Extracellular vesicle therapeutics for cardiac repair: A translational perspective

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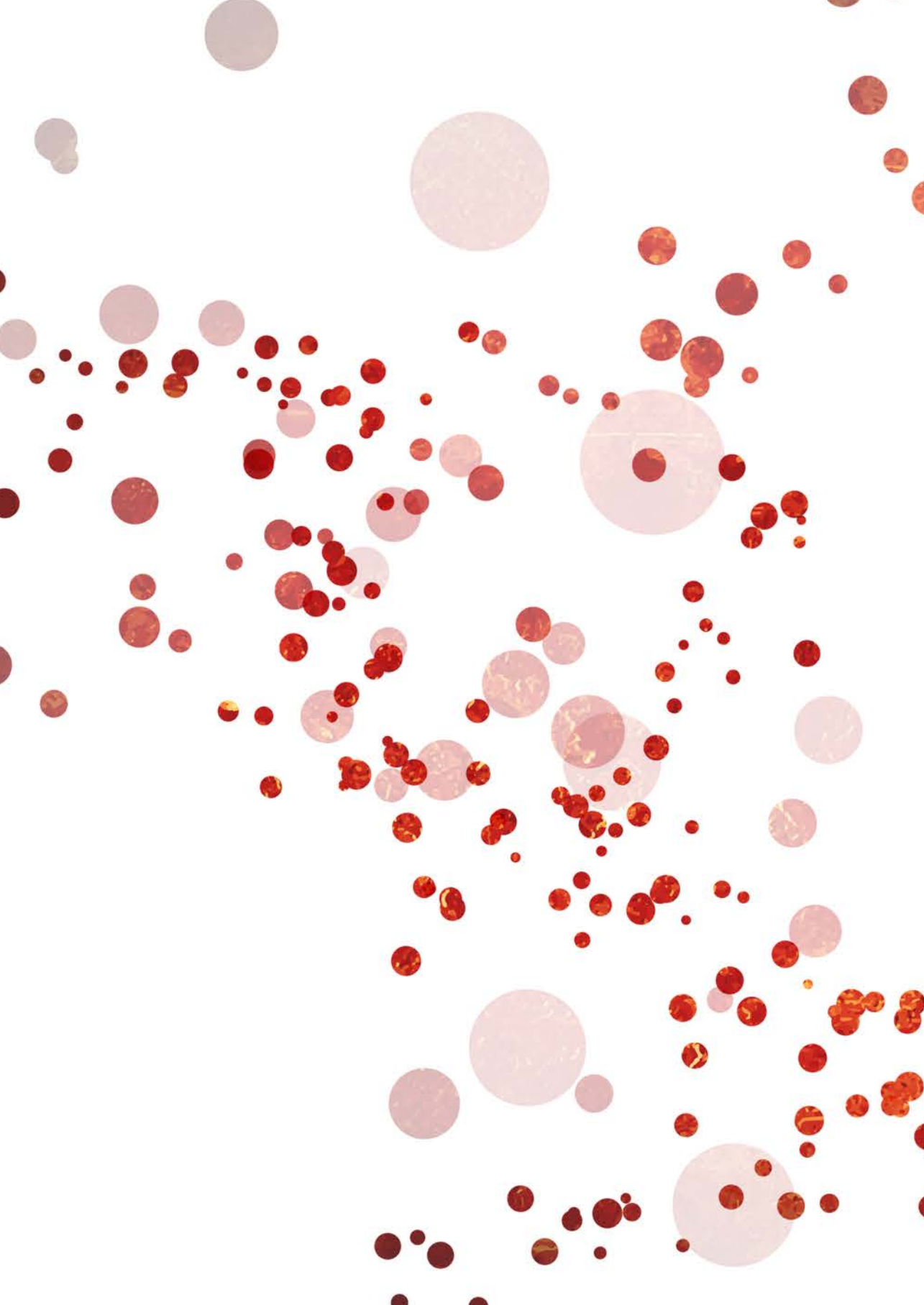

CHAPTER 3

Cardiac progenitor-derived extracellular vesicles do not reduce infarct size after myocardial infarction in mice, independently of isolation method

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In preparation

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ABSTRACT

Introduction

Local injection of extracellular vesicles (EVs) is a potential cell-free therapeutic approach for cardiac repair after myocardial infarction (MI). The enhanced cardiac function after progenitor cell transplantation is mainly ascribed to EVs released by the injected cells, containing bioactive paracrine factors. Previously, cardiac progenitor derived (CPC)-EVs have been shown to reduce infarct size 2 days after experimentally induced MI in mice. Ultrafiltration combined with size-exclusion chromatography (SEC) is a scalable EV isolation method that could potentially be used for the production of clinical-grade EVs. Recently, we have demonstrated that EVs isolated using size-exclusion chromatography-isolated EVs (SEC-EV) display enhanced functionality *in vitro* as compared to ultracentrifugation-isolated EVs (UC-EV). Here, we aimed to investigate whether SEC-EVs also have improved therapeutic efficacy when compared to UC-EVs *in vivo,* using two different preclinical models of MI.

Methods

CPCs were cultured in serum-free medium for 24 hours after which CPC-EVs were isolated using either UC or ultrafiltration, combined with SEC. Severe Combined Immunodeficient (SCID) mice underwent either a permanent ligation of the left anterior descending artery or ligation followed by reperfusion after 1 hour of ischemia and received treatment with UC-EVs or SEC-EVs by intramyocardial injection. After 2 days, a double blinded analysis was performed to assess infarct size (IS) and area at risk (AAR). Levels of plasma Troponin I and global longitudinal and reverse longitudinal strain rates were also measured. To confirm if the injected EVs displayed functionality *in vitro*, EVs were added to human microvascular endothelial cells (HMEC-1) *in vitro* and activation of extracellular signal regulated kinase 1/2 (ERK1/2) pathway was assessed.

Results

The isolated EVs were bioactive as shown by their ability to activate the ERK1/2 pathway in HMEC-1 cells *in vitro*. However, upon injection of either UC-EVs or SEC-EVs post MI, IS analysis showed no change in IS/left ventricular area 2 days after treatment in the permanent ligation model and the reperfusion infarct model, in which also IS/AAR did not change compared to PBS injected animals. Furthermore, strain analysis showed no significant differences in global or reverse longitudinal strain values between PBS, UC-EV, or SEC-EV treated groups in the reperfusion model. Plasma levels of Troponin I did not significantly differ between the groups in either infarct model.

Conclusion

As no change in infarct size was observed 2 days after treatment with UC-EV or SEC-EV compared to PBS in two different infarct models, we were not able to determine if the choice of EV isolation method influences therapeutic efficacy of EVs *in vivo*. These results are in contrast with previously reported studies using CPC-EVs that demonstrated reduced infarct size at 2 days post MI. Future studies should address if potential differences between CPC clones, culture media, or dosing could explain these different outcomes.

INTRODUCTION

Cardiovascular disease (CVD) is one of the most prevalent causes of death worldwide, with myocardial infarction (MI) accounting for one third of all deaths by CVD[1]. Although acute mortality decreased due to improved arterial reperfusion strategies, this also induces additional damage, referred to as ischemia-reperfusion (I/R) injury. Approximately 25% of patients that survive a MI are known to develop heart failure (HF)[2]. Currently, there is no curative treatment available for chronic heart failure patients besides heart transplantation. Progenitor cell therapy has been proposed as potential therapy to prevent HF development by reducing I/R injury and reducing infarct size. While cell types of different origins (e.g. mesenchymal stromal cells, bone marrow derived mononuclear cells) have been used, cardiac progenitors have gained attention because of their cardiac origin and the possibility to differentiate in all cardiac cell types[3,4]. CPC therapy in mice has been shown to increase ejection fraction and inhibit adverse ventricular remodeling post-MI[5]. However, despite functional improvement, a low cell engraftment was observed after intramyocardial injection. This could be due to an immediate wash-out of cells through the venous drainage after intramyocardial injection, which we previously described[6]. Using carriers such as gelatin microspheres reduced this wash-out of cells, but did not translate into increased beneficial effects on cardiac function[7]. Given the low retention despite functional improvement, it has been suggested that paracrine factors secreted by CPCs cause this beneficial effect on cardiac function. Indeed, administration of conditioned medium from progenitor cells also preserved cardiac function post-MI, thus resembling studies injecting the cells themselves[8]. Further studies showed that extracellular vesicles (EVs) are important carriers of bioactive paracrine factors responsible for this beneficial effect[9–11].

EVs are nanosized lipid bi-layered particles that play important roles in intercellular communication in both health and disease. After internalization by recipient cells, EVs are able to transfer their biological cargo (e.g. mRNAs, small RNAs, proteins, and lipids), resulting in phenotypical changes[12–15]. Recently, we demonstrated that EV secretion from CPCs is essential to reduce infarct size[16], since Rab27A knock down-CPCs were not able to reduce infarct size in mice after 2 days as compared to control CPCs. Indeed, direct CPC-EV treatment reduced infarct size 2 days after permanent ligation of the left anterior descending artery (LAD)[16]. Thus, CPC-EVs may be a potential off-the-shelf therapy to induce cardiac repair, aiming to treat chronically diseased patients.

Currently, ultracentrifugation, based on high-speed sedimentation, is the most commonly used isolation method for EVs. Although widely used, EV isolation using UC is a labor intensive method, yield is operator dependent, and is limited in its ability to isolate a sufficient numbers of EVs for clinical application[17,18]. In contrast, ultrafiltration combined with size-exclusion chromatography (SEC) is a scalable method that could potentially be used for clinical-grade production of EVs[19]. Moreover, we have previously shown that isolating EVs using SEC yields EVs with a higher bioactivity compared to UC in a functional assay *in vitro*[20]. To investigate whether these differences also translate to distinct behavior *in vivo*, we here sought to determine whether UC-EV and SEC-EV have different therapeutic efficacy in two commonly used infarct models in mice.

MATERIAL AND METHODS

Cell culture

Human microvascular endothelial cells (HMEC-1 cells) were cultured in HMEC-1 medium (83,8% MCDB-131 (Invitrogen, 10372019), 0.1% human EGF (Peprotech/Invitrogen, 016100-15-A,), 0.1% Hydrocortison (Sigma, H6909-10), 1% Penicillin/Streptomycin (Invitrogen, 15140122), 10% fetal bovine serum (FBS) (Life-Tech, 10270), and 5% L-glutamine (Invitrogen, 25030024). Human fetal heart tissue was obtained by individual permission using standard written informed consent procedures and prior approval of the ethics committee of the Leiden University Medical Center, the Netherlands. This is in accordance with the principles outlined in the Declaration of Helsinki for the use of human tissue or subjects. Cardiac progenitor cells (CPCs) were cultured in SP++ medium (22% EGM-2 (Lonza, CC-4176), 66% Medium 199 (Gibco, 31150-022), 10% FBS (Life-Tech, 10270), 1% Penicillin/Streptomycin (Invitrogen, 15140122), and 1% MEM nonessential amino acids (Gibco, 11140), as described before[3]. Cells were incubated at 37˚C with 5% CO_2 and 20% O_2 and passaged at 80-90% confluency after digestion with 0.25% trypsin-EDTA. Before EV collection, CPCs were cultured for 3 days on SP++ medium until 80-90% confluency, followed by 24 hours of culture on Medium 199 without any additives.

EV isolation

EV isolation was performed as described before[20]. In brief, conditioned medium (CM) was collected after 24 hours of culture in Medium 199 without any additives, centrifuged for 15 min at 2000 x *g* and passed through a 0.45 µm filter (0.45 µm Nalgene filter bottles) to remove cell debris. Next, the CM was equally divided, and half was used for EV isolation using ultracentrifugation (UC), and the other half for EV isolation using ultrafiltration followed by size-exclusion chromatography (SEC). For UC, EVs were pelleted by a 1 hour centrifugation at 100.000 x *g* using a type 50.2 Ti fixed-angle rotor. EVs were washed with PBS, followed by a second 100.000 x *g* centrifugation step. For SEC, CM was concentrated first using 100-kDA molecular weight cut-off (MWCO) Amicon spin filters (Merck Millipore). Next, the concentrated CM was loaded onto a S400 high-prep column (GE Healthcare, Uppsala, Sweden) using an AKTAStart (GE Healthcare) containing an UV 280nm flow cell. The fractions containing the EVs were pooled and again concentrated using a 100-kDA MWCO Amicon spin filter. After isolation, both EV preparations were passed through a 0.45 um filter. Number of particles was determined using Nanoparticle Tracking Analysis (Nanosight NS500, Malvern), using a camera level of 15 and a detection threshold of 5.

EV functionality

1.2 x 10^5 HMEC-1 cells were plated into a 48-wells plate the day before stimulation. HMEC-1 cells were starved using basal MCDB131 medium for 3 hours before stimulation. To stimulate HMEC-1 cells, 6 x 10^{10} EVs were added for 30 minutes to the starvation medium, unless stated otherwise. Cells were put on ice, lysed using lysis buffer (Roche, 04719964000), and centrifuged at 14.000 x *g* for 10 minutes to remove cell debris. Protein levels of phosphorylated extracellular signal regulated kinase 1/2 (pERK1/2) and total ERK 1/2 (ERK1/2) were determined using Western Blotting as described below.

Western blotting

Lysates were loaded onto pre-casted 4-12% Bis-Tris protein gels (ThermoFisher, NW04125BOX). The gels were run for 1 hour at 160V, followed by blotting of the proteins on PVDF membranes (Millipore, IPVH00010). Membranes were blocked with 5% BSA in TBS and incubated with antibodies against 42/44 pERK1/2 (1:1000) (Cell Signaling, 43705) or 42/44 ERK1/2 (1:1000) (Cell Signaling, 91025) dissolved in 0.5% BSA in TBS for 1 hour. Membranes were washed 3 times for 5 minutes with TBS-0.1%Tween, followed by incubation with secondary goat anti-rabbit HRP antibody (Dako, P0448) dissolved in 5% milk in TBS-0.1%Tween for 1 hour. Next, membranes were washed with TBS for 30 minutes and the proteins were detected with chemiluminescent peroxidase substrate (Sigma, CPS1120) using a Chemi Doc™ XRS+ system (Bio-Rad) and Image Lab™ software.

Animal experiments

All animal experiments were performed conform the 'Guide for the Care and Use of Laboratory Animals' and carried out after approval by the Animal Ethical Experimentation Committee, Utrecht University, the Netherlands. Healthy male NOD.CB17-Prkdcscid/NCrHsd mice (age 10-14 weeks, weight 20-30g) were obtained from Envigo and received standard chow and water *ad libitum* and were housed under standard conditions with 12-h light/dark cycles until experimental procedures. Animals were randomized to one of the three groups and all operators were blinded during surgery, intramyocardial injections, and subsequent analyses.

Induction of myocardial infarction and EV therapy

Mice were anesthetized by intraperitoneal injection of medetomidine (0.05 mg/kg body weight), midazolam (5 mg/kg) and fentanyl (0.5 mg/kg), followed by intubation and connection to a respirator with a 1:1 oxygen-air ratio (times/min). Body temperature was maintained at 37 °C during surgery using an automatic heating blanket. Left lateral thoracotomy was performed to access the heart. For permanent ligation, the LAD was ligated using an 8-0 Ethilon suture (Ethicon). For ischemia/reperfusion, the LAD was ligated for 1 hour using an 8-0 Ethilon suture (Ethicon) and a polyethylene-10 tube to ensure easy removal of the suture. Ischemia was confirmed by observing immediate blanching of the cardiac tissue upon LAD ligation. Two times five µl of either PBS, UC-EVs, or SEC-EVs were intramyocardially injected into the border zone using a 30G needle, either 15 min after permanent ligation or at the moment of reperfusion. The injected EV dose was 10×10^{10} particles. All surgeries and injections were performed by blinded operators. Next, the surgical wounds were closed, followed by a subcutaneous injection of antagonist consisting of atipamezole hydrochloride (3.3 mg/kg), flumazenil (0.5 mg/kg), and buprenorphine (0.15 mg/kg) as pain relief.

Termination

2 days post-injection, mice were euthanized after being anesthetized using sodium pentobarbital (60.0 g/kg). For the I/R injury model, blood was collected in EDTA tubes through orbital puncture. Next, the LAD was again ligated at the same place as MI induction.

The thoracic aorta was cannulated with a 21-24G catheter (Abbocath) using a 1 ml syringe. 4% of Evans Blue dye was injected until a clear demarcation between area-at-risk and healthy tissue was apparent. The heart was removed and washed with PBS several times to remove excess dye. Heart weight and tibia length were assessed.
For permanent ligation, mice were euthanized after being anesthetized using sodium pentobarbital (60.0 g/kg). Blood was collected through orbital puncture in EDTA tubes to determine plasma levels of Troponin I. The heart was flushed through the right ventricle using 5 mL of PBS. Heart weight and tibia length were assessed.

Infarct size
To determine infarct size, the hearts were cut into 3-4 equal slices using razor blades. Next, slices were incubated with 1% TTC solution at 37°C for 15-20 minutes followed by incubation in formalin for 5-10 minutes. Slices were weighed to be able to correct for differences in slice size. Images were taken using a SZH10 Olympus Zoom Stereo Microscope and IC Capture software, version 2.4. For ischemia-reperfusion injury, the infarct area (white), area-at-risk (red), and healthy area (blue) were assessed to calculate infarct size. Infarct size (IS) was expressed as a percentage of area-at-risk (AAR) and total left ventricle (LV). For permanent ligation, infarct area (white) and healthy area (red) were used to determine infarct size. Infarct size was expressed as percentage of total left ventricle.

Echocardiography
At baseline and 2 days after I/R injury, anesthesia was induced by inhalation of 2.0% isoflurane in a mixture of oxygen/air (1:1). Echocardiography was used to determine global longitudinal strain and reverse strain rate. Heart rate, respiration, and rectal temperature were constantly monitored, and body temperature was maintained at 36-38˚C using heat lamps. Images were taken and analyzed using the Vevo 2100 System and VevoLab Software (Fujifilm VisualSonics Inc., Toronto, Canada).

Statistical analysis
Statistical analysis were performed using SPSS and Graphpad Prism. Data are presented as mean ± SD. Data were tested for normal distribution. One-way ANOVA was used to compare multiple groups and data was corrected for multiple testing using Tukey post-hoc test.

RESULTS

Previously, we have shown that EV isolation technique can affect their functionality *in vitro*, as SEC isolation resulted in more functional EVs compared to UC isolation[20]. To investigate if the choice of EV isolation method also affects EVs' therapeutic efficacy *in vivo*, mice were subjected to permanent ligation (Figure 1A) and treated with either UC-EVs or SEC-EVs 15 minutes after ischemia induction. After 48 hours, surprisingly, infarct size as determined by IS/LV did not differ between PBS and SEC-EV-treated groups (32.6 ± 12.0 vs 29.1 ± 11.1%, p = 0.73; Figure 1B+C) nor between UC-EV and SEC-EV-treated groups (28.7 ± 8.7 vs 29.1 ±

11.1%, p=0.99). Next, in order to investigate if EVs potentially affect a different mechanism, mice were subjected to 60 minutes of ischemia, followed by reperfusion and treatment with either UC-EVs or SEC-EVs (Figure 1D). Also in this model, infarct size after I/R injury, as assessed by IS/AAR, did not differ between PBS and SEC-EV treated groups (46.9 ± 13.6 vs 49.4 ± 8.8%, respectively, p=0.92; Figure 1E+F) nor between UC-EV and SEC-EV treated groups (50.0 ± 20.2 vs 49.4 ± 8.8%, respectively, p=0.99). In addition, IS/LV was comparable between all groups (19.1 ± 7.9 for PBS vs 17.0 ± 8.8% for SEC-EV, p=0.88 and 22.3 ± 7.9 for UC-EV vs 17.0 ± 8.8% for SEC-EV, p=0.36; Figure 1G), as was AAR/LV (42.6 ± 15.5 for PBS vs 36.1 ± 22.7% for SEC-EV, p=0.67 and 45.3 ± 9.7 for UC-EV vs 36.1 ± 22.7% for SEC-EV, p=0.48; Figure 1H).

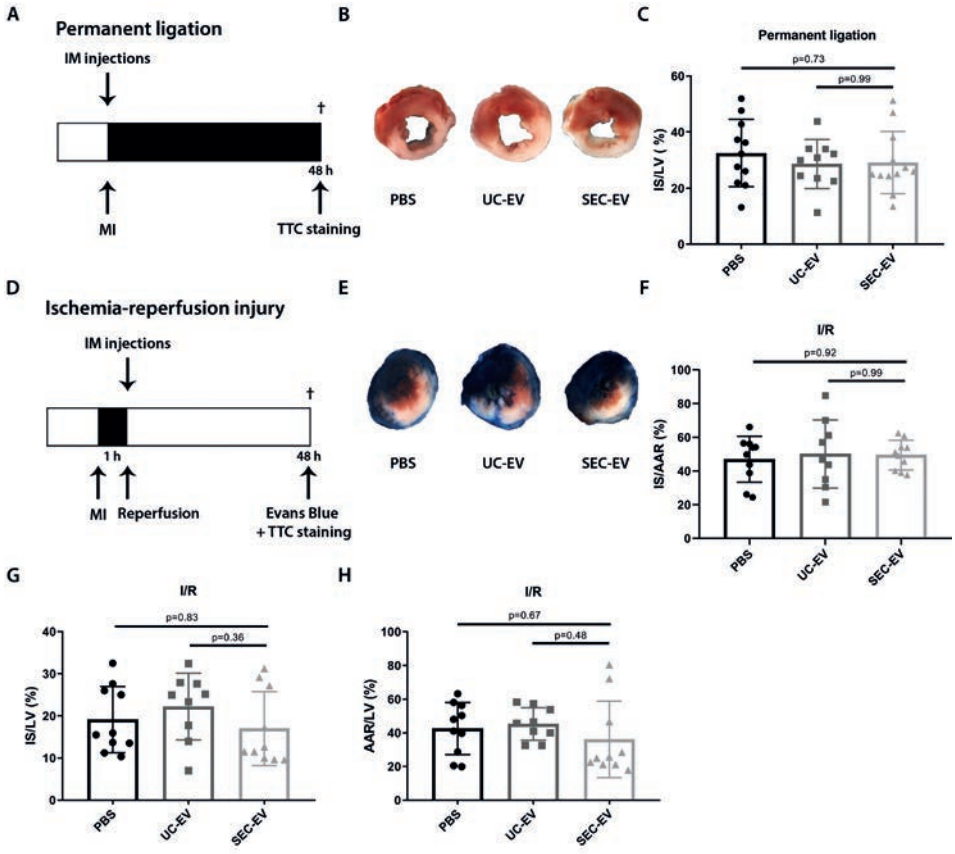

Figure 1 Infarct size is not affected by treatment with UC-EVs or SEC-EVs after experimentally induced ischemia in two mouse infarct models.
A) Timeline and experimental set-up of the permanent ligation model. B) Representative images of TTC stained heart sections. C) Infarct size was not different between PBS, UC-EV or SEC-EV treated groups after permanent ligation. D) Timeline and experimental set-up of the ischemia-reperfusion injury model. E) Representative images of Evans Blue and TTC stained heart sections. F) Infarct size expressed as IS/AAR was comparable between all the groups, as was IS/LV (G) and AAR/LV (H).

While volume measurements are used to assess cardiac function at a later stage, speckle tracking analysis is more sensitive to detect early myocardial deformations. Therefore, global longitudinal strain (GLS) and reverse longitudinal strain rates (rLSR) were assessed to evaluate myocardial contractility 2 days post-I/R injury. GLS did not differ between PBS and SEC-EV treated groups (-12.5 ± 5.1 vs -12.1 ± 3.9, p=0.99; Figure 2A) nor between UC-EV and SEC-EV treated groups (-10.7 ± 7.5 vs. -12.1 ± 3.9, p=0.85). Furthermore, also rLSR, a measure of diastolic dysfunction, was comparable between the groups (5.9 ± 3.5 for PBS vs 4.2 ± 2.3 for SEC-EV, p=0.62 and 6.0 ± 4.9 for UC-EV vs 4.2 ± 2.3 for SEC-EV, p=0.57). In summary, we did not observe early functional improvement after treatment with EVs.

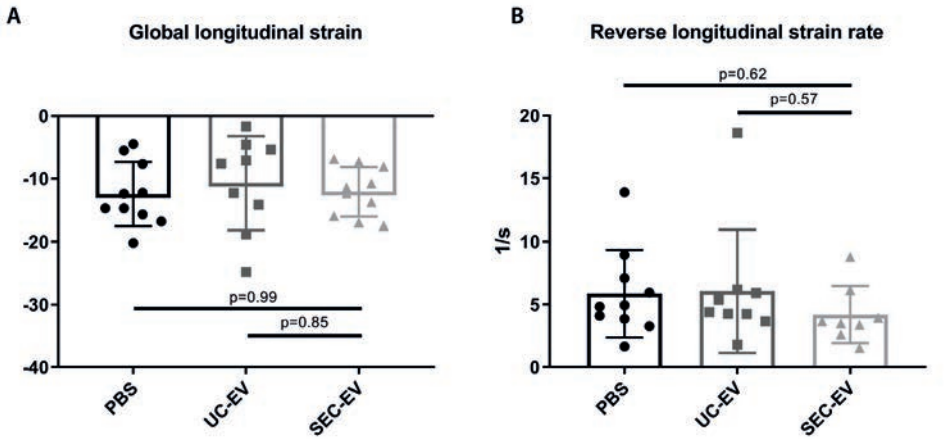

Figure 2 Global longitudinal strain and reverse longitudinal strain rate are unaffected after treatment with UC-EVs or SEC-EVs.
Strain analysis was performed 2 days post-I/R injury. No functional improvement was observed after treatment with either UC-EVs or SEC-EVs as assessed by global longitudinal strain (A) and reverse strain rate (B).

Cardiac troponin I is a well-known biomarker used for detection of acute myocardial injury. Therefore, plasma levels of cardiac troponin I were assessed to evaluate the amount of cardiac muscle damage in all three groups 2 days post-MI. Troponin I levels did not differ between PBS and SEC-EV treated groups in the permanent ligation model (41811 ± 18539 vs 34369 ± 16174 ng/L, respectively, p = 0.67; Figure 3A) nor between UC-EV and SEC-EV treated groups (44311 ± 27372 vs 34369 ± 16174 ng/L, respectively, p=0.51). Similar findings were observed for the I/R injury model (95.3 ± 78.0 for PBS vs 189.9 ± 291.7 ng/L for SEC-EV, p = 0.90 and 401.6 ± 824.7 for UC-EV vs 189.9 ± 291.7 ng/L for SEC-EV, p=0.62; Figure 3B). Next, the ratio between heart weight/tibia length (HW/TL) was calculated for each group. Interestingly, a significant decrease in HW/TL ratio was observed for the SEC-EV-treated group compared to PBS- and UC-EV-treated groups after permanent ligation (9.6 ± 1.2 for PBS vs 8.6 ± 0.7 for SEC-EV, p<0.05 and 9.7 ± 1.0 for UC-EV vs 8.6 ± 0.7 for SEC-EV, p<0.05; Figure 4A). Moreover, a similar trend was observed for SEC-EVs after I/R injury (7.2 ± 0.6 for PBS vs 6.8 ± 0.4 for SEC-EVs, p = 0.25 and 7.2 ± 0.6 for UC-EV vs 6.8 ± 0.4 for SEC-EV, p=0.29; Figure 4B).

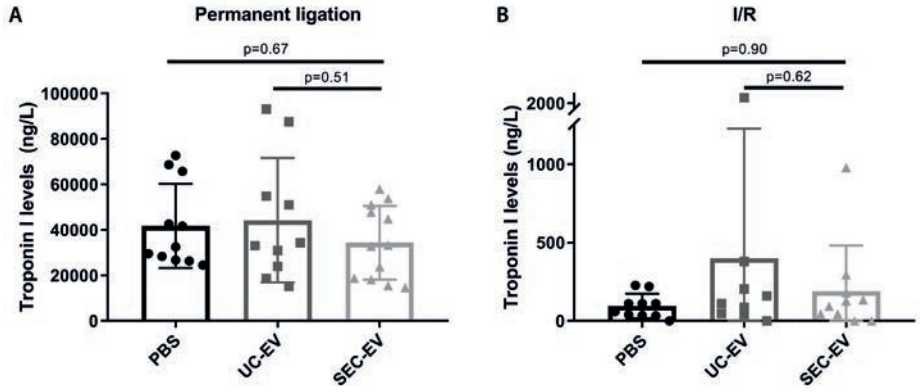

Figure 3 Plasma levels of Troponin I are unaffected by UC-EV or SEC-EV treatment.
Plasma levels of troponin I were determined 2 days post-MI. A) Plasma levels of troponin I after UC-EV or SEC-EV treatment are comparable to those after PBS treatment 2 days after permanent ligation. B) Plasma levels of troponin I were not different between groups 2 days post-I/R injury.

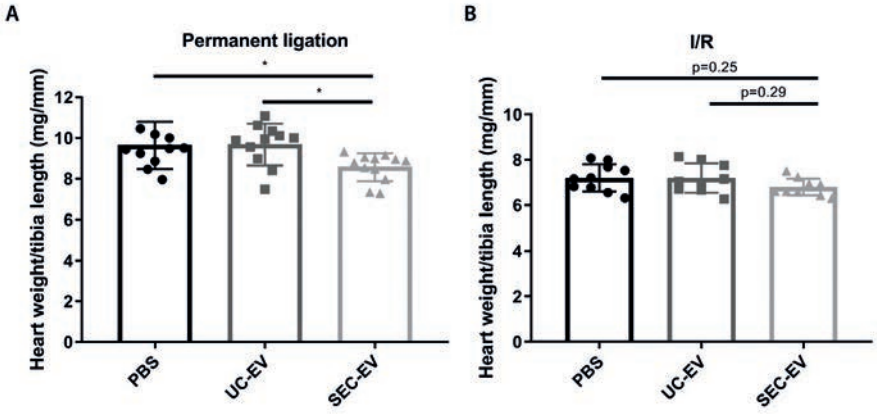

Figure 4 HW/TL ratio is decreased after treatment with SEC-EVs.
HW/TL ratio was calculated for all three groups. A) A significant decrease in HW/TL ratio is observed after treatment with SEC-EVs as compared to PBS and UC-EVs. B) A lower HW/TL ratio is observed after treatment with SEC-EVs in I/R injury, although not significant.

Since we observed that infarct size is not affected by SEC-EV or UC-EV treatment, we questioned whether the injected EV batches were indeed functional, also in an *in vitro* setting. HMEC-1 cells were stimulated with 6 x 10^{10} UC-EVs or SEC-EVs or PBS for 30 min to assess ERK1/2 activation. Comparable to our previous study[20], SEC-EV stimulation resulted in higher ERK1/2 phosphorylation, as compared to UC-EVs, thereby confirming their functionality *in vitro* (Figure 5).

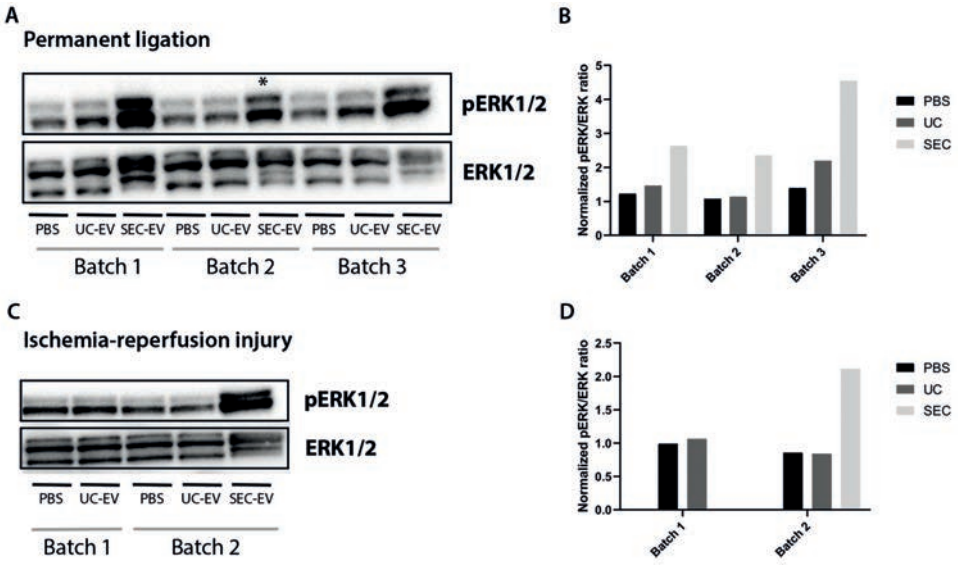

Figure 5 EVs' bioactivity is confirmed *in vitro*.
A) Bioactivity of all three EV batches was assessed using an *in vitro* assay. HMEC-1 were stimulated with 6 x 10^{10} EVs for 30 minutes, after which pERK1/2/ERK1/2 ratio was determined using western blotting. All three SEC-EV batches show increased ERK1/2 activation compared to PBS and UC-EV. * indicates the use of an EV dose of 0.95 x 10^{10}EVs to stimulate HMEC-1. B) The two batches of EVs used in I/R injury were tested for their ability to activate ERK1/2. Due to a too low amount of SEC-EVs left for *in vitro* studies, this value is missing for SEC-EV in batch 1. SEC-EVs were able to activate ERK to a higher extent compared to PBS and UC-EV.

DISCUSSION

Cellular therapeutics have been developed aiming to replace and/or repair the damaged myocardium after MI. Injection of CPC into the myocardium resulted in beneficial effects on cardiac function despite low cell engraftment, indicating the involvement of paracrine mediators[5]. CPC-EVs have been identified as paracrine secretions from CPC carrying important bioactive factors. Indeed, CPC-EV treatment resulted in significant reduction in infarct size 2 days after permanent ligation of the LAD in mice[16]. Hence, moving towards the use of EVs for therapeutic application, large scale production of EVs with maintained functionality is crucial. Therefore, standardized protocols and scalable isolation methods are needed. Previously, we have shown that isolation method can influence EV functionality *in vitro*, as SEC isolation resulted in more functional EVs compared to UC[20]. With the aim to investigate possible differences in functionality between EVs isolated with UC or SEC *in vivo*, we assessed infarct size after PBS, UC-EV, or SEC-EV treatment 2 days after permanent ligation or I/R injury in mice.

Surprisingly, EV treatments were not successful in exerting therapeutic effects since infarct sizes were comparable between UC-EV, SEC-EV and PBS treated groups after permanent ligation as well as after I/R injury. To investigate if EV treatment led to early functional differences, we measured GLS and rLSR, parameters that are considered to be sensitive

measures of early myocardial deformation[21]. Similar to infarct size measurements, no functional improvements were detected upon treatment with EVs based on global longitudinal strain and reverse strain rate. Interestingly, we did observe a significant difference in HW/TL ratio after SEC-EV treatment compared to PBS and UC-EV in the permanent ligation model. Moreover, the same trend was visible after I/R injury. HW/TL ratio is a measure of cardiac hypertrophy, as an increased ratio could indicate that hearts are compensating for volume lost by necrotic cardiomyocytes after MI[22,23]. Previous studies reported increased HW/TL ratios when measured 1 to 4 weeks post-MI, and in those studies effective treatment was able to reduce HW/TL ratios[24–27]. Although our study used a more acute time point, this may indicate that SEC-EV have effects on edema formation and/or inflammation. Future studies should be performed to investigate the role of SEC-EVs in this process in more detail.

Our data is in contrast with previous reports that did show reduced infarct size upon CPC-EV treatment[16]. There are several possible explanations for these different observations. First, we observed high variability in infarct size within the PBS treated group for both MI models. One might argue that detecting a small beneficial effect after EV treatment is difficult when such a high variability is present between initial infarct sizes. However, when comparing troponin levels, infarct sizes, and strain values to other reports, similar variability is generally observed[11,16,28–31]. Secondly, we employed a different CPC clone compared to our previous study[16]. Different Sca1+ CPC subpopulations have been discovered within the human heart that are either more committed to the vascular (N-glycosylated PECAM1+ CPC) or cardiomyocyte lineage (N-glycosylated GRP78+ CPC)[32,33]. Future studies should reveal if the CPC clone used in our current and previous studies represent different CPC subpopulations and if that could explain the differences in functional efficacy. Thirdly, differences in culture protocol could influence outcome. In order to make our EV preparation suitable for future human application, we isolated EVs from serum-free basal medium, which is different from other studies that mostly use 'EV-depleted' FBS-containing medium. In those studies, EVs are depleted from serum before culturing via an overnight ultracentrifugation step. Doubts have been raised on whether ultracentrifugation leads to efficient removal of serum-derived EVs and extracellular RNA[34–37]. Indeed, although ultracentrifugation highly reduced the amount of RNAs present in FBS, residual serum-derived RNAs could still be detected in EV preparations from EV-depleted FBS-containing medium[34]. Hence, serum RNA and/or proteins may be co-isolated when associated to other macromolecules that have similar sizes as EVs. Furthermore, it has been reported for neuroblastoma-derived EVs that serum-free culturing alters protein composition of EVs[38]. Whether CPCs produce EVs with different cargo in response to serum-free medium compared to EV-depleted medium remains unclear to date. Future research should reveal if this change to serum-free culture media in our study is responsible for the observed differences in functional outcome compared to other studies. Alternatively, differences in EV dose could explain our findings, despite the fact that our EV dose correlates to the amount of EV protein used in previous studies and that therapeutic effects have been obtained using similar numbers of EVs[10,11,16]. The optimal EV dose needed for therapeutic efficacy post-MI has not been adequately covered by previous studies. Thus, in our study we may administer either too low or too high doses in order to achieve

therapeutic benefits post-MI. Future studies should therefore focus on determining dose-response curves for EV therapeutics.
When assessing the functionality of the used EV batches *in vitro*, we found that SEC-EV were still able to activate ERK1/2 to a higher extent than PBS and UC-EV, which is in agreement with our previously reported study[20], thereby indicating some level of functionality before injection *in vivo*. Yet, one could question the predictive value of this *in vitro* assay for EV functionality *in vivo*, since our *in vitro* results do not correspond to our *in vivo* data. To date, we are not aware of an *in vitro* assay that is able to assess and predict EV functionality *in vivo*. The development of such an assay would be a valuable contribution when exploring the use of EV therapeutics after MI.
In conclusion, we observed no difference in infarct size between PBS, UC-EV and SEC-EV treated groups after permanent ligation nor after I/R injury, which is in contrast to previously reported studies. Future studies should address whether high variability within the MI models, or differences in CPC clones, serum-free culture media, or dosing could explain these differences.

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