Microsurgical technique for locoregional delivery of extracellular vesicles in a forelimb ischemia-reperfusion injury model

Omar A. Selim[1], Aida Sarcon[2], Elameen Adam[1], Ichiro Tsukamoto[1], Atta Behfar[3], Chunfeng Zhao[1], Steven L. Moran[1,4]
[1]Department of Orthopedic Surgery; [2]Department of General Surgery, [3]Department of Cardiovascular Medicine, [4]Division of Plastic Surgery;
Mayo Clinic, Rochester, MN
Email of presenting author: selim.omar@mayo.edu

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Introduction: Locoregional delivery via intra-arterial limb infusion (ILI) can present an efficient and clinically translatable approach for the targeted delivery of novel biologic therapeutics such as extracellular vesicles (EVs) to vascularized composite tissue allografts (VCA). Potential advantages of ILI include homogenous and wider distribution of the injectable substance into skeletal muscles and other extremity soft tissues including skin, nerves, and vessels. Additionally, intra-arterial perfusion can bypass the first pass effect and optimize EVs homing to target tissues. Several studies have demonstrated the efficacy and safety of intra-arterial infusion of stem cells and recombinant adenoviral vectors for critical limb ischemia and Duchenne Muscular Dystrophy, respectively.[1,2] Nevertheless, delivery of EVs using an intra-arterial method has not been reported and characterized before. In this proof-of-concept study, we describe a microsurgical approach to deliver off-the-shelf platelet-derived extracellular vesicles (pEVs) to the rat forelimb using selective brachial artery microcatheterization. We then characterized skeletal muscle EVs uptake in a tourniquet-induced forelimb ischemia-reperfusion injury (IRI) model. IRI is an inevitable consequence of upper extremity VCA due to the abundance of metabolically active skeletal muscle tissue that can potentially cause life-threatening renal failure and allograft functional impairment. Currently, the field of VCA lacks a standard donor preservation solution or treatment that effectively blunts IRI response in the recipient subject following allograft revascularization. Strategies explored to enhance survival of VCA allografts include cold static storage and *ex vivo* machine perfusion. A recent study demonstrated that *in situ* pre-treatment of donor vascularized composite allografts with a complement inhibitor delivered using limb perfusion significantly reduced inflammatory infiltrates upon revascularization of the donor tissue.[3] Thus, we also hypothesized that platelet-derived EVs can offer protection against reperfusion insult in our model following their intra-arterial limb infusion upon tourniquet release.

MATERIALS AND METHODS: Institutional IACUC approval has been for the *in vivo* experiments and procedures of this study. **(1)** *Animals and Study Design*: 13 male and female Sprague-Dawley (SD) rats were used in this pilot testing. Three rats and ten rats were tested for EVs tracking studies and biochemical analysis, respectively. **(2)** *Selective brachial artery microcatheterization and platelet-derived EVs delivery*: A silicone tourniquet was placed at the level of the shoulder joint and ischemia was confirmed by absence of brachial artery pulsations using digital doppler and presence of clinical pallor. The forelimb neurovascular pedicle was exposed using a 5 mm to 10 mm longitudinal skin incision parallel to the long axis of the brachium (**Fig.** I, **1A**). Next, the major vessels (basilic and cephalic veins and brachial artery) were bluntly dissected using sterile Q-tips and fine-tipped microsurgical forceps. A background material was placed posterior to the brachial artery to facilitate microcatheter introduction and provide tissue support (**Fig. 1C**). The field was constantly irrigated with 2% lidocaine to prevent vasospasm. An arteriotomy was carefully created in the anterior wall using the beveled tip of a 32G needle held using a Castroviejo microneedle holder (**Fig. I1D**). Next, a specialized vessel cannulation forceps (Roboz Surgical Instruments Co., Gaithersburg, MD) was used to introduce a polyimide microcatheter connected to a four-way stopcock (Doccol Corporation, Sharon, MA) with an outer diameter: 0.191 mm and inner diameter: 0.152 mm (**Fig. 1E**). Slipknots using 7-0 silk suture were applied to prevent backflow of injectable substance and secure the microcatheter in place (**Fig. 1F**). Five hundred μL of the injectable substance was then infused over a period of 15 minutes. At the end of the infusion, the catheter was withdrawn gently and the arteriotomy was repaired using a10-0 nylon suture (**Fig. 1G&H**) (3) *Validation of ILI technique for therapeutic delivery to skeletal muscles:* Initial validation of our technique was done by injecting 500-1000 μL of 1% Evans Blue dye as a surrogate marker of any biologic agent directly into the brachial artery under tourniquet isolation (n=2). At the end of the injection, dye biodistribution was determined by inspecting forelimb soft tissues. (4) *Biplane angiography:* To determine microcatheter position and further confirm accurate placement, 500 μL of Omnipaque™ 350 (iohexol) was injected following microcatheter insertion and blood flow was recorded using a biplane fluoroscopy system (Artis Zee, Siemens Healthineers, Gemany). Digital subtraction angiography (DSA) was performed to enhance visualization of the forelimb arterial tree. (5) *Characterization and fluorescent labeling of lyophilized commercially available pEVs formulation*: Labeling of platelet-derived EVs (Purified Exosome Product, Rion, Rochester, MN) using DiI (Tetramethylindocarbocyanine Perchlorate) was performed before ILI according to manufacturer's protocol. (6) *Biodistribution study* (n=3): 24 hours following ILI, rats were euthanized and forearm skeletal muscles from surgical and normal sides were harvested and scanned immediately under Xenogen IVIS® Spectrum (PerkinElmer, Waltham, MA) using an emission/excitation filter 535 nm/580 nm; (7) *Immunofluorescence and confocal microscopy:* Following IVIS scan, flexor digitorum profundus (FDP) muscles were snap frozen and 7 μm frozen sections of were obtained and stained for laminin. Cellular localization of DiI-labeled EVs was then performed using confocal microscopy (Zeiss LSM980) under 40X magnification. (8) *Serum biomarkers of muscle and kidney injury*: Three clinical markers of muscle tissue damage: creatine kinase (CK), lactate dehydrogenase (LDH), and aspartate transaminase (AST) and two renal function markers: blood urea nitrogen (BUN) and creatinine (Cr) were quantified spectrophotometrically using an automated chemistry analyzer (IDEXX Bioanalytics). (8) *Statistical analysis*: results were presented as mean ±standard deviation.

RESULTS: No mortality was associated with this rat cohort that underwent this procedure. ILI of Evans blue dye resulted in discoloration of the forelimb skin and skeletal muscles distal to the tourniquet placement site (**Fig 1, II**). DSA revealed placement of the microcatheter at the midhumeral level and visualization of the brachial artery and its two terminal branches: the median and ulnar arteries (**Fig 1, II**). Following ILI validation in our rat model, the same technique was utilized to deliver an off-the-shelf EVs formulation positive for exosomal markers (CD63, CD9, HSP90, and Alix) in a forelimb IRI model. Skeletal muscles of the surgical side showed more than twofold higher mean total radiant efficiency i.e. the relative fluorescence units of the analytic software, compared to the same muscles of the contralateral normal side (**Fig 2, I**). Confocal microscopy demonstrated localization of DiI-labeled pEVs in intermyofibrillar, subsarcolemmal, and perinuclear regions of skeletal muscle myofibers (**Fig 2, II**). Preliminary testing for serum biomarkers of muscle and renal injury (CK, LDH, AST, and BUN) were approximately 20-40% lower in rats that received platelet derived EVs compared to subjects that received saline containing exosome excipient (**Fig. 3**).

DISCUSSION: Delivery of EVs to skeletal muscles for non-regenerative applications is challenging. Although intra-arterial administration of therapeutics is regarded as a systemic route of delivery, our technique demonstrated excellent retention of EVs by skeletal muscles up to 24 h post infusion. CK, LDH, and AST are present in the cytosol of skeletal muscles and are significantly elevated following muscle membrane damage. Although the sample size is small, our preliminary results show diminution of these values compared to control upon administration in an IRI model. Future studies will compare outcomes and EVs uptake by skeletal muscles using other routes of delivery including intramuscular vs. intravenous injections, and timings of pEVs delivery (pre-ischemia and immediately upon limb reperfusion) to identify the optimal window for therapeutic efficacy of pEVs.

SIGNIFICANCE/CLINICAL RELEVANCE: A reproducible microsurgical technique reported herein can be utilized to effectively deliver EVs in intricate rodent VCA studies using a clinically relevant paradigm. Platelet-derived EVs can be administered intra-arterially to VCA grafts either *in situ* or *ex vivo* as a component of VCA preservation protocols. \

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