliferation of PBMCs and an active response of inflammatory cytokines once PBMCs were stimulated and absence of cATDPC-EV. In contrast, when cATDPC-EV were added, it dose-dependently inhibited the proliferation of allogeneic PBMCs. Importantly, cATDPC-EV were able also to suppress the secretion of pro-inflammatory cytokines such as IFNγ, TNFα, and IL12p40 produced by PMA and Io-stimulated PBMCs. IL10 is also reduced by the effect of cATDPC-EV, whereas secretion of IL6 and IL8 was not altered by the presence of cATDPC-EV. Noteworthy, cATDPC-VEV did not induce an inflammatory response to allogeneic PBMCs, suggesting an absence of alloreactivity.

Likewise, the success of cardiac repair from the administration of EV embedded within a cardiac matrix is based on the assumption that EV will simultaneously activate the migration of endogenous repair cells that can act directly in the damaged area. For this reason, the chemotactic response of allogeneic porcine endothelial progenitor cells (OECs) and mesenchymal stem cells (MSCs) to cATDPC-EV has been studied in vitro. Most notably, it was observed that cATDPC-EV are actively and dose-dependently promoting the migration of allogeneic OECs and MSCs into cATDPC-EV-containing droplets.

Next, porcine myocardial and human pericardial scaffolds were generated to compare which of the two was most optimal as a supportive matrix for EV administration. The mechanical properties of decellularized matrices at both the macroscopic, tensile and microscopic levels were evaluated by atomic force microscopy (AFM). The ultrastructure of the scaffolds and their protein composition were also analysed using

scanning electron microscope and mass spectrometry respectively. We developed different techniques for fluorescently labelling EVs in order to track them and assess retention once incorporated into the scaffolds. The staining of the EV with NIR815 was fine-tuned, which allowed us to observe on a macroscopic scale the degree of retention of the EV in the decellularized scaffolds. Staining with PKH26 also allowed us to detect the presence and distribution of EV within the scaffolds by means of confocal fluorescence microscopy. Finally, we verified the presence of EV in the scaffolds by scanning electron microscopy, which showed the optimum degree of EV retention inside both types of matrices analysed.

To analyse the immunomodulatory properties of matrices in combination with EV, their effect on the inflammatory cytokine response of PBMCs stimulated with PMA and Io was studied. Of note, the presence of cATDPC-EV-loaded pericardial scaffold decreased the levels of pro-inflammatory cytokine secretion (IFNγ and TNFα) and increased those of IL-6 compared to stimulated PBMCs alone or in the presence of the myocardial scaffold loaded with cATDPC-EV. Together with the fact that the pericardial scaffold is able to retain more volume of EV, this lower degree of detected immunoreactivity was convincing for pericardial scaffold to be chosen for testing in in vivo experiments. Regarding the analysis of the regenerative capacity of the scaffold enriched with EV in the MI pig model, the combined administration of the pericardial matrix with the AGTP was not studied in vivo, as previous studies by our group have shown this combination does not have a beneficial effect compared to the administration of the pericardial extracellular scaffold alone, and the inflammatory infiltrate observed in the ischemic area was much more evident in the combination approach (Gálvez-Montón C et al., Preclinical Safety Evaluation of Allogeneic Induced Pluripotent Stem Cell-Based Therapy in a Swine Model of Myocardial Infarction. Tissue Eng Part C Methods. 2017 Nov; 23 (11): 736-744).

Alternatively, an in vivo study was performed with a 6-day follow-up pig MI model. This study was performed to evaluate the safety and biodistribution of cATDPC-EV in the short term, and to be able to determine their potential anti-inflammatory effect demonstrated previously in vitro. Specifically, 8 pigs subjected to an MI were included. The animals were then randomized into 2 study groups: control (n = 4; implantation of a pericardial decellularized scaffold with hydrogel) or treatment (n = 4; implantation of decellularized pericardial scaffold enriched with EV isolated from cATDPCs of pig origin

labelled NIR815). The engineered scaffolds were implanted 30 min post-induction of MI, covering the ischemic area, and animals were recovered and followed up for 6 days.

We detected the presence of NIR815-labeled cATDPC-EV six days after implantation in both the implanted scaffold and in the infarcted area in treated animals by fluorescence imaging technology. The histological study allowed us to verify that all the scaffolds were correctly integrated with the myocardium, showing neoinnervation and neovascularization regardless of whether they were enriched with EV. However, the presence of cATDPC-EV induced higher vascular density within the scaffolds in the treated animals. In the MI area, treated animals showed less presence of infiltrated monocytes (CD163 +) in the infarct core, whereas no differences were found within the implanted scaffolds. In addition, there was a tendency towards a lower fibrosis in the infarct core of animals treated with cATDPC-EV.

3. Relevance to potential future implications

Heart failure is the final stage of many cardiovascular diseases, such as that caused by the scar that forms after an acute myocardial infarction. MI continues to have very negative effects, greatly limiting the quality of life for both men and women around the world. In this context, therapeutic strategies designed to limit post-ischemic remodelling ultimately leading to heart failure may prevent ventricular dilatation and preserve a supportive structure for effective cardiomyocyte contraction. In this sense, in the project we have developed a new therapeutic strategy that could improve the recovery of patients with post-infarction scars, to which the current therapy options are in most cases aggressive and / or limited.

The clinical implications of the project can preferably be divided into two main aspects:

1. Application of AGTP is advantageous and has potential clinical benefits in several important areas:

- Integration of both therapeutic approaches (cell therapy and tissue engineering) in a single procedure, significantly reducing the cost of technical processes, specialized facilities and qualified staff.

- Improvement of cell therapy efficacy by preventing (i) myocardial damage and risk of induced ventricular arrhythmias compared to intramyocardial cell injection; (ii) nonspecific migration of cells administered to remote organs after systemic / intravenous administration; and (iii) adverse immune response when allogeneic stem cells are administered.
- Stem cell retention on the ischemic myocardium, where the desired effects can be exerted, to favour vascular connections and cell migration from the adipose graft to the underlying myocardium. On the other hand, recruitment of host pro-regenerative cells with anti-fibrotic, anti-inflammatory, cardio-protective, pro-angiogenic function may also be crucial in the positive effects of AGTP.
- Minimization of unfavourable complications resulting from stem cell manipulation under *ex vivo* culture conditions. Likewise, open and semi-open cell culture systems may not comply with regulatory agency recommendations and are associated with a potential risk of contamination, chromosomal alterations and end-products not suitable for clinical use. Large-scale cell expansion greatly increases production costs due to the need for clinical grade media formulations and processes in controlled environments.
- MI scar reduction and improvement of heart function. Pre-clinical results show a significant reduction in infarct size and a lesser degree of scar fibrosis in terms of total collagen deposition following AGTP implantation. In our AdiFLAP clinical trial we observed a significant reduction in the rate of necrosis and a tendency to a decreased cardiac output after 3 months of evolution, with an excellent safety profile for arrhythmia events.
- Because this project has allowed the first clinical trial to be performed with the AGTP (NCT01473433, AdiFLAP trial), a new phase II clinical trial has been launched to demonstrate the effectiveness of this surgical technique (AGTP II trial, NCT02798276). This is a multicenter trial with an exponential increase in the number of participating patients. If the aforementioned positive results are confirmed in this second trial, the benefits obtained can be transferred into clinical practice, making a new therapeutic option available for those patients whose current treatments are inadequate.

2. The pre-clinical trials of EV administration in the pig model of MI have contributed to the development of specific fields by substantially increasing our knowledge in various areas:

- Materials science: the use of biomaterials in this project has met basic parameters such as mechanical stability, elasticity, porosity and geometric

requirements. The cardiac origin of the materials used has successfully mimicked the cellular environments required to induce and control cell functions, differentiation, and vasculogenesis, and has acted as an optimal vehicle for local administration of multifunctional EV. The combination of EV and scaffolds as a new tissue therapeutic strategy has become a major challenge successfully achieved throughout the project.

- Biomedical sciences: the in vitro and in vivo experiments performed during the project have been a crucial step in studying the role of the factors that affect the function of the EV and of the host's own cells after treatment with the new bioimplant. The advances made will help us better understand the underlying cellular and molecular mechanisms that govern cardiac regeneration. In line, the potential implications for medical methodologies may be many, and we hope that they will help promote the incorporation of new paradigms in the field of study and application of new, more reliable and efficient surgical interventions.
- Medical advancement: the main results of the project have substantially contributed to the establishment of a new therapeutic platform and to significantly improving the results of existing treatments for the repair of infarcted heart tissue. On one hand, the translation of pre-clinical studies into phase I-II clinical studies opens a whole new approach for cardiac regeneration. On the other hand, the new bioimplant that has been developed is easy to design and does not require too specific and expensive instrumentation to manufacture.

4. Scientific communications

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