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Biohybrid Cardiac ECM-Based Hydrogels Improve Long Term Cardiac Function Post Myocardial Infarction

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Abstract

Injectable scaffolds for cardiac tissue regeneration are a promising therapeutic approach for progressive heart failure following myocardial infarction (MI). Their major advantage lies in their delivery modality that is considered minimally invasive due to their direct injection into the myocardium. Biomaterials comprising such scaffolds should mimic the cardiac tissue in terms of composition, structure, mechanical support, and most importantly, bioactivity. Nonetheless, natural biomaterial-based gels may suffer from limited mechanical strength, which often fail to provide the long-term support required by the heart for contraction and relaxation. Here we present newly-developed injectable scaffolds, which are based on solubilized decellularized porcine cardiac extracellular matrix (pcECM) cross-linked with genipin alone or engineered with different amounts of chitosan to better control the gel's mechanical properties while still leveraging the ECM biological activity. We demonstrate that these new biohybrid materials are naturally remodeled by mesenchymal stem cells, while supporting high viabilities and affecting cell morphology and organization. They exhibit neither in vitro nor in vivo immunogenicity. Most importantly, their application in treating acute and long term chronic MI in rat models clearly demonstrates the significant therapeutic potential of these gels in the long-term (12 weeks post MI). The pcECM-based gels enable not only preservation, but also improvement in cardiac function eight weeks post treatment, as measured using echocardiography as well as hemodynamics. Infiltration of progenitor cells into the gels highlights the possible biological remodeling properties of the ECM-based platform.

Keywords: cardiac tissue engineering, injectable scaffold, extracellular matrix, biohybrid material.

1. Introduction

Recent regenerative therapy approaches for progressive heart failure following myocardial infarction (MI) have mainly focused on biomaterial-based injectable platforms or stem cell-based injection therapy.[1, 2] These two approaches can also be combined to achieve improved retention of the cells.[3] For the biomaterial-based approach, different materials, synthetic as well as natural, are used to reinforce the damaged LV, arrest inflammatory progression and possibly enhance in situ cell recruitment and tissue remodeling.[1, 4, 5]

Our group, as well as others, has previously demonstrated the importance of biomaterials originating from decellularized extracellular matrix (ECM) isolated from porcine heart tissue, in cardiac regenerative therapy.[6-9] ECM plays a key role in the regeneration of tissue as well as in the mechanical properties of specific tissues.[10, 11] Decellularized ECM is considered the biomaterial that best mimics the natural cell environment, while being bioactive, biodegradable and biocompatible. Most importantly, it's unique bioactive composition (collagen proteins, glycosaminoglycans, fibronectin, etc.) and 3D structure provides the cells with the right environmental and chemical cues for integration of the new construct into the host.[12-14]

The motivation behind the design of injectable solubilized ECM for cardiac therapy is to avoid invasive surgery and to facilitate cell delivery when applicable.[15-18] In MI induced animal models, such approaches have demonstrated improvement or preservation of cardiac function. As with many other natural hydrogels, however, solubilized porcine cardiac ECM (pcECM), is restricted by limited mechanical strength and tends to form a soft gel that is particularly prone to rapid degradation due to its natural protein composition.[19] Such a scaffold may encounter obstacles when used for the long-term reinforcement of the LV wall,[20] particularly when treating the more challenging chronic MI model. In this model, which is considered more

clinically relevant, treatment is given only after a mature scar tissue has already formed. Furthermore, the functional improvements achieved in acute models are rarely repeated in chronic models.

To address all these challenges, we have developed injectable biohybrid ECM-based hydrogel platforms for cardiac tissue regeneration. A process to dissolve porcine cardiac ECM was developed, and the resulted hydrogel was crosslinked with genipin, a natural crosslinker, and chitosan, a natural linear biocompatible polysaccharide known to increase the strength and stability of collagen gels,[21] which can be cross-linked to collagen in the presence of genipin.[22, 23] This allows for the preservation of the ECM biological composition and its ultrastructure, while increasing its mechanical strength, thus improving the efficacy of the injectable scaffold. We have developed *three* gel formulations, all based on whole pcECM that was formulated with genipin and different concentrations of chitosan (0-0.2 gr/gr), and evaluated them in terms of their mechanical properties and cell integration. The effect of the pcECM-based gels on cell morphology and biological activity was studied as well as their biocompatibility *in vitro* and *in vivo*. Most importantly, the therapeutic potential of the pcECM-based injectable formulations was evaluated in rat models for both acute MI and long-term chronic MI, in which animals were treated 4 weeks post MI and followed for 4 and 8 weeks post treatments. This mode of treatment ensures scar formation and demonstrates the long-term activity of the cardiac tissue.

2. Materials and methods

2.1 Experimental Design

The primary objective of the present study was to develop injectable scaffolds for cardiac tissue regeneration, which are based on pcECM and reinforced with other biomaterials to achieve superior mechanical properties. To achieve this, we designed three pcECM-based gel formulations and characterized them in terms of structure and rheological properties. We then examined the crosstalk between the gels and mesenchymal stem cells, thus addressing cell viability and morphology when cultured on the gels, as well as their ability to remodel the gels. The biocompatibility of the gels was studied *in vitro* and *in vivo*. Finally, extensive *in vivo* efficacy studies were conducted to assess the scaffold's potential. Sample size was determined using online power calculators (http://powerandsamplesize.com) or according to previous studies.

2.2 Preparation of soluble pcECM

The pcECM was decellularized and prepared with some modifications to our previously published protocols.[6] To summarize, left ventricular tissue was isolated from healthy commercial slaughter-weight pigs. The decellularization procedure consisted of two cycles of the following steps: Alternating hyper/hypo tonic NaCl solutions; enzymatic treatment using trypsin; and detergent washes with Triton-X-100. The decellularized pcECM was then frozen in liquid nitrogen, crushed and lyophilized to a dry fine powder. The powder was solubilized using slightly modified published methods [15, 18, 24]. Briefly, ECM (10 mg ml^{-1}) was solubilized in HCl (0.01 M) using sonication (1 min), followed by enzymatic digestion using pepsin (1 mg ml^{-1}). The solution was then adjusted to pH 5 with NaOH and kept cold (4°C).

2.3 Solubilized pcECM composition

The total collagen and GAG content in soluble pcECM was quantified using a Sirius red assay, and the Safranin-O method, respectively, as previously described.[25, 26] Protein composition analyses were performed by the Proteomics Center, Technion – Israel Institute of Technology using LC-MS/MS.

2.4 Production and characterization of different pcECM-based gel formulations

The soluble pcECM was assessed alone or when combined with chitosan (FMC Biopolymer, Norway) and genipin (Sigma-Aldrich). Three gel formulations were developed: Gel A- pcECM + genipin (0.01 gr $_{genipin}$ /gr $_{ECM}$), Gel B- pcECM + genipin (0.01 gr $_{genipin}$ /gr $_{ECM}$)+ chitosan (0.05 gr $_{Chitosan}$ / gr $_{pcECM}$), and Gel C- pcECM + genipin (0.01 gr $_{genipin}$ /gr $_{ECM}$) + chitosan (0.2 gr $_{Chitosan}$ / gr $_{pcECM}$). To allow self-assembly of the gels, samples of solubilized pcECM, with or without chitosan and genipin, were plated (37°C, 3 hrs) and PBS was added.

Scanning electron microscopy (SEM) analysis was performed on pcECM, pcECM gel, Gel A, Gel B, and Gel C. Gels were washed and submerged in distilled water, frozen and then lyophilized overnight. Samples were imaged using side- and back-scattered electrons (0.98 Torr, 20 kV), using an FEI-Quanta 200 electron microscope (OXFORD Instruments). SEM images were analyzed using Image J software to measure fiber diameters.

Rheological analyses of the pcECM-based gels were done using a DISCOVERY HR-2 Hybrid Rheometer (TA Instruments). Samples of the pcECM-based biomaterials were transferred into the rheometer and allowed to gel for 20 minutes. Gels were tested using parallel plate geometry

(40 mm diameter, 1.2 mm gap, 1% strain, 1 rad sec^{-1}, 37°C). Frequencies ranged between 0.1 to 600 rad sec^{-1}.

2.5 Culturing hMSCs on solubilized pcECM-based gels

Human bone marrow mesenchymal stem cells (Lonza, Switzerland) were cultured using αMEM, supplemented with FCS (10%), Pen-Strep (1%), Fungizone (0.4%), and basic fibroblast growth factor (5 ng mL^{-1}, Biological Industries, Israel). Media was replaced at least every 2 days. Cells were cultured in a humidified incubator (37°C, 5% CO2). MSCs were seeded in 24-well plates (75,000 cells per well) coated with 100 µl of the tested gel, and cultured for 28 days. Cell viability was determined using the AlamarBlue™ reagent (AbD Serotec) according to the manufacturer's protocol.

To study the morphology of hMSCs seeded on the gel, microscopy analyses were performed. The cells (75,000) were seeded on gels (100 µl) in glass bottom tissue culture plates (2 cm) and cultivated for up to 7 days. Samples were fixated in PFA (4%) and stained for actin fibers with phalloidin-FITC and for DNA with Hoechst 33258 (Sigma-Aldrich). hMSCs seeded on cover slips served as a control. Images were taken using LSM700 confocal microscope (Zeiss). For hematoxylin and eosin (H&E) staining, hMSCs cultured on gel were fresh frozen in Tissue-Tek® OCT compound (Sakura), cross-sectioned into slices (10 µm) and stained. Slides were visualized by inverted phase-contrast microscopy (Eclipse TE2000-E, Nikon Inc.). WET-SEM™ (QuantomiX®, Israel) analysis was also used to image hMSCs seeded on the gels. Gels (20 µl) were seeded with hMSCs (10,000) in QX-302 tissue capsules and cultured (24 hrs). Samples were then imaged by side- and back-scattered electrons (0.98 Torr, 20 kV) using an FEI-Quanta 200 electron microscope (OXFORD Instruments). For light sheet fluorescent microscopy

imaging (LSFM), Di0 (Life technologies) labeled hMSCs ($5x10^5$) were mixed with 200 µl of pre-gel A solution, allowed to gel at 37°c for 20 min in a 24-well plate. 1 ml of culture media was then added. One or 7 days post-seeding, samples were fixed with 4% PFA and stained with TAMRA labled RGD-CPB (Sigma), which is a collagen stain, according to the manufacturer's instructions. DNA was labeled with Hoechst 33258. Prior to imaging, samples were immersed in 1% low melting agarose solution (Bio-Rad) and allowed to gel in LSFM glass capillaries. Samples were then imaged using Lightsheet Z.1 (Zeiss).

2.6 MSCs remodeling of pcECM-based gels

The expression of ECM remodeling-related genes by hMSCs seeded on the pcECM-based gel was quantitatively studied for 30 days, by real-time RT-PCR. The following genes were studied: Collagen I (α1 chain), collagen III (α1 chain), matrix metalloproteinase 2 (MMP2), and type 1 tissue inhibitor of metalloproteinases (TIMP1). MSCs were seeded on the gel as described above (n=5). Total RNA was isolated from the seeded cells at different time points using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions, and reverse-transcribed in a PTC-200 PCR cycler using a Verso™ cDNA kit (Thermo-Scientific). Primers were designed to specifically amplify genes' cDNA as follows: 5′-TACAGCGTCACTGTCGATGGC-3′ and 5′-TCAATCACTGTCTTGCCCCAG-3′ for collagen Iα1; 5′-AATTTGGTGTGGACGTTGGC-3′ and 5′-TTGTCGGTCACTTGCACTGG-3′ for collagen III α1[27]; 5′-TTGACGGTAAGGACGGACTC-3′ and 5′-ACTTGCAGTACTCCCCATCG-3′ for MMP2; 5′-TACTTCCACAGGTCCCACAA-3′ and 5′-ATTCCTCACAGCCAACAGTG-3′ for TIMP1[28]; and 5′-CAACAGCGACACCCACTCCT-3′ and 5′-CACCCTGTTGCTGTAGCCAAA-3′ for glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) as an intrinsic housekeeping gene control. Reactions were run on the StepOnePlus system, and analyzed using StepOne software v. 2.2.2 (Applied Biosystems).

2.7 In vitro studies of pro-inflammatory signals

To evaluate the pro-inflammatory potential of the pcECM-based gels, *in vitro* macrophage stimulation assay was performed. Levels of secreted nitric oxide (NO) as well as the expression of the pro-inflammatory cytokines were measured. RAW cell line (TIB-71™; ATCC) was seeded to six-well culture plates and cultured in high-glucose DMEM (Sigma, 3 mL), supplemented with FCS (10%), Pen-Strep (1%), and Fungizone (0.4%). One day post-seeding, the medium was replaced with low-serum medium (2% serum). When 70% confluence was reached, cells were exposed to lyophilized Gel A, Gel C, and Poly(lactic-co-glycolic acid) (PLGA, Sigma-Aldrich). Lipopolysaccharide (LPS, 1 μg mL^{-1}) served as a positive control, and untreated cells served as a negative control (basal NO secretion). The treated RAW cells were incubated for 16 h, and the secreted NO was measured as the free stable nitrite form (NO_2^-) in the medium, using the Griess Reagent System (Promega), according to the manufacturer's instructions (n=4). In addition to NO secretion measurements, real-time RT-PCR was performed as described above to quantify the expression of the pro-inflammatory cytokines TNF-α and IL-1β using the following specific primers.

5'-GCCTCCCTCTCATCAGTTCT-3' and 5'-TGGTGGTTTGCTACGACGTG-3' for TNF-α. 5'-AGGATGAGGACATGAGCACC-3' and 5'-ATGGGAACGTCACACACCAG-3' for IL-1β.

2.8 Animal studies

Immunogenicity experiments were conducted in accordance with the Israeli Animal Welfare (Protection and Experimentation) Law, after obtaining the permission of the Technion's Animal Care Committee. Efficacy experiments were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC), National University of Singapore.

2.9 In vivo immunogenicity studies

The immunogenic potential of pcECM-based gels was further evaluated *in vivo*. Six week-old male C57BL mice were subcutaneously injected with Gel A, Gel C or alginate gels, as a negative control (n=6 mice per group per time point, Harlan Labs, Israel). Blood samples were taken from the facial vein for CBC and cytokines quantification prior to, and 1, 7, 14 and 28 days post-implantation. At these same time points, mice were sacrificed and their inguinal lymph nodes were harvested. The lymph nodes were homogenized, mRNA was extracted, reverse-transcribed and TNFα and IL-1β levels (normalized to GAPDH) were quantified as described above.

2.10 In vivo efficacy studies of pcECM-based gels

Male Wistar rats (weighing 300–350 gr) were induced with MI as follows: The rats were anesthetized using an isoflurane/oxygen mixture (5% isoflurane for induction and 2-3% for

maintenance), intubated and mechanically ventilated using an Inspira ASV ventilator (Harvard Apparatus). The heart was exposed, under sterile conditions, by left thoracotomy via the 5th intercostal space, followed by pericardiectomy. The left anterior descending coronary artery (LAD) was ligated proximally using a 7-0 Premilene® suture (BBraun, Germany). A successful ligation was confirmed by visible blanching of the left ventricle and significant ECG changes. The rats were randomly divided into two groups, for the acute and chronic MI models. In the acute model, mimicking the treatment of primary MI damage, rats were treated immediately after MI induction. Three experimental groups were studied: control (i.e., PBS treatment), Gel A treatment and Gel C treatment (n=8 rats per group). Rats were treated by injection of either pcECM-based gels or PBS (120 µl) to the margins of the infarcted area (using a 30G needle). The chest was closed in layers; the animals were extubated and transferred to a small-animal intensive care unit for recovery. Enrofloxacin (25 mg kg^{-1}) and Buprenorphine (0.05 mg kg^{-1}) were administered subcutaneously for up to 5 days post operation. All rats were sacrificed 4 weeks post treatment.

In the chronic model, treatment of MI following the formation of a mature scar tissue, rats were treated 4 weeks post MI induction. Five experimental groups were studied: Sham (i.e., no MI), control (i.e., PBS treatment), Gel A treatment, Gel B treatment and Gel C treatment (n=8 rats per group). Rats were treated by injection of either pcECM-based gels or PBS (120 µl) to the margins of the infarcted area (using a 30G needle). The chest was closed in layers and the animals were extubated and transferred to a small-animal intensive care unit for recovery. Enrofloxacin (25 mg kg^{-1}) and Buprenorphine (0.05 mg kg^{-1}) were administered subcutaneously for up to 5 days post operation. Rats were sacrificed 4 or 8 weeks post treatment.

2.11 Echocardiography

Transthoracic echocardiography was performed under general anesthesia prior to MI induction (i.e., baseline reads), and 4 weeks post treatment, using a Vivid S6 ultrasound system (General Electric, VingMed, Norway) equipped with an i12L-RS Linear Array transducer (5-13MHz). B- (Brightness, 2D) and M- (Motion) mode images were recorded. Acquired echo data was analyzed by EchoPac^{TM} software (Version 6.0, General Electric, Vingmed, Norway). The dimensional parameters monitored were left ventricle interior diameter in diastole and systole (LVIDd and LVIDs, respectively) and left ventricle posterior wall thickness in diastole and systole (LVPWd and LVPWs, respectively). Functional parameters monitored were Fractional Area Change (FAC), Ejection Fraction (EF) and Fractional Shortening (FS). Formulas used for calculations of echocardiographic data were as previously reported[29] and are detailed in Table S3.

2.12 Hemodynamic analysis

Hemodynamic assessments were made immediately prior to sacrifice as previously published.[30] Briefly, following median sternotomy, a 2 mm transit-time flow probe (Transonic Systems, Ithaca, NY, USA) was placed around the ascending aorta for cardiac output measurement used for volume calibration (α calibration). A pressure-volume (P-V) catheter (SPR-838 NR, Millar Instruments, Houston, TX) was inserted into the left ventricle, using the MPVS-Ultra system (Millar Instruments, Houston, TX) and PowerLab 16/30 (AD Instruments, Mountain View, CA). Volume calibration was performed using the 910-1048 Volume Calibration Cuvette (2-15 mm wells) (AD Instruments, Mountain View, CA), according to the

manufacturer instructions. A saline calibration (50-100µl of 30% w/v NaCl in DDW) was used to determine parallel volume (V_p).

Normal (baseline) hemodynamic parameters were recorded followed by inferior vena cava occlusion (IVCO) tests to assess pre-load independent left ventricular parameters, and finally, dobutamine stress tests were conducted. All cardiac parameters of at least five consecutive cardiac cycles within each recording, as previously reported [30] and as detailed in Table S4, were obtained from analyses and calculations using LabchartPro™ software (AD Instruments, Mountain View, CA). Average ± standard deviations are presented out of n=8 rats per group; comparison between treatment groups was based on two-way ANOVA with Tucky's HSD post-hoc correction (α=0.05). For the dobutamine stress test, a stepwise dosage increase was used at constant flow rates of 5, 10, and 20 µg/Kg/min, and with a 5 min interval between steps. Pressure-volume data and ECG heart rates were continuously recorded. During stress tests, SW, SV, CO and CI were calculated for each animal at each dobutamine concentration (and without dobutamine as a baseline recording).

2.13 Histopathological assessment

Upon rat sacrifice, hearts were harvested and transversely cut in the middle of both ventricles through the infarct zone. Both sections were processed on paraffin blocks, sectioned (5 µm, Leica RM2255/RM2235 Microtome) and stained by hematoxylin and eosin (H&E) and Masson Tri Chrome (MTC) for pathological evaluation and quantification of relative infarct size, respectively. MTC stained slides (n=5 animals per group, at least 2 sections from each animal) were analyzed for relative infarct size quantification (Supplementary Fig. S4) using the MIQuant semi-automated infract sized measurement software, as previously published [31].

Immunohistochemical (IHC) staining was performed using GATA4 (1:500, rabbit anti rat, Abcam) and α-SMA (1:50, mouse anti rat, Cell-Marque, CA) primary antibodies for cardiac progenitors and for smooth muscle cells and mature arterioles, respectively. All staining was performed using an auto-stainer according to the manufacturer's instructions (BondTM autostainers, Leica). Representative H&E and IHC stain-pictures out of at least two sections per antibody stained for each animal are presented.

2.14 Statistical analysis

Data is expressed as mean values ± standard deviation (n=5), unless otherwise noted. Analysis of variance (ANOVA) was used for comparison using Microsoft Office Excel™ 2010 software, and post-hoc Tucky's HSD was applied when indicated. $p<0.05$ was considered significant unless otherwise mentioned.

3. Results

3.1 Soluble pcECM and pcECM-based gel production and characterization

To produce cardiac ECM gel, slices of porcine left ventricular tissue were decellularized according to a protocol we developed (WO 2006095342A2, fig. 1A). The pcECM was then processed into fine powder (fig. 1B) and dissolved with pepsin in an HCl solution (fig. 1C). The resulting pcECM solution was successfully injected through a 24G CV catheter (fig. 1D) and gelled at 37°C (fig. 1E). Mass spectrometry analysis confirmed that the soluble pcECM is mostly composed of collagens I and III (table S1). Sirius red assay demonstrated that the collagen

content of the soluble pcECM is 93± 7.2%, and safranin O assay determined the glycosaminoglycan (GAG) content to be 0.74% ± 0.0024%.

To achieve control over the mechanical properties of the ECM gel, the addition of strengthening biomaterials was studied. Three gel formulations were developed: Gel A, in which pcECM is crosslinked with genipin (0.01 gr/gr), Gel B, in which pcECM is crosslinked with genipin (0.01 gr/gr) and chitosan (0.05 gr/gr), and Gel C, to which a higher concentration of chitosan is added (0.2 gr/gr chitosan, 0.01 gr/gr genipin). Gel structure was assessed using scanning electron microscopy (SEM), which revealed all gel formulations had preserved a fiber mesh structure. Each formulation was characterized by a unique fiber architecture, size and uniformity (fig. 1F-J, fig. S1). While Gel A exhibited a less organized structure of fibers, varying in size, and resembling the native ECM (fig. 1H, F and fig. S1), Gels B and C were characterized by thinner fibers that were arranged in a denser and more uniform structure. In comparison to Gel B, Gel C, which has the higher chitosan content, exhibited thicker fibers and larger pores (fig. 1I-J, Fig. S1).

The mechanical properties of the pcECM-based gels were studied by frequency sweep measurements, in which the gels were exposed to small-deformation oscillations covering a range of frequencies to assess the structural response to deformations with longer or shorter timescales. In all gel formulations, the storage modulus (G') was higher than the loss modulus (G'') over the entire frequency range, and both moduli were only slightly dependent on frequency. However, while Gel A exhibited G' values ranging between 2,000 and 6,000 Pa, Gel B and Gel C reached G' values of 13,600 and 36,800 Pa, respectively (fig. 1K).

3.2 Culturing hMSCs on pcECM-based gels

The properties of our pcECM-based gels were characterized in terms of cell adherence and long-term viability using human mesenchymal stem cells (hMSCs). hMSCs were seeded and cultured on the gels for up to 4 weeks, and their viability on different gel formulations and the standard culture dish, serving as control, was compared. As shown in figure 2A, both Gel A and Gel C supported the adherence of the hMSCs, which remained viable at an average density of 70,000 cells per 100 µl gel for 28 days. In contrast, hMSCs cultured on a standard culture dish survived only up to 20 days post-seeding (data not shown). H&E staining performed 28 days post-seeding further confirms the presence of the cells within Gel C (fig. 2B). The morphology of the seeded hMSCs was imaged by WET-SEM, confocal and light sheet fluorescent microscopy 1, 3, and 7 days post-seeding. The results demonstrated that hMSCs had populated the gel scaffold and aligned to the same direction in a tissue-like structure. This morphology, however, was not obtained with hMSCs that were seeded on a cover slip as a control (fig. 2C-H, movie S1).

The seeded hMSCs' ability to remodel the ECM-based gel was evaluated by their expression of ECM remodeling related genes (fig. 2I-L). In these studies Gel C was assessed since it contains both genipin and the higher concentration of chitosan, which are not biologically active as the pcECM. While collagen I, collagen III, and TIMP1 expression levels in the gel-seeded samples were significantly higher than in the hMSCs on the plate, the opposite trend was observed with MMP2 expression, where higher expression was measured on the plate. Maximal increase in the expression of all tested genes occurred 7 days post-seeding, and by day 20 post-seeding, the expression of remodeling genes decreased to basal levels.

3.3 Immunogenicity studies

The immunogenicity and pro-inflammatory potential of pcECM-based gel formulations was evaluated both *in vitro* and *in vivo*. *In vitro*, RAW macrophage cell line was exposed to lyophilized pcECM-based gels and the secretion of nitric oxide was measured as well as the expression of pro-inflammatory cytokines. As demonstrated in Figure 3A, Gel A and Gel C did not induce nitric oxide secretion. Lipopolysaccharide, on the other hand, induced secretion of substantial levels of nitric oxide. Similar results were obtained for RAW macrophage expression of pro-inflammatory cytokines TNF-α (fig. 3B) and IL-1β (fig. 3C) 16 hr post exposure.

Immunogenicity in the mouse model was evaluated by subcutaneous injection of Gel A, Gel C, or alginate gels that served as a negative control (fig. 3D). Complete blood count tests (CBC) revealed similar levels of neutrophils, hemoglobin, red blood cells, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, and hematocrit for all tested groups. Slightly higher white blood cell (WBC) levels ($p<0.05$) were observed in the Gel C treatment group compared to the control group 7 days post injection. However, these WBC levels decreased back to basal level 7 days later (fig. S2). Lymph node examination revealed no swelling or irritation in all tested groups (fig. 3E-F). In addition, real time RT-PCR analyses performed on mRNA isolated from the lymph nodes revealed slightly lower TNF-α expression levels, in pcECM-based gels as compared to the control (fig. 3G-H). IL-1β expression levels were elevated in the Gel A group after 4 weeks, however, this three-fold increase is minor in terms of IL-1β gene expression, since its expression usually increases more dramatically (>6 times) when an inflammatory reaction is induced.[32, 33] Blood cytokines levels were also similar to the control in both treatments (table S2).

3.4 In vivo efficacy studies - acute MI model

To study the biological efficacy of our ECM based gel formulation, two different MI rat models were used: acute and chronic. In the acute model, we assessed Gel A and Gel C in comparison to a negative control (PBS). The echocardiographic measurements revealed improvement in the acute model in both cardiac dimensions and cardiac function 4 weeks post treatment (fig. 4, table S3). The values of the left ventricular posterior wall thickness (LVPW) were significantly higher than the negative control, while the left ventricular internal dimensions (LVID) of the gel-treated groups were significantly lower than the control, both in diastole and systole (fig. 4A-D, $p<0.05$). Similarly, the values of all tested functional parameters, i.e., fractional area change (FAC), ejection fraction (EF) and fractional shortening (FS) for both gel treatment groups were significantly higher than the control (fig. 4E-G, $p<0.05$).

Our histological evaluation demonstrated that 4 weeks post treatment, Gel A remained mostly intact with minimal degradation, while Gel C was more degraded and cellularized (Figure 4H). Both pcECM-based gels were clearly detected by a certified pathological examination (H&E) due to the less ordered appearance of cells in their vicinity and the dense ECM organization compared to the infarcted area. Both gels were observed to be localized either within the area of infarction or in the subepicardium. The recruitment of smooth muscle cells (SMC), and GATA4 positive-reparative cells, was shown by immunohistochemistry staining using α-SMA and GATA4, respectively (Figure 4H).

3.5 In vivo efficacy studies – chronic MI model

In the chronic MI model, we first monitored the cardiac functional parameters (FAC, EF and FS) over the 4 weeks following treatment (8 weeks following MI). Our results revealed similar

significant functional improvements for all gel formulations for up to 8 weeks post MI, in contrast to the continuous deterioration measured in the control group (fig. 5A-C, respectively). An additional group of chronic MI mice treated with Gel C was followed for up to 12 weeks post MI. The FAC, EF and FS values for each animal were recorded and normalized to each animal's infarction but not treated baseline at 4 weeks post MI (i.e., prior to treatment). Averaging this paired change from baseline per group revealed significant improvements in all parameters indicated by average ratios above the 100% baseline, which contrasted with the deterioration of the control animal (below the 100% threshold, fig.5D). Furthermore, data analyses indicated significant marginal improvements in EF and FS change (i.e., difference between treated-group values to control) from 8 to 12 weeks post MI (data not shown). Semi-automated image analyses revealed no significant difference in the relative infarct size between treated groups and control (p>0.05, Supplementary Fig. S4). The histological evaluation of the heart 4 weeks post treatment in the chronic MI model (fig. 5E) presented similar results to those of the acute model (fig. 4H).

3.6 In vivo efficacy studies – Hemodynamics

To further investigate the effects of our gel formulations on the infarcted heart, hemodynamic studies were conducted. Standard hemodynamic measurements were recorded (fig. S3, table S4) at the end-point (8 or 12 weeks post MI) under both normal baseline conditions (table 1 and table S5, respectively) and preload independent conditions (Inferior Vena Cava Occlusion test, IVCOT; fig. 6). With the exception of stroke work (SW, table 1) and the ventricular elastance, the slope of the end systolic pressure volume relationship curve (ESPVR, fig. 6), systolic and diastolic parameters were similar between all gel-treated groups. Two general trends were observed: 1) equally significant improvement from the control towards sham values for all gel-

treated groups ($p<0.05$, e.g., SV, CO and PRSW), 2) non-significant differences ($p>0.05$, e.g., ESP, Max dp/dt and efficiency). In the case of SW and ESPVR, though, Gel C displayed a slight yet significant improved function when compared to the other gel groups.

Dobutamine hemodynamic stress test (DHST, fig. 7) results corresponded with the standard hemodynamic test results. In the DHST test, Gel C displayed a significantly higher SW profile at 8 weeks post MI, while both Gels A and B showed similar values as the non-treated control (fig. 7A). The CO and SV profiles at 8 weeks post MI were similar between all gel-treated groups, and were significantly higher than the untreated control group (fig. 7B-C, two-way ANOVA TK-HSD, $p<0.001$). The CI profile, however, was significantly higher in Gel B and Gel C groups, while Gel A showed similar values to the non-treated control (fig. 7D). Twelve weeks post MI Gel C still significantly improved cardiac function compared to the control (fig. 7E-H, table S5, $p<0.001$). Moreover, a dobutamine dose-dependent increase in values was observed in the treated hearts, whereas control values of all DHST parameters measured at 12 weeks corresponded with heart failure and displayed a constant, dobutamine dose-independent profile (fig. 7E-H).

4. Discussion

Among the different natural injectable materials studied for treating MI (such as alginate[34] and chitosan[35, 36]), decellularized ECM is one of the most promising biomaterials.[8, 16, 19, 37] ECM's dynamic reciprocal communication with the tissue's cellular component plays a fundamental role in tissue development, signaling and function.[38, 39] Though ECM components in different tissues may be similar, each tissue holds a unique combination and 3D structure of macromolecules that provide the cells with the required cues and mechanical

support.[40] Cardiac acellular ECM should, therefore, be an ideal candidate biomaterial to serve as a scaffold for myocardial tissue engineering. The procedure used for decellularization is critical in order to preserve the original composition of the cardiac ECM. Our patented decellularization procedure [41] avoids the use of SDS, thus allowing improved preservation of ECM biological activity.

In this study, we explored the feasibility of injectable hybrid scaffolds reconstituted from decellularized pcECM and strengthened with the natural materials genipin and chitosan for myocardial regeneration. Our method for ECM isolation and solubilization allowed for the preservation of important ECM components such as collagen I and III as well as GAGs. These components are known to not only be crucial for cell–ECM interactions but also for the gelling process.[12, 38, 39] To achieve long-term mechanical stability, the use of injectable ECM, as with other natural based gels, requires mechanical reinforcement—critical for the proper remodeling of the damaged LV tissue. The natural gel, comprising pcECM alone, was very soft and unstable and thus, was not durable enough when cultured with cells (Figure 1E), which is similar to the findings of Grover et al.[19]. These weak mechanical properties might also fail to provide the longstanding support required by the heart, possibly leading to the breakdown of the gel during heart contraction and relaxation.[20] To strengthen the mechanical properties of the ECM gel and gain better control of the gel stability and degradation over time, the addition of strengthening biomaterials was studied. We studied the reinforcement of pcECM with two materials: genipin, a natural crosslinker, and chitosan, a natural linear polysaccharide. The rationale for using these two natural materials lies in their biological origin and safe use, which may facilitate their approval for future clinical application. Chitosan is known for its biocompatibility, biodegradability and its ability to cross-link collagen. Furthermore, in structure,

chitosan resembles ECM's glycosaminoglycans.[23, 42] Genipin was chosen for its ability to cross-link collagen gel with mechanical strength comparable to chemically cross-linked collagen, but with significantly lower cytotoxicity.[22, 23] The three ECM-based gel formulations that were developed were primarily characterized in terms of their structure. All the gel formulations preserved a fiber mesh structure that is known to allow cellular infiltration and attachment.[43] However, different fiber size, architecture, and uniformity characterized each formulation. Gel A resembled native ECM in its disorganized structure and high variation in fiber size. In contrast with Gel A, the gels that contained chitosan, (Gels B and C), had thinner fibers and a more dense and uniform structure. These findings are concomitant with Yan et al.[44], who demonstrated that the addition of chitosan to genipin cross-linked bovine collagen leads to a more organized, dense structure, and that the pore size increases with higher concentrations of chitosan.

Other researchers have chosen to strengthen their ECM gels with synthetic materials such as polyethylene glycol[19] and the common chemical crosslinker, glutaraldehyde (GA),[45] reaching G' values of up to 720 and 136 Pa, respectively, though compromising the gel's biocompatibility. Our work, demonstrates G' values ranging between 2,000 and 6,000 Pa, for the genipin cross-linked pcECM (Gel A), and G' values of 13,600 and 36,800 Pa for the pcECM-based gels that are also strengthened with chitosan (Gel B and Gel C, respectively). These results indicate that alternating the gel formulations using different ratios of ECM, chitosan and genipin enable modulation of pcECM-based gel's mechanical properties. Furthermore, considering that scaffold mechanical properties can affect cell viability, morphology and differentiation,[46] these findings suggest that by fine-tuning the gel formulations, one may control the cell fate and cell interactions.

We further characterized the ability of our pcECM-based gels to support cell adherence and viability in the long-term using hMSCs. To demonstrate that the pcECM-based gel formulations preserve the biological activity of the pcECM, we have chosen to study Gel C, which in addition to genipin also contains the higher concentration of chitosan (thus the lower amount of pcECM), and therefore, may be the least biologically active among our three gels. MSCs were chosen for these studies as they have the potential to differentiate into the three most predominant cell types identified in the heart: myofibroblast,[47] cardiac endothelial cells[48] and cardiomyocyte-like cells.[49, 50] In addition, it has been shown that improvement in the recovery of the left ventricle is achieved when MSCs are transplanted into animal models and even human patients after MI.[51, 52] From our studies, it is evident that seeded hMSCs can adhere, survive and proliferate in all ECM-based scaffolds. Moreover, hMSCs aligned themselves all to the same direction in a tissue-like structure, and remodeled the ECM-based gel. For these studies, we focused on the following genes: Collagens I and III, the main cardiac ECM constituents; MMP2, a main ECM decomposing factor, and TIMP1, a major MMP inhibitor . Together, these genes help create a general picture of the "building versus breaking" status of the cells on the gel. Our results clearly indicate that the pcECM-based gel (Gel C) encourages hMSCs to increase collagen production and diminish collagen digestion. However, due to the *in vitro* setting, which lacks additional biological stimuli associated with the *in vivo* environment, these remodeling processes were temporary. These findings suggest that similar to endogenous ECM, scaffolds of pcECM-based gels might also play a role in reparative processes and provide essential environmental cues, in addition to providing mechanical support.

To validate the biocompatibility of our gels, *in vitro* and *in vivo* studies were conducted. *In vitro*, the pcECM–based gels did not stimulate a macrophage cell line, as indicated by basal levels of

NO secretion as well as pro-inflammatory cytokines expression. Similar results were obtained *in vivo*, where—following gel injection—the expression levels of pro-inflammatory cytokines in the inguinal lymph nodes did not increase as much as expected in an inflammatory reaction, [32, 33] and the cytokines levels in the blood were similar to control. In addition, CBC measures, previously reported as another relevant indicator for toxicity,[53] did not change throughout the experiment. Taken together, the data demonstrates that although our pcECM-based gel formulations originate from a porcine source and are combined with chitosan and genipin, the gels are non-immunogenic and are thus good candidates for safe implantation as cardiac scaffolds. These results support our previous work that demonstrated the biocompatibility of a porcine cardiac ECM scaffold[6] as well as the findings of previous studies testing the biocompatibility of decellularized ECM gel,[15, 54] genipin[55] and chitosan-based gels.[55-57]

The biological efficacy of our ECM based hybrid gel formulation was studied in acute and chronic MI rat models. In the acute model, treatment was given almost immediately after the MI, as is often done in the research of injectable scaffolds.[58-60]. In the chronic model, which is more clinically relevant, treatment was given after a mature scar tissue has already formed, posing a more challenging clinical scenario. In reported studies, treatment has been given 2-3 weeks post MI and followed for up to 6 weeks. [16, 61] We have chosen to treat the animals 4 weeks post MI, thus allowing the formation of a mature scar tissue. Moreover, to address the long-term efficacy of the treatment, we followed the animals for up to *8 weeks* post injection, i.e., 12 weeks post MI. In the acute model, echocardiographic measurements revealed improvement in both cardiac dimensions and cardiac function 4 weeks post treatment compared to the control. Furthermore, no significant difference was observed between the two treatments of genipin-crosslinked pcECM gels, with or without chitosan. The impressive efficacy of these

gels was repeated in the more challenging chronic MI model, in all the groups treated with pcECM-based gels, up to 8 weeks post MI. Furthermore, the functional improvement was clearly detected even 12 weeks post MI, when measured after the treatment with the strongest gel formulation (Gel C).

Our histological evaluation revealed no significant differences in the relative infarct size. However, while Gels A and B were more intact, Gel C, with the higher content of chitosan, was more degraded and was populated with higher cell densities. The recruitment of smooth muscle cells (SMC), supporting neo-vascularization and GATA4 positive-reparative cells, was seen in all treatment groups for both the acute and the chronic model. SMC were previously shown to support the maturation of newly formed blood vessels,[62, 63] suggesting vascularization of the transplanted scaffold. Similar results, of an increase in α-SMA positive cells, have also been demonstrated following the injection of other biomaterials. [5, 64] GATA4 positive cells were shown to up-regulate paracrine factors that extended survival of neighboring cardiomyocytes.[65] Hence, this recruitment of various cells can slow down or even prevent adverse remodeling of the infarcted heart.[65-67] Quantifying such cell recruitment, however, is extremely challenging given the lack of clear boundaries between the gel and the infarct zone. Nevertheless, a similar study performed on the same pcECM material in a patch treatment modality—which boundaries are much clearer—further supports such a biological mechanism of action and identified the role of GATA4+ cells in this process.[68] Altogether, these findings strongly suggest that our gel formulations not only provide the mechanical support needed to stop deterioration and preserve cardiac function, but also induce an ongoing biological process which possibly leads to tissue repair, and consequently, improved cardiac function.

The efficacy of our gel formulations as injectable scaffolds for cardiac tissue regeneration was further evaluated using hemodynamic tests. These tests are considered the gold standard for providing comprehensive assessment of ventricular performance, and can be tested at steady state, at decreasing preloads or during stress [30, 69]. While most of the measures indicated similar significant improvements in cardiac function for all gel treated groups, as was seen in the echocardiography measurements, in the case of SW and ESPVR, the strongest gel (Gel C) improved function the most as compared to the other gel treated groups (table 1, fig. 6). These subtle differences in the *in vivo* performance of the three gel formulations were repeated in the dobutamine hemodynamic stress tests (DHST). When systemically administered, dobutamine greatly increases myocardial oxygen consumption and enhances LV contractility. Simulating exercise activity, it allows for the measurement of cardiac function under stress. The results of the DHST test showed a similarly impressive improvement in cardiac function for all gel treated groups for most of the tested parameters. However, a significantly higher profile was obtained for the Gel C treated group in the SW measurements at eight weeks post MI, compared to the other gels. Gel C efficacy was also preserved for up to 12 weeks post MI, significantly improving cardiac function. The efficacy of Gel C treatment was demonstrated not just by higher values in all DHST parameters but also by the dobutamine dose-dependent increase in values, which was observed only in the treated hearts.

The effects obtained by our three gel formulations appeared similar when analyzed using echocardiography. Nevertheless, histological analyses and hemodynamic measurements hinted at differences in biological function of the different pcECM-based gels. Histological analyses revealed that a higher concentration of chitosan enhances cell recruitment and gel degradation. Hemodynamic measurements revealed its contribution to cardiac function as depicted by stroke

work measured at the baseline and in the DHST as well as by ESPVR measured at preload independent conditions. The difference between gel functions clearly demonstrates the importance of fine-tuning the gel formulations. Fine-tuning not only affects gel architecture and mechanical properties but may also, consequently lead to improved cardiac function. Although various studies demonstrated improvement in cardiac function following treatment with various natural or synthetic injectable materials,[36, 70] we have not found a comprehensive study including two models and echocardiographic and hemodynamic analyses assessing the efficacy of a composite, naturally driven biomaterial in the literature. Notably, no reports of animals treated in the chronic phase (4 weeks after MI) are available. Instead as in most studies, the treatment is given only 1 or 2 weeks post MI. Singelyn et al. has demonstrated the efficacy of solubilized cardiac ECM with no additives in rat and porcine models achieving cardiac function improvement following scaffold injection. [16] Our work, which demonstrates significant improvement in heart function following treatment with three biohybrid pcECM-based injectable scaffolds, further emphasizes the advantages and importance of this unique biomaterial platform, and offers a new approach to efficiently control the gel's mechanical properties. Hence, strengthening pcECM gel with natural biocompatible polymers not only improves the mechanical properties but leads to different biological outcomes and to superior performance.

5. Conclusion

Our three newly-developed pcECM-based gels exhibit promising results as an injectable scaffolds for cardiac regeneration. The improved physical properties, beneficial biological functionalities and biocompatibility were demonstrated herein both *in vitro* and *in vivo*. Above all, our pcECM-based gels not only preserved heart functions but also alleviated MI damage in

rat hearts, even after the formation of a mature scar tissue, leading to a substantial therapeutic outcome using all gel formulations and particularly Gel C. Altogether, the presented data clearly point at our pcECM-based gels as prospective scaffolds for the treatment of end-stage heart failure.

Disclosure

The authors declare no conflicts of interest in this work.

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Supplementary data

Fig. S1. Average fiber diameter of ECM-based gels.

Fig. S2. Complete blood counts of C57 black mice following subcutaneous injection of Gels.

Fig. S3. Gel injection and hemodynamic analyses.

Table S1. Protein Composition of ECM gel.

Table S2. Biocompatibility studies – Blood cytokines.

Table S3. Terminology and calculations used for echocardiography analyses.

Table S4. Terminology for hemodynamic analyses.

Table S5. Baseline hemodynamic parameters, eight weeks post treatment.

Movie S1. MSCs seeded in pcECM gel.

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Significance Statement

This work describes the development of new injectable scaffolds for cardiac tissue regeneration that are based on solubilized porcine cardiac extracellular matrix (ECM), combined with natural biomaterials: genipin, and chitosan. The design of such scaffolds aims at leveraging the natural bioactivity and unique structure of cardiac ECM, while overcoming its limited mechanical strength, which may fail to provide the long-term support required for heart contraction and relaxation. Here, we present a biocompatible gel-platform with custom-tailored mechanical properties that significantly improve cardiac function when injected into rat hearts following acute and chronic myocardial infarction. We clearly demonstrate the substantial therapeutic potential of these scaffolds, which not only preserved heart functions but also alleviated MI damage, even after the formation of a mature scar tissue.

Tables

Table 1: Baseline hemodynamic parameters, four weeks post treatment.

Group Parameter	Sham	Control	Gel A	Gel B	Gel C
HR (bpm)	343 ± 17*	289 ± 30†	312 ± 29	290 ± 34†	325 ± 38
ESP (mmHg)	73 ± 6	75 ± 6	67 ± 6	55 ± 9	74 ± 6
EDP (mmHg)	9.5 ± 1.5	9.5 ± 4.5	6.9 ± 1.7	6.5 ± 1.1	9.4 ± 2.6
SV (uL)	215.7 ± 5.7*	60.6 ± 30.6†	134.6 ± 26.1†*	112.5 ± 22.6†*	161.1 ± 8.2†*
SW (mmHg/uL)	15741 ± 944*	4599 ± 2856†	9028 ± 1983†	6361 ± 1791†	11351 ± 3758*
CO (mL/min)	75.6 ± 1.7*	17.9 ± 9.5†	43.6 ± 1.7†*	33.4 ± 6.3†*	48.7 ± 7†*
CI (L/min*Kg)	183 ± 3*	46 ± 24†	104 ± 17†*	85 ± 13†*	119 ± 17†*
Max dP/dt (mmHg/s)	5275 ± 1033	4039 ± 1230	3805 ± 559	3214 ± 704†	5615 ± 1524
Min dP/dt (mmHg/s)	- 4865 ± 1499*	- 2574 ± 445†	- 2879 ± 538†	- 1917 ± 557†	- 3227 ± 1184

* $p < 0.05$ vs. Control

† $p < 0.05$ vs. Sham

One-way ANOVA, TK-HSD

Figure captions

Figure 1: pcECM gelation, optimization and characterization. (A) Acellular pcECM. (B) Lyophilized and minced acellular pcECM. (C) Soluble pcECM. (D) A drop of pre-gel leaving the catheter, marked by the white arrow. (E) pcECM gel at 37°C. (F-J) SEM imaging of (F) pcECM, (G) pcECM gel, (H) Gel A, (I) Gel B, (J) Gel C. Scale bar 50μm. (K) Frequency sweep

rheological characterization, changes in storage (G') and loss (G'') moduli in ascending frequencies.

Figure 2: MSCs cultivation on pcECM-based gels. (A) Cell viability as measured with Alamar BlueTM on pcECM-based gels: Gels A & C over 28 days. (B) H&E staining of Gel C 28 days post-seeding. Scale bar 50μm. (C) WET-SEMTM imaging of hMSCs on Gel C 24 hr post-seeding. Scale bar 20 μm. Confocal microscopy imaging of hMSCs on Gel C (D-F) compared to control (G), one (D), three (E) and seven (F, G) days post-seeding; green: phalloidin –FITC (Actin), blue: Hoechst (DNA). scale bar 50 μm. (H) Light sheet fluorescent microscopy imaging of hMSCs on Gel C 7 days post-seeding, Blue: Hoechst (DNA), Green: Di0 (cell membrane), Red: TAMRA-RGD-CPB (collagen). (I-L) Relative expressions of ECM remodeling genes by hMSCs grown on Gel C or standard tissue culture dish. (I) Collagen I (J) Collagen III (K) Tissue inhibitor of metalloproteinases type 1 (TIMP1) (L) Matrix metalloproteinase-2 (MMP2).

Figure 3: Immunogenicity studies. (A-C) Macrophage stimulation assay: RAW cells were exposed to LPS, Gel A, Gel C or PLGA for 16 hr (NT; non-treated). The secretion levels of nitric oxide were measured by the Griess method n=4,* $p<<0.001$ (A). The expression levels of TNF-α (B) and IL-1β (C) mRNA, quantified by RT real-time PCR, n = 6, *$p<<0.001$. *In vivo* immunogenicity study: C57 black mice were subcutaneously injected with 100 μl of pre-gel or alginate gels as control (n = 6 per group), and their immune response was monitored along four weeks. (D) Subcutaneous Gel C marked by a black arrow. (E) Exposed inguinal lymph nodes; no inflammation observed, (F) extracted lymph nodes; normal size. The expression level of TNF-α (G), and IL-1β (H) mRNA in lymph nodes, quantified by RT real-time PCR. Gene expression values are presented with standard error.

Figure 4: *In vivo* efficacy studies – acute MI model. Wistar rats were induced with MI and immediately treated with Gel A (A), Gel C (C) or PBS as negative control (NC). Cardiac dimensions and cardiac function were assessed through echocardiography 4 weeks post treatment. Left ventricular posterior wall thickness in diastole (LVPWd, A) and systole (LVPWs, B); left ventricular internal diameter in diastole (LVIDd, C) and systole (LVIDs, D); Fractional area change (FAC, E), ejection fraction (EF, F) and fractional shortening (FS, G). Values are normalized to baseline. *$p<0.05$. (H) Histological and immunohistochemical stains 4 weeks post treatment. Representative images for hematoxylin & eosin (H&E), α-smooth muscle actin (α-SMA) and GATA4. Scale bars: 10 μm, 50 μm and 10 μm, respectively.

Figure 5: *In vivo* efficacy studies – chronic MI model. Wistar rats were induced with MI and treated 4 weeks post MI with Gels A, B, C or PBS as control. Cardiac function was assessed through echocardiography up to 8 (A-C) or 12 (D) weeks post MI. Fractional area change (FAC, A, D), ejection fraction (EF, B, D) and fractional shortening (FS, C, D) were measured. Values are normalized to baseline. (E) Histological and immunohistochemical stains 4 weeks post treatment: Representative images for hematoxylin & eosin (H&E), α-smooth muscle actin (α-SMA) and GATA4. Scale bars: 10 μm, 50 μm and 10 μm, respectively.

Figure 6: Inferior Vena Cava Occlusion Test (IVCOT) hemodynamic parameters. Wistar rats were treated with Gels A, B, C or PBS as control, four weeks post MI and cardiac function was assessed through IVCOT hemodynamic parameters; end systolic pressure volume relationship (ESPVR, A), preload recruitable stork work (PRSW, B), maximal velocity of pressure change –

end diastolic volume relationship (MaxdP/dtEDV,C), and cardiac efficiency (Efficiency, D). *p<0.05.

Figure 7: Dobutamine hemodynamic stress tests (DHST) show gel formulation dependent efficacy. Eight (A-D) or 12 (E-H) weeks post MI, rats' cardiac function was measured under escalating doses of dobutamine. Stroke work (SW, A and E), stroke volume (SV, B and F), cardiac output (CO, C and G), and cardiac index (CI, D and H). Two-way ANOVA category level table is displayed for A-D at a $p<0.001$ significance level.