Heparin-Conjugated Bioactive Glue for Regeneration of Lubricin-infiltrated Meniscus Tears by Recruitment of Stem/Progenitor Cells

Biomedical Engineering

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Abstract

Meniscus injuries occur in over one million Americans each year and are one of the most important contributing factors to knee osteoarthritis (OA). The presence of lubricin is in meniscal injuries is detrimental to the avascular healing of meniscus due to the lubricating properties that negate regeneration. By utilizing the heparin binding domain at the N-terminal of lubricin, we attempted to circumvent the effects of lubricin through the use of heparin-conjugated fibrin gel cross-linked with genipin to establish a successful avascular tissue healing effect. Menisci from mature bovine knee joints were treated with varying mixtures of gels and used for lap shear tests. Separate tissue was incised, loaded with gels, and cultured. After 4 weeks, samples were harvested and underwent histology, biochemical assays, and multi-scale mechanical tests. Compared to the other material combination gels, conjugated Hep-Fib-Gen consistently outperformed the other groups. Reduction in shear strength by 14%-33% was observed in other groups with lubricin coated tissues, while conjugated Hep-Fib-Gen display ~68% increase in shear modulus. Additionally, when lubricin coated, conjugated Hep-Gen-Fib displayed superior tissue integration. This study suggested that heparin conjugation into Fib-Gen hydrogel strengthened initial bonds in lubricin coated meniscus tears, leading to improved avascular healing, and mechanical properties. In conclusion, conjugated Hep-Fib-Gen may serve as efficient bio-glue to support healing of clinically relevant meniscus tears by endogenous stem cell recruitment.

Contents

1. Introduction

1.1. ***Rationale***

Knee meniscus is vital to structural support for articular joints by playing important roles in load bearing, transmission and distribution, and shock absorption [1, 2]. Knee menisci are subjected to various types of physiological loading such as shear, tension, and compression forces [1, 2]. The complex biological and biochemical composition of the meniscus allows for these properties to be incorporated cohesively into a fibrocartilage tissue. The meniscus is sectioned into two major components: the vascular section and the avascular section. There is an additional transitional section between these major regions that possesses a combination of properties from both regions. The outer third meniscus are populated with spindle-shaped fibroblast-like cells and the inner third avascular zone with rounded chondrocyte-like cells. Tears in the vascularized outer third region of meniscus can be often surgically repaired by suture-fixation, while the clinical outcome remains controversial. In contrast, tears in the inner third of the meniscus have poor intrinsic healing properties that lead to minimal healing due to the avascularity of the region. Consequently**,** these tears frequently progress to extend into the middle-third region, followed by meniscus deterioration. Partial or total meniscectomy is often performed to alleviate symptoms caused by the irreparable meniscus injuries with over 750,000 patients each year in the U.S. [3, 4] However, meniscectomy significantly increases the occurrence of osteoarthritis (OA) later in life by elevating joint contact stress. Our recent study has suggested that temporally controlled delivery of a profibrogenic cue, connective tissue growth factor (CTGF), and a chondrogenic factor, transforming growth factor beta 3 (TGFβ3) via poly(lactic-co-glycolic acids) (PLGA) microspheres (μS) can lead to seamless healing of avascular meniscus tears by regulating recruitment and step-wide fibrocartilaginous differentiation of endogenous stem/progenitor cells [5, 6]. Clinically, however, torn menisci in human patients frequently show lubricin expression penetrating deeply into the torn surfaces of meniscal tissues [7]. Given that there is an inevitable period of time (>2 - 3 weeks) between meniscus injury and the first treatment, the injured tissue surfaces have a discrete layer of lubricin that coats the torn edges of the meniscus [7]. Lubricin is a natural lubricant in synovial fluids that promotes frictionless joint movement and inhibits cell and tissue adhesion. Thus, lubricin penetration into torn surface is likely attributed to poor re-integration of meniscus tears, supported by our experimental evidence.

Accordingly, the purpose of this project is to investigate how to prevent the potentially harmful effects of lubricin exposure on stem/progenitor cell-derived healing of torn menisci. We here applied heparin-conjugation into our fibrin-based bioactive glue delivering CTGF and TGFβ3 to guide regeneration of lubricin-penetrated avascular meniscus tears by stem cell recruitment. We hypothesize that the conjugation

of heparin into a fibrin-based bio-glue will sequester lubricin through interacting with its heparin-binding domains and thus promote fibrocartilaginous tissue healing induced by endogenous stem/progenitor cells stimulated by CTGF/TGFβ3 μS.

1.2. ***Background***

1.2.1. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) often refer to bone marrow derived mononuclear cells that 1) adhere to cell culture polystyrene, 2) are capable of self-replication and 3) can differentiate into multiple cell lineages [8, 9]. In recent years, several stem- or progenitor-like cells have been isolated from various adult tissues, including but not limited to synovial membrane, adipose tissue, skeletal muscle, periosteum, tooth, dental pulp. Many of stem- or progenitor-like cells share commonalities with bone-marrow-derived MSCs, although several differences have been noted. Bone marrow MSCs natively form connective tissues including bone, cartilage, adipose tissue, tendon, muscle, and many craniofacial structures [8, 9]. MSCs have remarkable, but probably not unlimited, capacity for self-replication [10]. It is for certain that MSCs are capable of differentiation into multiple cell lineages including, but not limited to, chondrocytes, osteoblasts, adipocytes, etc. [2, 8-13]. MSCs have been treated as the yardstick for the regeneration of musculoskeletal tissues and have been utilized in the regeneration of other tissues such as cardiac and neural tissues. MSCs can be isolated from the patient who needs the treatment, and therefore can be used autologously without the issue of immunorejection. MSCs have also been used allogeneically and been shown to heal large defects [2, 8-13]. MSCs are one of the adult or somatic stem cells, and therefore not associated with ethical concerns in comparison with embryonic stem cells [10]. In this project, MSCs are utilized as an endogenous source for meniscus regeneration. Our in-situ regeneration represents an emerging novel strategy to guide tissue regeneration by recruitment of body's own stem/progenitor cells. As an accessible MSC source for meniscus regeneration, our approach is designed to recruit synovial MSCs (syMSCs) into the defects in avascular zone of knee meniscus.

1.2.2. Controlled delivery of CTGF and TGFβ3 for meniscus regeneration by recruitment and differentiation of syMSCs

To guide regeneration of avascular meniscus tears, we devised step-wise approach for MSC differentiation into fibrochondrocytes-like cells controlled by temporal delivery of CTGF and TGFβ3. This strategy was inspired by the phenotype transition observed in meniscal development [14]. At embryonic day 16 (E16), menisci are immature fibrous tissues predominantly expressing collagen type I that gradually transform into zone-specific fibrocartilage with increased expressions of collagen type II and aggrecan in the inner zone by 9 months of age [14]. Taking lessons from the meniscus development and our previous study, we

devised a novel approach to improve avascular meniscus healing by inducing recruitment and step-wise differentiation of syMSCs (**Fig. 1**). Temporally controlled delivery of CTGF, a chemotactic/profibrogenic cue, and TGFβ3, chondrogenic cue, successfully recruited syMSCs into the defect sites and formed integrated intermediate fibrous matrix, followed by fibrocartilaginous integration with functional restoration. The sustained release is vital allowing TGFβ3 to provide the stimulus for the differentiation of MCSs into chondrocytes necessary for the generation of zone-specific fibrocartilage tissue [17].

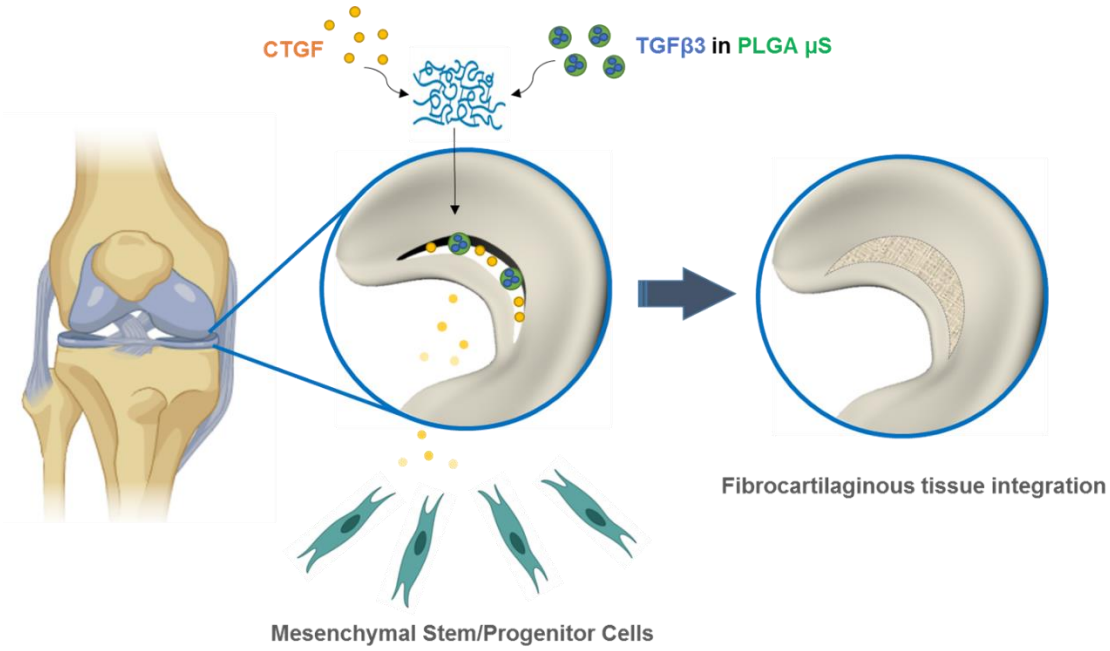

Figure 1. *In situ* regeneration strategy for avascular meniscus tears by recruitment of syMSCs, followed by step-wise differentiation [5, 6].

To enable single application with timely release of CTGF and TGFβ3, we delivered CTGF-loaded in fibrin-based bio-glue for a short-term release (<7 days) and TGFβ3 as encapsulated in PLGA μS for a prolonged release. The encapsulation of growth factors in PLGA microspheres (μS) has been widely applied for a sustained and controlled release. As PLGA undergoes hydrolysis-based degradation in physiological buffer, encapsulated growth factors or bioactive cues are released from the μS in a sustained manner [2, 8]. The release rate can be further adjusted by controlling the degradation rate of PLGA through applying various monomers ratio and compositions [2, 8].

1.2.3. *Lubricin*

Lubricin is a surface-active mucinous glycoprotein created in the synovial joint that is integral to cartilage integrity by coating the knee joint and providing boundary lubrication and preventing cell and protein

adhesion [15]. Lubricin's known physiological role is to promote healing and reduce friction-induced damage of articular cartilage, protecting against degenerative joint diseases like osteoarthritis [15]. However, the lubricating effect of lubricin may have a detrimental effect on mesenchymal stem-cell regeneration of fibrocartilaginous tissue due to the anti-adhesive properties.

1.2.4. Genipin-crosslinked Fibrin Gel with Heparin conjugation

Fibrin is a natural hydrogel or bio-glue with notable biocompatibility that have been extensively used for various tissue engineering and drug/cell deliveries. As similar to natural blood clotting process, fibrinogen is crosslinked by thrombin to form fibrin gel. One of the outstanding challenges of fibrin gel is its weak mechanical properties and fast degradation in vivo. To elevate the essential properties of fibrin, we applied genipin as an additional crosslinker. Genipin is a natural water-soluble and biocompatible crosslinking agent that produces very stable products. When combined with fibrin, genipin stabilizes the gel structure and delays the degradation of fibrin. In this project, we further conjugated genipin-crosslinked fibrin with heparin. As an anticoagulant, heparin has been used to prevent the formation of blood clots [16]. Additionally, heparin has been incorporated in fibrin gel to support sustained release of growth factors with heparin binding affinity [16]. Here we conjugated heparin in genipin-crosslinked fibrin to promote initial bonding of fibrin-based bio-glue to lubricin-coated surface of torn menisci given the existence of a heparin binding site (K134RSPKPPNKKKTKKV148) in lubricin [15]. Binding of lubricin and heparin will potentially bypass the detrimental effects of lubricin on healing of avascular meniscus tears.

2. Methods

2.1. Synthesis of Heparin-conjugated Fibrinogen

Heparin was covalently bonded to plasminogen-free fibrinogen (Sigma) using a procedure that employed standard carbo-diimide chemistry as depicted in **Fig. 2**. Heparin powder was dissolved in 50 mL of DI water. N-hydroxysuccinimide solution (0.005 g of NHS in 10 mL DI; 0.04mM) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride solution (0.015 g EDCA in 10 mL DI; 0.08 mM) were added to the solution. The final solution of 100 mL was achieved by adding additional DI water. After waiting 12 hours of reaction at 4º C to activate the carboxylic acid groups of heparin, fibrinogen (200 mg) was dissolved in phosphate-buffered saline (PBS 20 mL) and reacted with the activated carboxylic acid groups of heparin for 3 hours at 4º C. The resultant white powder was completely dissolved in PBS and dialyzed through a porous membrane bag to remove residual heparin at 4ºC for 24 hours. Finally, heparin-conjugated fibrinogen was lyophilized for 48 hours to produce a powder product [16].

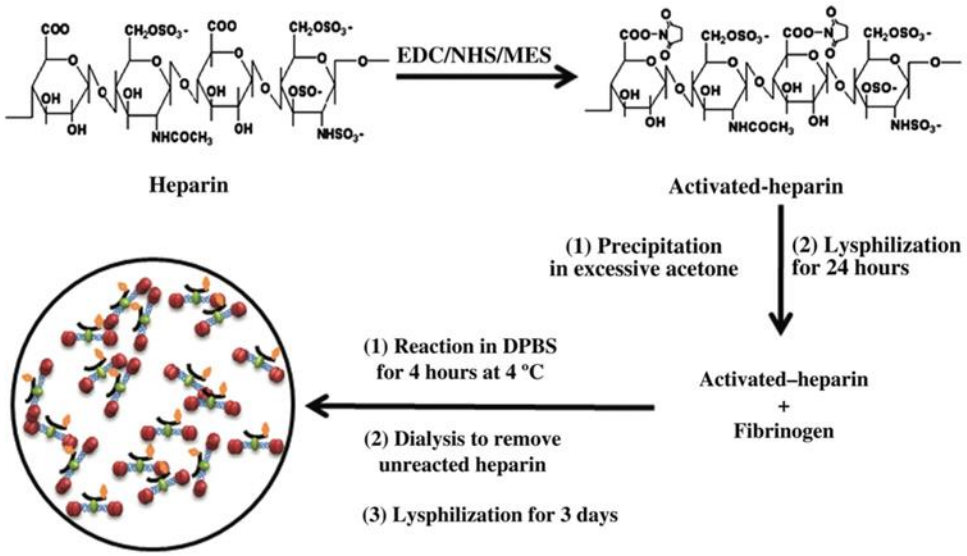

Figure 2. Process of heparin conjugation to fibrinogen [16].

2.2. TGFβ3 encapsulation in PLGA μS

PLGA (66,000-107,000 Mw) with a PLA/PGA ratio of 75:25 was purchased from Sigma (St. Louis, MO). PLGA μS encapsulating recombinant human TGFβ3 were prepared by a modified double-emulsion technique, a well-established control-delivery vehicle demonstrating preserved bioactivity of growth factors. Briefly, 500 mg PLGA was dissolved into 5 mL chloroform followed by adding 250 μL of diluted TGFβ3. This solution was then emulsified (primary emulsion) by ultrasonicating for 5 minutes to reduce the size of μS. The primary emulsion (w/o) was then added to 10 mL 4% (w/v) PVA (poly vinyl alcohol) solution to form the second emulsion (w/o/w) by 2 minutes ultrasonication followed by 1-minute vortexing. This double emulsion solution was then added to 250 mL of 0.3% PVA solution followed by continuous stirring for 2 hours to evaporate the solvent. Finally, the microspheres (μS) were filtered, washed with DI water, resuspended in DI water and then lyophilized.

2.3. Lap shear test

We performed lap shear tests to evaluate initial bonding strength of bio-glue to meniscal tissues as per our well-established protocol [5, 6]. Briefly, 20 μl bio-glue was applied in between bovine meniscus tissue strips coated with lubricin, followed by applying displacement at 0.02 mm/s using BioDynamics testing system (TA instruments, New Castle, DE) (**Fig. 3**). From the load vs. displacement curve, the lap shear modulus was as shear stress (kPa; N/mm^2; exerted load divided by adhesion area) divided by shear angle (rad). Lap shear strength was obtained as the maximum shear modulus at break.

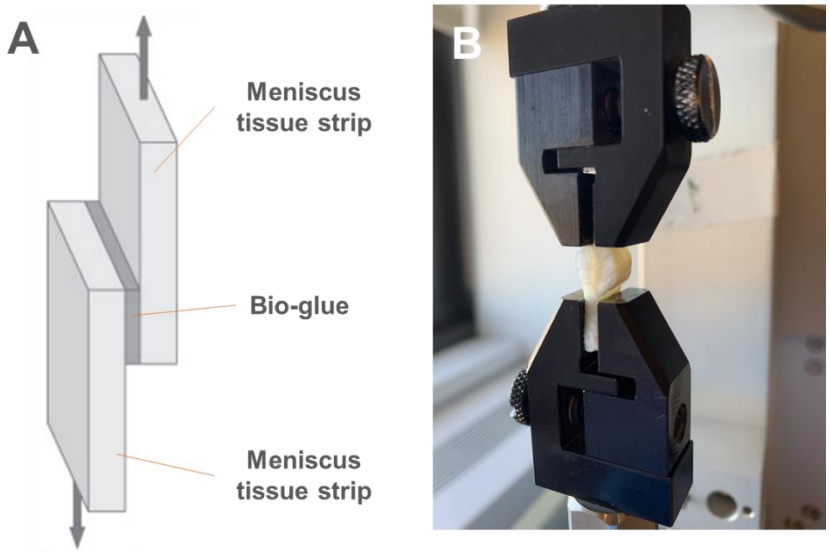

Figure 3. Experimental configuration for lap shear test (**A**). Actual specimen mounted in BioDynamics test system (**B**) (Author).

2.4. Explant model for avascular meniscus healing by syMSCs recruitment

A meniscus explant model was used to study *in vitro* healing of avascular meniscus tears (**Fig. 4**). Menisci were isolated from skeletally mature bovine knee joints from a local butcher shop. An Institutional Animal Care and Use Committee (IACUC) approval was exempted as there is no live animal involved in the experiments as per Public Health Service (PHS) policy. The isolated menisci were rinsed with 10X antibiotics (5 mins; 2 times), 1X antibiotics (5 mins; 2 times), and then washed in PBS. The inner third zone of menisci were cut and prepared as wedge-shaped tissue explants in a thickness of 2–3 mm. Then full-thickness longitudinal incisions were made in the middle of the inner third zone, and various gel combinations, including i) fibrin (Fib), ii) fibrin + genipin (Fib-Gen), iii) heparin mixed with fibrin + genipin (free Hep-Fib-Gen), and iv) heparin conjugated fibrin + genipin (Conjugated Hep-Fib-Gen) were applied to glue the incised tissues. Briefly, 50 mg/mL of each gel solution and 50 U/mL thrombin mixed with genipin (2.5 mg/mL), CTGF (100 ng/mL) and TGFβ3 encapsulated PGLA µS (10 mg/mL) were co-injected at a total volume of 50 µl in between the incised tissue surfaces using FibriJet® dual-injector with a blending applicator. Then the meniscus explants were placed on the monolayer cultured P5 - P6 human syMSCs, established from our previous work. Fibrogenic induction supplement (50 µg/mL ascorbic acids) was applied to the samples for 2 weeks followed by chondrogenic induction supplements (1% 1 × ITS + 1 solution, 100 µg/ml sodium pyruvate, 50 µg/ml L-ascorbic Acid 2-phosphate, 40 µg/ml L-proline, and 0.1 µM dexamethasone) for an additional two weeks to the explant culture. The samples were observed

weekly using bright-field microscopy. Fibrocartilaginous tissue integration was evaluated by H&E and Saf-O/Fast Green staining and mechanical tests as described below.

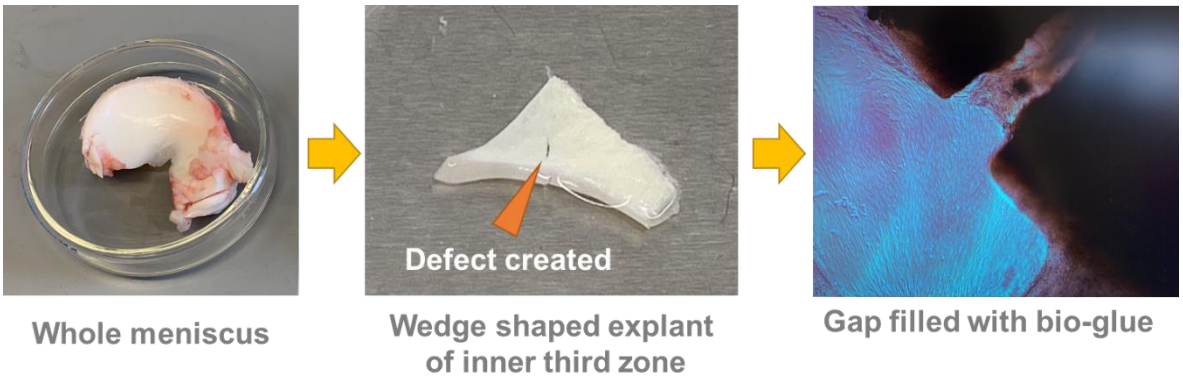

Figure 4. Preparation of meniscus explant healing model for avascular meniscus tears (Author).

2.5. Tensile Tests of Meniscus Explants

Tensile tests were conducted to evaluate the functional properties of healed meniscus. Following a well-established testing protocol for meniscus explants [5, 6], samples for the pull-out tests were prepared using a cryotome as 500~600 μm in thickness and a width of 1 mm as per previous works. Upon mounting with tensile jigs in an isotonic saline bath at RT, a 0.02-N tare load was applied to the samples and then the samples will be elongated at 10%/min until failure. From the force vs. elongation curve, the ultimate strength and tensile modulus were obtained. Briefly, the tensile modulus was calculated as the slope of stress (force/cross-sectional area) vs. strain (displacement/initial length), and the ultimate strength represent maximum load divided by cross-sectional area. All pull-out tests were performed using BioDynamics test system (TA instruments, New Castle, DE).

2.6. Statistical Analysis

For all the quantitative data, following confirmation of normal data distribution, one-way analysis of variance (ANOVA) with post-hoc Tukey HSD tests were used with p value of 0.05. Sample sizes for all quantitative data were determined by power analysis with one-way ANOVA using a level of 0.05, power of 0.8, and effect size of 1.50 chosen to assess matrix synthesis, gene expressions, and mechanical properties in the regenerated meniscus tissues and controls upon verification of normal data distribution.

3. Results

Meniscus explant samples showed surface coating of lubricin when incubated in synovial fluids for 24 hours (data not shown). In our lab shear tests with lubricin-coated meniscus tissue samples, conjugated

Hep-Fib-Gen showed a significant increase in shear modulus as compared to Fib, Fib- Gen, and free Hep-Fib-Gen (**Fig. 5A**) (n = 8 – 15 per group; p < 0.0001). Fib-Gen and free Hep-Fib-Gen resulted in marginal increases in the shear modulus as compared to Fib control, as tested with lubricin-coated meniscal tissue strips (**Fig. 5A**). As compared to the shear strengths tested without lubricin coating, Fib, Fib-Gen, and free Hep-Fib-Gen showed approximately 14% - 33% reduction when tested with lubricin coated tissues (**Fig. 5B**). In contrast, conjugated Hep-Fib-Gen led to ~68% increase in lap shear modulus with lubricin coating as compared to without coating (**Fig. 5B**) (n = 8 – 15 per group; p < 0.0001).

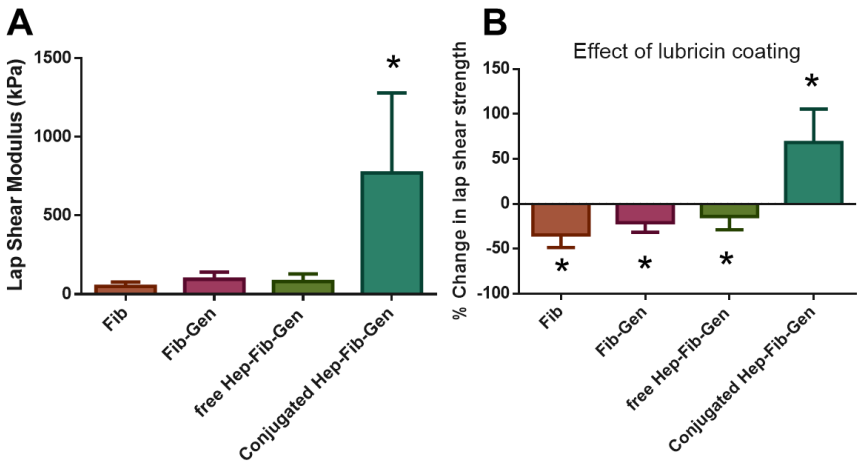

Figure 5. Lap shear modulus (**A**) (n = 8 - 15 per group: p<0.0001 compared to all the groups). % Change in shear strength by lubricin coating compared to no coated samples (**B**) (n=8-15 per group; p<0.001 compared to no-coating control) (Author).

By 4 weeks of explant culture, all the tested bio-glues delivered with CTGF and TGFβ3, including Fib, Fib-Gen, free Hep-Fib-Gen, and conjugated Hep-Fib-Gen, displayed improved tissue integration of avascular meniscus tears through MSC recruitment (**Fig. 6A**), consistent with our previous works. However, lubricin coating resulted in larger remaining gaps between incised tissues with Fib, Fib-Gen and free Hep-Fib-Gen as compared to control without lubricin coating (**Fig. 6A**). Only the conjugated Hep-Fib-Gen showed a notable improvement in tissue integration of lubricin-coated avascular meniscus tears as compared to control without lubricin coating (**Fig. 6A**). Quantitatively, the tensile modulus of healed meniscus was significantly higher with conjugated Hep-Fib-Gen delivered with CTGF and TGFβ3 as compared to Fib, Fib-Gen, and free Hep-Fib-Gen (**Fig. 6B**) (n = 8 – 15 per group; p < 0.001). When

compared to control without coating, lubricin coating resulted in ~200% increase in tensile modulus with conjugated Hep-Fib-Gen, whereas Fib, Fib-Gen, and free Hep-Fib-Gen showed significant decreases in tensile modulus (**Fig. 6C**). Similarly, tensile strength was significantly decreased with lubricin coating in Fib group (**Fig. 6D**). Fib-Gen and free Hep-Fib-Gen showed marginal increase in tensile strength with lubricin coating as compared to control without coating (**Fig. 6D**). Conjugated Hep-Fib-Gen showed a significantly higher tensile strength with lubricin coating as compared to all the other bio-glue groups (**Fig. 6D**) (n = 8 – 15 per group; $p < 0.001$).

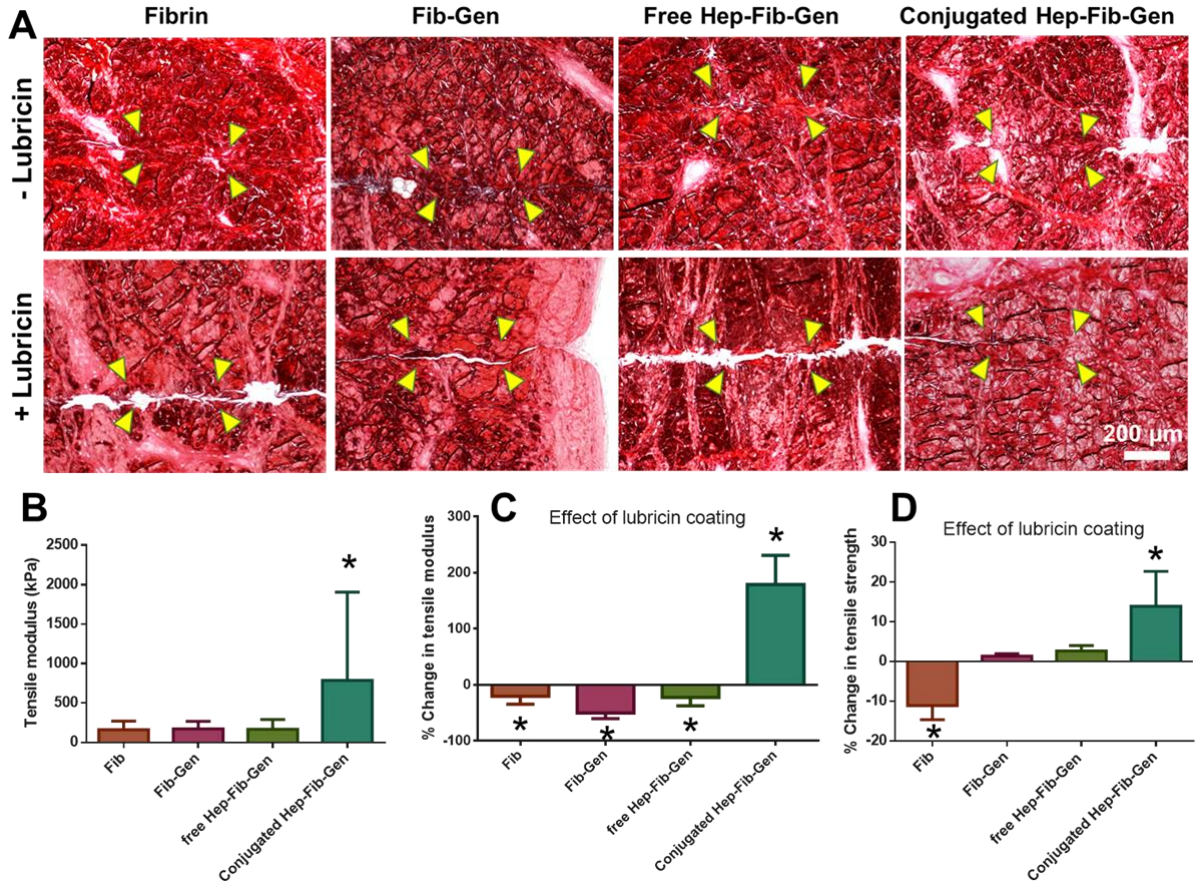

Figure 5. Healing of avascular meniscus tears by MSC recruitment with CTGF and TGFβ3-µS delivered through various bio-glues for 4 wks: Histology with Picrosirius Red (PR) (**A**), and tensile modulus (**B**) (n = 5 per group; *:p<0.01 compared to all groups), % changes in tensile modulus (**C**) and strength (**D**) by lubricin pre-coating (n = 5 per group; *:p<0.01 compared to control with no coating) (Author).

4. Discussion

To support the body, the knee meniscus is a crucial component of load bearing, transmission and distribution, and shock absorption [1, 2]. Knee meniscus tears are common injuries that inhibit this fibrocartilage structure and damage its effectiveness. In order to combat these tears, clinically invasive techniques have been primarily employed as a remedy. A novel approach to induce the avascular healing of the meniscus was introduced by harnessing mesenchymal/progenitor stem cells and stimulating them with CTGF and TGFβ3. The sustained release of CTGF and TGFβ3 from PLGA microspheres allows recruitment of the MSC and step-wise differentiation into zone specific cells, such as fibroblast-like and chondrocyte-like cells, to form a layered, diverse fibrocartilage tissue. Previously, these methods exhibited regeneration of the avascular meniscus similar to native tissue types and presented a regenerative therapy that could be a consistent delivery for healing of avascular meniscus tears and reduce the incidence and severity of degenerative osteoarthritis in patients. However, the common presence of lubricin, a mucinous glycoprotein that provides boundary lubrication and prevents cell and protein adhesion, in meniscus tears inhibits the significance of this method [15].

Our results display that through the conjugation of heparin with fibrinogen and genipin the mechanical levels of the gel have been increased and the stability of the gel has been improved due to the addition of genipin, a guaranteed cross-linker. This remedied a previous flaw in the use of fibrin as a delivery system due to a rapid degradation rate of fibrin that was not suitable for a controlled release profile of PLGA microspheres and growth factors. Additionally, when cultured with MSCs and growth factors, significant healing was observed with the heparin conjugated fibrinogen genipin gel despite the presence of lubricin and its hindering properties. The tensile strength tests of the gels after culturing also showed how heparin conjugated fibrinogen genipin retained high tensile modulus in lubricin samples, significantly higher, when compared to fibrin, fibrin genipin, and heparin fibrin genipin. Our hypothesis was supported that the incorporation of heparin into a fibrin-based bio-glue will sequester lubricin through interacting with its heparin-binding domains and thus, promote fibrocartilaginous tissue healing induced by MSCs with CTGF/TGFβ3uS. Despite these findings, there are shortcomings of our results.

4.1. Limitations

One of the limitations of this study is a relatively large variance in the initial mechanical properties of conjugated heparin fibrin genipin as compared to fibrin or fibrin genipin, which may require further refinement of purity and accuracy control in the synthesis process. Additionally, due to inconsistency of the lap shear machine, results produced ranged with varying numbers also possibly due to the conditions

of the test and environment. Further mechanical testing would be recommended to reinforce the conclusions reached.

5. Conclusion and Future Studies

Our findings built off an established novel approach to induce healing of avascular meniscus tears by recruitment and step-wise differentiation of endogenous stem cells. Developing this approach, we expanded the effectiveness to counter the presence of lubricin. In human patients, there is an inevitable time interval (over 2 ~ 3 weeks) between the meniscus injury and the first doctor's visit. Therefore, the injured tissue surfaces are very likely infiltrated by lubricin through exposure of torn area to synovial fluids from the surrounding knee joint. Our previous study consistently showed detrimental effects of lubricin coating on the healing of avascular meniscus tears by bio-glue delivered with CTGF and TGFβ3. In this study, our data suggest that heparin conjugation coupled with fibrin-genipin hydrogel strengthened the initial bonding of lubricin-coated meniscal tissues, consequently enhancing avascular meniscus healing. Given that lubricin binds to cartilage and meniscus surface through C-terminal, the heparin binding domain at N-terminal of lubricin may form stronger initial bonding with heparin covalently bonded into fibrin. Heparin conjugated fibrinogen with genipin displayed nearly seamless healing, despite the barrier that lubricin presented. Conversely, when compared to lubricin coated samples with fibrin, fibrin genipin, or free heparin fibrin genipin gels, such healing is not observed, and noticeable separation is still present in the tissue samples. This strongly suggests that heparin conjugated reinforced and strengthened the binding potential of the fibrin genipin bio-glue. Heparin conjugation also further enhanced mechanical properties of fibrin cross- linked with genipin. Genipin cross-links through ring opening attacked by amine groups, followed by covalent bonding and re-closure of rings and so on. As fibrin as well as heparin have amine group, pre-conjugation with heparin is suggested to result in further improved mechanical properties. While the synthesis of conjugated heparin is inconsistent, continued testing with varying concentrations of genipin could display different effects on the stability and consistency of conjugated heparin fabrication and the integrity of the hydrogel. Additionally, further testing with various kinds of tears, such as complex tears, radial tears, and flap tears, could identify the regenerative capabilities of this novel approach. Clinical testing in animal models would also provide more insight on the methods true healing abilities.

In conclusion, heparin conjugated fibrinogen may serve as efficient bio-glue to support avascular healing of clinically relevant meniscus tears by endogenous stem cell recruitment. This data serves as a significant building block off of previously established methods from our previous works by bypassing the detrimental effects of lubricin in meniscus tears.

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