Hydrogels With Integrin-Binding Angiopoietin-1-Derived Peptide, QHREDGS, for Treatment of Acute Myocardial Infarction

Reis et al: Peptide Modified Hydrogel for Treatment of MI

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DOI: 10.1161/CIRCHEARTFAILURE.114.001881

Journal Subject Codes: Basic science research:[130] Animal models of human disease, Heart failure:[11] Other heart failure, Treatment:[27] Other treatment
Abstract

**Background**—Hydrogels are being actively investigated for direct delivery of cells or bioactive molecules to the heart post-myocardial infarction (MI) to prevent cardiac functional loss. We postulate that immobilization of the pro-survival angiopoietin-1-derived peptide, QHREDGS, to a chitosan-collagen hydrogel could produce a clinically translatable thermo-responsive hydrogel to attenuate post-MI cardiac remodeling.

**Methods and Results**—In a rat MI model, QHREDGS-conjugated hydrogel (QHG213H), Control gel, or PBS was injected into the peri-infarct/MI zone. By in vivo tracking and chitosan staining, the hydrogel was demonstrated to remain in situ for 2 weeks and was cleared in ~3 weeks. By echocardiography and pressure-volume analysis, the QHG213H hydrogel significantly improved cardiac function compared to the controls. Scar thickness and scar area fraction were also significantly improved with QHG213H gel injection compared to the controls. There were significantly more cardiomyocytes (CMs), determined by cardiac troponin-T staining, in the MI zone of the QHG213H hydrogel group; and hydrogel injection did not induce a significant inflammatory response as assessed by PCR and an inflammatory cytokine assay. The interaction of CMs and cardiac fibroblasts with QHREDGS was found to be mediated by β1-integrins.

**Conclusions**—We demonstrated for the first time that the QHG213H peptide modified hydrogel can be injected in the beating heart where it remains localized for a clinically effective period. Moreover, the QHG213H hydrogel induced significant cardiac functional and morphological improvements post-MI relative to the controls.

**Key Words:** hydrogel, collagen, chitosan, myocardial infarction
Cardiomyocyte (CM) death, myocardial remodeling, and scar tissue formation following myocardial infarction (MI) results in severe cardiac dysfunction and serious health problems. If not treated, and often with treatment, heart failure progresses. Direct transplantation of various cell types and/or bioactive molecules has shown promise, however both strategies have been hampered by low injection site retention and even lower long-term survival of the cells/bioactive molecules. To mitigate the problems these treatments are combined with hydrogels to confine the cells/bioactive molecules to the site of injection and promote their long-term survival therein.

The acute phase post-MI might be the most appropriate time to utilize hydrogel-based therapy because therein hydrogel injections can prevent cardiac remodeling, deliver cells to replace the damaged tissue, and/or recruit endogenous stem cells. Chitosan and collagen are natural, biodegradable, biocompatible polymers that have been explored for their potential use in the treatment of cardiac dysfunction. Typically, collagen and chitosan, alone or in combination, are cross-linked using exogenous, sometimes toxic, chemical cross-linkers to improve the hydrogel mechanical properties. However, we have previously shown that chitosan-collagen composites gel due to ionic interactions at physiological temperature and pH to form mechanically stable hydrogels that are appropriate for in vivo application. Furthermore, the collagen-chitosan interaction within the gels resembles the collagen-glycosaminoglycan interaction found in vivo in the extracellular matrix (ECM). Thus, chitosan-collagen may mediate physiological cell-matrix interactions.

The functional success of hydrogel-based cardiac and cell therapies can be improved by modifying biomaterials with bioactive molecules because bioactive molecules (cytokines, growth factors, etc.) have the potential to increase transplanted cell survival, reduce resident cell apoptosis, recruit desired regenerative cells, and promote stem/progenitor cell differentiation.
One such bioactive molecule is the growth factor angiopoietin-1 (Ang1). In endothelial cells, Ang1 binds the Tie2 receptor\textsuperscript{16–18} but in cells such as neonatal rat cardiomyocytes (NCMs) that lack the Tie2 receptor, Ang1 binds to integrins\textsuperscript{19} and activates pro-survival pathways\textsuperscript{20}. We identified the short sequence QHREDGS as the integrin-binding motif of Ang1, and the QHREDGS peptide was found to support CM attachment and survival similar to full-length Ang1\textsuperscript{5,21}. It is therefore possible that the QHREDGS peptide could retain/restore cardiac contractile function post-MI by promoting CM survival. Importantly, the QHREDGS peptide is water-soluble, stable, fully-synthetic with a precisely defined composition, and does not require a specific orientation to function.

We therefore incorporated the pro-survival peptide QHREDGS into our chitosan-collagen hydrogel to design a novel hydrogel-based cardiac regenerative therapy. We used \~85\% deacetylated, chitosan-glutamate salt (UPG213), in the form of a linear polysaccharide. We covalently immobilize the QHREDGS peptide to this chitosan backbone using EDC/S-NHS chemistry. EDC is a zero-length cross-linker; therefore no other chemical moieties aside from the QHREDGS peptide are added to chitosan. The hydrogel is made by mixing collagen and the QHREDGS-chitosan without the addition of any exogenous chemical cross-linkers to facilitate gelation and without formation of permanent cross-links between chitosan and collagen.

The peptide was immobilized onto chitosan by covalent cross-linking because as an integrin-binding ligand, QHREDGS does not need to be internalized to function\textsuperscript{12}, and immobilization has been demonstrated, in previous studies, to both reduce the amount of bioactive molecules required and prolong signaling by stabilizing the receptor/ligand complex\textsuperscript{22–26}. In our previous study using the QHREDGS-modified chitosan-collagen hydrogel, we demonstrated increased CM survival in \textit{in vitro} culture and negligible hydrogel degradation therein, as well as \textit{in vivo}
biocompatibility in a sub-cutaneous model. This indicates that peptide release, which depends upon hydrogel degradation, was not required for improved CM survival.

Based on these findings, we sought to investigate the effect of our QHREDGS-modified chitosan-collagen hydrogel in vivo post-MI. We examined the in vivo biodistribution, localization, and lifespan of the hydrogel in the heart, and its effect on cardiac remodeling in a rat acute MI model at 6 weeks, when pathological remodeling is considered to be complete.

**Methods**

All animal experimental procedures were approved by the Animal Care Committee of the University Health Network and the University of Toronto Committee on Animal Care, according to the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care.

See Supplementary Material for additional methods.

**Peptide-modified chitosan-collagen hydrogel (QHG213H)**

QHREDGS peptide (Biomatik) was conjugated to chitosan (UP-G213, Novamatrix) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemistry as described previously. Briefly, 2.5mg/mL 1:1 (mass:mass) chitosan-collagen hydrogel was prepared to produce Control (no peptide) or QHG213H hydrogel (651±8nmol peptide/mL gel) and kept on ice for up to 3h prior to use.
In vivo studies

Animal model

Lewis rats (200–250g) were obtained from Charles River Laboratories (Saint-Constant, QC, Canada), and MI was generated under general anesthesia by occluding the left anterior descending (LAD) coronary artery as previously described9,30–32. Three weeks post-injection, rats were assessed by echocardiography and only those exhibiting 20-40% fractional shortening were included in the study.

Experimental groups and injection timeline

Syringes with PBS (MI Only), Control gel (no peptide), or QHG213H (peptide-modified) hydrogel were prepared prior to surgeries as previously described12. Treatments were randomized among the animals and the surgeon was blinded to the treatment given. Injections were performed using a 28-guage needle (BD Biosciences) inserted into the peri-infarct left ventricle (LV) wall and directed towards the developing scar (MI zone). A total of 50μL was injected into 3 locations immediately following LAD ligation, prior to closure of the thorax.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0 and GraphPad Prism 5.0. Differences between experimental groups were analyzed using one- or two-way ANOVA with Bonferroni post-hoc tests unless otherwise specified. P<0.05 was considered significant for all statistical tests. Results were plotted with GraphPad Prism 5.0.
Results

*In vivo hydrogel degradation*

DyLight800-conjugated hydrogel was injected into rat LVs. After 1h, animals were imaged and labelled hydrogel was seen to localize in the heart with unconjugated dye and gel washout evident in the chest ([Error! Reference source not found.]A). After 24h, labelled hydrogel was visible in the heart but no longer in the chest. At 3, 5, 7, and 14 days post-injection, labelled hydrogel was visible in the heart but the extent of staining decreased with time. By day-14, labelled hydrogel was only visible with increased exposure. To confirm heart localization and to quantify the amount of gel remaining, hearts were excised at 1h and 14 days and fluorescence intensity was measured. By day-14, ~40% of the gel present at 1h remained in the heart ([Error! Reference source not found.]B). Heart sections were also excised at 3 weeks and stained for chitosan, wherein ~20% of animal hearts stained positive ([Error! Reference source not found.]A).

**Functional Data**

All animals had similar echocardiograms prior to surgery. LAD ligation caused significant and progressive ventricular dysfunction, decreased fractional shortening, and increased LV internal diameter at systole (LVIDs) and diastole (LVIDd) at 3 and at 6 weeks post-MI ([Error! Reference source not found.]A-C). Injection of the Control gel significantly increased the cardiac shortening fraction and the LVIDs compared to the MI Only group at 3 and 6 weeks, with additional improvements in these parameters with QHG213H gel injection ([Error! Reference source not found.]A-B). Most of the decrease in fractional shortening for the MI Only and Control gel groups occurred in the first three weeks after MI, as there was no significant
reduction in fractional shortening between 3 and 6 weeks for either group, while the decrease was lesser and more gradual with QHG213H gel injection (Error! Reference source not found.A, D). The LVIDd was not markedly affected by either treatment (Error! Reference source not found.C).

At 6 weeks post-MI, load-dependent measurements of ejection fraction, dP/dt Max and dP/dt Min were all significantly improved in the QHG213H gel group relative to either the Control gel or the MI Only group (Error! Reference source not found.A-C). Control gel injection significantly improved these parameters relative to the MI Only group (Error! Reference source not found.A-C). The end systolic LV volume was lowest in the QHG213H group, followed by the Control gel group, which was significantly lower than the MI Only group (Error! Reference source not found.D). No difference was observed in the end-diastolic LV volumes between groups (Error! Reference source not found.E).

Load-independent measurements of end systolic pressure-volume relationship (ESPVR, Error! Reference source not found.A) and preload recruitable stroke work (PRSW, Error! Reference source not found.B) were also significantly improved with hydrogel injection relative to the MI Only group, and addition of the peptide further improved these parameters (Error! Reference source not found.A-B).

Gross morphology and histology

Untreated MI Only hearts appeared larger than Control and QHG213H gel injected hearts, and the MI Only cross-sections showed the most remodeling (Error! Reference source not found.A). The largest scar fractional area and the smallest scar thickness were in the MI Only group. The Control gel group had significantly smaller scar areas and thicker scars than the MI
Only group, parameters that were significantly improved in the QHG213H gel group (*Error! Reference source not found.*B-C).

Differences in scar thickness and area were also evident by Masson’s trichrome staining, which showed more healthy (red) tissue in the MI zone of the QHG213H group relative to the controls (Supplementary Figure 1A). Heart sections were stained for SMA (smooth muscle cell marker of mature vasculature), CD31 and FVIII (endothelial cell marker for all vasculature, including immature neovasculature) (Supplementary Figure 1B-D). The mean vessel density and diameter of all vasculature (FVIII+, Supplementary Figure 2 top panel) and mature vasculature (SMA+, Supplementary Figure 2 bottom panel) were quantified in the MI and border zones. While no difference in vascular metrics was seen in the MI zone or in the border zone FVIII+ vessels, there were significantly larger border zone SMA+ vessels in the QHG213H gel group compared to the controls.

Cardiomyocyte survival mechanism

TUNEL and cardiac troponin-T (cTnT) staining was used to determine if the functional and morphological improvements seen with Control and/or QHG213H gel injection could be attributed to improved CM survival. Fewer TUNEL+ (apoptotic) cells were visible within the border zone of the Control and QHG213H gel groups compared to the MI Only group (*Error! Reference source not found.*A-B). In the MI zone, there was no difference in the apoptotic cell fraction among the treatment groups but the QHG213H gel group had a higher proportion of cTnT+ cells (CMs) than the controls (*Error! Reference source not found.*C-D).

To gain further insight into the CM survival mechanism, RNA isolated from MI and border zone tissue excised 24h post-injection was subjected to qPCR. We did not observe any
significant differences between the treatment groups, but there were some large fold changes that may warrant further investigation. Specifically, there was a 4.50- and 2.29-fold increase in interleukin (IL)-6 and IL-1β (pro-inflammatory cytokines), respectively in the QHG213H gel compared to the MI Only group. Also, there was a 7.69-fold decrease in caspase-9 (CASP9, pro-apoptotic); a 2.32-fold decrease in PI3K (pro-survival); and a 2.17-fold increase in BCL2 (anti-apoptotic) gene expression in the QHG213H gel compared to the MI Only group (Supplementary Figure 3). For all the aforementioned genes, peptide addition to the gel resulted in a larger fold change than the Control gel with the exception of BCL2, which was down-regulated by Control gel treatment and up-regulated by QHG213H gel treatment (Supplementary Figure 3). We also performed an inflammatory cytokine array on the border zone tissues extracted at 24h but did not detect any significant differences in protein expression, indicating a lack of significant inflammatory reaction (Supplementary Figure 4).

Western blot analysis was performed to determine if integrin-linked kinase (ILK) and MAPK were involved in the CM survival mechanism as both proteins have been reported to be up-regulated by QHREDGS. In the border zone, ILK protein levels and MAPK phosphorylation did not differ between groups but there was a significant increase in MAPK protein expression in the QHG213H gel group relative to the controls (Supplementary Figure 5).

To identify the receptor mediating the CM- and cardiac fibroblast-QHREDGS interaction, major cell types in the native myocardium, polyethylene glycol (PEG) hydrogels were generated with and without immobilized QHREDGS peptide. PEG was used because it is non-fouling and non-adhesive in short-term culture. The peptide concentration in the PEG hydrogel was 11.7±4.5mg/mL (9.7±3.7nM). The addition of QHREDGS to PEG significantly increased the number of adherent cells relative to PEG alone (Error! Reference source not found.A&B).
Pre-incubation of either cell type with an anti-\(\beta_1\)-integrin antibody reduced adhesion to the QHREDGS-PEG hydrogel to that of PEG alone.

**Discussion**

Hydrogel degradation is most often characterized *in vitro* or by histological staining of excised *in vivo* samples\(^ {36,37}\) because tracking hydrogels in the heart is extremely difficult due to the dynamic nature of the beating heart, the depth of the heart within the animal, and the small volume of material injected therein (~20-200\(\mu\)L\(^ {36,38}\)). The near-infrared dye DyLight800 and the Kodak In-Vivo FX Pro Imaging System were used in this study to provide online information regarding hydrogel localization and lifespan. Near-infrared dyes permit deep tissue imaging without affecting the surrounding tissue; a potential advantage in comparison to the long-term use of radiolabels or the use of contrast agents (e.g. iron oxides or gadolinium) which might be taken up by macrophages, skewing the degradation profiles, although PET and MRI imaging have much higher resolution\(^ {38}\).

By imaging the injected labelled hydrogel over 14 days, we confirmed localization and retention of the hydrogel within the heart (Figure 1A). We were unable to perform quantification because the 2D imaging system could not provide volumetric information for the injected material and images were distorted by heart movement over the 40s imaging period. Therefore, we quantified degradation from excised tissue samples using the assumptions: (i) the bulk of unbound dye was removed from the gel by 1h post-injection and (ii) the gel present at 1h post-injection represented the total amount of successfully localized gel. We determined that ~60% of
the gel was degraded/removed from the heart by day-14 (Error! Reference source not found.).

Notably, there was no observable fluorescent signal in the processing organs (e.g. kidneys or liver) 24h post-injection. The collagen and chitosan-Dylight800 that leaked into the chest cavity prior to gelation (Error! Reference source not found., 1st panel) was cleared in 24h (Error! Reference source not found., 2nd panel). These results are consistent with reports that polysaccharides (dextrans and pollulans of 5-850kDa) are cleared in under 24h39. Even the largest sugars had a tissue clearance rate of 10μL/h and an excretion rate of 100μL/h, which suggests that a polysaccharide like chitosan (150-600kDa) would be cleared in hours, thus further motivating its incorporation into a stable hydrogel. Additionally, FITC-labelled chitosan (∼100kDa) injected directly into murine peritoneum was found to rapidly distribute to the urine and kidneys40. Within 1h, ∼25% of the injected chitosan was detected in the urine40. By 14h, chitosan was completely absorbed from the peritoneal cavity, >90% of the injected chitosan was detected in the urine and <6% remained in the kidneys40.

Upon gelation, the hydrogel would have undergone degradation then clearance. Due to the absence of exogenous crosslinking, hydrogel degradation and chitosan release are depended upon the digestion of the collagen fibers holding the hydrogel together. The degraded collagen and chitosan would then be quickly cleared and expelled from the body.

We injected into the peri-infarct and MI-border region to prevent the damaged area from expanding. In rat and dog studies, CM apoptosis was shown to continue through the first 4 months post-MI, which correlated with deterioration of pump function34,41. We performed TUNEL staining at 6 weeks to determine if there was expansion of the scar and border zone, which would induce apoptosis in the expanding region. Injection of either the Control or
QHG213H gel attenuated apoptosis in the border zone (Error! Reference source not found.). We observed no difference in the MI zone (scar) with the treatments, however by 6 weeks the scar tissue was fully formed and non-apoptotic.

Importantly, the amount of QHG213H hydrogel that localized in the heart and its lifespan therein was sufficient to improve cardiac functional outcomes relative to the controls at 6 weeks post-MI (Error! Reference source not found., Error! Reference source not found., & Error! Reference source not found.). Specifically, QHG213H gel treatment resulted in a 62% improvement in ejection fraction and a 35% improvement in fractional shortening from the MI Only group (Error! Reference source not found. & Error! Reference source not found.), which translates into an improvement in ejection fraction and fractional shortening from severe to mild impairment, on a normal–mild–moderate–severe scale of impairment42. At 6 weeks post-MI, we observed a significant decrease in the systolic dimension and volumes but did not find any difference in the diastolic parameters. This may be because while systolic function decreases significantly due to CM necrosis post-MI, a longer time frame (e.g. 12 weeks) may be required to observe changes in diastolic parameters due to matrix remodeling and scar expansion.

One explanation for the functional results is that the QHG213H hydrogel stabilized the infarct wall and increased its thickness thus altering the surrounding tissue properties and reducing wall stress. This is unlikely because the storage modulus of the Control and QHG213H gels were measured at ~55Pa and ~45Pa respectively, well below the stiffness of native rat myocardium (1 to 140kPa, diastole to systole)12,43. Also, there was no difference in end LV diastolic volume between groups, which would be expected if the hydrogel were aiding LV mechanics and altering the geometry (Error! Reference source not found.).
Alternatively, the QHG213H hydrogel may act at the cellular level to promote survival. We investigated the number of CMs and apoptotic cells in the various areas of the infarct at 6 weeks. There was significantly more MI zone CMs in the QHG213H gel group compared to controls. There was no difference in apoptotic cells in this same region. It is possible that the increased CM numbers in the QHG213H injected group were due to the increased size of the mature vessels in the QHG213H gel group border zone (Supplementary Figure 2). By delivering more nutrients and oxygen to the infarcted tissue, these vessels may have promoted cell survival. Interestingly, apoptosis was significantly reduced in the Control and QHG213H gel group border zone compared to MI Only. The chitosan-collagen gel may therefore have a long-term pro-survival effect on the progressing front of apoptosis post-MI.

We observed no difference in total vascularization (neo- and mature) but a significant increase in large (SMA+) vessels within the border zone with QHG213H gel treatment. This may be because Ang1 angiogenic effects are mediated through the receptor Tie2, while the peptide QHREDGS is the Ang1 integrin-binding site, and may not elicit identical angiogenic responses. Other Ang1-derived peptides (e.g. Vasculotide) have been demonstrated to bind Tie2 and are angiogenic. Moreover, the Ang1-CM interaction is integrin-dependent and Tie2-independent; while QHREDGS has been demonstrated to interact with integrins on endothelial cells ($\alpha_5\beta_1$ and $\alpha_6\beta_3$) and induced pluripotent stem cells ($\beta_1$-type) to promote cell adhesion and survival. Thus, the QHG213H gel treatment may have promoted the survival of existing vessels post-MI.

We also investigated the acute phase (24h) post-MI wherein critical apoptotic and immune/inflammatory responses predominate. Both our PCR (Supplementary Figure 3) and
inflammatory cytokine array data (Supplementary Figure 4) indicated no significant difference in the cytokines measured. This suggests that the hydrogel treatments did not elicit an inflammatory response that differs significantly from PBS injections post-MI within the first 24 hr after an MI.

We have recently shown that the integrin subunits α5, β3, and β1 and the downstream effectors ILK and MAPK are involved in QHREDGS-mediated pro-survival pathways in stem cells and endothelial cells33,45. While we did not detect up-regulation of these genes at 24h (Supplementary Figure 3) nor up-regulation of ILK protein or MAPK phosphorylation between groups, we did observe a significant increase in MAPK protein expression in the QHG213H gel group compared to the controls (Supplementary Figure 5). Timing of the assessment is critical as phosphorylation and translation can occur within minutes to hours, whereas transcription occurs within hours to days. Once again, an extensive and individualized time course for each event would be required to fully delineate the mechanism by which the QHG213H gel promotes CM survival post-MI.

By conjugating QHREDGS to a PEG hydrogel we found that both the CMs and cardiac fibroblasts interact with QHREDGS through β1-integrins (Error! Reference source not found.). We have previously demonstrated that QHREDGS interacts with endothelial cells through α5β1 and α5β3 integrins to promote cell survival45. It has also been reported that Ang1-mediated effects on monocyte adhesion and chemotaxis are both Tie2- and integrin-independent46. Hence, QHREDGS may bind and promote the survival of CMs, cardiac fibroblasts and endothelial cells through integrin-binding but may not affect monocyte/macrophage function, which would account for the absence of a significant inflammatory response.

Conclusions
This is the first study to evaluate the effect of the Ang1-derived peptide QHREDGS on remodelling post-MI. Injection of the QHG213H hydrogel into the LV of rats with acute MI showed significant improvements in cardiac morphology and functionality at 6 weeks relative to the controls. Scar thickness improved by 53%, fractional scar area decreased by 34%, fractional shortening improved by 35% and ejection fraction improved by 62% compared to the MI Only group. The injected hydrogel was demonstrated to remain in situ for 14 days and was ~80% cleared by 3 weeks. Based on our results, cardiac cells bind via β1-integrins to the QHREDGS peptide. The novelty of our work lies in the expanded insight into the integrin-mediated effects of the Ang1-derived peptide, QHREDGS, on cardiac cells post-MI in vivo. The developed hydrogel - collagen:chitosan mixture with covalently-immobilized QHREDGS - could form the basis of a new cardiac therapy that is fully chemically-defined, based on biocompatible and biodegradable molecules.

Acknowledgments

We thank Dr. Peter Liu for helpful discussions.

Sources of Funding

This work is funded by the Heart and Stoke Foundation GIA T6946, the Canadian Institutes of Health Research (CIHR) Operating Grant (MOP-126027), NSERC-CIHR Collaborative Health Research, Grant (CHRPJ 385981-10), NSERC Discovery Grant (RGPIN 326982-10), NSERC Discovery Accelerator Supplement (RGPAS 396125-10) and National Institutes of Health grant 2R01 HL076485. L.R. is funded by NSERC PGS-D Fellowship.
Disclosures

None.

References


Figure Legends

Error! Reference source not found. DyLight800-labelled gel was detected in live animal hearts (A) at 1 hour (hr) up to 14 days (d) post-injection with increased exposure (↑exp.). Insets. Enlarged and enhanced view of the heart region indicated by the white square. At 3 weeks (Wk), heart sections were stained for chitosan (purple dots). Whole heart fluorescence was quantified (B) from homogenized hearts excised at 1hr (N=3) and 14d (N=4) post-injection.

Error! Reference source not found. (A) QHG213H gel injection significantly enhanced the %fractional shortening (%FS) at 21 days (3 weeks) and 42 days (6 weeks) post-MI compared to the Control gel and MI Only groups. The Control gel injection induced a significant improvement in %FS relative to MI Only group. (B) Injection of gel with or without peptide significantly reduced LV systolic dimension (LVIDs) at 3 and 6 weeks relative to MI Only. The QHG213H gel significantly improved the LVIDs relative to the Control gel. (C) No significant difference in LV diastolic dimension (LVIDd) was evident between groups. (D) Repeated measures ANOVA results for factor “days after MI” for data from (A-C). Data analyzed by two-way repeated measures ANOVA and expressed as Mean±SD, N=7/group.*P<0.01, **P<0.001 vs MI Only; #P<0.01 vs Control gel.

Error! Reference source not found. Steady-state pressure-volume analysis was used for load-dependent cardiac functional analysis. (A-C) Injection of the Control gel significantly improved cardiac function compared to MI Only, and injection of the QHG213H gel further improved function. (D) End systolic LV volume was significantly lower in the QHG213H gel group vs either control, and in the Control gel group vs MI Only group. (E) The end LV diastolic volume was unchanged between groups. Data expressed as Mean±SD, N=7/group, *P<0.05, **P<0.01 vs MI Only; #P<0.05 vs Control gel.

Error! Reference source not found. Occlusion pressure-volume analysis was used for load-independent cardiac functional analysis. Significant differences were seen in both (A) end systolic pressure-volume relationship and (B) preload recruitable stroke work between the QHG213H gel group and either control, as well as between the Control gel group and the MI Only group. Data expressed as Mean±SD, N=7/group, *P<0.05.

Error! Reference source not found. (A) Representative whole heart and heart sections (from just below suture to apex of heart, 5 sections per heart) illustrate differences in gross morphology between groups (scale= 1mm). Significant differences in (B) fractional MI scar coverage and (C) MI scar thickness between the QHG213H gel and the Control gel and MI Only groups, as determined from (A) gross section images. Data expressed as Mean±SD, N=7/group, *P<0.05, **P<0.01 vs MI Only; #P<0.05 vs Control gel.
Error! Reference source not found.
Heart sections were stained for TUNEL (red), cTnT (green), and DAPI nuclear stain (blue). (A) Representative images showed fewer TUNEL+ cells in the Control gel and QHG213H gel groups than the MI Only group in the border zone (Scale bars=100μm). (B) Quantification of the fraction of TUNEL+ cells (# of TUNEL+ cells/# of DAPI+ cells) in the border zone. The Control and QHG213H gel groups had a significantly smaller TUNEL+ cell fraction relative to the MI Only group. (C) There was no difference in the TUNEL+ cell fraction between groups in the MI zone. There was a significant increase in cTnT expression in the QHG213H group relative to either control. Data expressed as Mean±SD, * P<0.05, ** P<0.01, N=7/group.

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Rat NCMs (A) or cardiac fibroblasts (B) were seeded onto PEG only (no peptide) or PEG-QHREDGS disks with or without pre-incubation with an anti-β1-integrin (CD29) antibody. After 50min, the attached cells were counted. The data is normalized to the PEG only gels. Both NCMs and cardiac fibroblasts have significantly more attached cells to PEG-QHREDGS surfaces over PEG alone, but the difference is abolished when pre-incubated with anti-β1-integrin antibody. Data expressed as Mean±SE, ** P<0.01, N=6/group.
A. Fractional shortening (%)

- MI Only (PBS)
- Control
- QHG213H

Days after injection: 0, 21, 42

B. LVIDs (cm)

- MI Only (PBS)
- Control
- QHG213H

Days after injection: 0, 21, 42

C. LVIDd (cm)

- MI Only (PBS)
- Control
- QHG213H

Days after injection: 0, 10, 20, 30, 40

D. Repeated Measures ANOVA (sig.)

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% FS

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LVIDs

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LVIDd

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<th>0 v 42 days</th>
<th>21 v 42 days</th>
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<td>&lt;0.001</td>
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Hydrogels With Integrin-Binding Angiopoietin-1-Derived Peptide, QHREDGS, for Treatment of Acute Myocardial Infarction

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_Circ Heart Fail._ published online January 28, 2015;
_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

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http://circheartfailure.ahajournals.org/content/early/2015/01/28/CIRCHEARTFAILURE.114.001881

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1. Supplementary Material

Supplementary Methods:

1.1. Peptide-modified polyethylene glycol (PEG-QHREDGS)

QHREDGS peptide was conjugated to polyethylene glycol (PEG) as previously described \(^1\), with some modification. Briefly, 8.0mg acrylate-PEG-NHS was incubated with or without 29mM (24mg/mL) QHREDGS solution in a final volume of 200µL. To the lyophilized powder, 80mg polyethylene glycol diacrylate and 0.8mg 2-Hydroxy-2-methyl-propiophenone was added; 150µL of this solution was added to a circular silicon mold (11mm diameter, 1mm thick) placed atop a glass microscope slide and a polyvinyl coverslip was placed on top of the mold. After 1h at 4°C the cover-slip was gently peeled off the hydrogel, the mold was removed, the hydrogel was sterilized, then placed in sterile PBS for 1h at 37°C.

NCMs or cardiac fibroblasts, isolated as previously described \(^2,3\), were washed and resuspended in equal volumes of PBS or 50µg/mL anti-β\(_1\)-integrin antibody (α-CD29; BD Biosciences, Mississauga, ON) and incubated at 37°C for 20min. The NCMs or cardiac fibroblasts were spun for 5min at 200g, resuspended in CM media, seeded onto the PEG-hydrogels (22,000 cells per hydrogel) in a volume of 10µL and incubated at 37°C for 50min. After 50min, the hydrogels were washed, fixed, stained and imaged as previously described \(^1\).

1.2. In vivo studies

1.2.1. Assessment of cardiac function (6 Week time point)

N=7 animals per group were used for a long term 6 week study. Cardiac function was evaluated by echocardiography (Sequoia C256 System, Siemens Medical; 15 MHz linear array transducer) before MI (pre-ligation baseline), 3 and 6 weeks post-MI. M-mode images were
obtained in the parasternal short-axis view at the level of the papillary muscles. The measurements were performed by a single, blinded examiner. LV internal diastolic dimension (LVIDd) and internal systolic dimension (LVIDs) were measured. Three consecutive cardiac cycles were recorded and averaged. Percent fractional shortening (%FS) was calculated as follows: 

\[
\%FS = \frac{(LVIDd - LVIDs)}{LVIDd} \times 100
\]

The 6 week time point was selected as it is assumed the heart has completed remodeling by this time. PBS, Control gel (no peptide), or QHG213H (peptide-modified) hydrogel was injected into 3 locations in the LV wall (50µL total) surrounding the infarct using a 28-gauge needle. End (6 weeks after treatment) cardiac function was assessed with a pressure-volume catheter, as described. Briefly, the rats were anesthetized and intubated with mixed oxygen and room air by a rodent ventilator (Harvard apparatus, Canada). A pressure-volume catheter-tipped pressure transducer (2F, Millar Instruments, USA) was inserted into LV cavity via right carotid artery. Volumes were measured by conductance, and end-systolic and -diastolic values were recorded. Ejection fraction, dP/dt Max, and dP/dt Min were measured, and end systolic pressure-volume relationship (ESPVR) and preload recruitable stroke work (PRSW) were calculated.

After pressure-volume analysis, hearts were perfusion fixed with 10% formaldehyde, excised, and sectioned into five 2-mm thick slices as previously described.

1.2.2. 24h time point

N=6 animals per group were used in a short 24h study to elucidate the mechanism by which the injected hydrogel preserved cardiac function. Surgery and injections were performed in the same manner as described above and animals were monitored overnight. The animals were anaesthetized 24h after injection with isoflurane, intubated, ventilated, and maintained with 2% isoflurane. The heart was accessed in the same manner as performed for LAD surgery and
arrested using 10% KCl injected directly into the LV. Hearts were then immediately excised and
the right ventricle and atria were removed. The LV was then sectioned into a MI region, border
region, and remote region. These sections were placed in separate cryo-tubes and snap frozen in
liquid N$_2$. Frozen tissues were subsequently ground using standard mortar and pestle with liquid
N$_2$ and kept at -80°C prior to RNA extraction using TRIZOL reagent according to
manufacturer’s instructions (Life Technologies).

1.2.3. Hydrogel lifespan

To assess the in vivo lifespan of our hydrogel, chitosan was conjugated with the near-infrared
dye DyLight800 (Thermo Scientific, Waltham, MA) and injected into the heart of live animals.
Briefly, 1 vial of DyLight800 label (suitable for 1mg of protein) was dissolved in 57.7μL PBS
and mixed immediately with 92.3μL of chitosan at 20mg/mL in 0.9% normal saline (final
reaction volume of 150μL and chitosan concentration of 12.31mg/mL). The mixture was
protected from light, placed on a microtube shaker at 650rpm for 1h, and then stored at 4°C until
use. Upon use, the DyLight800-labelled chitosan was used to make hydrogels as per the standard
protocol $^6$, kept on ice, and then 50μL of the labelled gel was injected directly into 3 locations of
the healthy LV wall of N=7 Lewis rats. Rats (anesthetized under isoflurane) were then imaged
using a Kodak In-Vivo FX Pro Imager (Kodak Molecular Imaging Systems), with a 5sec
exposure X-ray image overlaid with a 40sec exposure fluorescent image (760nm excitation,
830nm emission). Full-sized images were processed to illustrate fluorescent decrease over time
by applying identical threshold and gamma adjustment to all images using Kodak MI 4.0
software (Kodak Molecular Imaging Systems). Inset images were processed to highlight
fluorescent localization by applying the optimal brightness/contrast and threshold to each
individual image using ImageJ (NIH). N=3 rats were sacrificed 1h after injection, and N=4 were
monitored up to 14 days post injection. Excised hearts were stored at -80°C until use, at which time they were homogenized and fluorescently imaged to quantify degradation.

1.3. Immunohistochemistry

Paraffin embedding and sectioning was done on samples prepared as described above by the Pathology Research Program (PRP) at the University Health Network. Additionally, they performed immunohistochemical staining for hematoxylin and eosin (H&E), Mason’s trichrome, smooth muscle actin (SMA), Factor VIII (FVIII), and CD31. Sections were also stained for the presence of chitosan using a published protocol based on Cibacron Brilliant Red-3BA (CBR-3BA, Sigma Aldrich) and Weigert’s Iron Hematoxylin .

Cardiac troponin-T (cTnT) and TUNEL staining was done on sections deparaffinised and rehydrated with subsequent baths of 100% xylene, 100% EtOH, 95% EtOH, 75% EtOH, and distilled water (3 baths each for 3min per change). Antigen retrieval was through microwaving, immersion in Tris-EDTA buffer for 5min at room temperature (RT), permeabilization with 0.1% Triton-X, 0.1% Sodium Citrate in Tris-buffered saline (TBS) solution (10min, RT), and blocked with Dako Serum Free Protein Block (Dako Canada, Inc., Burlington, ON X0909; 30min, RT). After each step 3x3min TBS washes were performed. Primary antibody (mouse monoclonal cTnT antibody, Thermo Scientific MS-295-P, diluted 1:200) was applied for 2h at RT, followed by 3x3min TBS wash, and then secondary antibody (Alexafluor 594 goat anti-mouse IgG (H + L), Invitrogen) at 1:400 dilution for 1h at RT. TUNEL label and enzyme (Roche Applied Science, Indianapolis, IN, 11767291910) were applied as per manufacturer’s instructions and DAPI (Sigma Aldrich) nuclear stain was applied at a 1:1000 dilution for 10min. Sample were mounted with Dako Fluorescence Mounting Medium (Dako Canada, Inc., S3023), and imaged.

1.4. Quantitative PCR

Supplementary Material 4
Whole heart or heart sections frozen at -80°C were ground using a mortar and pestle and liquid N\textsubscript{2}. TRIZOL Reagent was used for RNA extraction, following the manufacturer’s protocol. cDNA synthesis and qPCR were performed as previously described \textsuperscript{8}. All oligonucleotide sequences used are listed in \textbf{Supplementary Table 1}. Data was standardized between samples to reflect gene expression changes of the progressing scar, quantifying gene expression in the border zone compared to the established MI zone. Fold changes in gene expression between groups were analyzed and genes showing a >2-fold or <0.5-fold change in the QHG213H gel group over the MI Only (PBS) injection group were considered significant.

\textbf{1.5. Western Blotting & Inflammatory Cytokine Array Analysis}

Whole heart or heart sections frozen at -80°C were ground using a mortar and pestle and liquid N\textsubscript{2}. Protein was isolated using Pro-Prep Protein Extraction Solution (iNtRON Biotechnology, Inc., Seongnam, KR) according to the manufacturer’s instructions. Western blots were performed as previously described \textsuperscript{1}.

Protein isolated from border zone tissues and used for Western blots were also analyzed using a rat inflammatory cytokine array kit (RayBio, AAR-CYT-1-8) as per the manufacturer’s protocol. Assayed array membranes were exposed as described for western blots, and data was collected by performing densiometry using the Dot Blot Analyzer plug-in in ImageJ.

\textbf{1.6. Image analysis techniques}

Morphometric analysis was performed as described previously \textsuperscript{4,5}. TUNEL/DAPI cell counts were made using a macro developed in Olympus CellSens Dimension software and tested on control images. TUNEL/cTnT MI border zone quantification was based on 2 independent images on either side of the MI zone in each animal, and MI zone quantification performed by averaging at least 4 independent images within the scar. All counts and normalizations were performed for
each image and averaged to get a single value per sample. Quantifying differences in vasculature in the MI zone and border zone of hearts excised 3 and 6 weeks post-MI was done using SMA and FVIII stained sections as described previously\(^9\).

Automation of the image quantification process eliminates human errors and bias between images, ensuring consistent quantification among all images and more accurate results.
Supplementary References


Supplementary Figure Captions:

Supplementary Figure 1: Histological staining 6 weeks post-MI

(A) Mason’s trichrome, (B) smooth muscle actin (SMA), (C) CD31, and (D) Factor VIII (FVIII) stained sections of hearts excised 6 weeks post-MI. Black outlined sections in (A) are enlarged in (B-D). A large collagenous scar is seen in all groups (A), but there appears to be more SMA, CD31 and FVIII staining in the Control gel and QHG213H gel groups compared to MI Only (PBS) group, suggesting increased vascularization (B-D). Scale bars = 1 mm (A), 50 µm (B-D), N=7/group.

Supplementary Figure 2: Vascularization at 6 weeks post-MI

Vascularization within the MI and border zones was quantified at 6 weeks from heart cross sections. Vessel density and diameters were quantified from SMA stained sections to assess mature vasculature, and from FVIII stained sections for neovascularization. The QHG213H gel group had significantly larger mature SMA+ vessels in the border zone. Data expressed as Mean±SD; N=7/group.

Supplementary Figure 3: qPCR of MI and border zone tissues from hearts excised 24h post-MI

Hearts subjected to MI and immediate treatment were excised 24h later, sectioned into MI, border, and remote regions, snap frozen then ground and used for qPCR. Overall, 27 genes conventionally associated with apoptosis, necroptosis (Necrop), survival, anti- and pro-inflammatory responses, as well as cardiac specific genes were assessed. Data was standardized in samples to reflect changes in gene expression in the progressing border zone to the established MI zone. No significance was determined between groups. Data expressed as Mean±SD, N=5/group.

Supplementary Figure 4: Cytokine expression in border zone tissues from hearts excised 24h post-MI

Inflammatory cytokine expression analysis within border zone tissues revealed no significant differences in any of the 19 cytokines analyzed (select data shown). Data expressed as Mean±SD, N=5/group.

Supplementary Figure 5: Western blot analysis of border zone tissue from hearts excised 24h post-MI

Western blot analysis of border zone heart tissues showed no differences in the levels of ILK or phospho-MAPK (PMAPK) protein expression between groups. The MAPK protein expression was significantly increased in the QHG213H gel group compared to the Control gel and MI Only (PBS) groups. Data expressed as Mean±SD, * P<0.05, N=5/group.
## Supplementary Tables:

### Supplementary Table 1: Genes and primers used in qPCR

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Supplementary Figures:

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Supplementary Figure 3: qPCR of MI and border zone tissues from hearts excised 24h post-MI

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