Delayed Onset of Physiological Loading for Avascular Meniscus Healing: Effect of Magnitude, Direction & Mode of Stimulation

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INTRODUCTION: Inner third avascular region of the meniscus tissue has challenging to repair tear due to poor intrinsic capacity. To promote regenerative healing of avascular meniscus tears, we have developed a fibrin-based bioactive glue, sequentially released connective growth factor (CTGF) and transforming growth factor β3 (TGFβ3), designed to timely control the recruitment and differentiation of human synovial mesenchymal stem/progenitor cells (syMSCs). Although our previous *ex vivo* and *in vivo* studies with bioactive glue has demonstrated the enhanced healing of avascular meniscus tears, the functional properties of healed menisci were yet suboptimal to native tissues, likely associated with immature tissue matrix. To achieve a maturated meniscus healing *ex vivo*, we designed the meniscus-specific bioreactor applying the physiological loading, including the compressive, tensile, hoop forces on our well-established wedge-shaped meniscus explant healing model. Our recent study has shown that a delayed onset of physiological loading (10% femoral compression), starting at 5 wks after bioactive glue application for a duration of 3 wks, was the most effective for the initial cell infiltration and matured tissue integration, as compared to earlier onsets. To delineate the effect of complex physiological loadings on maturation of meniscus healing, this study investigated the effect of mechanical loading with different magnitudes, modes, and directions in our bioactive glue-guided healing of avascular meniscus tears via syMSC recruitment. **METHODS:** *Meniscus-specific bioreactor:* We custom-built the meniscus-specific bioreactor based on the 3-axis control system (400 × 300 × 500 mm^3, stroke range of 150-mm (x,y,z), 10 μm resolution). All the parts (stimulation- , holder-, well plater-holder) were 3D printed by using biocompatible poly(lactic acids) (PLA). The stimulation parts were designed in the shape of femoral condyle that can apply loadings on 6 different samples simultaneously with individual controls of z-directional heights, enabling consistent loadings on multiple samples (**Fig. 1A**). To implement the compression, shear, tension similar to femoral loading environment to the meniscus, stimulation parts were moved in a diagonal direction by adjusting the z-, y-axis via custom G-code programming (**Fig. 1B & C**). Meniscus explants were secured in custom-designed PDMS molds fitting in 6-well plates (**Fig. 1D & E**). To apply different hoop strain/stress (circumferential directional loading), two different designs of PDMS molds were prepared; one with x-directional anchor (**Fig. 1E**) and the other with no x-directional confinement (**Fig. F**). Finite element analysis (FEA) was performed using ABAQUS (Dassault Systems, USA) to estimate the stress and strain distributions depend on different magnitude and type of the physiological loading, using quadratic tetrahedron elements and mechanical properties adopted from literature (**Fig. 1G-H**). *Meniscus explant repair:* As per our prior works, a full-thickness longitudinal incisions were created in inner third zone bovine meniscus explants, and fibrin gel crosslinked with genipin (FibGen) with 100 ng/ml CTGF and 10 mg/ml of TGFβ3 encapsulated in PLGA μS was applied to the defect site with syMSCs suspended in media. *Mechanical stimulation:* The five different types of dynamic strain: 5%, 10% and 15% of femoral compression (P5, P10, and P15, respectively), 10% femoral compression with 4% hoop strain (P10 / H4), and 10% femoral compression with 10% shear strain on articular surface (P10 / S10). All mechanical stimulations were applied at 1Hz frequency for 30 mins every third day in a week during 5 wks – 8 wks of culture after bioactive glue application. All the tissue samples were harvested at 8wks and analyzed with histology, tensile tests, and 3D volumetric collagen orientation using our tissue clearing method with confocal microscopy. **RESULTS:** *FEA outcome:* The FEA result showed that our bioreactor applying femoral loading (F_{fem}) exerts complex mechanical loading to the meniscus explant including hoop strain/stress (circumferential directional), horizontal and vertical strain/stress related to the compression, tension, and shear stress (**Fig. 1G**). P15 yielded the highest z-directional stress (**Fig. 1H**), and P10/H4 presented the highest y-directional deformation (**Fig. 1I**). *Meniscus healing:* Quantitatively, tensile properties and strengths were significantly higher in the P5, P10, and P10/H4 groups than P15 and P10/S10 (**Fig 2A, B**). Similarly, GAG and Collagen contents were significantly higher in P5, P10, and P10/H4 than P15 and P10/S10 (**Fig 2C,D**). Tissue clearing with collagen hybridizing peptide labeling and confocal microscopy showed highly reorganized collagen fibers in P5, P10, and P10/H4 groups as compared to no stimulation, P15, P10/S10 groups (**Fig 2E**). In the P5 and P10, collagen fibers were mostly reorganized in z-direction, whereas P10/H4 showed y-directional (circumferential) fiber orientation at the healing zone (**Fig. 2E**) (3D images not shown). The tensile tests showed weak tissue bonding in the no stimulation and P15 groups and the newly formed integrated tissues in P5, P10, and P10/H4 groups (**Fig. 2F**). P10 samples showed more resilient tissue integration than all the other groups (**Fig 2. F**). **DISCUSSION:** Our prior study showed that delayed onsets of mechanical stimulations enhanced mechano-regulated maturation of meniscus healing while early onsets to immature tissue matrix disrupt healing. This study suggests the effect of magnitude, mode and direction of mechanical stimulation on the fiber maturation in meniscus healing. Our data show that femoral loading of 15% and additional 10% shear loading resulting in excessive stress/strain disrupted meniscus healing and maturation. Interestingly, an addition of 4% hoop strain significantly promoted fiber orientation in the circumferential direction, suggesting that the loading direction may affect directional maturation of collagen fibers in the healing meniscus matrix. These findings have important implications for exploring optimal external stimuli for mature healing of avascular meniscus through recruitment of endogenous stem/progenitor cells. Moreover, in-depth understanding of roles of type- & direction-specific mechanical stimulations in matured meniscus healing may provide guidance for optimal designing of physical therapy after meniscus repair. The limitations of this study include the lack of *in vivo* factors potentially affecting meniscus healing, such as inflammation and interactions with other joint tissues (e.g., synovial membrane, cartilage, and infrapatellar fat pad). To address these limitations, our separate study is developing an *in vitro* joint model, enabling co-culture of meniscus explants with engineered synovium and fat pad to be integrated into our meniscus-specific bioreactor. **SIGNIFICANCE:** Our findings on matured-healing of avascular meniscus tears by injectable bioactive glues in combination with timely controlled and directional-specific mechano-regulation have significant implications in developing a translational approach to achieve functional healing of avascular meniscus tears by endogenous stem/progenitor cells.

IMAGES AND TABLES:

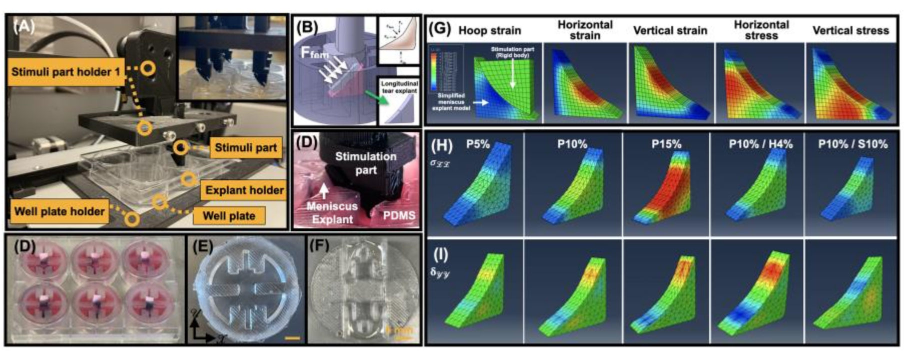

Fig. 1 A custom-built meniscus-specific bioreactor to apply complex physiological joint loadings to a total 6-meniscus explants simultaneously. (A) The 3D-printed PLA construct 3-axis system with 6 condyle-shaped loading units, (B) allowing multi-directional motions which biomimetic of the physiological load and longitudinal tear, (C,D) PDMS molds which (E,F) two different type to control the X-directional stress/strain, fitting to 6-well plates with bio-glue treated on the bovine meniscus explant defect region enabling 6 samples being loaded simultaneously. (G) FE analysis results that bioreactor allows to exert complex joint loadings, including physiological loading such as hoop strain/stress, horizontal and vertical strain/stress, (H) σ_{zz} and (I) δ_{yy} depend on different magnitude- & directional-specific mechano-regulation (P: Physiological loading; H: Hoop Strain; S: Shear).

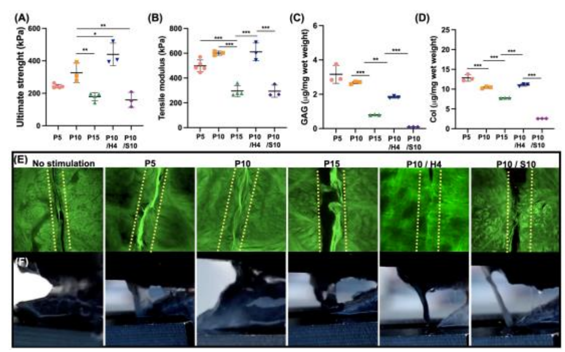

Fig. 2. (A-D) Results of the ultimate strength, tensile modulus, GAG and Col contents of the meniscus explant after exerting the different type of stimulation (P: Physiological loading; H: Hoop Strain; S: Shear), (E) confocal microscope image of each stimulation groups with collagen hybridizing peptide labeling after optical clearing to visualize the 3D volumetric collagen fiber structures, (F) photograph of the tensile test after 8 wks. culture