Therapeutic Efficacy of Cryopreserved, Allogeneic Extracellular Vesicles for Treatment of Acute Myocardial Infarction

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Summary
Extracellular vesicles (EV) that are derived from endothelial progenitor cells (EPC) have been determined to be a novel therapy for acute myocardial infarction, with a promise for immediate “off-the-shelf” delivery. Early experience suggests delivery of EVs from allogeneic sources is safe. Yet, clinical translation of this therapy requires assurances of both EV stability following cryopreservation and absence of an adverse immunologic response to EVs from allogeneic donors. Thus, more bioactivity studies on allogeneic EVs after cold storage are necessary to establish quality standards for its widespread clinical use. Thus, in this study, we aimed to demonstrate the safety and efficacy in delivering cryopreserved EVs in allogeneic recipients as a therapy for acute myocardial infarction.

In this present study, we have analyzed the cardioprotective effects of allogeneic EPC-derived EVs after storage at −80°C for 2 months, using a shear-thinning gel (STG) as an in vivo delivery vehicle. EV size, proteome, and nucleic acid cargo were observed to remain steady through extended cryopreservation via nanoparticle tracking analysis, mass spectrometry, and nanodrop analysis, respectively. Fresh and previously frozen EVs in STG were delivered intramyocardially in a rat model of myocardial infarction (MI), with both showing improvements in contractility, angiogenesis, and scar thickness in comparison to phosphate-buffered saline (PBS) and STG controls at 4 weeks post-MI. Pathologic analyses and flow cytometry revealed minimal inflammatory and immune upregulation upon exposure of tissue to EVs pooled from allogeneic donor cells.

Allogeneic EPC-EVs have been known to elicit minimal immune activity and retain therapeutic efficacy after at least 2 months of cryopreservation in a post-MI model.

Key words: Endothelial progenitor cell, Shear-thinning hydrogel, Cardiomyopathy, Exosome, Stem cell therapy

Heart disease remains a global health epidemic, with current therapies limited to interventions mainly targeting large vessel disease via percutaneous coronary interventions (PCI) or surgical coronary artery bypass grafting. While mortality from acute myocardial infarction has improved in the post-PCI era, the development of ischemic heart failure remains largely unchanged, which, in turn, suggests a need for therapy focused on the microvasculature. Extracellular vesicles (EVs) have garnered significant interest as a novel therapy for vascular regeneration after ischemic injury. Numerous studies have demonstrated therapeutic efficacy of EVs derived from progenitor or stem cells in recapitulating the beneficial effects of their parent cells without the pitfalls inherent to cell-based therapy, namely, cell viability, engraftment, and immunologic concerns.

EVs are lipid membrane-bound particles that are actively released from cells and carry a cargo of RNA species, proteins, and bioactive lipids. EVs are taken up by host cells via a process similar to endocytosis wherein they regulate cellular signaling pathways, resulting in angiogenic, proliferative, regenerative, and anti-apoptotic effects. We have demonstrated the successful sustained delivery of EPC-derived EVs to post-MI ischemic myocardium with preservation of structure and function. Successful translation of EVs into a clinically relevant therapy requires the ability to easily package, store, and deliver a consistent dose from allogeneic donor progenitor cells.
While studies involving new applications of EVs have been increasing exponentially in the field of cardiovascular research, data remain lacking in terms of the implications of storage techniques and use of allogeneic donors on EV function and effect within the ischemic myocardium. This detailed understanding of cryopreservation and allogeneic pooling on EPC-EVs has critical implications for its translation into clinical therapy.

Our previous studies have shown the efficacy of a shear-thinning hydrogel as an injectable carrier for EV delivery in a rat model of MI. In these studies, assessments at 4 weeks post-MI demonstrated improved hemodynamics and reduced scar formation. Our present study evaluates the clinical translatable efficacy of EV-based therapy in the heart. In this study, we aim to determine the effects of long-term (>2 months) storage at ∼80°C on the physical, biochemical, and functional properties of EPC-EVs, while assessing the safety of EPC-EV therapy derived from allogeneic cell donors. We hypothesized that EPC-EVs from allogeneic sources stored at ∼80°C for up to 2 months would retain their physical, biochemical, and functional integrity.

Methods

Animal use: All experiments conform 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, MA).

Cell culture: Human umbilical vein endothelial cells (HUVECs, Lonza) were used prior to passage six (p6) and grown in Endothelial Cell-Growth Medium-2 (EGM-2, Lonza). Rat aortic endothelial cells (RAECs, Cell Applications, San Diego, CA) were used prior to p6 and cultured in RAEC Growth Medium (Cell Applications). H9C2 myoblasts (ATCC) were cultured in 10% Fetal Bovine Serum (FBS) Dulbecco’s Modified Eagle Medium (DMEM) and used up to p23. All cells were maintained at 37 °C in a humidified incubator with 21% O2 and 5% CO2.

Primary cardiac fibroblasts (CFs) were isolated from the EPC donor animals as per the methods described by Seluanov, et al., with the modification that Liberase TM (Roche) was used to digest tissue and that cells were cultured as the adherent fraction in 5% FBS EGM-2 (Lonza, Basel, Switzerland) without heparin or hydrocortisone. After 4 days, cells were washed twice to remove non-adherent cells.

Isolation of extracellular vesicles from endothelial progenitor cells and dosing: Four days post-isolation, EPCs were changed to serum-free EGM-2 without heparin or hydrocortisone. After 48 hours, the conditioned medium (CM) was collected and cleared of cellular debris and large subcellular contaminants by centrifugation at 2000 xg for 30 minutes. The conditioned medium was then filtered through a 0.22 μm polycarbonate filter (PVDF) membrane. CM was then incubated in a 1:2 ratio with 36% PEG, 1.5 M NaCl overnight at 4°C, then pelleted by centrifugation at 10,000 xg at 4°C for 2 hours, and re-suspended in 0.22 μm-filtered Dulbecco’s Phosphate Buffered Saline (DPBS). EVs from donor animals were pooled and used for therapy at the time of isolation for the fresh EV treatment arm or stored at −80°C until use for the frozen EV treatment arm.

EVs were re-suspended at a concentration of 9.33 × 1011 particles/mL for in vitro use and at 1.87 × 1011 particles/mL for in vivo use. In case those samples were too dilute, they were repelled with ExoQuick-TC (System Biosciences, Palo Alto, CA) as per the manufacturer’s instructions.

Characterization of EPC-derived EV size and structure: EVs suspended in DPBS were analyzed using the NanoSight NS500 with green laser at 532 nm (Malvern Instruments, Malvern, UK). Samples were injected manually, and data acquisition was performed at ambient temperature. Data were analyzed using Nanoparticle Tracking Analysis software (NTA 2.2; Malvern).

Characterization of EPC-derived EV protein and total nucleic acid content: Protein content of previously frozen EPC-derived EVs was analyzed by mass spectrometry using MaxQuant version 1.5.1.2 (Max Planck Institute of Biochemistry, Munich, Germany). EPC-derived EV samples (n = 4) were isolated as previously mentioned and stored at −80°C prior to analysis. Network and pathway analyses were performed using MetaCore (Thomson Reuters, New York, NY), and statistical analysis was performed using the Perseus framework.

RNA was isolated from fresh and previously frozen EPC-derived EVs using the mirVana miRNA kit (Thermo Fisher Scientific, AM1560). Total nucleic acid purity and stability measurements of the samples were made immediately following isolation using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Values are reported as 260/280 ratios and nucleic acid concentrations in μg/μL.

Tubule formation assay: 10 μL of Matrigel (Corning, Cat #354234) was pipetted into the inner chamber of the μ-Angiogenesis slides (Ibidi, Cat# 81506). After a 30-minute polymerization period at 37°C, experimental conditions and 1.2 × 103 HUVECs were added to each well. Serum-free EGM was used as a positive control. After 14 hours of incubation, vascular mesh formation was visualized for each treatment group, with the EVOS XL Imaging System (Invitrogen) under bright field at 4x magnification. Vascular meshes were quantified using the count tool on ImageJ, and the response of HUVECS to fresh
and frozen EVs was normalized to the vehicle control.

**Shear-thinning gel synthesis:** STG was formed by modifying hyaluronic acid (HA; Lifecore, Chaska, MN) with adamantane (Ad; Acros Organics) and β-cyclodextrin (CD), as described previously. The sodium salt of HA was dissolved in deionized water at 4.5% w/w, exchanged against Dowex-100 resin, neutralized by tetrabutylammonium hydroxide (TBA), frozen, and lyophilized to form HA-TBA. For Ad-HA, HA-TBA was coupled with 1-adamantane acetic acid. For CD-HA, CD was modified with a hexane diamine linker and coupled to HA-TBA. Hydrogels of 4% w/w were prepared from lyophilized polymers of Ad-HA and CD-HA dissolved in PBS and mixed. All reagents were manufactured by Sigma-Aldrich unless otherwise indicated.

**Rat model of myocardial infarction:** An established rat model of MI induced by permanent occlusion of the left anterior descending artery (LAD) was used. Animals were then randomized into four groups: PBS control (n = 8), STG control (n = 12), STG+Fresh EV (n = 4), and STG+Frozen EV (n = 5). Animals were induced with 5% isoflurane, intubated, and mechanically ventilated with 1-

**Immunohistochemical analysis:** To assess angiogenesis in the infarct border-zone, 10 μm sections taken at the midpapillary level were stained with primary antibodies such as Biotinylated Griffonia Simplificofila Lectin I (GSL I) isolectin B4 (Vector Laboratories, B-1205-.5, 1:200), anti-alpha smooth muscle Actin antibody Alexa Fluor 594 conjugate (SMA-AS594; Abcam, ab202368, 1:300), and wheat germ agglutinin, Alexa Fluor 647 conjugate (WGA-AF647, Thermo Fisher Scientific, 1:300). Slides were then stained with secondary antibodies against native streptavidin protein conjugated with fluorescein isothiocyanate (FITC, Abcam, ab136201, 1:1000). Images were taken on the Leica DM5000b (Leica, Wetzlar, Germany) at 20x magnification using a DFC350 FX monochrome camera (Leica). Three high-power fields per animal were taken of the peri-infarct myocardium, defined as one field away from WGA-labeled scar. Images were quantified for total vasculature using ImageJ. Capillaries were identified as areas expressing isolectin B4 (1-B4) alone, while areas co-expressing I-B4 and SMA were identified as arterioles.

**Measurement of hemodynamics:** Hemodynamic measures were obtained 4 weeks following LAD ligation. After induction of anesthesia with 5% isoflurane, the rat was intubated and mechanically ventilated. Following confirmation of adequate surgical anesthesia maintained with continuous inhaled 2% isoflurane, a 2 Fr pressure-volume (PV) catheter (Millar, Houston, TX) was inserted into the left ventricle in a retrograde fashion via the right common carotid artery, in a closed chest approach. End-systolic elastance (Ees) was determined by temporary occlusion of the inferior vena cava using the methods described by Pacher, et al.

**Masson’s staining and scar thickness:** Hearts were explanted, embedded in OCT (Tissue-Tek, Torrance, CA), and frozen on dry ice. Thereafter, 10-μm transverse sections were taken via cryosectioning at the midpapillary muscle level. Sections were stained using Masson’s Trichrome (Sigma-Aldrich). Scar thickness, a measure of left ventricular (LV) remodeling, was taken at the midpoint of the scar using ImageJ (NIH).

**Histologic H&E analysis of inflammation:** Male Wistar rats (250-275 g) were randomly divided into five groups: sham control (n = 4), PBS control (n = 5), STG control (n = 4), PBS+EV (n = 5), and STG+EV (n = 4) in the absence of a myocardial infarction. A left thoracotomy was performed to enter the chest (see protocol details above). Upon exposure to the heart, 100 μL PBS, 100 μL STG, 9.33 × 10^5 EVs in 100 μL PBS, and 9.33 × 10^5 EVs in 100 μL STG were delivered with two injections around the left ventricle (LV), along with a sham treatment group of needle alone. Non-MI and MI animals were sacrificed 5 and 4 days, respectively, post-injections. Fresh hearts were explanted and fixed in 10% formalin at 4°C overnight for paraffin embedment. Hearts were sectioned and stained with H&E. The distribution of the immune cell infiltrate was analyzed with a light microscope by our expert collaborators in the Veterinary School of the University of Pennsylvania Pathology Core. Each slide was graded on a scale from 0 to 3 for inflammatory changes in comparison to healthy tissue.

**Flow cytometry for detection of in vivo immune response:** Various immune cell types were analyzed through flow cytometry in the absence of a MI (Supplemental Figure 1). At day 1 or day 7 post-injection of sham, PBS, STG, PBS+EV, and STG+EV into the LV, animals were euthanized, and their hearts explanted and washed in PBS. The LV was isolated and digested in collagenase II in PBS with calcium chloride and magnesium chloride (Worthington Biochemical Corporation). Cells were then isolated from the digested heart through a 70-μm cell strainer and aliquoted at 2 × 10^6 per sample for flow cytometry. These samples were then further spun down at 400 xg for 5 minutes and resuspended in 100 μL of FACS-FC Buffer (2.5% Fetal Bovine Serum (Life Technologies) in PBS with CD32 (1:500)). Following a 5-minute incubation at 4°C, the samples were stained for CD43 (1:500) (BD Biosciences), CD161 (1:300) (BD Biosciences), CD4 (1:500) (Biolegend), CD3 (1:150) (BD Biosciences), CD11b (1:150) (BD Biosciences), CD8a (1:150) (BD Biosciences), CD45RA (1:500) (BD Biosciences), and CD45 (1:150) (BD Biosciences). The antibody cocktail was prepared in Brilliant Stain Buffer (BD Biosciences), and each sample was stained with 50 μL of the antibody cocktail. Afterward, the stained cells were incubated at 4°C in the dark for 30 minutes. The cells were washed three times with 2 mL of FACS buffer (2.5% Fetal Bovine Serum in PBS). Following the last wash step, the samples were resuspended in 0.5-1 mL of FACS buffer and analyzed through a flow cytometer (BD LSR II) within an hour.
Statistical analysis: The primary endpoint of the in vivo experiment was contractility, measured by end-systolic elastance (Ees). Secondary endpoints 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 were expressed as mean ± standard error of the mean (SEM). Comparison across experimental groups was performed by one-way analysis of variance (ANOVA). When a significant difference between groups was performed by Tukey's HSD test. P-values < 0.05 were considered statistically significant. Graphs show mean values with SEM.

Results

EV size, proteome, and total nucleic acid cargo remained steady through extended cryopreservation: The mean particle sizes of fresh EVs and EVs frozen for 2 months were found to be statistically similar, as determined by NTA, 123.2 ± 4.8 nm versus 125.5 ± 0.9 nm (P = 0.38), respectively (Figure 1A). Similarly, the modes showed no statistically significant difference between the two groups — 91.3 ± 2.8 vs. 93.3 ± 5.2 nm, P = 0.47, with similar median values of the fresh versus frozen groups, 108.5 versus 111.5 nm, P = 0.058, respectively. Within the EV population, the fraction falling within the size range of exosomes (50-150 nm), which are thought to be the most biologically active subgroup, was essentially unchanged after 2 months of freezing: 79.1% versus 78.1%, P = 0.51, in the fresh versus frozen groups, respectively.

Mass spectrometric analysis revealed that the frozen EVs expressed a number of known EV markers as well as factors known to be contained within EVs, including angiogenic growth factors, matrix proteins, and enzymes (Figure 1B). EV markers such as CD9, CD63, and CD61 were also detected in high concentrations following storage at −80°C. In addition, growth factors commonly associated with angiogenesis such as ANPEP, NRP1, and ANXA2 were present at similarly high concentrations following storage. Matrix proteins such as MMP9 and TIMP2 were also part of the EV proteomic profile. Spectral counts of proteins can be seen in Supplemental Table I. These proteins are the major mediators of change in the extracellular matrix following ischemic myocardial injury, which are known to be involved in several acute inflammatory processes.

Total nucleic acid analysis over 8 weeks showed that total nucleic acid from frozen EVs maintained similar measures of purity, as compared to fresh EVs (Figure 1C). There was minimal degradation over time, with serial measures of total nucleic acid concentration demonstrating stability at up to 8 weeks of storage at −80°C (Figure 1)
EV therapy post-storage promotes angiogenesis: A 5% in vivo dose of EVs was given per treatment well of HUVECs seeded on Matrigel (Figure 2A). HUVECs treated with fresh EVs showed an average of 3.2 ± 0.3-fold ($P = 0.03$) increase in cellular mesh formation in comparison to HUVECs in EBM, the negative control. HUVECs treated with EVs previously frozen for at least 2 months also outperformed the EBM group by a factor of 3.0 ± 0.62, ($P = 0.04$) on average.

Angiogenesis in the in vivo model was assessed via immunohistochemical analysis of hearts explanted at 4 weeks post-MI (Figure 2B, C). There was increased total vessel and capillary formation for both EV+STG treatment groups over STG or PBS alone (Figure 2B). Total vessel counts - arterioles and capillaries - of PBS versus STG animals averaged 1400 ± 110 vessels/mm² versus 2100 ± 77 vessels/mm², respectively ($P = 0.06$). Treatment of previously frozen EV+STG therapy led to 2600 ± 150 vessels/mm², ($P = 0.0001$ compared to PBS), while fresh EV+STG therapy yielded 2300 ± 120 ($P = 0.0007$ compared to PBS). Total vessel count in the frozen EV+STG group was not statistically significantly different when compared to the fresh EV group, $P = 0.46$ (Figure 5A).

Similar results were observed for capillary counts, with the PBS control group averaging 1200 ± 92 capillaries/mm², STG cohort with 1800 ± 100 capillaries/mm² ($P = 0.04$ versus PBS), fresh EV+STG cohort with 2100 ± 95 capillaries/mm² ($P = 0.0002$ versus PBS), and previously frozen EV+STG cohort with 2300 ± 140 capillaries/mm² ($P < 0.0001$ versus PBS, $P = 0.03$ versus STG). The two EV+STG treatment groups were similar in capillary counts ($P = 0.38$).

Cryopreserved EV therapy preserves post-MI contrac-
tility and scar thickness in myocardium: Delivery of both fresh and previously frozen EVs in STG conferred hemodynamic benefits, with the fresh EV+STG treatment group showing a contractility of 0.60 ± 0.03 mmHg/uL (P = 0.0005, 0.0003 versus PBS, STG controls, respectively) while the cohort receiving previously frozen EV+STG treatment had a contractility of 0.57 ± 0.05 mmHg/uL (P = 0.0008, 0.0004 versus PBS and STG groups, respectively) (Figure 3). The previously frozen EV+STG treatment group had similar contractility to the fresh EV+STG group (P = 0.97). Contractility of PBS-injected animals was 0.29 ± 0.04 mmHg/uL at 4 weeks post-infarct, whereas animals receiving STG treatment had a contractility of 0.29 ± 0.03 mmHg/uL (P > 0.99).

Explanted hearts were cryosectioned, and Masson’s staining was used to differentiate healthy myocardium from scar. The thickness of the ventricular wall in the region of the post-infarction scar was measured and is referred to as scar thickness. Scar thickness for animals treated with fresh and frozen EVs was both significantly greater than those given PBS alone — 0.68 ± 0.04 mm (P = 0.03) and 64 ± 0.07 mm (P = 0.21), respectively (Figure 4). The PBS control group had the thinnest scars, averaging 0.49 ± 0.04 mm, whereas animals with STG injections had average scar thickness of 0.56 ± 0.05 mm. Animals receiving previously frozen EV+STG therapy had scar thickness values similar to those receiving fresh EVs (P = 0.96).

Pooled EV in STG shows similar immune response in myocardium compared to saline: Immune cell activity after treatment injections were investigated with H&E analysis of cardiac tissue 5 days after injection of sham, PBS, STG, PBS+EVs, or STG+EVs into the LV myocardium. A scattered pattern of inflammatory cell infiltrate, predominantly histiocytes and lymphocytes with lesser granulocytes, around the sites of injection was observed. There was no evidence of surrounding myocyte death or damage. A quantitative pathology score demonstrated no significant difference in inflammatory changes across all control and treatment groups (P = 0.55, Figure 5).

Flow cytometric analysis revealed that STG+EV therapy generally elicits similar levels of immune cell infiltration in the LV in comparison to saline (Figure 6). During the acute stage (day 1), STG and STG+EV groups showed similar increases in leukocytes, neutrophils, and T cells as PBS. Immune cells persisted until day 7, with STG, STG+EV, and PBS groups showing significant increases in leukocytes, B cells, and CD4+ T cells. In comparison, EVs in PBS showed lower levels of leukocytes, neutrophils, natural killer cells, and T cells in comparison to saline injection alone at day 1. Similar results were seen at day 7, with EVs mitigating the influx of both innate and adaptive immune cells across the board in comparison to PBS - such results may be indicative of the immunomodulatory role that EVs can play in the myocardium following an infarct.

Figure 3. Fresh and cold-stored EV therapies promote in vivo contractility post-infarct. Delivery of therapy using either fresh or previously frozen EVs has led to improved contractility in comparison to controls. Fresh EVs showed no added benefit in contractility compared to previously frozen replicates (P = 0.97).

Figure 4. Scar thickness is preserved after treatment of fresh and frozen EPC-EVs in STG. A: Representative panel of frozen midpapillary sections from hearts sacrificed at 4 weeks post-MI and injection with either fresh or frozen EVs. Stained with Masson’s Trichrome. B: Hearts treated with fresh EPC-EVs delivered in STG demonstrated greater scar thickness compared to those in the PBS control group (P = 0.031). There is no significant difference in scar thickness between the fresh and frozen treatment groups (P = 0.96).
Discussion

This study aimed to improve our understanding of the effects of long-term storage and allogeneic pooling on the physical, biochemical, and functional properties of EVs in the context of a unique delivery method within an STG. Previous studies have demonstrated preservation of EV markers CD9, CD63, and CD81 following storage at −80°C for several months.20) Our deeper analysis of EPC-EV cargo and structure post-storage builds upon these findings, as proteomic and total nucleic acid cargos, as well as median particle size, were all shown to remain steady through a minimum 2-month period of cryopreservation.

In addition to physical properties, previous studies have also assessed the stability of the functional effect of cryopreserved EVs in in vitro models of therapy. In one study assessing EV storage, Lorinz, et al. showed that the antibacterial capacity of EVs significantly decreases over a period of 28 days in storage at 20, 4, and −20°C. However, storage at −80°C preserved EV bioactivity in comparison to storage at higher temperatures, though the antibacterial effect of EVs still tended to decrease with increased storage times.21) Similarly, we found that storage at −80°C preserves angiogenesis in an in vitro endothelial cell tube formation assay.

While physical properties and in vitro function of frozen EVs have been demonstrated, to our knowledge, no studies exist to date that attempt to capture the cryopreservability of EV function in in vivo models of therapy.
In our rat model of MI, we observed similar, statistically significant increases in both myocardial vascular density and LV contractility in rats treated with both fresh and frozen EV+STG, compared to controls. As observed previously, our STG alone had angiogenic properties due to the HA component of the gel. HA induces the RHAMM-TGFβ interaction, an angiogenic mechanism largely studied in literature. Moreover, ventricular analysis of rat hearts at 4 weeks post-MI revealed similar preservation of infarct wall thickness between STG+EV treatment groups. These findings may indicate decreased LV dilatation and mitigation of adverse remodeling, which may be due to the negative regulation of fibroblasts. Recent studies of EV mechanisms in ischemic cardiac injury have shown that EVs may negatively regulate fibrosis via miR-155 signaling. Our EV therapy may suppress fibroblast signaling and proliferation, thereby inhibiting scar formation. These results, when taken together, only demonstrate that EV physical integrity, angiogenic potential, and functional benefits are well preserved when EVs are stored at −80°C for a minimum of 2 months. Such findings give further credence to the translation of EV therapy to a clinical setting, in which maintaining a supply of fresh EVs would be technically and financially prohibitive.

In addition to the question of cryopreservability of EV therapy, there have previously been widely held assumptions of the EV as non-immunogenic, evading detection by the immune system, and many groups - including our own - have used EVs pooled from multiple donors in animal studies with an overall beneficial effect. One study by Marino, et al. implicated exosomes as transferring donor MHC molecules to recipient cells, thus serving as the mechanism for triggering early acute rejection of solid-organ transplants. These MHC-expressing exosomes were capable of stimulating T cell immune responses both in vitro and in vivo in a mouse model. Another study demonstrated that transplantation of allogeneic EPC-derived endothelial cells expressed only low levels of MHC class I and no constitutive MHC class II and were protected against destruction via complement-mediated lysis or cytotoxic T cell activity. Although this data suggests that EVs derived from EPCs would similarly have low immunogenic potential, to our knowledge, no studies have rigorously examined the immune effects of donor-pooled EPC-EVs.

H&E analysis on injured myocardium demonstrated modest infiltration of inflammatory cells related to the injection itself with minimal concomitant immunologic reaction, cell death, and damage associated with EV therapy. Such findings showed that our EV therapy yielded no gross increases in cellular inflammation in comparison with saline, or even sham (needle), groups.

However, interestingly, upon flow cytometric analysis, we found notable differences in leukocyte populations between groups. Particularly, EPC-EVs in PBS showed modulation in both the innate and adaptive immune responses following myocardial injury associated with injection. Such findings are found to be consistent with previous studies that have shown that EVs may have immunoregulatory effects through a host of different cellular pathways. These immunomodulatory effects, however, were not seen in our STG+EV therapy, which showed similar levels of immune cell infiltration as PBS control. Given this disparity in STG+EV therapy versus EVs in PBS alone, it is possible that a higher initial bolus of EVs-given during the acute phase of the immune response - is necessary to realize a notable decrease in inflammatory milieu post-injection. Since the STG serves as a slow-release agent for EVs, we may be missing such immunomodulatory effects that occur during the acute phase.

However, despite potentially foregoing these anti-inflammatory properties, our STG+EV therapy has previously been shown to confer hemodynamic benefits that exceed EV therapy alone. This may indicate that the other downstream effects of our therapy - such as angiogenesis and mechanical support of the ventricular wall - may be enough to supersed the trade-off in immunomodulatory and anti-inflammatory effects of the EV itself. Furthermore, key components of the adaptive immune system, such as CD4+ and CD8+ T cells, and B cells, were similar between PBS, STG, and STG+EV groups. These data suggest that, though we do not evoke immunoregulation, there is no increased immunogenic response in the setting of our novel therapy.

In summary, our findings suggest that when applied to a clinical setting as a potential therapeutic agent after MI, EVs frozen at −80°C for 2 months or longer will deliver the same effect as EVs that are freshly isolated. This has positive implications for the feasibility of administering frozen EVs as an off-the-shelf treatment without a decrement in therapeutic effect and without the need for elaborate processing and storage procedures to maintain EV integrity. Additionally, the EVs showed minimal directed immune response in the absence of MIs, allowing for the therapeutic use of allogeneic donor cells. This study provides exciting and necessary evidence that EV therapy from multiple allogeneic donors can be safely processed, stored, and delivered in a clinical setting with retained effectiveness when maintained in these relatively straightforward conditions.

Acknowledgments

The authors would like to thank the Proteomics Core Facility at the Children’s Hospital of Philadelphia (CHOP) research institute for assistance with proteomic data acquisition and analysis.

Disclosure

Conflicts of interest: None.

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