Cardiac Uptake of Mesenchymal Stem Cell-Derived Extracellular Vesicles after Intramyocardial or Intravenous Injection in Murine Myocardial Infarction

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Research Article

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Abstract

Background: In animal models, human bone marrow mesenchymal stem cell-derived extracellular vesicles (MSC-EV) have beneficial effects in cardiovascular disease (CVD) only when administered via intramyocardial injection. The biodistribution of either intravenous (IV) or intramyocardial injection of MSC-EV in the presence of myocardial injury is unknown.

Methods: MSC-EV were isolated and labeled with DiD lipid dye. FVB mice underwent left coronary artery ligation followed by either peri-infarct intramyocardial or IV injection of $3 \times 10^6$ or $2 \times 10^9$ particles of DiD-labeled MSC-EV or a DiD-saline control. The heart, lungs, liver, spleen and kidneys were harvested 2 hours post-injection and were submitted for fluorescent molecular tomography imaging.

Results: Myocardial uptake of MSC-EV was only visualized after intramyocardial injection of $2 \times 10^9$ MSC-EV particles ($p = 0.01$), and was not detected after IV injection of MSC-EV ($p > 0.9$), compared to controls. There was no significantly detectable MSC-EV uptake in other organs after intramyocardial injection. After IV injection of $2 \times 10^9$ particles of MSC-EV, the liver ($p = 0.02$) and spleen ($p = 0.04$) appeared to have MSC-EV uptake.

Conclusions: This study confirms the role for intramyocardial injection in maximal and effective delivery of MSC-EV. Our ongoing studies aimed at developing bioengineered MSC-EV for targeted delivery to the heart may render MSC-EV clinically applicable for CVD.

Background

Stem cell-derived extracellular vesicles (EV) as a therapeutic in CVD have been found to have beneficial effects in animal models (1–4). However, a significant barrier to clinical use is the method of delivery. Currently, the most effective delivery method is via intramyocardial injection, which requires thoracotomy. Less invasive methods of delivery, such as IV, may not confer meaningful benefits in CVD (5, 6).

MSC-EVs and other types of EVs been delivered via intramyocardial, IV, intracoronary, and intrapericardial routes (7, 8). IV and intracoronary injections have had mixed results; intrapericardial is not as well studied (6, 8–12). Additionally, when EVs are injected intravenously, they have a short circulation half-life and are readily taken up by macrophages, which congregate in the liver or spleen (13–16). Other factors to consider are the EV progenitor cell origin, and the presence of cellular damage as injured cells more readily take up EVs than healthy cells (15, 17).

Previous biodistribution experiments have investigated the uptake of EVs in mouse models and detected no cardiac uptake with IV injection – these models included MSC-EVs delivered via tail vein injection in a murine radiation model and cardiosphere-derived EVs delivered via retro-orbital injection in wild-type non-infarcted mice (15, 16). Only with intramyocardial injection were cardiosphere-derived EVs localized to the heart in the non-infarcted mice (16). No cardiac injury was present in these models. Therefore, the aim of
this study was to investigate whether intramyocardial versus intravenous administration affect MSC-EV uptake in the presence of myocardial injury.

**Methods**

**Human Bone Marrow Mesenchymal Stem Cell (HBMSC) Culture**

HBMSC were purchased from Lonza (Walkersville, MD, USA, PT-2501), grown in Mesenchymal Stem Cell Growth Medium BulletKit (MSCGM) (Lonza, PT-3001) to passage 7.

**MSC-EV Isolation**

At passage 7 and 80–90% confluence, the MSCGM was removed and was replaced with fresh MSCGM. The cells were placed in an airtight humidified hypoxia chamber containing 95% N₂ and 5% CO₂ at 37°C for 24 hours. Afterwards, the media was collected, and was centrifuged at 2000 x g. The supernatant underwent ultracentrifugation (WX Ultra Centrifuge with Sorvall AH-629 rotor) at 100,000 x g for 70 minutes. The MSC-EV were then washed with Dulbecco's Phosphate Buffered Saline (PBS) and centrifuged at 100,000 x g for 70 minutes. The MSC-EV were re-suspended in PBS with 1% dimethylsulfoxide, and stored at -80°C (1, 15).

**MSC-EV Characterization Studies**

The MSC-EV were evaluated by electron microscopy after fixation in 2% paraformaldehyde. With the NanoSight NS500 (Malvern Instruments, Malvern, UK), the size, number and distribution of the MSC-EVs were determined. The following MSC-EV markers were evaluated via western blot: CD81 (Cell Signaling, 52892S), CD9 (Cell Signaling, 13403S), Alix (Cell Signaling, 92880S), GAPDH (Cell Signaling, 97166S), heat shock protein 70 (HSP70) (Cell Signaling, 4872T), and albumin (Cell Signaling, 4929S).

**Labeling of MSC-EV**

Fluorescent-labeled MSC-EVs as well as a negative control were prepared. PBS was added to the MSC-EV to make a total volume of 1 mL, and to this 5 µl of Vybrant DiD Cell-Labeling Solution (Invitrogen, V22887) was added (15). For the negative control (DiD-saline), 5 µl of the labeling solution was added to 1 mL of PBS. These solutions were incubated at 37°C for 30 minutes. The solutions were then transferred to 2 separate ultracentrifuge tubes with an additional 30 mL PBS each. The solutions were washed two times with 30 mL PBS and underwent two ultracentrifuge cycles at 100,000 x g for 1 hour each. After the final wash, the DiD-labeled MSC-EV and negative control were re-suspended with PBS, and aliquoted for intramyocardial or IV injection.

**Animals**
Female and male FVB/NCrl mice (6–8 weeks old) from Charles River (Stock No. 207) were used in this study. The animals were housed at the Coro Building Barrier facility, acclimatized appropriately and fed a normal diet. All experimental procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee.

**Echocardiogram**

The mice underwent pre-operative echocardiogram. Under 2% isoflurane anesthesia, left heart systolic function was evaluated via two-dimensional parasternal long axis views with left ventricular trace measurements. The Shapiro-Wilk Test and Kruskal-Wallis H Test were used for statistical analysis.

**Surgical Procedure: Left Anterior Descending Coronary Artery (LAD) Ligation and Injection**

Anesthesia was induced and the mice were intubated and ventilated. The heart was exposed with a left thoracotomy, and the LAD was ligated 2–3 mm below the left atrial appendage (18). Successful ligation was confirmed with subsequent blanching and dyskinesia.

The mice were then allocated to one of six groups – 1) intramyocardial injection with DiD-saline (n = 5), 2) intramyocardial injection with $3 \times 10^6$ particles DiD-labeled MSC-EV (n = 4), 3) intramyocardial injection with $2 \times 10^9$ particles DiD-labeled MSC-EV (n = 5), 4) IV injection with DiD-saline (n = 5), 5) IV injection with $3 \times 10^6$ particles DiD-labeled MSC-EV (n = 4), and 6) IV injection with $2 \times 10^9$ particles DiD-labeled MSC-EV (n = 5). The MSC-EV particles were quantified by nanoparticle tracking analysis.

Immediately after LAD ligation, the injection was performed prior to closing the thoracotomy. To perform the intramyocardial injection, a Neuros Syringe (Hamilton, 1183U32) was used to inject 5 µL of DiD-labeled MSC-EV or DiD-saline into the peri-infarct area. To perform the IV injection, a 0.5 mL syringe was used to inject 200 µL of DiD-labeled MSC-EV or DiD-saline into the tail vein.

The thoracotomy was closed and the mice were successfully weaned from anesthesia and extubated.

**Organ Harvest and Fluorescent Molecular Tomography (FMT) Imaging**

Two hours post-injection, the mice were euthanized. The heart, lungs, liver, kidneys and spleen were removed and imaged with the FMT 4000 imaging system (PerkinElmer). The fluorescence was quantified with TrueQuant v3.0 (PerkinElmer) by measuring the counts/energy, normalized by the geometric size captured of the organ. The Shapiro-Wilk Test, Kruskal-Wallis H Test and Dunn's Multiple Comparison Test were used for statistical analysis.

**Results**

*MSC-EV Characterization Confirmed the Presence of EV Markers*
The MSC-EVs were visualized via electron microscopy (Fig. 1A). Nanoparticle tracking analysis determined MSC-EV concentration, size and distribution (Fig. 1B). Western blot identified CD81, CD9, Alix and GAPDH (Fig. 1C) (15, 19). Neither HSP70 nor albumin were identified.

**Normal Pre-operative Systolic Function Confirmed by Echocardiogram**

Normal left heart systolic function was confirmed via echocardiography, with left ventricular ejection fractions (LVEF) identified within normal parameters. There were no significant differences in LVEF between the animal groups (p = 0.9).

**Cardiac Uptake of MSC-EV only after Intramyocardial Injection**

In the intramyocardial injection groups, myocardial MSC-EV uptake 2 hours post-injection was seen only with the injection of 2*10^9 particles, with uptake significantly increased from control (p = 0.01) (Fig. 2). Intramyocardial injection of 3*10^6 particles of DiD-labeled MSC-EV did not demonstrate fluorescent uptake (p > 0.9) (Fig. 2). No significant fluorescence was detected in any other organs 2 hours post-intramyocardial MSC-EV injection (Fig. 3A).

Following IV injection of MSC-EV, no significant fluorescent uptake was detected in the myocardium (Fig. 2) or in the lungs or kidneys (Fig. 3B). As expected, significant increases in fluorescence were detected in the liver (p = 0.02) and spleen (p = 0.04) after IV injection of 2*10^9 particles MSC-EV particles compared to control (Fig. 4).

**Discussion**

To the best of our knowledge, prior to this study, the biodistribution of MSC-EVs in a myocardial ischemia model had not been investigated before. Here we demonstrated that intramyocardial injection most effectively delivered the MSC-EV dose to the ischemic myocardium, and that IV injection did not result in detectable levels of MSC-EV in the heart despite the presence of myocardial injury.

Previous studies have shown that cellular injury can increase the uptake of EVs. For example, in murine models of glycerol-induced acute kidney injury and radiation injury, MSC-EVs had heightened accumulation in the kidneys and hematopoietic organs, respectively, after IV injection (15, 20). However, our model does not demonstrate increased uptake in the heart after acute myocardial infarction and immediate IV injection. Significant uptake by the liver and spleen may explain the lack of uptake of MSC-EV by other organs after IV injection.

Numerous studies have shown EV therapeutic benefits in CVD consistently with only intramyocardial injection but not intravenous injection (1, 5, 6, 11, 21). One explanation could be that EVs administered systemically are not able to reach their destination due to impaired blood flow from ischemia. Also, the
coronary capillaries have tight junctions that may limit EV uptake by the myocardium. Lastly, it is unclear when the cardiomyocytes and cardiac endothelial cells release injury signals that could potentially attract the EVs with subsequent increased uptake into the myocardium.

Limitations of this study include the lack of evaluation of the consequences of intramyocardial versus IV MSC-EV administration beyond 2 hours. Also, the EV dosages administered in fluorescence uptake studies are markedly supra-therapeutic given the limits of the detection devices – thus the lack of immunofluorescence may not equate to the absence of EVs. However, we can still conclude that intramyocardial injection results in the maximal dose delivered to the heart.

**Conclusion**

In conclusion, intramyocardial injection appears to be the optimal mode of delivery of MSC-EV to ischemic myocardium. This study will help future experiments treat CVD with MSC-EVs by optimization of the route of administration. The murine model used in this study can be developed to be a high throughput vehicle for testing the efficacy of various kinds of MSC-EVs, as well as testing bioengineered EVs to optimize cardiac uptake.

**List Of Abbreviations**

- **EV**, extracellular vesicles
- **MSC-EV**, human bone mesenchymal stem cell-derived extracellular vesicles
- **IV**, intravenous
- **HBMSC**, human bone marrow mesenchymal stem cell
- **MSCGM**, Mesenchymal Stem Cell Growth Medium BulletKit
- **PBS**, Dulbecco's Phosphate Buffered Saline
- **HSP70**, heat shock protein 70
- **LAD**, left anterior descending coronary artery
- **FMT**, fluorescent molecular tomography
- **LVEF**, left ventricular ejection fraction

**Declarations**
Ethics approval and consent to participate: N/A

Consent for publication: N/A

Competing interests: None

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Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Authors’ contributions: CMX designed and conducted the experiments, collected and analyzed data, and prepared the manuscript; SAS contributed to the experiments and data collection; RBT contributed experiment conception and initial development of the animal model; MS contributed to data collection; FWS contributed to the conception; MRA contributed to the intellectual content, data analysis, and edited the manuscript. All authors read and approved the final manuscript.

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References


Figures

Figure 1

**Human bone marrow mesenchymal extracellular vesicle (MSC-EV) characterizations. A.** Electron microscopy image of MSC-EV (scale bar = 200 nm; magnification 54,800x). **B.** MSC-EV fractions determined by nanoparticle tracking analysis, demonstrating mean particle size to be 217.3 nm ± 10.9 nm. **C.** Western blot images of CD81, CD9, Alix, GAPDH, HSP70, and albumin of MSC-EV lysates and human bone mesenchymal stem cell (HBMSC) lysates. HSP70, a promiscuous cytosolic protein, and albumin were not identified. The absence of albumin confirmed the purity of the isolated MSC-EVs.

Figure 2

**Fluorescence molecular tomography of the post-myocardial infarction heart after intramyocardial and IV injections of MSC-EV. A.** Uptake of human bone mesenchymal stem cell-derived extracellular vesicles (MSC-EV) was only detected after intramyocardial (upper panels) injection of $2 \times 10^9$ particles. No fluorescence was detected in the heart after tail vein (lower panels) injection of either $3 \times 10^6$ or $2 \times 10^9$ particles MSC-EV. **B.** Significantly higher fluorescence was detected only after intramyocardial injection of $2 \times 10^9$ DiD-labeled MSC-EV compared to control ($p = 0.01$). No significant fluorescence uptake was seen after IV injection of either low ($3 \times 10^6$ particles) or high dose ($2 \times 10^9$ particles) of MSC-EV ($p = 0.6$, $p > 0.9$, respectively). The Shapiro-Wilk Test, Kruskal-Wallis H Test and post hoc Dunn's Multiple Comparisons Test were used for statistical analysis.

Figure 3

**Organs with no detectable immunofluorescence** after **A.** intramyocardial injection of DiD-labeled human bone marrow mesenchymal stem cell-derived extracellular vesicles (MSC-EV) and **B.** tail vein injection of
MSC-EV (see Fig. 2 for heart). After intramyocardial injection, no MSC-EV uptake was visualized in the lungs, liver, kidneys or spleen. After tail vein injection, no MSC-EV uptake was visualized in the heart, lungs or kidneys. The Shapiro-Wilk Test and Kruskal-Wallis H Test were used for analysis.

**Figure 4**

**Immunofluorescence detected in the liver and spleen after IV injection of MSC-EV. A.** Representative images of the liver (upper panels) and spleen (lower panels) depicting increased organ fluorescence after tail vein injection of $2 \times 10^9$ particles of DiD-labeled MSC-EV. **B.** Quantification of immunofluorescence in the liver and spleen, which showed significantly increased levels of fluorescence after the MSC-EV injection of $2 \times 10^9$ particles, compared to control ($p = 0.02$, $p = 0.04$, respectively). The Shapiro-Wilk Test, Kruskal-Wallis H Test and post hoc Dunn's Multiple Comparisons Test were used for statistical analysis.