Timing underpins the benefits associated with injectable collagen biomaterial therapy for the treatment of myocardial infarction

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A R T I C L E   I N F O
Article history:
Received 20 August 2014
Accepted 3 November 2014
Available online 22 November 2014

Keywords:
Collagen
Extracellular matrix
Fibrosis
Heart
Hydrogel

A B S T R A C T
Injectable hydrogel biomaterials are promising therapies to promote repair and regeneration post-myocardial infarction (MI). However, the timing of delivery and the mechanisms through which biomaterial treatments confer their benefits are translational issues that remain to be addressed. We assessed the efficacy of an injectable collagen matrix at 3 different delivery time points post-MI. Infarcted mice received the matrix or control (saline) treatment at 3 h, 1 week or 2 weeks after MI. The earlier treatment delivery better prevented negative ventricular remodeling and long-term deterioration of cardiac function (up to 3 months), whereas waiting longer to administer the matrix (1 and 2 weeks post-MI) reduced the therapeutic effects. Collagen matrix delivery did not stimulate an inflammatory response acutely and favorably modulated inflammation in the myocardium long-term. We found that the matrix interacts with the host tissue to alter the myocardial cytokine profile, promote angiogenesis, and reduce fibrosis and cell death. This work highlights that the timing of delivery can significantly affect the ability of an injectable hydrogel to protect the post-MI environment, which will be an important consideration in the clinical translation of cardiac biomaterial therapy.

1. Introduction
Heart failure is a burgeoning disease and ischemic cardiomyopathies such as myocardial infarction (MI) are a major cause of this complex clinical syndrome [1]. Despite prompt intervention to restore perfusion post-MI, the loss of viable myocardium and adverse ventricular remodeling leads to dilated and functionally incompetent myocardium in many patients [1–4]. The remodeling is at first compensatory, but as it progresses, it results in deformed geometry, increased wall tension and impaired contractility [5]. Today, no therapies exist to reverse ventricular remodeling or heart failure. Cell therapy has yet to demonstrate clinically meaningful regeneration; therefore, alternative or concomitant therapies are being explored [6]. It is plausible that functionally stabilizing the heart by targeting early post-infarction processes may limit or circumvent the need for regenerative therapies in many patients.

Acellular biomaterials, many based on natural extracellular matrix (ECM) components, are emerging as new treatments for MI. These have evolved rapidly into increasingly complex therapies being tested in clinically relevant models of MI and heart failure [7,8]. Several materials have been shown to improve or preserve cardiac function in rodent and swine MI models, as assessed by left ventricular ejection fraction (LVEF), and improved ventricular geometry such as end-diastolic (ED) or end-systolic (ES) diameters [7,8]. Injectable hydrogels are particularly attractive for cardiac applications owing to their ease of use and the possibility of minimally-invasive delivery to the infarcted myocardium. Despite their promise, the optimal timing for delivery and the mechanisms through which biomaterial treatments confer their benefits for the treatment of MI remain unknown.

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http://dx.doi.org/10.1016/j.biomaterials.2014.11.004
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As MI involves distinct necrotic, inflammatory, proliferative and maturation phases, prior delineation of the ideal time of delivery is important to ensure optimal efficacy of the biomaterial therapy [7]. For example, early application should not prevent the acute inflammatory process, since early inflammatory cytokine signaling is required to initiate the healing process [9,10]. If therapy is applied too late, then major tissue damage and fibrosis have occurred, and there may be little myocardium to preserve and/or salvage. Given that a biomaterial’s properties and the components from which it is made can differentially target and regulate cell and tissue functions [11], the ideal timing of delivery is likely to differ for different biomaterial therapies. These issues should be addressed in order to avoid shortcomings similar to those experienced with the clinical translation of stem cell therapies [12,13]. For example, the optimal time to deliver cells post-MI has only recently been investigated in the clinic, such as the TIME and SWISS-AMI trials [14,15]. Clinical translation of biomaterial therapies should, at a minimum, involve evaluation of these parameters in animal MI models prior to moving forward with patient trials. To our knowledge, the only study to consider the impact of time of delivery for a natural material was performed by Landa et al. using an alginate-based injectable hydrogel [16]; however, the study did not include an early time point (7 vs. 60 days post-MI were evaluated), and the mechanisms of action underlying the benefits were not elucidated.

We have previously reported that therapy using an injectable collagen-based matrix could mediate apoptosis, vascularization, and regeneration to confer functional recovery in models of ischemia, necrosis and/or infarction [17–19]. In this study, we sought: 1) to determine whether timing of administration affects the ability of our collagen matrix to preserve or improve cardiac function post-MI; and 2) to examine the mechanisms by which the matrix treatment mediates cardiac repair. We demonstrate that the collagen matrix can mediate multiple repair processes and prevent progressive cardiac decompensation post-MI, and that the magnitude of the therapeutic benefit achieved is dependent on the timing of treatment delivery.

2. Materials and methods

2.1. Study design

2.1.1. Rationale
The objective of the study was to determine the ideal time-point for the delivery of an ECM-based hydrogel biomaterial to improve cardiac function in the infarcted mouse heart. A chronic LAD permanent occlusion MI model was used. Three different delivery time-points were tested: 3 h, 7 d and 14 d post-MI (Fig. 1A). The primary end-point was the effect of treatment on cardiac function (%LVEF) at 4 wk post-treatment. Following this determination, mechanisms underlying the observed therapeutic benefits were investigated through histology, immunohistochemistry and molecular analyses. Further follow-up analysis of the 14d treatment cohort was not pursued due to lack of efficacy.

2.1.2. Randomization & blinding
All surgeries were performed by the same animal technician blinded to treatment allocation. Mice were assigned a code number associated with the study protocol but not treatment. The treatment delivered to each mouse was recorded...
2.2. Experimental MI model and cardiac echocardiography

All procedures were performed with the approval of the University of Ottawa Animal Care Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experimental MI was induced in 7–8-wk old female C57BL/6J mice (Charles River Laboratories) as previously described [17]. Briefly, mice were anaesthetized under 2.5% isoflurane, intubated and kept under mechanical ventilation. A left-sided open thoracotomy was performed and the left anterior descending coronary artery was permanently ligated with a 7-0 silk suture 2 mm below the tip of the left atrium. MI was confirmed by myocardial blanching in the region supplied by the artery. Short acting buprenorphine was administered at least an hour prior to surgery, and long-acting buprenorphine was administered subcutaneously immediately before surgery for peri-operative analgesia. Cardiac function was assessed by echocardiography on long-axis views with a Vevo770 system (VisualSonics) in B mode with the use of a 707B series real-time microvascularization (RMV) scanhead probe.

2.3. Preparation and injection

Preparation of the matrix has been described previously [18]. Briefly, 1M 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 1:1 (molar equivalent) cross-linking mixture of N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC/NHS; 13 mm) was added to 0.375% rat tail collagen type I (w/v, BD Biosciences) with 100 µL of 40% chondroitin sulfate-C (CS-C; w/v) (Wako Chemicals) and kept on ice. The final pH was adjusted to 7.2–7.4 using NaOH. The collagen-based matrices are liquid at the time of injection but upon exposure to physiological temperatures (~37°C) solidify into a gel. Using an ultrasonound guided (long axis view) closed-chest procedure, mice were assigned to randomly receive one of the following treatments (3–4 injections; 50 µL total) into the infarct/border zone: 1) phosphate buffered saline (PBS) or 2) injectable matrix, delivered at 3 h, 7 d or 14 d post-MI (Fig. 1A). The syringe was secured in a micro-maneuverable (VisualSonics), and both the needle and RMV scanhead probe were aligned along the heart long-axis before the injection procedure. The needle was retracted from the ultrasound field-of-view (FOV) with the use of the micromaneuverable until the needle tip was in the desired location within the myocardium. The collagen matrix was then injected into the border and infarct zones of the anterior wall. Mice were observed for 4 wk post-treatment, after which they were sacrificed for histological assessment. Groups of mice in the 3 h cohort were sacrificed at 2 d or 3 months post-treatment to assess the short-term effect of the treatment on neovascularization, inflammation and cell death, and the effect of treatment on long-term functional preservation, respectively.

2.4. Cytokine array and immunohistochemistry

At 4 wk post-treatment, infarct tissue from a subset of mice in the 3 h and 7 d treatment groups (PBS and matrix) was isolated for cytokine array (RayBiotech). The tissue was micro-dissected from freshly harvested hearts and frozen with liquid nitrogen until use. Protein was extracted using the lysis buffer provided with the cytokine array kit. The protein in each sample was quantified using a BCA Kit (Thermo scientific) and appropriately normalized. Fluorescence intensity was assessed and cytokine expression was quantified and normalized using manufacturer supplied software.

For all histology and immunohistochemistry, hearts were collected, perfused with 2–3 mL of saline, snap frozen in OCT and stored at -80°C. Slides were prepared with 8 µm sections at different levels. Masson’s trichrome or hematoxylin-phloxine saffron (HPS) staining was performed to measure relative final scar size by the midline–arc method, as previously described [20]. Unless otherwise specified, all primary antibodies for immunohistochemistry were purchased from Abcam and all Alexa fluor (488, 546) secondary antibodies from Invitrogen. To assess vascularization, tissue sections were stained with antibodies against CD31 (for endothelial cells; Santa Cruz Biotechnology) and aSMA (for vascular smooth muscle cells). Cell death and apoptosis was identified using a TUNEL kit (Roche) and anti-active caspase 3 antibody, respectively, while cell proliferation was assessed with an anti-Ki67 antibody. Additionally, Ly6G was used to stain for neutrophils, CD68 or F4/80 for macrophages, and F4/80 + CD206 or F4/80 + CD16 for M2 and M1 macrophages, respectively. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Imaging was performed with a Zeiss Z1 fluorescence microscope and ZenBlue (2011–12) digital image software. For quantification, 6 random microscopic FOV of the infarct area (except where otherwise indicated) were counted per sample in a blinded fashion.

2.5. Bone marrow-derived macrophage culture

Bone marrow-derived macrophages (BMDMs) were generated from the bone marrow of 7–9 week old female mice tibia, as previously described [21]. Matrix-coated 6-well plates were prepared and cells were maintained in DMEM with 10% FBS, 1% L-glutamine containing M-CSF on standard tissue culture polystyrene (TCP) or collagen matrices for one week. To assess immunomodulatory effects of BMDMs exposed to the matrix, supernatant was collected after one week. Produced cytokines were quantified in culture supernatants using the Mouse Inflammation Array (Raybiotech), following procedures as described above.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software. Data is presented as mean ± standard error (SE). Unless otherwise specified, comparisons of data between multiple groups were performed by a one-way analysis of variance with a post-hoc Tukey test to establish differences between individual groups, while comparisons between two groups were analyzed using a two-tailed student’s t-test. Statistical significance was given for p < 0.05.

3. Results

3.1. Effect of timing of delivery on the efficacy of collagen matrix treatment post-MI

The left anterior descending coronary artery was ligated to induce experimental MI in mice, which were randomized to 1) treatment type and 2) treatment timing. Mice received intramyocardial injections of either the collagen matrix or PBS (control), delivered at 3 h, 7 d or 14 d post-MI (Fig. 1A). MI surgery was performed with 100% survival rate, while treatment delivery was associated with survival of 91%, 86% and 83% for mice treated at 3 h, 7 d and 14 d, respectively, with no differences in mortality between matrix and PBS treated mice at any time-point. Cardiac function (LVEF) was assessed at baseline (pre-treatment) and 4 wk post-treatment.

The efficacy of the collagen matrix in improving cardiac function was dependent on when the biomaterial was delivered after MI. At 4 wk post-treatment, mice that received biomaterial therapy at 3 h post-MI had superior cardiac function (%LVEF) compared to mice with biomaterial treatment given at 7 d and 14 d post-MI, and compared to all PBS-treated mice, regardless of time-point. Biomaterial treatment at 7 d moderately improved %LVEF compared to 7 d PBS treatment, whereas no improvement was seen for mice with 14 d biomaterial treatment (Fig. 1B). No statistically significant differences in %LVEF were observed at 4 weeks post-treatment between the mice receiving PBS at 3 h, 7 d, or 14 d. These results identify that earlier delivery of the collagen matrix offers superior benefits to cardiac function. Since biomaterial therapy at 14 d post-MI was ineffective, this delivery time-point was not further evaluated in this study.

3.2. Vascular density and cell death in the myocardium after matrix treatment

To provide mechanistic insight into the effect of delivery time on the efficacy of biomaterial treatment, we performed a histological comparison of the 3 h and 7 d treatment groups. We first assessed vascular density and apoptosis at 4 wk post-treatment in tissue sections. Biomaterial treatment at either time-point was equivalently effective at supporting greater total arteriole density (Fig. 2A, B); however, the 3 h biomaterial treatment was superior at promoting a higher density of capillaries (CD31+ structures with identifiable lumen) compared to all other groups in the infarcted myocardium (Fig. 2C). Although exact cell identity was not evaluated, overall apoptosis was assessed by quantifying active caspase 3+ cells at 4 wk post-treatment. Only matrix delivery at 3 h post-MI had a long-term effect of reducing the level of apoptosis in the infarcted myocardium compared to PBS (Fig. 2D, E). Since altered cytokine expression has been implicated in myocardial remodeling and decompensation are long-post-MI [122], we assessed whether our matrix influenced the cytokine milieu. Infarct/peri-infarct tissue expression of most of the cytokines assessed were unaffected 4 weeks after 3 h matrix delivery, while 1 wk delivery resulted in...
several significant changes (see Supplemental Fig. 1). Notably, FGF-2 levels were significantly higher in mice receiving matrix treatment at 3 h compared to all other groups (Fig. 2F).

3.3. Effects of matrix treatment on chronic inflammation in the MI heart

A chronic inflammatory state is a major determinant of ventricular remodeling and aggravated cardiac injury post-MI [22]. Therefore, we assessed the level of on-going inflammation in the heart at the time of animal sacrifice (4 wk post-MI) by staining for persisting macrophages (CD68+ cells). Biomaterial therapy led to a reduction in macrophage number in the infarct area, whether it was delivered at 3 h or 1 wk after infarction (Fig. 3A, B). A reduction in TNF-α at 4 wk post-treatment was also observed in MI hearts that received biomaterial treatment at 3 h post-MI (Fig. 3C).

To further understand how the collagen matrix is able to mediate inflammation, we assessed the cytokine secretion profile of macrophages cultured on the biomaterial in vitro. Bone-marrow derived macrophages from mice were seeded onto the biomaterial or standard tissue culture polystyrene for 1 wk and, as shown in Fig. 3D, this resulted in a marked difference in cytokine secretion assessed in the conditioned media. Biomaterial culture resulted in a decrease in the secretion of pro-inflammatory cytokines such as MIP-1α [23], MIP-1γ and CCL5 [24,25]; an increase in the anti-inflammatory IL-4 [26] and angiogenic CXCL5 [27] cytokines; an
increase in TIMP-2; and reductions in the soluble TNF receptors, sTNFR1 and sTNFR2 (Fig. 3D).

### 3.4. Remodeling and cardiac function after early matrix treatment

The earliest time-point of biomaterial delivery (3 h post-MI) conferred superior functional benefit, and thus we sought to further characterize the effects in this treatment group through the assessment of final scar size, and other parameters of cardiac performance and negative remodeling. Biomaterial delivery at 3 h post-MI significantly reduced scar size by approximately 40% compared to PBS controls when assessed at 4 wk post-treatment (Fig. 4A, B). This was consistent with the observed improvement in cardiac performance, as measured by % fractional area change.

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Fig. 3. Matrix therapy reduces chronic inflammation and alters cytokine secretion in macrophages. (A) Representative images of CD68+ macrophages in myocardial tissue sections (scale bar = 20 μm). (B) Quantification of macrophage density in the infarcted myocardium at 4 wk in the 3 h and day 7 treatment groups. *p < 0.05 vs. PBS, **p < 0.01 vs. PBS; n = 3–6. (C) Fluorescence intensity (FU) of TNF-α assessed by cytokine array from infarcted tissue for the 3 h and day 7 treatment groups. *p < 0.05 vs. PBS; n = 5–6. (D) Fold-change in cytokine secretion for BMDMs cultured in vitro on Matrix vs. TCPS. *p < 0.05 and **p < 0.01; n = 3–4. (PBS, phosphate buffered saline; TCPS, tissue-culture polystyrene; TNF-α, tumor necrosis factor-α; BMDM, bone marrow derived macrophage; MIP, macrophage inflammatory protein; CCL5, chemokine (C–C motif) ligand 5; TIMP2, tissue inhibitor of metalloproteinase 2; IL4, interleukin 4; CXCL5, chemokine (C–X–C motif) ligand 5; sTNFR, soluble tumor necrosis factor receptor).
(Fig. 4C), cardiac output (Fig. 4D) and stroke volume (Fig. 4E). Additionally, mice treated with the collagen matrix therapy at 3 h also had reduced end-systolic volume (Fig. 4F), a metric of global cardiac remodeling. Since interstitial fibrosis penetrating into the bordering and remote regions of the heart following MI can lead to dyskinetic contraction and diastolic stiffness, and perpetuate remodeling [28], we quantified the level of fibrosis in the regions bordering the infarct. Matrix treatment delivered at 3 h post-MI was associated with a reduction in fibrosis assessed at 4 wk post-treatment compared to PBS-treated animals (Fig. 4G, H). Together our results suggest that early delivery of our injectable matrix can protect the myocardium by limiting negative remodeling and functional deterioration.

3.5. Vascularity and cell death in response to early matrix treatment

To better understand the effect of 3 h post-MI biomaterial therapy on the host tissue response, we assessed vascular density, angiogenesis and cell death at 2 d after treatment delivery. Given that this time-point is 5 days earlier than the next treatment time-point tested (7 d), this would provide insight into the bioactive benefits of delivering the matrix sooner post-MI (Fig. 5A). At 2 d post-treatment, no difference in the number of α-smooth muscle actin+ (α-SMA) arterioles was found in the infarct area of control-versus matrix-treated mice (Fig. 5B, F). However, mice that received matrix treatment had a higher density of CD31+ capillaries in the infarcted myocardium compared to PBS-treated mice (Fig. 5C, F). The greater number of capillaries is likely a result of increased angiogenesis, since more proliferating (Ki67+) CD31+ endothelial cells were observed in the infarct area of matrix-treated hearts (Fig. 5D, G). Moreover, proliferating CD31+ endothelial cells represented a higher proportion of the total number of proliferating cells in the myocardium in the matrix-treated animals (Fig. 5E). The ability of the matrix to limit cell death, a hallmark of cardiac ischemic injury, was also evaluated early post-treatment. Matrix-treated mice had less TUNEL+ apoptotic cells in the infarcted myocardium at 2 d post-treatment compared to PBS-treated mice, demonstrating the ability of the matrix to reduce cell death in the post-MI heart (Fig. 5H, I).

![Fig. 4. Early matrix treatment reduces scar size, improves cardiac performance and limits ventricular remodeling and fibrosis. (A) Representative Masson-trichrome or HPS-stained sections of hearts at different levels from the apex at 4 wk post-treatment (for treatment delivered at 3 hr post-MI). (B) Quantification of final scar size for the 3 h treatment groups at 4 wk post-treatment. *p < 0.05; n = 3–5. (C-F) % Fractional area change, cardiac output (ml/min) and stroke volume (µl) and LV-volume at end-systole (µl) for the 3 h treatment groups assessed at 4 wk post-treatment. **p < 0.01, ***p < 0.0001, †p = 0.0545; n = 15–16. (G) Representative Masson-trichrome images of fibrosis (blue = collagen) in the MI border region at 4 wk post-MI (scale bar = 100 µm). (H) Assessment of fibrosis in the MI border zone at 4 wk post-MI. ***p < 0.001; n = 5–6. (PBS, phosphate buffered saline). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](#)
3.6. Early matrix treatment effects on the acute inflammatory response

Biocompatibility is an important concern for biomaterial therapies; therefore, matrix effects on the acute inflammatory response (at 2 d post-treatment) were assessed in myocardial tissue sections. Two major cell types recruited during the initial inflammatory phase were examined: neutrophils (Ly6G<sup>+</sup> cells) and macrophages (F4/80<sup>+</sup> cells). Given our in vitro results with cultured macrophages, we also stained for two important subsets of macrophages: the pro-wound healing M2 phenotype (CD206<sup>+</sup> cells) and the pro-inflammatory M1 phenotype (CD86<sup>+</sup> cells). Matrix treatment did not stimulate the recruitment of additional neutrophils to the border zone or the infarct area (Fig. 6A). Similarly, the recruitment of macrophages to both the infarct and border zone was not different between PBS- and matrix-treated mice (Fig. 6B).
was no difference in the number of recruited M2 macrophages between treatment groups (Fig. 6C); however, more M1 macrophages were present in the infarct and bordering areas for the 3 h treatment groups at 2 days post-treatment. *p < 0.05 and †p = 0.07 vs. PBS; n = 4. (E) Representative immunofluorescence images of infiltrating neutrophils (Ly6G⁺), macrophages (F4/80⁺), M2 macrophages (CD206⁺) and M1 macrophages (CD86⁺) in the infarcted myocardium (Scale bar = 100 μm). (PBS, phosphate buffered saline).

3.7. Long-term heart function after early matrix treatment

To determine whether the benefit of early matrix treatment is stable and confers long-lasting functional improvement to the myocardium, a subset of mice was followed for 3 months post-treatment (Fig. 7A). Cardiac function (%LVEF) was assessed at baseline (3 h post-MI), and 1, 4, 8 and 12 wk post-treatment. Three hours after MI was induced (baseline), there was no difference in cardiac function (%LVEF) between the PBS and matrix treated mice. Two animals from the PBS-treated group died prior to the 12 wk end-point, whereas all matrix-treated mice survived in the 12 wk cohort. The improved cardiac function conferred by matrix treatment at 3 h post-MI was stable for the 12 wk period, while those receiving PBS treatment demonstrated progressive functional deterioration (Fig. 7B). At 3 months post-treatment, matrix-treated mice also demonstrated superior cardiac output (Fig. 7C) and stroke volume (Fig. 7D). Mice receiving matrix treatment also demonstrated improvements in end-diastolic and end-systolic volume at 12 wk (Fig. 7E, F) that did not achieve significance, possibly due to low sample size. Thus, when the matrix is delivered early after MI, it offers long-term stability to cardiac function and anatomy.
Early matrix therapy preserves cardiac function long-term in mice. (A) Schematic of experimental design for the 3rd end-point for the 3 h treatment cohort. Cardiac function was assessed by echocardiography at baseline (at pre-treatment), and at weeks 1, 4, 8 and 12 post-treatment. (B) %LVEF measured for 12 weeks. *cardiac function was assessed by echocardiography at baseline (at pre-treatment), and at weeks 1, 4, 8 and 12 post-treatment. (C) Assessment of (C) cardiac output (ml/min); (D) stroke volume (ul); and left ventricular volume at (E) end-diastole (ul) and (F) end-systole (ul) at 12 wk post-treatment. *p < 0.005; n = 3–4. (MI, myocardial infarction; PBS, phosphate buffered saline; ECHO, echocardiography; LVEF, left ventricular ejection fraction).

4. Discussion

This study highlights that the timing of delivery is an important consideration for the therapeutic function of an injectable biomaterial for treating MI. Specifically, we report that the delivery time-point underpins the efficacy of a collagen matrix hydrogel to prevent negative remodeling, reduce scar size, and improve function of the infarcted heart. Possible mechanisms contributing to the observed benefits may include an altered tissue level cytokine profile leading to superior short- and long-term neovascularization, less cell death, and favorable regulation of inflammation.

Determining the ideal time-point for the administration of therapy to treat MI is an important clinical issue owing largely to the dynamic and time-dependent processes that occur in the myocardial environment post-MI, yet its impact on the efficacy of injectable hydrogels for treating MI is poorly studied [6–8]. In the literature, the time-point for the injection of hydrogel therapies ranges from immediately post-MI, to 1 week to 2 months post-MI [6–8]. The clinical translation of studies on treatment delivery immediately post-MI may be limited as it is unlikely that any patient could be treated within this time frame. Therefore, we chose our earliest delivery time-point to be 3 h post-MI, which is arguably more clinically relevant. Our results demonstrate that the greatest therapeutic benefit was achieved when the collagen matrix was delivered at this early time-point. Specifically, the deterioration of cardiac function and contractility (EF, SV, CO, FS) and remodeling (scar size, ESV, EDV, fibrosis) was prevented, and cardiac function was stabilized long-term. In comparison, the benefits of matrix treatment were reduced when administered at later time-points (1 and 2 weeks post-MI). Therefore, it appears that this particular collagen matrix can prevent further cardiac decompensation, but cannot restore function that is already lost.

To our knowledge, only one other study has investigated multiple time-points for an injectable natural material for the treatment of MI. Although a very early time-point was not investigated, Laluka et al. injected alginate hydrogels into both recent (1 wk) and mature (2-month) infarcts and reported more favorable effects on infarct wall thickness and parameters of remodeling for the earlier treatment [16], a result similar to that observed in our study. In contrast, a study using a synthetic polyethylene glycol hydrogel reported that superior therapeutic benefit was achieved with later delivery (1 wk post-MI) compared to immediate injection, which was attributed to less rapid degradation of the PEG material when delivered at the later time-point resulting in increased scar thickness and improved cardiac function [29]. However, Rane et al. found that passive structural reinforcement by a PEG hydrogel delivered at 1 wk post-MI was insufficient to improve cardiac function [30]. The different outcomes may relate to differences in the PEG hydrogels tested — one was polymerized with an enzymatically degradable peptide sequence and injected in the presence of MMP-1 — and highlights the need to better understand how specific biomaterials are influencing the cardiac environment.

Numerous other injectable hydrogels for the treatment of MI have been shown to improve or preserve cardiac function in rodent or swine models [7,8]. Notably, a material's bioactivity is critical in stimulating a repair/regeneration response in the infarcted heart [30], which may be achieved with the use of ECM-derived hydrogels [31,32] and/or co-delivery of a biomaterial with growth factors and cytokines such as TIMP-3, VEGF or FGF-2 [33–35], for example. Such strategies are expected to provide natural binding sites and/or specific cues to promote cell-ECM signaling and direct repair and regeneration [11,36]. Nevertheless, the mechanisms responsible for the benefits of ECM-derived injectable biomaterials in treating the MI heart remain largely unknown. Given the importance of cell-ECM interactions, we investigated which processes in the progression of post-MI left ventricular remodeling were being altered by our matrix therapy.

The therapeutic function of our collagen matrix was greatest when delivered at 3 h post-MI, which coincides with the early inflammatory phase of infarction. Interfering with early post-MI inflammation has been shown to delay the removal of necrotic cell debris and exacerbate the deterioration of cardiac function [37]; therefore, it was important to assess the matrix's effect on the inflammatory response. At 2 days post-treatment, the number of Ly6G<sup>+</sup> neutrophils and F4/80<sup>+</sup> macrophages recruited to the infarct and border zone was not different between PBS- and matrix-treated hearts, suggesting that matrix therapy did not impede the process of debris removal. It also did not perpetuate inflammatory
cell invasion, as indicated by the reduction in CD86+ M1 macrophages. In fact, the matrix reduced chronic inflammation as shown by a lower level of TNF-α and less macrophages in the myocardium at 4 weeks post-treatment. This evidence supports the suitability of the collagen matrix for cardiac therapy, and suggests that it may promote pro-wound healing macrophage function.

TNF-α is a pleiotropic cytokine that is not constitutively expressed in the heart, but is released by the myocardium as a stress response to ischemic injury [22]. TNF-α appears to be cytoprotective through TNFR2 at low physiological concentrations, while it is pro-inflammatory and pro-apoptotic through TNFR1 at high concentrations [22]. Reduced TNF-α expression in matrix-treated hearts may be contributing to the decreased cell death observed at both short- (2 days) and long-term (4 weeks) follow-up by activating the cytoprotective TNF-α pathway. In vivo studies using BMDMs were performed to further explore the influence of the collagen matrix on the function of macrophages, which play an important role in cardiac repair. Macrophages not only mediate inflammation, as they are also involved in tissue remodeling and regeneration [38], and have been shown to provide signals needed for vascularization of the regenerating mouse heart [39]. Culturing macrophages on the matrix resulted in a reduction in the secretion of pro-inflammatory cytokines (MIP-1α, MIP-1β, CCL5) and soluble TNFR1 and TNFR2, while increased levels of anti-inflammatory and angiogenic cytokines (IL-4 and CXCL5), and TIMP-2 were observed. Over-expression of TIMP-2 has been shown to improve survival and remodeling post-MI in mice [40], while increased concentrations of soluble TNF receptors have been associated with adverse remodeling and ventricular rupture post-MI [41]. Taken together with the in vivo observations, it appears that the matrix can promote anti-inflammatory and pro-wound healing macrophage function.

We also found a significant increase in FGF-2 at 4 weeks post-treatment in animals receiving matrix therapy at 3 h post-MI. FGF-2 has potent anti-apoptotic and angiogenic properties and has been associated with reduced infarct expansion and preserved LV function post-MI [42]. This is consistent with the increased FGF-2, reduced cell death, improved neovascularization and superior LV function that were observed with matrix treatment. Matrix therapy promoted a greater density of capillaries and arterioles in the myocardium at 4 weeks post-treatment. The increased number of proliferating CD31+ endothelial cells observed in tissue 2 days after treatment suggests that the matrix is stimulating the formation of new blood vessels, and not only possibly protecting existing ones.

It appears, as has been suggested previously [17,30], that the therapeutic effects of our matrix for treating the MI heart rely on its ability to interact with cells and guide host tissue responses. Modifications to ECM proteins, including collagens, initiated during the inflammatory phase post-MI, result in ECM changes that disrupt cell-ECM interactions required for cell signaling, function and survival [43,44]. It is probable that the delivery of our matrix therapy at 3 h post-MI preserves or restores important cell-ECM interactions prior to the proliferative and/or maturation phase of MI, and thus promotes tissue repair, limits the adverse remodeling process and improves tissue function.

Although the data presented demonstrates the ability of our injectable collagen hydrogel to interact with the host tissue post-MI and preserve cardiac geometry and function, there are limitations to this work. For one, this study used an echo-guided intra-myocardial injection technique to deliver the hydrogel post-MI. This method allows visualization of the infarct region to better direct hydrogel injections, but it would also constitute a more invasive approach for patients compared to some alternatives. For example, percutaneous transendocardial delivery may prove less invasive for patients and can be visualized with NOGA mapping. Although this is technically unfeasible in rodents, it may constitute a viable option as this work moves into larger animal models. Additionally, there was limited molecular analysis to precisely characterize the mechanisms mediating the benefits of the matrix therapy and the interactions involved, and not all possible effects of the matrix were investigated. For example, fibroblast activity and MMP/TIMP regulation are important determinants of post-MI remodeling [45], and biomaterial therapy strategies that target these processes have been shown to ameliorate cardiac function [46,47]. Given the observed reduction in fibrosis in our early (3 h) matrix treatment group, it is possible that the matrix delivered at this time affected fibroblast function and differentially regulated MMP/TIMP activity, which may also have contributed to the overall therapeutic benefit. A more detailed analysis of this and other mechanisms constitutes a viable opportunity for future investigation. Nevertheless, the results presented provide strong evidence that the benefits of therapy delivered soon after MI at 3 h are achieved through the regulation of multiple processes (e.g. cell survival, inflammation, angiogenesis, fibrosis).

5. Conclusion

In this study, we demonstrate that an injectable collagen hydrogel is a promising therapy for treating the infarcted heart, and that it is most efficacious when administered soon after the onset of ischemia and inflammation. The material positively regulated multiple repair processes to stabilize cardiac function, and the functional benefits were maintained up to 3 months. This study confirms the importance of timing of delivery for biomaterial therapy, and provides a strong rationale to begin testing the safety and efficacy of this material in a large animal model of myocardial infarction.

Acknowledgments

The authors thank Rick Seymour, Kimberly McEwan, Dr. Donna Padawan and Suzanne Crowe for technical assistance, and the animal care staff at the University of Ottawa for their help in animal surgery and maintenance. This work was supported by the Heart and Stroke Foundation of Canada (HSFC; grant-in-aid T1693 to E.J.S.), by the Canadian Institutes of Health Research (CIHR; grant FRN 125678 to E.J.S.), and by the National Natural Science Foundation of China (grant NSFC 81261120557 to Z.Z., C.D.). N.J.R.B. and B.M. were supported by awards from the University of Ottawa’s Faculty of Medicine Endowed Funds for Cardiac Research and the UOHI Foundation, T.S. by Master’s studentship award from the HSFC, T.S. and D.K. by Canadian Graduate Scholarships from the CIHR, and B.M. by a Research Fellowship from the HSFC.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jbiomaterials.2014.11.004.

References


