Delayed delivery of endothelial progenitor cell-derived extracellular vesicles via shear thinning gel improves postinfarct hemodynamics

Jennifer J. Chung, MD, Jason Han, MD, Leo L. Wang, PhD, Maria F. Arisi, BA, Samir Zaman, BA, Jonathan Gordon, BA, Elizabeth Li, BA, Samuel T. Kim, BA, Zoe Tran, BA, Carol W. Chen, MD, MSCE, Ann C. Gaffey, MD, MTR, Jason A. Burdick, PhD, and Pavan Atluri, MD

ABSTRACT

Background: Extracellular vesicles (EVs) are promising therapeutics for cardiovascular disease, but poorly-timed delivery might hinder efficacy. We characterized the time-dependent response to endothelial progenitor cell (EPC)-EVs within an injectable shear-thinning hydrogel (STG+EV) post-myocardial infarction (MI) to identify when an optimal response is achieved.

Methods: The angiogenic effects of prolonged hypoxia on cell response to EPC-EV therapy and EV uptake affinity were tested in vitro. A rat model of acute MI via left anterior descending artery ligation was created and STG+EV was delivered via intramyocardial injections into the infarct border zone at time points corresponding to phases of post-MI inflammation: 0 hours (immediate), 3 hours (acute inflammation), 4 days (proliferative), and 2 weeks (fibrosis). Hemodynamics 4 weeks post-treatment were compared across treatment and control groups (phosphate buffered saline [PBS], shear-thinning gel). Scar thickness and ventricular diameter were assessed histologically. The primary hemodynamic end point was end systolic elastance. The secondary end point was scar thickness.

Results: EPC-EVs incubated with chronically versus acutely hypoxic human umbilical vein endothelial cells resulted in a 2.56 ± 0.53 versus 1.65 ± 0.15-fold increase (P = .05) in a number of vascular meshes and higher uptake of EVs over 14 hours. End systolic elastance improved with STG+EV therapy at 4 days (0.54 ± 0.08) versus PBS or shear-thinning gel (0.26 ± 0.03 [P = .02]; 0.23 ± 0.02 [P = .01]). Preservation of ventricular diameter (6.20 ± 0.73 mm vs 8.58 ± 0.38 mm [P = .04]; 9.13 ± 0.25 mm [P = .01]) and scar thickness (0.89 ± 0.05 mm vs 0.62 ± 0.03 mm [P < .0001] and 0.58 ± 0.05 mm [P < .0001]) was significantly greater at 4 days, compared with PBS and shear-thinning gel controls.

Conclusions: Delivery of STG+EV 4 days post-MI improved left ventricular contractility and preserved global ventricular geometry, compared with controls and immediate therapy post-MI. These findings suggest other cell-derived therapies can be optimized by strategic timing of therapeutic intervention. (J Thorac Cardiovasc Surg 2020;159:1825-35)

Extracellular vesicles (EVs) are lipid membrane-bound particles that are actively released from cells and carry cargo of various RNA species, proteins, and bioactive lipids.1 EVs have garnered significant interest as a novel therapeutic agent for vascular regeneration after ischemic injury, including myocardial infarction (MI).2 Numerous

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Central Message

EPC-derived extracellular vesicles in a shear thinning gel dramatically improve post-MI hemodynamics when delivered 4 days versus immediately post-infarct.

Perspective

Endothelial progenitor cell-derived extracellular vesicles have great potential for widespread therapeutic applications in cardiovascular recovery after ischemic injury, but the optimal timing of intervention to maximize benefits remains unknown. This study demonstrates superior functional and mechanical properties with delayed injection of STG+EV 4 days after acute myocardial infarction.

See Commentaries on pages 1836 and 1838.

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Address for reprints: Pavan Atluri, MD, Division of Cardiovascular Surgery, Department of Surgery, University of Pennsylvania, 6 Silverstein Pavilion, 3400 Spruce St, Philadelphia, PA 19104 (E-mail: pavan.atluri@uphs.upenn.edu).

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publications have reported that EVs derived from progenitor or stem cells can recapitulate the beneficial effects of these cell therapies.\textsuperscript{1,3,4} However, there are few data on the effect of the timing of intervention on outcomes after EV therapy. Ischemia alters cell metabolism and function and can lead to irreversible injury and cell death. Surviving cells are exposed to a wide range of evolving inflammatory, neurohormonal, and paracrine effects that drive cardiac fibrosis and remodeling.\textsuperscript{5} Because the substrate upon which EVs act is the recipient cell, we hypothesized that the cell microenvironment would affect EV uptake and therapeutic efficacy after MI.

We have previously described how EVs derived from endothelial progenitor cells (EPCs) produce the same therapeutic benefits as EPCs themselves, namely improved hemodynamics, increased peri-infarct vascular density, and mitigation of deleterious post-MI remodeling.\textsuperscript{6} In our previous work, EPC-EVs were delivered within a shear-thinning gel (STG+EV), which is formed from biocompatible components binding via guest-host interactions.\textsuperscript{5}\textsuperscript{6}\textsuperscript{8} These bonds can be broken with shear stress and allow injection through a syringe, resulting in more precise targeting of EVs to the ischemic border zone post-MI, as well as sustained delivery over at least 21 days. Using this novel construct of STG+EV, we designed the current study to parallel the phases of inflammation and healing after an ischemic insult—acute inflammation, proliferation, and fibrosis\textsuperscript{9}—to determine how changes in the timing of delivery of EPC-EVs affect functional results of this therapy.

**METHODS**

**Animal Use**

All experiments conformed to the National Institute of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. *Rattus norvegicus* (Wistar) rats were obtained from Charles River Laboratories, Inc (Boston, Mass).

**Isolation of Endothelial Progenitor Cells**

Adult Wistar rats (350-375 g) received an intraperitoneal pentobarbital injection at 100 mg/kg. After cessation of pedal reflex, euthanasia was performed by transection of the carotid artery, and introduction of pneumothorax. After exsanguination, the long bones were collected, and bone marrow was harvested.

Bone marrow mononuclear cells were isolated using density-gradient centrifugation on Histopaque-1083 (Sigma-Aldrich, St Louis, Mo; 10831) and plated on vitronectin-coated 10-cm culture dishes. EPCs were cultured as the adherent fraction in 5% fetal bovine serum EGM-2 (Lonza, Basel, Switzerland) without heparin or hydrocortisone. After 4 days, cells were washed twice to remove nonadherent cells.

**Isolation of EVs from EPCs and Storage**

Four days postisolation, EPCs were changed to serum-free EGM-2 without heparin or hydrocortisone. After 48 hours, the conditioned media was collected, centrifuged at 2000g for 30 minutes, then clarified through a Sterirucip 0.22 μm polyvinylidene difluoride filtration unit (Millipore Sigma, Burlington, Mass). Clarified conditioned media was then incubated in a 1:2 ratio with 36% polyethylene glycol, 1.5 M NaCl overnight at 4°C, pelleted using centrifugation at 10,000g at 4°C for 2 hours, and resuspended in sterile-filtered Dulbecco’s phosphate buffered saline. EVs were stored at −80°C until use. EVs were resuspended at a concentration of 9.33 \times 10^10\textsuperscript{3} particles/mL for in vivo use, and at 1.87 \times 10^11\textsuperscript{3} particles/mL for in vitro use (Figure 1). Samples that were too dilute were repelleted with ExoQuick-TC (System Biosciences, Palo Alto, Calif) per manufacturer’s instructions.

**Shear-Thinning Gel Synthesis**

Shear-thinning gel (STG) was formed by modifying hyaluronic acid (HA) with adamantane (Ad) and β-cyclodextrin (CD). The sodium salt of HA was dissolved in deionized water at 2% w/w, exchanged against Dowex-100 resin, neutralized using tetrabutylammonium hydroxide, frozen, and lyophilized to form HA-tetrabutylammonium hydroxide. Hydrogels of 4% w/w were prepared from lyophilized polymers of Ad-modified HA and CD-modified HA dissolved in phosphate buffered saline (PBS) and mixed. All reagents were manufactured by Sigma-Aldrich.

**Tubule Formation Assay**

To study the effects of prolonged ischemia on cell response to EPC-EV therapy in vitro, a subset of human umbilical vein endothelial cells (HUVECs) were exposed to hypoxia (5% O\textsubscript{2}) for 7 days. On day 7, a Matrigel tubule formation assay was performed in hypoxic conditions.
and response to EPC-EV therapy was compared between HUVECs that had been chronically hypoxic versus naive cells. We pipetted 10 μL of Matrigel (Corning, Corning, NY; Cat #354234) into the inner chamber of u-Angiogenesis slides (Ibidi, Fitchburg, Wis; Cat #81506). After a 30-minute polymerization period at 37°C, experimental conditions and 1.2 × 10^6 HUVECs were added to each well. Groups included chronically hypoxic or naive cells with 4.68 × 10^8 EVs (2.5 μL of 1.87 × 10^11 particles/mL stock in 50 μL endothelial basal medium 2), or vehicle control (2.5 μL Dulbecco’s phosphate buffered saline in endothelial basal medium 2). Serum-free EGM-2 was used as a positive control. After 14 hours of incubation in 5% CO₂, vascular mesh formation was visualized with the EVOS XL Imaging System (Invitrogen, Carlsbad, Calif) with brightfield 4× magnification. Vascular meshes were quantified using ImageJ (National Institutes of Health [NIH], Bethesda, Md), and the response of cells to EVs was normalized to the vehicle control.11,12

**Labeled EV Uptake by HUVECs in Acute Versus Chronic Hypoxic Conditions**

EV action is predicated upon the uptake of secreted particles into a host cell. We therefore assessed the variation in HUVECs’ ability to uptake EVs after a 4-day exposure to hypoxia (5% O₂) compared with that of naive HUVECs. EVs were labeled with 2 μM 1,1,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) for 5 minutes at 37°C, and then 15 minutes at 4°C. Excess dye was removed with Exosome Spin Columns (molecular weight, 3000; Fisher Scientific, Waltham, Mass; Cat #4484449). We delivered 9.36 × 10^8 DiI-labeled EVs in serum-free EGM-2 to 1 × 10^6 HUVECs seeded in 8-well chamber slides. After 3 and 14 hours of incubation in 5% O₂, slides were washed in PBS 3 times, membranes were stained with WGA-AF647 (#W32466; Thermo-Fisher, Eugene, Ore) for 15 minutes at room temperature, and nuclei were stained with 4′,6-diamidino-2-phenylindole. Images were taken using the Leica DM5000b upright fluorescence microscope at (Leica, Morrisville, NC) 40× magnification and processed using ImageJ (NIH). Mean fluorescent intensity was measured and normalized by the quantified area.

**Histological Analysis**

Hearts were explanted, embedded in OCT (Tissue Tek, Torrance, Calif) and frozen on dry ice. Using cryosectioning at the midpapillary muscle level we took 10-μm transverse sections. Sections were stained using Masson’s Trichrome (Sigma-Aldrich). Scar thickness, a measure of left ventricle (LV) remodeling, was determined by averaging the thickness at 5 equidistant points along the scar, using ImageJ (NIH). The LV diameter was measured from the midpoint of the septum to the midpoint of the opposite wall, along a perpendicular line, to determine postinfarction ventricular dilatation.

For hematoxylin and eosin (H&E) staining, hearts were explanted and fixed in 10% formalin at 4°C overnight, then embedded in paraffin. Hearts were sectioned and stained with H&E. Slides were analyzed with light microscopy at 4× and 10× magnification to assess cellular infiltration and inflammation.

**Rat Model of MI**

An established rat model of MI induced by permanent occlusion of the left anterior descending artery was used. Animals were randomized into 1 of 6 groups: PBS control (n = 10); STG control (n = 10); and STG+EV delivered at 0 hours (n = 11), 3 hours (n = 12), 4 days (n = 12), or 2 weeks (n = 11) after MI. Animals were induced with 5% isoflurane, intubated, and mechanically ventilated, with 1% to 3% isoflurane to maintain a surgical anesthetic depth. Before incision, 0.05 mg/kg of buprenorphine was injected subcutaneously. Using a left fourth interspace thoracotomy, the left anterior descending artery was identified and ligated 1 mm below the left atrial appendage with a resultant anterolateral MI encompassing...
30% of the LV. At time of treatment, 100 μL of STG containing $9.33 \times 10^9$ EVs was delivered via 5 × 20 μL intramyocardial injections around the border zone of the infarcted area. The EPCs and EVs were allogenic and pooled from multiple donors. The 3-hour, 4-day, and 2-week groups all underwent a second thoracotomy at the designated time point.

Measurement of Hemodynamics

Hemodynamic measures were obtained 4 weeks after treatment. After induction of anesthesia with 5% isoflurane, the rat was intubated and mechanically ventilated. After confirmation of adequate surgical anesthesia maintained with continuous inhaled 2% isoflurane, a 2-French pressure-volume catheter (Millar, Houston, Tex) was inserted retrograde into the LV via the right common carotid artery, in a closed chest approach. Steady-state hemodynamic parameters and end systolic elastance (Ees), were determined using the methods described by Pacher and colleagues.14

Statistical Analysis

The primary end point was contractility, measured according to Ees. Secondary end points were in vitro angiogenesis and scar thickness. Treatment groups were designated and coded by random identifiers. Investigators were blinded to treatment group during data acquisition and analysis. Values are expressed as mean ± standard error of the mean. Comparisons across experimental groups was performed using 1-way analysis of variance. When a significant difference between groups was identified, pairwise comparison was performed using the Tukey honestly significant difference test. Graphs show mean values with standard error of the mean.

RESULTS

Chromically Hypoxic Cells Have a More Robust Angiogenic Response to EPC-EVs

The in vitro hypoxic angiogenesis assay was designed to elucidate the effect of chronic hypoxia on cellular responses to EVs. A state of chronic hypoxia, such as that experienced by myocytes after an MI, was simulated by culturing cells in 5% O₂ for 7 days before a hypoxic Matrigel assay. The angiogenic stimulus of EPC-EVs to chronically versus acutely hypoxic HUVECs revealed a $1.65 \pm 0.15$ versus $2.56 \pm 0.53$-fold increase ($P = .05$) in a number of vascular meshes in the chronic hypoxia compared with the naive group (Figure 2).

Chronic Hypoxia Increases EPC-EV Uptake by HUVECs

We next examined the effect of hypoxia on cells’ ability to take up EVs. DiI-labeled EPC-EVs were incubated with HUVECs that had been maintained at either 5% O₂ or at 21% O₂ (naive). After 14 hours, preconditioned HUVECs showed significantly increased uptake of EVs compared with the naive cells ($0.11 \pm 0.02$ vs $0.07 \pm 0.01$; $P = .01$; Figure 3).

Inflammatory Cell Infiltrate Most Pronounced at 4 Days Post-MI

To better understand the evolution of the infarct and border zone regions at each of the time points analyzed in this study, rat hearts were explanted at 0 hours, 3 hours, 4 days, and 2 weeks after MI. H&E stained sections showed a robust inflammatory cellular infiltrate present at 4 days post-MI with clear delineation between the border zone and areas of irreversible cell injury and death, with areas of necrosis within the infarct. This necrosis progresses to fibrosis and thinning of the left ventricular wall and scar formation by 2 weeks (Figure 4).
Hypoxic human umbilical vein endothelial cells (HUVECs) show robust Endothelial progenitor cell (EPC)-extracellular vesicle (EV) uptake ability. EPC-EV uptake by preconditioned HUVECs (4 days, 5% O₂) was compared with that of naive HUVECs at 3 and 14 hours after EPC-EV incubation in a hypoxic environment. After 14 hours, preconditioned HUVECs exhibited a greater number of EVs than naive (P < .05) and preconditioned (P < .01) cells in 3 hours of hypoxia. DAPI, 4’-Diamidino-2-phenylindole; Dil, 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate.

**STG+EV Delivery at 4 Days Improves Hemodynamics**

Pressure-volume catheter analysis showed significant hemodynamic differences between the delayed treatment groups and controls. Ees, the slope of the end systolic pressure-volume relationship measured during occlusion of the inferior vena cava, and is regarded as a volume-independent measure of contractility (Figure 5 and Figure E2). Improvements in Ees were also markedly improved with therapy at 4 days (P = .007). Furthermore, measures of LV relaxation were also markedly improved with therapy at 4 days and Figure E2). Improvements in Ees were most pronounced after injection of STG-EV therapy at 4 days post-MI, with statistically significant improvement over PBS and STG controls (0.54 ± 0.08 vs 0.26 ± 0.03 [P = .02]; 0.23 ± 0.02 [P = .008]; Table 1). An alternate measure of contractility, maximum change in pressure over change in time also showed improvement in the 4-day group over PBS (5432 ± 309 mm Hg/s vs 3886 ± 397 mm Hg/s; P = .03). The 4-day group also had statistically significantly higher ejection fraction (EF) compared with PBS and 0-hour groups (59.6 ± 1.4% vs 46.9 ± 2.6% [P = .004]; 42.8 ± 2.8% [P < .0001]).

Similarly, with regard to cardiac output, the 4-day group showed statistically significant increases over PBS (64,459 ± 6308 vs 38,874 ± 4929; P = .02), and 0-hours (37,558 ± 3549; P = .007). Furthermore, measures of LV relaxation were also markedly improved with therapy at 4 days (−5443 ± 303), with statistically superior minimum change in pressure over change in time (dp/dt<sub>min</sub>) compared with PBS (−3154 ± 347; P < .0001) and STG (−3942 ± 401; P = .04), as well as 0 hours (−3558 ± 465; P = .003) and 3 hours (−3762 ± 282; P = .01). The 2-week group also had superior dp/dt<sub>min</sub> over PBS (P = .0001), 0 hours (P = .003), and 3 hours (P = .01).

To characterize the changing effects of treatment over time, a cohort of 4-day and 2-week animals underwent hemodynamic assessment at 4 and 6 weeks post-MI. Over time, the 4-day group showed a mild decrease in EF at 4 versus 6 weeks post-treatment (59.6 ± 1.4 vs 53.7 ± 2.6; P = .04). In contrast, the 2-week group showed a more significant decrement in EF from 4 weeks post-MI (2 weeks post-treatment) to 6 weeks post-MI (4 weeks post-treatment; 61.4 ± 3.0 vs 47.6 ± 2.3; P = .01; Figure E1). LV end systolic volume in the 2-week group also trended higher over this time period (Δ = 61.7 ± 32.4 μL; P = .07), suggesting ongoing remodeling and LV dilatation.

**STG+EV Reduces Infarct Thinning and Preserves Ventricular Geometry**

Frozen section analysis of hearts at 4 weeks post-MI with Masson’s Trichrome showed distinct transmural infarct in all hearts included in the analysis (Figure E3). All STG+EV treatment groups showed attenuation of infarct thinning and had statistically significantly increased scar thickness compared with PBS alone. The 4-day group had the greatest scar thickness (0.89 ± 0.05 mm), which was statistically significantly increased compared with PBS (0.62 ± 0.03 mm; P < .0001) and STG (0.58 ± 0.05 mm; P < .0001; Figure 6). Additionally, ventricular geometry was better preserved in the 3-hour (6.09 ± 0.44 mm) and
4-day (6.20 ± 0.73 mm) groups, as indicated by smaller ventricular diameters compared with PBS (8.58 ± 0.38 mm; \( P = .03, .04 \)) or STG (9.13 ± 0.25 mm; \( P = .01, .01 \)).

**DISCUSSION**

Our group has previously shown that STG+EV is a novel therapeutic agent with hemodynamic benefits when delivered to ischemic myocardium immediately after MI. We showed that the EPC-derived EV reliably confers the same advantages of EPC therapy while overcoming many of the challenges to cell therapy and significant barriers to clinical translation. The use of STG further enhances the effect of the EPC-EVs by allowing precise targeting and sustained release of particles in addition to having intrinsic proangiogenic properties and providing mechanical stabilization of the peri-infarct region.

This study further informs and enhances the clinical applications of the STG+EV therapy by elucidating how timing of intervention affects resident cardiac cells’

**FIGURE 4.** Representative hematoxylin and eosin staining of infarct over time. Inflammatory cell infiltrate was evident at all time points. At 4 days there was delineation of scar and border zone regions. Thinning of the left ventricular wall from necrosis progressed in the 2-week group. Black arrows indicate border zone. Blue arrows indicate the infarct. Imaged at 4 and 10 times magnification under brightfield. Scale, 500 µm.
interaction with delivered EPC-EVs, and ultimately, the downstream functional and mechanical effect of therapy. By comparing outcomes after delivery of therapy at 4 time points after MI, we found the timing of delivery of therapy was administered. Treatment at 4 days post-myocardial infarction shows greatest improvement in measures of end systolic elastance (Ees), ejection fraction (EF), cardiac output (CO), stroke work (SW), and dP/dt max. PBS, Phosphate buffered saline; STG, shear thinning gel; dP/dt, change in pressure over change in time; LVESV, left ventricular end systolic volume; LVEDV, left ventricular end diastolic volume.

FIGURE 5. Hemodynamics panel. Data plotted as mean ± standard error of the mean. Treatment groups indicate time points post-myocardial infarction at which therapy was administered. Treatment at 4 days post-myocardial infarction shows greatest improvement in measures of end systolic elastance (Ees), ejection fraction (EF), cardiac output (CO), stroke work (SW), and dP/dt max. PBS, Phosphate buffered saline; STG, shear thinning gel; dP/dt, change in pressure over change in time; LVESV, left ventricular end systolic volume; LVEDV, left ventricular end diastolic volume.

This suggests that the effective payload of 4-day injections might be higher than that of 0-hour injections because of more robust uptake by the resident cells in the ischemic myocardium.

Histologic analyses of hearts explanted at 4 weeks after MI showed differences in scar thickness and ventricular geometry across treatment groups and controls, with less thinning of the infarct and mitigation of LV remodeling as indicated by preserved LV diameters in the 4-day group. Hemodynamic assessment consistently showed significant improvements in the group that received
therapy 4 days postinfarction. This time point correlates with the proliferative phase of the response to ischemic injury, and we hypothesized that the local environment within the myocardium at this point would be ideal for STG+EV delivery. H&E analysis of sections from the 4 days post-MI showed significant infiltration of inflammatory cells, which are crucial for clearance of dead cells from the infarct, and play an important role in tissue repair and regulation of the proinflammatory mediators. Because of the predominance of inflammatory cells at 4 days post-MI and the strong effect of STG+EV therapy seen with intervention at this time point, it is possible that EPC-EVs play a role in immune modulation. Additional studies to further evaluate the interaction of EPC-EVs and immune cells after ischemic injury would be useful.

TABLE 1. Summary table of hemodynamic data for each treatment group

<table>
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<tr>
<th>Treatment group</th>
<th>Ees, mm Hg/µL</th>
<th>dP/dt maximum, mm Hg/s</th>
<th>EF, %</th>
<th>CO, µL/min</th>
<th>Stroke work, mm Hg/µL</th>
<th>dP/dt minimum, mm Hg/s</th>
<th>Pmax, mm Hg</th>
<th>LVESV, µL</th>
<th>LVEDV, µL</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.26 ± 0.03</td>
<td>3886 ± 397</td>
<td>46.9 ± 2.6</td>
<td>38,874 ± 4929</td>
<td>6298 ± 892</td>
<td>–3154 ± 347</td>
<td>78.5 ± 3.8</td>
<td>167.6 ± 15.3</td>
<td>297 ± 29.9</td>
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<tr>
<td>STG</td>
<td>0.23 ± 0.02</td>
<td>4780 ± 411</td>
<td>52.3 ± 2.9</td>
<td>42,749 ± 4172</td>
<td>9844 ± 790</td>
<td>–3942 ± 401</td>
<td>94.7 ± 5.3</td>
<td>175.6 ± 27.8</td>
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<td>0 H</td>
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<td>4064 ± 501</td>
<td>42.8 ± 2.8</td>
<td>37,558 ± 3549</td>
<td>7044 ± 923</td>
<td>–3558 ± 465</td>
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<td>204.9 ± 23.5</td>
<td>339.1 ± 35</td>
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<tr>
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<td>–5453 ± 168</td>
<td>113.3 ± 6.9</td>
<td>248.5 ± 31.6</td>
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</table>

Values are displayed as mean ± standard error of the mean. Ees, End systolic elastance; dP/dt, change in pressure over change in time; EF, ejection fraction; CO, cardiac output; Pmax, maximum power; LVESV, left ventricular end systolic volume; LVEDV, left ventricular end diastolic volume; PBS, phosphate buffered saline; STG, shear thinning gel.

FIGURE 6. Delayed delivery of shear thinning gel (STG) and extracellular vesicles limits infarct thinning. A, Representative heart sections at 4 weeks post-myocardial infarction were stained with Masson’s Trichrome. B, Scar thickness was calculated. The 4-day group (P < .0001) had the greatest scar thickness compared with controls. PBS, Phosphate buffered saline.
Ultimately, the hemodynamic effects STG+EV on the primary outcome of Ees was greatest in the 4-day group and was the only treatment group with statistically significant improvement over PBS and STG controls. In additional measures of LV function, including maximum change in pressure over change in time, dp/dt_{min}, EF, and cardiac output, treatment at 4 days appears to confer greater benefits than controls or other treatment time points. At the 4-day time point, cells in the ischemic border zone have likely initiated compensatory measures and upregulated prosurvival pathways. On the basis of our in vitro data and preexisting scientific knowledge regarding the progression of postinfarct inflammation and healing, delivery of STG+EV into this milieu should optimize the ability of cells to respond to the proangiogenic cargo of the EPC-EVs. STG delivery at this time also serves a critical role of stabilizing the weakened peri-infarct myocardium as cell death and necrosis progresses within the infarct.

The 2-week group initially showed robust hemodynamics when assessed 4 weeks post-MI, however, when reassessed 2 weeks later, at 6 weeks post-MI (4 weeks post-treatment) there was deterioration of function. This is not surprising because of histologic evidence that there is significant cell necrosis and evidence of the initial stages of fibrosis already present 2 weeks after MI. We hypothesize that STG delivered at this time point has significant bulking and mechanical unloading properties that might be especially important at this point in infarct evolution, when mechanical stress can further injure and deform vulnerable regions and lead to fibrosis. It is probable that the results at the early sac point reflect effects of retained STG components, whereas the hemodynamics seen 4 weeks after treatment are a more accurate reflection of the ultimate effect of STG+EV treatment.

CONCLUSIONS
Overall, this study provides evidence that delayed intervention after MI with intramyocardial injection of STG+EV therapy into the infarct border zone confers hemodynamic and structural benefits with delivery 4 days post-MI. We have shown evidence of a proangiogenic effect of EPC-EVs that is more pronounced with chronically rather than acutely ischemic cells. This might be in part because of the increased affinity for cell uptake of EPC-EVs after a prolonged period of hypoxia. The mechanical offloading effects of STG likely also play a key role in preservation of scar thickness and ventricular systolic and diastolic function. Further studies to elucidate the mechanisms contributing to the differential effects of treatment at variable time points will help to guide engineering of the STG with regard to stiffness and degradation profile, as well as pinpointing the optimum time for delivery of EPC-EVs for maximum beneficial effect. As the possible therapeutic applications of EVs continues to expand, it is necessary to rigorously assess the optimal intervention strategy. This study helps to pinpoint the time at which post-MI intervention with STG+EV therapy can produce the most significant beneficial effect for preservation of ventricular contractility and structural integrity. The finding of optimal effectiveness occurring 4 days after MI improves the feasibility of clinical translation compared with immediate intervention.

Limitation
There are a few important limitations to note. Because of the numerous time points being evaluated, an STG-only control at each time point was not feasible and would have required a prohibitively large number of animals. Furthermore, there are benefits and shortcomings to defining a study end point on the basis of time from MI as opposed to time from therapy. We chose a 4-week post-treatment, rather than post-MI, end point for all groups, to have a consistent time period over which the effects of the STG+EV therapy could be realized.

Conflict of Interest Statement
Authors have nothing to disclose with regard to commercial support.

References
Discussion

Dr Joseph Cleveland. Our third paper this morning is entitled, “Fools Rush In: Delayed Delivery of a Shear-Thinning Hydrogel and Endothelial Progenitor Cell-Derived Exosomal Therapy Improves Hemodynamics Following Myocardial Infarction” presented by Dr Chung and colleagues from the University of Pennsylvania.

Dr Jennifer Chung (Philadelphia, Pa). All right. Good morning and thank you to the association for the opportunity to present our research this morning.

Extracellular vesicles are lipid-membrane bound particles that are actively released from the cells and are thought to carry important cargo including various RNA species, proteins, and bioactive lipids. They have recently garnered significant interest as novel therapeutics for tissue and vascular regeneration after ischemic injury. EVs derived from progenitor cells have been found to deliver the therapeutic effects of parent cells without the challenges associated with the progenitor stem cell production, viability, or potential drawbacks of stem cell therapy.

We have previously demonstrated that EVs derived from EPCs delivered within a shear-thinning gel and delivered within the ischemic myocardium immediately after MI has beneficial effects resulting in improved hemodynamics, increased angiogenesis, and preserved ventricular geometry. The shear-thinning gel in this study is formed by biocompatible materials, namely hyaluronic acid modified with adamantane and β-cyclodextrin. These components bond via reversible guest host interactions, which are easily broken with the application of shear stress, inducing a reduction in viscosity, and allowing injection via syringe. When the shear stress is removed, the gel quickly reheels within the tissue.

The therapy we are studying is a construct of endothelial progenitor cell-derived extracellular vesicles encapsulated within the shear-thinning gel. Despite promising previous results, we believe that the beneficial effects of EPC-EV therapy could be further optimized by intervention at specific time points in the post-MI period. Ischemia alters cell metabolism and function and cells are exposed to a wide range of inflammatory neurohormonal and paracrine effects after MI. We hypothesize that the local cell environment will affect EV uptake and the therapeutic uptake and therapeutic efficacy and sought to determine the optimal timing for delivery of STG EV therapy post-MI to maximize therapeutic effect on left ventricular function.

Our in vitro studies examined the effect of varying periods of hypoxia on cellular functions of angiogenesis and ability to take up exosomes. We incubated HUVECs in chronically hypoxic conditions for 4 to 7 days and compared them with normoxic cells. The hypoxic versus naive HUVECs were then incubated with EVs and hypoxic conditions to assess angiogenesis via a Matrigel assay. Additionally, we then measured uptake of EVs in these cells over a 14-hour period.

In the in vivo model, MI was induced with ligation of the LAD, producing an approximately 30% infarct at the anterolateral LV. Animals were then randomized into 6 groups. The controls consisted of PBS or STG injection and the treatment groups were divided into 4 different time points post MI. Each group received 5 × 20 μL injections of the assigned treatment into the border zone area of the heart at the designated time point. The times chosen were correlated with known phases of inflammation and healing post-MI, namely the acute inflammation proliferative phase and the fibrotic phase after MI. Hemodynamics were then assessed via PV loop catheterization at 4 weeks post-MI and hearts were explanted for histologic analysis.

The in vitro Matrigel assay showed that cells that were chronically hypoxic shown in the left lower quadrant had more robust angiogenic response to treatment compared with cells that were naive and transferred acutely into hypoxic conditions. Furthermore, we found that with the labeled exosomes, which are shown in red, that there is an increase in exosome uptake in the chronically hypoxic group over 14 hours of the assay and perhaps most significantly, when looking at invasive hemodynamics, we found that there was improvement with delayed delivery of the therapy of the STG/EV at 4 days measured according to end systolic elastins, which is a measure of volume-independent contractility.

Furthermore, our histologic analyses showed that the 4-day group had the greatest attenuation and thinning of the LV wall and had the greatest scar thickness across all groups.

In conclusion, our study showed that delayed intervention with STG/EV therapy post-MI confers hemodynamic and structural benefits. These findings have great implications for determining the optimum timing of EV therapeutic treatment in the treatment of ischemic injury. As shown by the differential uptake, one contributing mechanism for this
phenomenon might be that there is differential affinity of myocyte uptake of exosomes and extracellular vesicles with varying durations of hypoxia.

And I’d like to acknowledge my PI, Dr Atluri, and the rest of our lab. I’m happy to take questions.

Dr Cleveland. Have you also studied reperfusion? So if you reperfuse the LAD and also gave timed injections of the novel therapy, does that change the results in any way?

Dr Chung. That’s a great question. That’s actually something that we are exploring now. Our initial model was just a permanent ligation without reperfusion, but we are investigating an ischemia reperfusion model and also other novel therapeutics that specifically deal with the kind of consequences of reperfusion.

Dr Cleveland. Is there any downside to the therapy?

Dr Chung. We haven’t quite been able to parse out, so the therapy, the intramyocardial injection, allows us to specifically examine at the borders and you can see a very distinct area of blanching and identify the border zone. There is likely some degree of injury that happens with the actual injection and we haven’t exactly, we haven’t characterized that specifically, but that’s why we do the PBS control, which also has some of the injection damage.

Dr Cleveland. Thank you.

Dr Chung. Thank you.
FIGURE E1. Comparison of ejection fraction (EF) and left ventricular end systolic volume (LVESV) progression from 4 to 6 weeks post myocardial infarction (MI). Intervention at 4 days post-MI slows progression toward heart failure compared with 2 weeks by better preserving EF and preventing left ventricular dilation.

FIGURE E2. Inferior vena cava occlusion yields changes in pressure-volume loops that yield end systolic pressure-volume relationship (ESPVR). The slope of ESPVR is the end systolic elastance, a volume-independent measure of contractility. EDPVR, End diastolic pressure-volume relationship; LV, left ventricular; PBS, phosphate buffered saline.
FIGURE E3. Representative immunohistochemistry panel for scar and border zone regions of shear thinning gel (STG)-extracellular vesicle treatment groups 4 weeks post-myocardial infarction (MI). Populations of cardiomyocytes (troponin T) and cardiac fibroblasts (vimentin) were studied in sections of infarcted hearts at 4 weeks post-MI with the designated treatment group. Intervention at 4 days post-MI had the greatest myocyte preservation within the infarcted region compared with other treatment groups. Images were visualized using 20× magnification. DAPI, 4',6-Diamidino-2-phenylindole; PBS, phosphate buffered saline.