# Human Mesenchymal Stem/Stromal Cell-Derived Small Extracellular Vesicles Diffusivity in Meniscus Tissues

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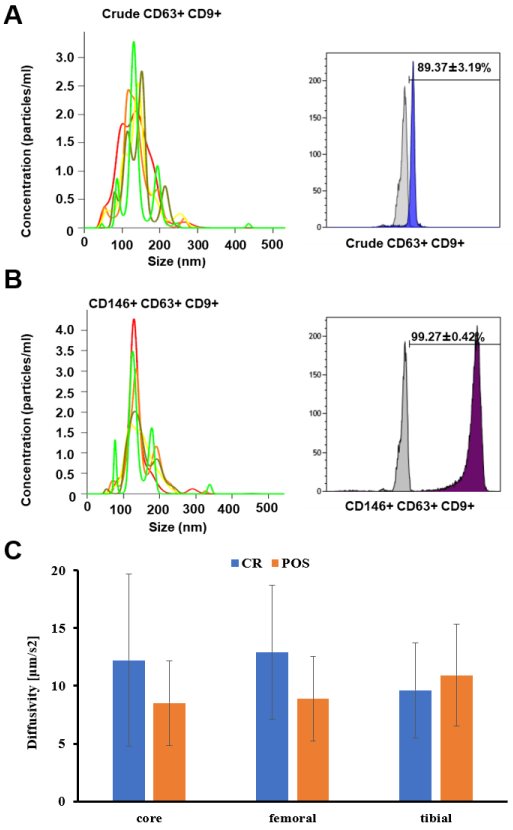
**INTRODUCTION:** The pathogenesis and progression of knee inflammatory pathologies is modulated partly by residing immune cells in the synovium/infrapatellar fat pad (IFP). Mesenchymal stem/stromal cells (MSCs) are a promising therapeutic alternative as they can modulate local immune responses and have trophic effects. Specifically, the CD146 signature is correlated with innately higher MSC immunomodulatory and secretory capacity, and thus therapeutic potency in vivo. We and others have isolated and characterized small extracellular vesicles (sEVs) from various MSC sources, confirming MSC paracrine strong anti-inflammatory, anti-fibrotic, and angiogenesis-remodeling capacities[1-3]. In order to design therapeutic protocols based on intra-articular injection of such agents, their transport properties within the pertinent tissues need to be characterized. In this study, we adopt a quantitative fluorescence imaging approach to investigate the diffusivity of sEVs isolated from Crude and CD146+ endometrial-derived MSC (eMSC) populations into the porcine knee meniscus. Since structural, compositional and morphological differences exist across meniscal regions, this analysis focused on extracellular vesicle transport across the meniscus core, as well as the tibial and femoral cartilaginous layers.

**METHODS:** ***Extracellular Vesicle Production and Labeling:*** eMSC sEVs were isolated from Crude and CD146+ eMSC cultures. Pre-enriched exosome populations were incubated with the Dynabeads®-based Exosome-Human CD63 Isolation/Detection Reagent (Invitrogen) and purified according to manufacturer's instructions for magnetic selection. CD9 (Invitrogen) expression was used to validate exosome presence in CD63+-gated particles by flow cytometry. Nanoparticle tracking analysis (NTA) (NanoSight NS300, Malvern) was performed for quantity and size determination. For eMSC sEV tracking, exosomes were stained with PKH67 green fluorescent membrane staining kit (Fluorescent Cell Linker Kits, Sigma) according to manufacturer's instructions. ***Specimen Preparation:*** Porcine meniscus tissues were obtained frozen from a commercial source (Animal Technologies, Tyler, TX). Tissue samples were taken from the femoral external layer, the tibial layer, and the meniscus core. A corneal trephine was used to punch cylindrical specimens in the axial orientation having a diameter of 5 mm; a custom 3D printed chamber (Precisionary Instruments, Inc.) was then used to cut the specimens to a height of 0.5 mm. Specimens were confined within two porous plates and an impermeable spacer and equilibrated overnight in a solution containing the stained Crude or CD146+ sEVs in deionized water, similar to our previous study[4]. A total of 8 (n = 8) meniscal samples were tested per tissue region (core, femoral and tibial layers) and probe (Crude or CD146+ sEVs). ***Diffusivity Measurement:*** A custom fluorescence recovery after photobleaching (FRAP) technique, developed (and validated) in our lab[4], was used to measure the diffusion coefficient of the probe in the meniscal tissue. Experiments were carried out at room temperature (22°C) using a Nikon A1-R-SI laser scanning confocal microscope. Images were analyzed using a custom MATLAB-based algorithm[4]. ***Statistical Analysis:*** A two-way ANOVA analysis was conducted with factors being tissue region (core, femoral or tibial layer) and probe (Crude or CD146+ sEVs). Interactions and main effects were investigated. A post-hoc Tukey test investigated for differences in the magnitude of diffusion coefficients across tissue regions, while a 2-sample t-test investigated for differences among probes. Grubbs' test was used to check for outliers. Level of significance of 0.05 (p = 0.05) was used for all tests.

**RESULTS:** Upon ultracentrifugation and CD63+ immunoselection, the CD9 typical surface marker showed high purity (>90%) in Crude and CD146+ eMSC sEVs. Both Crude and CD146+ eMSC sEVs showed <200nm sizes by NTA analysis (Figure 1A). For all measurements conducted, no outliers were identified. Values for diffusion coefficients are reported in Figure 1C. Probe diffusivity ranged from 4 to 30 mm/s$^2$ across all the tests conducted. A 2-way ANOVA indicated that interactions among probes and tissue regions were not significant (p > 0.05). Similarly, tissue region did not significantly affect probe diffusivity (p > 0.05). In contrast, differences (p = 0.025) were observed across probes, with Crude eMSC sEVs diffusion coefficients significantly larger than those observed for CD146+ eMSC sEVs.




**DISCUSSION:** The range of diffusion coefficient values found in this study are in agreement with our previous work, which investigated the diffusivity of several fluorescently-labeled dextran molecules (3kDa – 150 kDa, ~14-85 nm) in porcine meniscus tissue [5]. Our findings suggest that Crude and CD146+ eMSC sEVs display good diffusion across all 3 regions of the meniscus. This study serves as a proof-of-concept to investigate the innate capacity of MSC-derived sEVs to diffuse in cartilaginous tissues, an important property of sEVs necessary to exert effective and long-lasting immunomodulatory and trophic effects in the tissue microenvironment. Also, given the limited vascularity of the meniscus, which relies primarily on diffusion for nutrient supply and tissue viability, these findings offer promise for the development and characterization of CD146+ sEVs as possible transport vehicles for therapies targeted at vulnerable parts of the tissue.

**SIGNIFICANCE/CLINICAL RELEVANCE:** CD146+ sEVs could be considered as an adjunct in the delivery of orthobiologics and other cell-derived therapies to facilitate cartilaginous tissues healing.

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**Figure 1.** Crude and CD146+ eMSC EXO characterization. (A, B) Nanoparticle tracking analysis showed that isolated vesicles from eMSC conditioned media are <200nm diameter size, consistent with the known size of sEVs. Crude eMSC sEVs (A) and CD146+ eMSC sEVs (B) CD63+-selected sEVs showed purity by high positivity for exosome marker CD9. (C) Diffusion coefficients of Crude and CD146+ eMSC sEVs across meniscal regions. Bars in the diagram indicate standard deviation.