Extracellular Vesicles Derived from Three-Dimensional Culture of Adipose-Derived Stromal Cells Using Coaxial Bioprinting Method Showed Superior Effects on Tendon Healing Compared to Two-Dimensional Monolayer Culture

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INTRODUCTION: Patellar tendinopathy is a degenerative tendon disorder that causes pain and functional impairment, affecting approximately 10% of knee-related diagnoses. Excessive inflammation is often observed and ectopic bone is occasionally found in clinical samples. Current treatment options often fail to promote regeneration. Stem cell-derived extracellular vesicles (EVs) have demonstrated regenerative potential on tendon healing, with increasing evidence suggesting that stem cells cultured in a three-dimensional (3D) environment produce higher quantities of EVs and exhibit enhanced therapeutic effects compared to those cultured in a two-dimensional (2D) monolayer. However, no studies have yet explored whether EVs derived from 3D-cultured stem cells (3D-EVs) provide superior therapeutic effects for tendinopathy compared to EVs derived from 2D-cultured stem cells (2D-EVs). This study aimed to examine the effects of 3D-EVs compared to 2D-EVs on the responses of inflammatory tendon-derived stem / progenitor cells (TDSCs) in vitro and tendon healing in an animal model of tendinopathy.

METHODS: The study was approved by the animal research ethics committee of the authors' institution (Ref. AUP#24-244-MIS). Human adipose-derived stromal cells (ADSCs) were cultured in 3D core-shell alginate hydrogels using coaxial bioprinting method or 2D monolayers (Figure 1A). For in vitro experiments, TDSCs were treated with IL-1β, along with 2D-EVs and 3D-EVs. We examined cell proliferation with cck8 and Edu (n=3/group), migration with transwell and wound healing assays (n=3/group), inflammatory, and tenocyte, and non-tenocyte markers with qPCR (n=5/group) and multi-lineage differentiation potential (n=3) using standard assays. For in vivo experiment, 2D-EVs and 3D-EVs loaded in a biomaterial were injected into the tendons of a collagenase-induced rat tendinopathy model (6-8 weeks, male) at day 3 after collagenase injection. Male rats were selected to eliminate the effects of estrogen on tendon healing. Tendon samples were collected at weeks 2 and 8 post-treatment for micro-CT imaging (n=8/group), histology (n=6/group), immunohistochemistry (n=6/group), gait analysis (n=6/group), and biomechanical testing (n=10/group).

RESULTS: The 3D cell culture supported continuous collection of conditioned medium over 16 days, while 2D culture at the same seeding density became confluent in just 5 days, limiting the collection of conditioned medium for EV isolation. However, both 2D-EVs and 3D-EVs exhibited similar sizes, morphologies, EV markers, and uptake by TDSCs (Figure 1B-E). 3D-EVs significantly enhanced cell proliferation, migration, and reduced the expression of inflammatory cytokines in inflammatory TDSCs compared to 2D-EVs (all $p < 0.05$ compared to the 2D EV group). Additionally, 3D-EVs induced higher expression of tenocyte markers, lower expression of non-tenocyte markers in inflammatory TDSCs compared to 2D-EVs. Besides, 3D-EVs enhanced tenogenic differentiation and suppressed osteogenic and chondrogenic differentiation of inflammatory TDSCs. H&E staining (Figure 2), Masson's trichrome staining, and biomechanical testing demonstrated that 3D-EVs promoted superior tendon healing (all $p < 0.05$ compared to the biomaterial-only group). The pain-associated alterations in gait patterns were also restored following 3D-EV treatment. Administration of 3D-EVs significantly reduced ectopic bone formation (Figure 3), excessive proteoglycan deposition, and protein expression of TNF-α, IL-1β and IL 6 (all $p < 0.05$ compared to biomaterial-only group).

DISCUSSION: 3D-EVs produced by coaxial bioprinting method showed superior effects on tendon healing compared to 2D-EVs. These effects may be mediated by the suppression of inflammation and restoration of functions of inflammatory TDSCs.

SIGNIFICANCE/CLINICAL RELEVANCE: This study addresses the limitations of EVs in clinical applications, especially their limited therapeutic efficacy.

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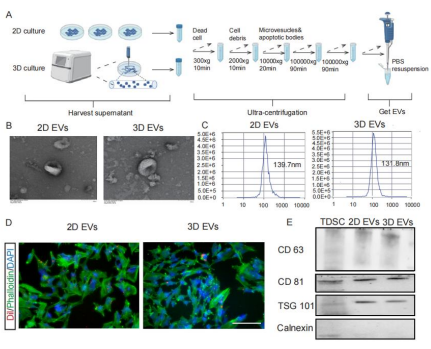

Figure 1. Characterization of 2D-EVs and 3D-EVs. (A) Procedures of EV isolation from conditioned medium. (B) TEM images of 2D-EVs and 3D-EVs. Scale bar: 100 nm. (C) NTA of particle size of 2D-EVs and 3D-EVs. (D) Cellular uptake of labelled 2D-EVs and 3D-EVs into TDSCs after incubation for 24 hours. Scale bar: 200 μm. (E) Western blotting of CD63, CD81, TSG101, and calnexin in protein extracts of TDSCs, 2D-EVs, and 3D-EVs.

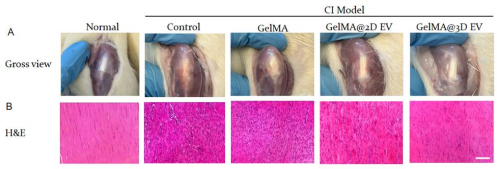

Figure 2. Gross view and H&E staining of healing patellar tendon following injection of 2D-EVs or 3D-EVs loaded in GelMA. (A) Gross view at week 2 post-injection. (B) H&E staining at week 2 post-injection. Scale bar: 100 μm. n=6/group.

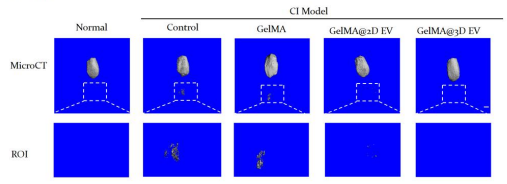

Figure 3. Ectopic bone in healing patellar tendon at week 8 post-injection of 2D-EVs or 3D-EVs loaded in GelMA. (A) microCT imaging of ectopic bone showing bone volume Scale bar: 5mm; n=8/group.