# Biochemical and Mechanical Modulations of Age-Dependent ECM-Based Hydrogel Systems for Meniscus Zone-Specific Repair

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**INTRODUCTION**: Repairing injuries within the meniscal avascular zone poses a significant challenge due to its limited intrinsic healing capacity **[1]**. The meniscus represents a complex tissue wherein biological and mechanical properties intricately vary across distinct zones **[2]**. It is well understood that the extracellular matrix (ECM) components of the meniscus undergo changes during tissue development, thereby influencing cellular phenotype and function **[3]**. Our recent work introduced bovine meniscus decellularized meniscus ECM (MeDEM)-based hydrogel systems, and demonstrated that DEM components could enhance meniscus cell proliferation and differentiation **[4]**. However, the intricate interplay between biochemical and mechanical alterations in the DEM during tissue development and their modulation of cellular responses remains elusive. Hence, this study delves into the impact of age-dependent DEM across a range of physiologic stiffnesses on cellular behavior, coupled with comprehensive proteomic analysis of the materials. Moreover, addressing meniscus tears might require injectable materials characterized by diverse properties tailored to specific meniscus tissue zones. Here, we further advance our investigation by developing a stiffness-tunable MeDEM based injectable hydrogel system by coupling DEM methacrylated hyaluronic acid (MeHA) and assessed how these hydrogels regulate cell phenotypes, thereby offering insight into their therapeutic potential.

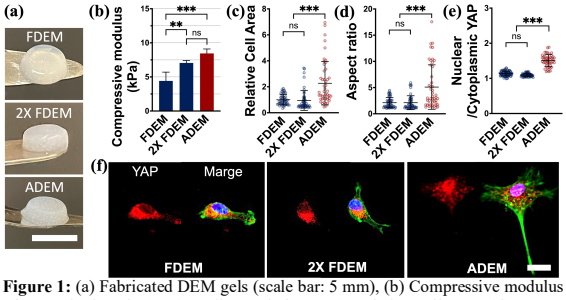


**Figure 1:** (a) Fabricated DEM gels (scale bar: 5 mm), (b) Compressive modulus of DEM hydrogels (n=5). Cell morphology: (c) Relative cell area, (d) Aspect ratio, (e) YAP nuclear localization (ns: not significant, **: p<0.01, ***: p<0.001), (f) Immunofluorescence images (Red: YAP, Green: F-actin, and Blue: DAPI; scale bar: 20 $\mu m$).

**METHODS:** Fetal (3rd trimester) and adult (<30 months) bovine menisci were decellularized using our established protocol **[5]**. FDEM (Fetal MeDEM), 2X FDEM (twice the concentration of FDEM), ADEM (Adult MeDEM), or FADEM (FDEM and ADEM mixed in a 1:1 ratio) pre-gels were prepared by digesting in an acetic acid solution **[4-6]**. 'Soft' (35% modified) and 'stiff' (100% modified) MeHA were synthesized and additionally, a combination of 'Soft and Stiff' MeHA in a 1:1 ratio was created. To achieve stiffness-tunable hydrogels, 1.5% FADEM was blended with 1.0% Soft, Stiff, or Soft/Stiff MeHA, with the inclusion of Irgacure 2959 for UV light-induced crosslinking. Subsequently, DEM-based MeHA hydrogels were crosslinked in a cylindrical mold under UV light at 37 ℃ **(Fig. 1a)**. The mechanical properties of the hydrogels were evaluated to measure their compressive moduli **[6]**. For proteomic characterization, gene ontology (GO) analysis was conducted on mass spectrometry data from four different donors per group to explore the correlation of specific proteins involved in both the mechanical and biological characteristics of the age-dependent DEM systems. Juvenile bovine mesenchymal stem cells (MSCs; P2) were seeded onto the hydrogels and cultured in basal growth media. At day 3, cells were stained with Phalloidin, primary antibodies against YAP, and DAPI, and images were captured. Cell area, aspect ratio, and YAP nuclear localization were quantified using ImageJ. Expression of Collagen type-I (COL1A2), SOX-9, and TGF were determined via RT-PCR on Day 7 with GAPDH as a housekeeping gene.

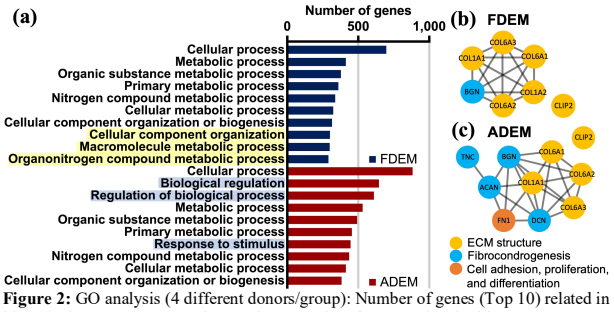


**Figure 2:** GO analysis (4 different donors/group): Number of genes (Top 10) related in biological process (a), Protein-protein networks of genes related with ECM structure, Fibrocondrogensis, cell adhesion, proliferation, and differentiation in FDEM (b) and ADEM (c).

**RESULTS:** ADEM hydrogels had a higher stiffness compared to FDEM hydrogels, and increasing FDEM concentration yielded a comparable compressive modulus to ADEM hydrogel **(Fig. 1b)**. Enhanced cell area, aspect ratio, and YAP nuclear localization were evident on ADEM hydrogels, while an elevated concentration of FDEM (resulting in increased stiffness, 2X FDEM) yielded no significant changes, suggesting the dominance of biochemical effects in the age-dependent DEM system. Proteomic analysis revealed an abundance of proteins related to biological regulation, response to stimuli, and biological processes in the ADEM system as compared to FDEM **(Fig. 2a)**. Remarkably, among the top 30 proteins constituting the majority of DEM systems, fibrochondrogenesis-related genes were prominent in ADEM **(Fig. 2c)**, including key regulators of cell adhesion, proliferation, and differentiation, including Fibronectin (FN1), forming a highly interconnected network **(Fig. 2c)**. Next, to enhance material injectability and better match specific tissue zones, stiffness-tunable DEM-based MeHA hydrogels were developed, exhibiting a wide range of stiffnesses **(Fig. 3a)**. MSCs cultured on FADEM-based stiffness-modulated MeHA demonstrated elongation and alignment on the stiff system **(Fig. 3b)**, with elevated YAP nuclear localization **(Fig. 3c)**. Notably, Stiff MeHA-based FADEM groups exhibited heightened COL1A2 expression **(Fig. 3d)**. Interestingly, chondrogenic markers SOX9 and TGF were higher across all MeHA-supplemented groups. Remarkably, the introduction of MeHA to DEM not only boosted chondrogenic gene expression but notably, stiff MeHA stimulated fibrochondrogenic gene expression in MSCs **(Fig. 3d)**.

**DISCUSSION:** This study explored the potential of age-dependent MeDEM hydrogel systems for meniscus repair, focusing on their biomechanical and biological effects. Notably, ADEM hydrogels exhibited increased stiffness, and adjusting FDEM concentration yielded similar stiffness levels. Interestingly, the 2X FDEM hydrogels showed minimal effects on cell behavior, suggesting biochemical factors might outweigh mechanical influences from this fetal source material. Proteomic analysis highlighted distinct GO profiles in FDEM and ADEM systems, with fibrochondrogenesis-related genes abundant in ADEM, indicating age-dependent DEM processing results in meniscus-derived ECM of differing composition. The innovative development of stiffness-tunable DEM-based MeHA hydrogel systems allowed precise control over stiffness, revealing the role of stiffness in regulating cellular behaviors. Stiff MeHA upregulated fibrous and chondrogenic gene expressions, while soft and soft/stiff MeHA enhanced chondrogenic gene expression in MSCs. Overall, our findings emphasize the importance of considering both biochemical and mechanical cues for effective meniscus repair strategies, with implications for tailored zone-specific meniscus repair. Current research endeavors are directed towards utilizing RNA-seq to investigate the impact of age-dependent ECM on transcriptome profiling in meniscus cells, to unveil underlying molecular mechanisms. Additionally, ongoing in vivo animal tests are being conducted to assess the therapeutic potential of these findings.

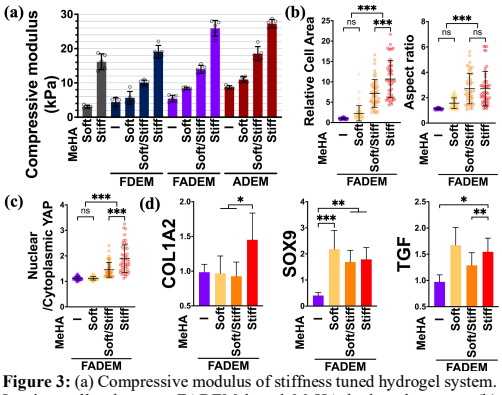


**Figure 3:** (a) Compressive modulus of stiffness tuned hydrogel system. In vitro cell culture on FADEM based MeHA hydrogel system: (b) Relative cell area, Aspect ratio, and (c) YAP nuclear localization, (d) Gene expression (*: p<0.05, **: p<0.01, ***: p<0.001, ns: not significant).

**SIGNIFICANCE:** The newly developed tunable DEM-based MeHA hydrogel system holds promise for effectively addressing zone-specific repair and regeneration in meniscus injuries.

**REFERENCES:** [1] Yan+, *Front Cell Dev Biol* 2021; [2] Murphy+, *J Mech Behav Biomed Mater* 2019; [3] Acun+, *Biomaterials* 2021; [4] Lee+, ORS 2023; [5] Lee+, ORS 2022; [6] Gao+, *Adv Funct Mat* 2017; [7] Mauck+, *J Biomech Eng* 2000. **ACKNOWLEDGEMENTS:** This work was supported by the National Institutes of Health (K01 AR077087, R56 HL163168).