DISSERTATION

CARTILAGE REPAIR USING TRYPSIN ENZYMATIC PRETREATMENT COMBINED WITH GROWTH-FACTOR FUNCTIONALIZED SELF-ASSEMBLING PEPTIDE HYDROGEL

Submitted by

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ABSTRACT

CARTILAGE REPAIR USING TRYPSIN ENZYMATIC PRETREATMENT COMBINED WITH GROWTH-FACTOR FUNCTIONALIZED SELF-ASSEMBLING PEPTIDE HYDROGEL

Treatment of cartilage defects remains challenging in the orthopedic field. Several techniques are currently available to treat cartilage defects, with subchondral bone microfracture being the most commonly used marrow stimulation technique. However, despite satisfactory results in the short-term, clinical and functional outcomes of microfracture treated patients tend to decline over time. Improving microfracture technique using tissue engineering principles may be a more attractive way to treat cartilage defects compared to other more complex and expensive alternatives. Self-assembling peptide hydrogel has been extensively studied as a scaffold for cartilage repair. This hydrogel is biocompatible within the joint environment and has been shown to increase cartilage healing and improve clinical and functional outcomes in both rabbit and equine models of cartilage repair. Recently, a clinically applicable technique was described using trypsin enzymatic pretreatment of the surrounding cartilage combined with local delivery of heparin binding insulin growth factor-1 (HB-IGF-1). The results of this study demonstrated improved cartilage integration *in vitro* when this technique is utilized. Thus, in the present study we evaluated the combination of trypsin enzymatic pretreatment with a self-assembling peptide hydrogel functionalized with growth factors to improve cartilage repair. First, the effect of trypsin enzymatic pretreatment alone or combined with self-assembling peptide hydrogel functionalized with HB-IGF-1 and/or platelet-derived growth factor- BB (PDGF-BB) was tested

using a rabbit model (48 rabbits). Subsequently, trypsin enzymatic pretreatment combined with self-assembling peptide hydrogel functionalized with HB-IGF-1 and PDGF-BB was used to augment microfracture augmentation in an equine model of cartilage defects (8 horses). In the small animal model, trypsin enzymatic pre-treatment resulted in an overall increase in defect filling, as well as improvements in subchondral bone reconstitution, surface regularity, cartilage firmness, reparative tissue color, cell morphology and chondrocyte clustering. The presence of PDGF-BB alone improved subchondral bone reconstitution and basal integration, while the combination of HB-IGF-1 and PDGF-BB resulted in an overall improvement in tissue and cell morphology. In the equine model, microfracture augmentation using trypsin enzymatic pretreatment combine with self-assembling peptide hydrogel functionalized with growth factors (HB-IGF-1 and PDGF-BB) resulted in better functional outcomes, better defect healing on second look arthroscopy at 12 months, as well as improved reparative tissue histology and increased biomechanical proprieties of the adjacent cartilage compared to defects treated with microfracture only. In conclusion, trypsin enzymatic pretreatment combined with self-assembling peptide hydrogel functionalized with growth factors (HB-IGF-1 and PDGF-BB) resulted in successful microfracture augmentation. These therapeutic approaches can result in a more cost effective way to improve cartilage healing in patients undergoing subchondral bone microfracture.

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SUBCHONDRAL BONE MICROFRACTURE FOR THE TREATMENT OF CHONDRAL DEFECTS: LITERATURE REVIEW

Introduction

Articular cartilage is a highly specialized tissue composed of a complex extracellular matrix and few chondrocytes. Its main function is to provide a low friction surface and facilitate load distribution to the subchondral bone. The three main components of the extracellular matrix are water, collagen and proteoglycan. Water is responsible for up to 80% of the total cartilage volume, existing in form of gel in the interfibrillar space. The frictional resistance to water flow and pressurization are considered to be the main mechanisms by which cartilage absorbs load. Collagen is the most abundant macromolecule found in the extracellular matrix, with collagen type II constituting 90 to 95% of the total collagen content. Other types of collagen such as collagen type I, IV, V, VI, IX and XI are also present and help to form and stabilize collagen type II. Proteoglycans are the second largest macromolecule present in articular cartilage and are composed of one or more linear glycosaminoglycan chains attached to one core protein. Aggrecan is the most abundant proteoglycan in cartilage and functions by interacting with hyaluronan to form large proteoglycans aggregates. Aggrecan is negatively charged and gives the articular cartilage its osmotic properties, which contributes to its ability to resist compressive loads. Articular cartilage can be divided in 4 distinct zones. The most superficial 10 to 20% of the articular cartilage is considered the superficial zone. This layer consists of tightly packed collagen type II, collagen type IX and flat shaped chondrocytes positioned parallel to the joint surface. The

superficial zone is responsible for protecting deeper layers from shear forces. The transitional zone sits just below the superficial zone and provides an anatomic and functional transition between the most superficial and the deeper layers. The transitional zone occupies 40 to 60% of the total cartilage volume. This region is rich in proteoglycans and contains thicker collagen fibrils organized obliquely to the joint surface, and is the first zone responsible for resisting compressive forces. The deep zone represents 30% of the articular cartilage volume and provides additional resistance to compressive loads. In this zone we find the largest diameter collagen fibrils, highest proteoglycan content, and lowest water content. Also, chondrocytes are arranged in columns parallel to the collagen fibers and perpendicular the joint surface. These characteristics give the deep zone a great capacity to resist compressive forces. Immediately below the deep zone is the tidemark, which separates the deep zone from the calcified cartilage and functions to secure the cartilage to the subchondral bone. Within the articular cartilage there also exists three distinct regions which are defined by their proximity to chondrocytes as well as by differing composition and diameters of collagen fibrils. The pericellular matrix is a thin layer surrounding the chondrocyte and is responsible for signal transduction between matrix and chondrocyte. The territorial matrix is thicker and protects the cartilage cells from mechanical load with its basket-like collagen network. The interterritorial region is the largest of the three regions and is a main contributor to the known biomechanical properties of cartilage(1).

Articular cartilage has very limited capacity to heal (2-4). Several factors such as low cellularity, dense extracellular matrix and lack of blood supply are considered responsible for decreased capacity of the articular cartilage to heal(1, 5). Additionally, chondrocytes are entrapped in a complex extracellular matrix, which prevents their migration to damaged sites(1).

Acute or chronic repetitive trauma, osteoarthritis, osteochondritis dissecans and metabolic factors are considered possible etiologies for cartilage defects (4, 6). Cartilage lesions affect approximately one million people per year in the United States and results in approximately 200,000 surgical procedures(7). About 60% of patients undergoing knee arthroscopy are found to have some type of cartilage lesion(8-10). Additionally, cartilage defects are observed in about half of the cases of diagnosed anterior cruciate tears (11, 12). Surgical procedures to treat cartilage defects has a mean annual incidence of 90 surgeries per 10,000 patients, with 5% growth per year(13).

A recent consensus statement from the United Kingdom knee surgeons suggests that the currently available cartilage resurfacing techniques should be reserved to treat isolated, symptomatic full-thickness cartilage defects(14). Therefore, despite the common incidence of cartilage defects, less than 10% of patients are considered ideal candidates for treatment with the currently available techniques(8-10).

The three main categories by which cartilage resurfacing techniques can be grouped are as follows: palliative (chondroplasty/debridement), reparative (drilling and microfracture), and restorative (autologous chondrocyte implant, osteochondral autograft/allograft transplantation). The decision regarding which treatment to choose should be based on defect size, number and geometry, as well as patient demographic characteristics (age, BMI), concomitant pathologies, physical activity, expectation and compliance to rehabilitation program(14, 15). Palliative treatment of the cartilage defect is still the most common approach; it is two times more common than reparative techniques and fifty times more common than restorative techniques(13). For older patients, palliative and reparative techniques are performed more commonly than restorative techniques(15). Microfracture falls within the category of marrow stimulation techniques, which also includes

procedures such as subchondral bone abrasion and drilling. In all of these procedures, the marrow cavity is accessed allowing progenitor cells migrate to the defect and participate in the healing process(4). Microfracture is the most common marrow stimulation technique in use today(13, 16). Dr. Steadman developed microfracture in the early 1980's based on modification of the Pridie drilling technique and basic science principles. This technique takes advantages of the body's own ability to heal and is considered simple, has low morbidity and does not burn bridges for future treatments(17, 18).

Basic Science

The first experimental study to validate subchondral bone microfracture was done by Frisbie and coworkers (1999) using an equine model. Bilateral 1 cm^2 cartilage defects were created on the medial femoral condyle of the femur and in the radial carpal bone of ten horses. One defect on the radial carpal bone and medial femoral condyle was randomly selected to receive subchondral bone microfracture, while the contralateral defect served as the control. All horses were exercised using a high-speed treadmill in the post-operative period. Five animals were euthanized at 4 months, and another five at 12 months. Clinical, radiological, necropsy, histological and histomorphometric evaluation was performed, as well as quantification of collage type I and II. Significant improvement in defect filling was noticed in microfracture treated defects. Repair tissue filling was similar between 4 and 12 months, suggesting no increase in reparative tissue is observed after 4 months. Additionally, at 4 months, the amount of collagen type II was increased in the microfracture treated defects of the medial femoral condyle compared to control. This difference was not statistically significant at 12 months. The authors concluded that beneficial effects are appreciated with subchondral bone microfracture and no apparent deleterious effects were noted.

Interestingly, despite the apparent beneficial effects, no significant difference was seen in the histologic appearance of the reparative tissues of defects treated with subchondral bone microfracture compared to the control(19).

In a subsequent study, the early events in cartilage healing after microfracture technique was evaluated. Bilateral 1cm^2 cartilage defects were created on the medial femoral condyle of 12 horses. Two horses were euthanized at 2, 4 and 6 weeks. The remaining six horses were euthanized at 8 weeks after defect creation. Gross examination of the joints and histological evaluation of the reparative were performed. The expression of cartilage extracellular matrix content (collagen type I, collagen type II and aggrecan) was evaluated by in situ tissue hybridization, immunohistochemistry and reverse transcription coupled polymerase chain reaction (rtPCR). In this study, microfracture significantly increased collagen type II expression as early as 6 weeks post defect creation, however aggrecan content was not different between the treatment and control groups(20). These findings were further supported by a transcriptional profile comparing the differences between articular cartilage and repair tissue in the equine medial femoral condyle. The authors reported that although the cells occupying the reparative tissue appeared to be of mesenchymal origin, full differentiation to the chondrocyte phenotype was not achieved(21).

The role of calcified cartilage in the healing of cartilage defects treated with microfracture was also evaluated. In this study, the authors compared microfracture technique in chondral defects that either had the calcified cartilage completely removed or left intact. Again, a cartilage defect was created in both medial femoral condyles of 12 horses. Similar to the first study, clinical, radiographic, necropsy and histological evaluations were performed. In this investigation, however, arthroscopic evaluation was performed at 4 months and an osteochondral biopsy was harvested for histology. Interestingly, there was 50% improvement in overall cartilage repair as

well as 28% more defect filling when the calcified cartilage was removed. Also, the presence of calcified cartilage had a strong negative correlation with cartilage attachment to the bone. The authors concluded that the removal of calcified cartilage during subchondral bone microfracture resulted in increased reparative tissue volume and improved attachment of the cartilage to the subchondral bone(22).

Significant variation in case selection, surgical technique, and post-operative rehabilitation has been demonstrated in a survey of 131 surgeons performing microfracture in Canada. This survey revealed that post-operatively, 89% of the surgeons do not use continuous passive motion and about 20% allow full weight bearing soon after surgery. Furthermore, careful removal of the calcified cartilage before performing the microfracure, a known factor affecting outcome, is not performed by 31% of the surgeons(23). Inconsistency in microfracture technique was objectively assessed using fresh human cadaver limbs. This study showed that surgeons tend to misjudge the penetration depth and the inter-hole distance, making the holes too deep and/or to close together. These inconsistencies were speculated to impact clinical outcome(24).

There are substantial differences in techniques for subchondral bone microfracture. Microfracture technique using small- (1 mm) or large- (1.2mm) diameter awls was compared to debridement alone in a sheep model of cartilage defect. In this study, both microfracture techniques resulted in improved histological cartilage repair compared to debridement alone, with the small-diameter awl resulting in improved overall histological tissue quality and surface regularity compared to the large-diameter awl(25). Additionally, Chen and coworkers (2009) showed in a rabbit model that subchondral bone defects differ substantially with regards to marrow access depending on whether they were created with an awl or a drill. Subchondral bone microfracture performed with an awl was shown to result in fracture and compacted bone around the holes, sealing them from the

marrow access and potentially impairing healing. On the other hand, subchondral bone microfracture performed with a drill at a depth of 6 mm was shown to increase blood clots and improve healing(26). In a subsequent study, the authors were able to show that deeper holes resulted in improved cartilage repair using the same animal model with a longer follow up period(27). Although the previous papers are widely referenced, the clinical translational information in these papers should be carefully evaluated. The authors opted to conduct their research in a rabbit model, which substantially differs from the human clinical condition with regards to cartilage and subchondral bone thickness. The exact difference between 2 mm and 6 mm hole depth, or between the use of an awl and drill to create microfracture are unknown in humans.

Subchondral bone sclerosis, similar to what is observed in late stage osteoarthritis (OA), may reach a critical limit at which point conventional microfracture creates bone compaction and fissures instead of marrow access(28). Considering these limitations, a patented "hollow awl" was developed and tested in human knees undergoing total knee replacement (terminal stage OA). The authors claimed the use of this device resulted in more patent marrow channels, while in conventional microfracture numerous crush particles occluding the marrow channels were observed. As a consequence, an increasing in bleeding (clot volume) and amount of mesenchymal stem cells were found when using the "hollow awl" to create the defect(29). Because this study was conducted using patients with terminal stage OA where subchondral bone density is increased, the "hollow awl" may not actually demonstrate the same meaningful clinical difference in cases of more acute cartilage damage.

Clinical Outcome

In the initial report describing microfracture outcomes in people, 298 patients underwent subchondral bone microfracture to treat cartilage defects in the knee, followed by an extensive rehabilitation program. Second look arthroscopy was performed in 77 knees, which demonstrated subjectively improved cartilage healing(18).

Later, the same group published a more comprehensive study on the treatment of isolated traumatic cartilage defects (without concomitant joint pathology such as ligament or meniscus injury) with microfracture. Seventy-two patients (45 year-old or younger) met the inclusion criteria. Follow-up was recorded from 71 of the 75 knees enrolled in the study (95%), and a follow-up period of 11 years was reported. Patients were evaluated prior to surgery and annually thereafter using Lysholm knee questionnaire and Tegner activity scale. Additionally, at the end of the study, the patient filled out both WOMAC and SF-36 questionnaires. Two patients were removed from analysis as they needed additional intervention and were considered failure. Overall, this study concluded that microfracture had low morbidity and minimal risk, as no complications were reported. Microfracture improved all evaluated parameters compared to pre-operative scores. The WOMAC pain score showed that 30% of the knees were considered pain-free, while 50% presented mild and 13% moderate pain. Increased pain after surgery was observed in only 4% of the knees. Age was considered a negative predictor of success, with patients younger than 35 years old improving 23% more than patients with age between 35-45 years old. The location of the lesion had a trend toward being a negative prognostic indicator, while defect size and chronicity were not considered to be associated with outcome. The most significant improvement was found to occur in the first year post-surgery, however maximum improvement was not observed until 2 to 3 years

after surgery. Overall, the authors concluded microfracture treatment resulted in good to excellent results for the majority of the patients and should be indicated as first-line of treatment for traumatic isolated full-thickness cartilage defects(30).

Several other studies report data on patient satisfaction as well as clinical outcome after subchondral bone microfracture in osteoarthritic knees. In one study, eighty-one patients with full thickness chondral defects in moderately osteoarthritic knees were evaluated. All patients were older than 40 years old (49.4 year-old on average) with an average defect size of 2.2cm^2. The patients filled out self-reported questionnaires assessing pain, swelling, functional outcomes, and patient satisfaction before and after surgery. The authors reported 54% improvement in Lysholm knee function score and 55% improvement on Tegner activity scale. There was a trend toward lesser improvement on Lysholm scores for defects larger than 4cm^2. Overall patient satisfaction was 8.3 on a 10-point scale with an average 2.6 year follow up time(31).

In a second paper, forty-four patients, with an average age of 57 years old, were evaluated with second look arthroscopy, knee radiography, and a patient reported outcome questionnaire after one year of surgery. In this study, defect mean size was 3.9 cm^2. Significant improvement in functional outcome was observed following treatment, with 36% patients reporting excellent and 53% reporting good results. Reparative tissue was present in more than 90% of the defect area in 55% of the cases. Twenty-one percent of the cases had reparative tissue covering 80 to 89% of the defect area and 17% of the cases the reparative tissue was covering between 50 to 79% of the defect. Only 3% of the cases had defect healing in less than 50% of the defect area. Additionally, there was a 32% improvement in joint alignment after microfracture treatment. Differing levels of collagen type II were found amongst the various cartilage biopsies 1 year after microfracture though most of the samples had less than 50% the defect staining positively for collagen type II.

This correlates well with the Westernblot results, which demonstrated that collage type II accounted for 44% of the normal cartilage on average. A positive correlation between amount of collagen type II and clinical outcome was found, though this was not statistically significant. The authors also recognized that cartilage lesions smaller than 3 cm^2 healed better than larger ones(32). Mithoefer and coworkers (2005) published clinical, functional and MRI outcomes in 48 patients subjected to microfracture for the treatment of focal cartilage defects with a minimum follow-up of 2 years (average follow up of 41 months). The average patient age was 41 years old with an average defect size of 4.8 cm^2. Twenty-four patients underwent MRI evaluation in the post-operative period. Sixty-seven percent of the patients reported good to excellent results, while 25% reported fair and only 8% reported poor results. Authors reported significant improvement in SF-36 and IKDC scores following microfracture. However, 69% of the patients showed decreases in IKDC scores after 24 months. All patients with poor defect filling showed decreased functional scores at twenty-four months, while only 3 patients with good defect filling had decreased functional outcome at the same time-point. Also, patients with higher scores on the physical component of the SF-36 demonstrated better defect filling grades on MRI. Poor cartilage integration was observed on MRI evaluation of 92% of the patients. The authors concluded that microfracture treatment of focal chondral defects resulted in significant functional improvement. The greatest improvement was observed in patients with higher degrees of defect filling(33). Unfortunately, actual means and variation were not provided, nor were statistically significant comparisons between all time points. This makes it difficult to conclude the clinical significance of these findings.

The affect of defect location on clinical, functional and MRI outcomes after subchondral bone microfracture was assessed in 85 patients (mean age 39.5 year-old). Patients were evaluated pre-

operatively and at 6, 12, 18 and 36 months after surgery. Clinical and functional outcomes were assessed using Cincinnati Knee and ICRS scores. MRI evaluation was performed pre-surgery and at 18 and 36 months post-surgery. Improvement in clinical and functional outcome was observed within first 18 months. Defects on the patella, tibia and trochlea demonstrated a decline in the clinical and functional outcomes between 18 and 36 months, while defects on the femoral condyles reported stable clinical and functional outcomes. Also, defects located in the femoral condyles had improved MRI scores compared to defects in other locations at 36 months. Clinical and functional outcomes correlated well with MRI defect filling in this study(34).

Ninety symptomatic patients with single cartilage defects and no concomitant joint pathology (meniscus/ligament injury) were enrolled in a therapeutic case series study (level IV). Following microfracture treatment, significant improvement was observed in all functional parameters evaluated with a mean follow up of 5 years. The authors observed that younger patients (< 35 years old) with body mass index less than 25 and defect size less than 2 cm^2 had better results. Defect location seemed to affect functional outcomes, with defects on the non-weightbearing surface resulting in better results. Also, symptom duration of less than 12 months was significantly correlated with better response (35).

Miller and coworkers (2010) published the long-term outcomes following microfracture treatment. Three hundred and fifty patients with an average age of 47.6 years old were enrolled in this study. A significant improvement in functional outcome was observed, with a maximum improvement at 2 years followed by a steady decline over the subsequent years. However, at the final follow up (10 years) the patients were still reporting higher functional outcomes scores compared to before surgery(36).

Decline in clinical outcome was not supported by another long-term study evaluating 110 patients over a period of 10 to 14 years. Clinical and functional outcome were considered improved at 5 years following surgery and maintained this improvement until the end of the study. Patient satisfaction was 68 (on the VAS scale of 0 to 100) at the end of the study and did not differ from the 5 year follow-up score. However, patients with long-standing knee symptoms (> 36 months), mild degenerative changes in the surrounding cartilage, concurrent meniscectomy, and more severe clinical signals prior to surgery and had worseoutcomes (37).

Moreover, long-term radiologic and magnetic resonance evaluation of microfracture was studied in 15 patients. In general, patients had poor functional outcomes in this study. Additionally, MRI showed poor defect filling in about 76.9% of the patients. The defect had increased in size in 10 patients at the average follow up time of 56 months(38). It is important to note the much lower rate of clinical improvement experienced by the patients in this study the compared to what is usually demonstrated in the literature. One reason for that is the inclusion of patients with joint mal-alignment in this study, which is generally a contra-indication to performing microfracture.

Clinical Outcome in Athletes

The first study evaluating microfracture for the treatment of cartilage defects in professional or recreational athletes came from Dr. Steadman's group. In this study, they looked at functional outcomes and lesion appearances in 38 professional and 140 recreational athletes for an average follow up period of 37 months. This study showed improvement in clinical symptoms and function following microfracture. The authors noticed significant clinical improvement at one year and maximal improvement at 2 years after surgery, after which the scores tended to plateau over the next 4 to 5 years. Pain scores slightly worsened between the third and fourth year post surgery in

the recreational athlete group. Seventy-seven percent of the professional athlete patients were able to return to compete, with 71% of those performing at the same level, 25% at a slightly lower level and only 4% at significantly lower level than pre-injury(39).

Similar return to play was founded by Steadman and coworkers (2003) when they evaluated microfracture for treatment of single symptomatic cartilage lesions in 25 National Football League players. The average age was 26 years old and average defect size was 3.8cm^2. All players showed significant improvement in pain, swelling and functional outcomes. Also, it was observed that 76% of the patients returned to play for an average of 57 games (4.6 seasons) (40).

In another study, athletes subjected to high-impact sports that received microfracture to treat single symptomatic cartilage lesions were evaluated for a minimum of 2 years follow up. Average patient age and defected size were reported to be 38 years old and about 5 cm^2, respectively. Sixty-six percent of the patients reported excellent or good results following microfracture. Functional outcomes, evaluated by self-reported questionnaires, were improved in most of the patients after treatment. However, 47% of the patients experienced a decrease in function after the initial improvement and only 44% of the athletes were able to return to high-level activity. Most importantly, the authors noticed that athletes were more likely to successfully return to high-impact activities if they were younger (<40 years old), had a lesion size of less than 2cm^2, had short duration of symptoms, and/or if microfracture was used as the first line of treatment(41).

Interestingly, in 2009, two very similar studies were published evaluating microfracture treatment in professional NBA basketball players(42, 43). Both studies enrolled 24 players with an average age at time of injury of 26 and 28 years old, respectively(42, 43) and the percentage of players not returning to play was 21% and 33% respectively(42, 43). Age (> 30 years old) and number of seasons played pre-injury were negative predictors for return to play(43). From those whom were

able to return to play, a significant decrease was found in minutes played per game. Seventy-six percent of the players missed at least one game in the post-surgery season due to pain in the operated knee(43). Player performance declined in the first season post-surgery but returned to nearly pre-injury levels by the second season(42). Decline in performance was less accentuated when corrected by minutes played per game(43).

Long-term outcomes for microfracture technique in professional and recreational athletes were evaluated in a study with 61 patients. Average patient age was 31.4 years old and average defect size was 4 cm^2. Patients were evaluated at 2 and 5 years post-operatively, as well as at the final follow-up time (15 years on average). No major complications were reported, however 11% of the patients had persistent pain within 5 years of surgery and were considered failures. Sixty percent of the athletes returned to the same pre-injury performance level at 2 years after surgery, however only 20% were able to perform at pre-injury level in the final follow up. Also, 40% of the patients had progressive osteoarthritic changes at the final follow up. This finding was significantly higher in older patients with large or multiple lesions. The authors concluded that microfracture is a viable first-line of treatment in young patients with small single lesions, leading to favorable results in short and long term outcomes. However, deterioration of the clinical outcomes should be expected after 2 and 5 years post surgery, especially for older athletes with large and multiples lesions(44).

Microfracture Compared to Other Cartilage Resurfacing Techniques

Microfracture was compared with first generation autologous chondrocyte implantation (ACI) using the patient reported Activity Rating Scale as well as objective evaluations of the mobility, strength and hop performance. Overall, functional outcome was similar between the two

techniques at 2 years, however ACI resulted in slower recovery at 9 and 12 months compared to microfracture(45).

Knusten and coworkers (2007) conducted a randomized clinical trial to compare microfracture to first generation ACI. The authors evaluated 80 patients at 2 and 5 years post-surgery. At the 2 year follow up, the microfracture group demonstrated significantly better scores for the SF-36 physical component. At five years, microfracture treatment had a trend ($p=0.054$) toward statistically significantly improved scores for the SF-36 physical component compared to ACI treatment. ACI had a tendency to fail (as defined by a specific set of criteria) at earlier time points compared to microfracture, with no difference in survivors between both techniques. The authors conclude microfracture should be considered as first line of cartilage repair, as it is minimally invasive and lower cost(46).

Similarly, a study was done by Saris and coworkers (2008) comparing microfracture and first generation ACI. Functional outcome and reparative tissue histology were evaluated in 118 patients. The authors concluded functional outcome was similar between techniques at 12 and 18 months post-surgery, however ACI resulted in a histologically superior quality of the reparative tissue(47). The functional outcomes of 5-year follow-up in the same subset of patients were later published(48). Clinical improvement after both techniques persisted up to 5 years post-surgery. When evaluating the subset of patients with symptom duration of less than 3 years, ACI proved to be clinically superior. Both techniques had similar levels of failure at 5 years, but contrary to what was previously reported(46), microfracture seems to fail earlier, around 3 years post-surgery, while ACI tends to fail later, between 4 to 7 years. Adverse events related to the surgery, which included joint pain, swelling, or effusion, were higher on the ACI group at 3 years, but not different from the microfracture group at 6 years(48).

Hyalograft C surgical technique (second-generation ACI) was compared to microfracture for the treatment of chondral defect in another study. Eighty patients (40 in each group) were evaluated pre-surgery, and at 2 and 5 years post-surgery. The average patient age was 29.8 years old and defect size ranged from 1 to 5cm^2. At the 5 year follow up, significantly superior results in activity (Tegner scale) and function (IKDC) were seen in patients treated with Hyalograft C surgical technique compared to microfracture. The authors concluded that both treatments resulted in satisfactory functional outcomes, and that second generation ACI is a good and potentially durable option for the treatment of cartilage defects(49). Of note, the rehabilitation protocol in this study was the same for both groups with patients starting weight bearing at 4 weeks post-operatively. The original recommendation for weight bearing for microfracture starts at 6 to 8 weeks. Also, actual mean values and variation were not provided in this paper, making it difficult to make any conclusions regarding clinical significance observed between both techniques.

A few years later, this same group published another paper comparing Hyalograft C surgical technique to microfracture(50). In this study, they selected a group of 41 young (about 20 years old) high-level male soccer players. Twenty-one patients were treated with the second generation ACI technique while 20 were treated with microfracture. Defect size was not statistically significant different between groups, with an average of 1.9 cm^2 for microfracture group and 2.1 cm^2 for the Hyalograft C group. The authors reported a faster recovery in the microfracture group compared to the second-generation (ACI) with average times of 6.5 months and 10.2 months, respectively. A 9.8% decrease in IKDC subjective score was observed in the microfracture group between 2 and 5 years post-surgery. The Tegner scale also showed a statistically significant decrease in activity level in the microfracture group from 2 to 5 years. Comparing the groups, Hyalograft C resulted in significantly improved subjective IKDC scores and subjective functional

levels (EQ-VAS questionnaire) compared to microfracture. The authors concluded that while microfracture allows faster return to activity, the clinical outcomes deteriorate over time. O the other hand, Hyalograft C results in durable good clinical outcomes(50).

Matrix-assisted chondrocyte implantation, MACI, (third generation ACI) was compared to microfracture in a set of 60 patients (40 MACI and 20 microfracture) with cartilage defects greater than 4 cm^2. Patients were evaluated for clinical and functional outcomes prior to and at 6, 12 and 24 months post-surgery. Although minimal, a statistically significant difference in duration of the symptoms prior surgery was found between groups. MACI patients were on average younger than microfracture patients (33 years old for MACI *vs.* 37.5 years old for microfracture), however this difference was not statistically significant. MACI resulted in 33% improvement in Lysholm scores compared to microfracture at 24 months. There was a 14% decline in Lysholm score between 12 and 24 months for the microfracture group. Activity level and ICRS sores were also improved in the MACI treated patients compared to microfracture. The authors concluded that MACI treatment resulted in superior clinical and functional outcomes when compared to microfracture for defects greater than 4 cm^2(51).

Two studies using the same set of patients compared microfracture (control group) to third generation ACI (MACI®). One study published the results of the 2 year follow up(52), while the other reported results from a 5 year follow up(53). The authors reported clinically relevant improvement in pain and function at the 2 and 5 year follow up in MACI® treated patients compared to microfracture treated patients with defects over 3cm^2. No differences in tissue repair quality or procedure safety between groups were observed(52, 53). Of note, several authors in these studies reported to have conflict of interest involving the company who owns the rights over the MACI® product.

Similarly, microfracture was used as control group for another third generation ACI product (NeoCart®). Twenty-one patients were enrolled in the NeoCart® group and only nine were enrolled in the microfracture group (control). The author concluded that both techniques resulted in clinical improvement and improved functional outcome at 3, 6, 12 and 24 months post-surgery, however the NeoCart® technique was associated with better results compared to microfracture(54). However, again, one or more of the authors had a conflict of interest.

A prospective study in 20 patients (10 in each group) compared microfracture to a third generation ACI (CaReS®) for the treatment of patellar cartilage defects. Clinical and functional outcomes were assessed prior to and at 36 months post-surgery. Significant improvement was observed for both groups, but no statistical significance difference between treatments could be appreciated(55).

In another study, 52 athletes (mean age of 24.3 years old) with symptomatic full cartilage defects were randomly selected to be treated with osteochondral autologous transplantation (OAT) or microfracture. The mean duration of the symptoms prior to surgery was 21.32 months and mean follow up was 37.1 months. Patients were evaluated for clinical and functional outcome parameters using self-reported questionnaires (HSS and ICRS). Both treatments resulted in significant improvement compared to before surgery. However, OAT resulted in 96% of the patients reporting good to excellent results, compared to only 52% in the microfracture group. Also, clinical and functional outcome parameters showed better results in the OAT group at 12, 24 and 36 months. Second look arthroscopy showed significantly more patients with excellent repair of the cartilage defect in the OAT group. Biopsy specimens obtained from 25 patients (11 treated with OAT and 14 with microfracture) at an average follow up period of 12.4 months showed hyaline cartilage formation in all OAT treated defects as compared to fibrocartilage formation in the microfracture

treated defects. The authors concluded that OAT showed significant superior results compared to microfracture in the treatment of cartilage defects in young, active athletes(56).

Microfracture was compared to OAT and cartilage debridement for the treatment of cartilage defects concomitantly with anterior cruciate ligament (ACL) reconstruction. Ninety-seven patients (mean age 34 years old) were evaluated for a 3-year follow up period. OAT resulted in improved subjective International Knee Documentation Committee subjective scores compared to microfracture or debridement. Although there was no difference between groups in return to same pre-injury activity level (57).

These findings were different than reported by Ulstein and coworkers (2014). The authors studied 25 patients undergoing microfracture (11) and OAT (14) to treat chondral defects. All patients were evaluated before treatment and at a final follow up (9.8 years in average). No statistically significant difference was observed between groups for subjective (Lysholm/KOOS) and objective (isokinetic muscle strength) functional outcomes(58). However, despite a lack of statistical significance, radiographic signs of OA were present in 5 of 11 patients treated with microfracture versus only 2 of 12 in the OAT group ($p=0.193$). The authors concluded that at long-term follow up, both techniques resulted in similar clinical and functional improvement. However, neither of the techniques resulted in return of normal knee function (58).

Differences between microfracture and osteochondral autologous transplantation were however observed in a longer-term follow up study. A cohort study including 102 patients (median age= 36 years old and defect size less than 5 cm^2) compared OAT to microfracture at several time points with a minimum follow up of 15 years. The authors reported significant clinical improvement for OAT over microfracture at 6 months, 12 months, 5 years and 10 years post-surgery. Interestingly, at 15 to 18 years this difference did not reach statistical significance(59).

In another study, microfracture was compared to OAT and autologous chondrocyte implantation (ACI), with a minimum of 3 years follow up. Thirty knees were treated with microfracture, while 22 knees were treated with OAT and 18 knees were treated with ACI. All treatments resulted in improvement after surgery with no statistically significant differences on functional scores. Follow up MRI was performed on 88% of the knees at 12 to 14 months post-surgery and no statistically significant difference between treatments was observed for Outerbridge MRI scores. Second look arthroscopy was performed in 74% of the knees at 12 to 18 months post-surgery and no statistically significant difference between treatments groups was observed for ICRS evaluation. The authors concluded that no clear benefit was observed for OAT or ACI over microfracture(60).

Systematic Reviews and Meta-analysis

A systematic review published in 2019, included 18 studies published between 2013 and 2018, comprised a total of 1,830 defects and 1,759 patients. Mean patient age was 36.6 years old. Microfracture was used to treat grade III and IV (Outerbridge or ICRS) symptomatic cartilage defects, with an average size of 3.4 cm^2. The average follow up period was 6.5 years. Improvement in Lysholm scores ranges from 19 to 37%, whereas improvement in IKDC scores ranged between 62 to 66%. The authors concluded that microfracture provides good function and pain relief at the midterm period and satisfactory results thereafter(61).

Meta-analysis evaluation of 6 studies (399 patients), comparing microfracture to autologous chondrocyte implantation (all three generations) at 1, 2 and 3 years follow-up was performed. When considering all three ACI generations together, non-significant superiority of ACI over microfracture was observed. When first generation ACI was omitted from the meta-analysis, a large size effect was founded favoring ACI, with differences becoming smaller over the years(62).

Oussedik and coworkers (2015) showed similar findings using systematic review analysis of 34 studies. Data from a total of 1,622 cartilage defects treated with microfracture, first, second or third generation ACI were compared. The author concluded that while microfracture is effective in smaller lesions, matrix associated ACI results in better clinical outcomes in lesions greater than $4cm^2$ (63).

Mithoefer and coworkers (2009) conducted a meta-analysis to compare cartilage resurface techniques (microfracture, osteochondral autologous transplantation and autologous chondrocyte implantation) in athletes. Overall, data from 1,363 patients were pooled for analysis. Average defect size was 3.6 cm^2 across all groups, and ACI treated patients had the largest average defect size (5.1 cm^2) of the three groups. Average follow up period was 42 months. Osteochondral autologous transplantation resulted in a statistically significantly higher rate of return to sport, however autologous chondrocyte implantation treated patients were able to continue in sport for longer. No difference between treatment groups was observed in rate of return to pre-injury level. Patients treated with ACI took twice as long to return to sport than patients treated with microfracture or OAT(64).

More recently, another meta-analysis was published comparing cartilage resurface techniques in athletes. Data from 2,549 patients in 55 cohort studies were analyzed. Overall return to sport was 76%, with osteochondral autologous transplantation resulting in 60% higher rate of return to sport compared to microfracture (65).

Meta-analysis was used to compare activity-related outcomes between microfracture, OAT and ACI. Twenty studies, including 1,375 patients in total were included for analysis. There was a significant improvement in Tegner scores (at 1 year post-surgery) and IKDC scores (at 2 years post-surgery) for ACI treated patients compared to microfracture treated patients. Osteochondral

autologous transplantation was superior to microfracture for Lysholm scores at 1 year and Marx score at 2 years. No statistically significant difference was observed at the final follow up (average 3.7 years), except for osteochondral autologous transplantation resulting in better Marx scores than microfracture. A significantly higher number of complications were observed for the autologous chondrocyte implantation(66).

A systematic review of randomized clinical trials comparing at least two different cartilage resurfacing techniques included 10 papers. At the 10 year follow up period, there was more failure in the microfracture group compared to OAT and more in the OAT group compared to ACI. Cartilage lesions larger than 4.5 cm^2 had better outcomes when treated with ACI or OAT than with microfracture. The authors concluded no single treatment could be recommended over the others for the treatment of cartilage lesions in the knee(67).

Dibartola and coworkers (2016) used a meta-analysis approach to correlate histological outcomes with different cartilage resurfacing techniques. In total, 33 studies were included in this analysis. The authors concluded that microfracture had poorer histological outcomes and the lowest percentage of hyaline cartilage present in the reparative tissue compared to other cartilage resurfacing techniques. Also, no correlation between ICRS histological scores and clinical outcomes where found, however presence of hyaline cartilage was associated with improved clinical outcomes (68).

Cost-Effectiveness Studies

Cost-effectiveness of microfracture was compared to osteochondral autologous transplantation using systematic review approach. Data from 3 studies and 134 patients were evaluated. Average follow up was 8.4 years. Cost-effectiveness was defined as total cost by 1 point in improvement.

Microfracture was more cost-effective than OAT for Lysholm and HHS score, while osteochondral autologous transplantation was more cost-effective than microfracture for Tegner and ICRS scores. The cost of return to play was 46% cheaper for osteochondral autologous transplantation compared to microfracture(69).

A similar study design was used to compare the cost-effectiveness of microfracture and autologous chondrocyte implantation. Data from 4 studies and 319 patients were included in this analysis, with a maximum follow up period of 5 years. Depending on which patient reported outcome measurement was evaluated, microfracture was 46% to 75% more cost-effective than autologous chondrocyte transplantation, with the maximal difference observed for the SF-36 scores and minimal difference observed for the Tegner scores(70).

Shrock and coworkers (2017) compared the cost-effectiveness of microfracture to osteochondral autologous transplantation, first generation and matrix-assisted autologous chondrocyte implantation. Twelve studies (6 level I and 6 level II) were included in this analysis. Data from 730 knees were evaluated for a follow up period raging from 19 to 38 months. Despite matrix-assisted autologous chondrocyte implantation resulting in the highest functional outcome, microfracture was still considered the most cost-effective technique(71).

Conclusion

Subchondral bone microfracture is a well-established cartilage resurfacing technique. Animal studies and clinical evidence suggest microfracture results in a functional fibrocartilage reparative tissue covering the defect site. Clinically, microfracture combined with adequate rehabilitation protocols results in satisfactory outcomes in strictly selected cases. However, there is a wide range of reported clinical outcomes in the literature, which can be explained by the variation in case

selection, surgical techniques, post-operative rehabilitation programs and assessment of the outcomes(23).

Patient selection seems to play major role in the success of the microfracture treatment. Young patients (<40 years old), those with acute focal chondral lesions less than $4cm^2$, those with a body mass index < 30 and those with no angular deviation of the knee tend to benefit more from microfracture treatment. While microfracture outcomes seem to reach maximal improvement at 2 years post-surgery, there is substantial evidence that outcomes gradually decline thereafter. This is especially true in more activity-demanding patients (high-level athletes).

Although other cartilage resurfacing techniques have been developed (such as autologous chondrocyte implantation and osteochondral autologous/allogenic transplantation), microfracture is still more cost-effective and demonstrates similarly satisfactory clinical outcomes. More recently, the concept of combine tissue engineering principles (scaffold, cells and growth-factor) with microfracture (microfracture augmentation) became a very interesting approach to improving cartilage repair in a cost-effective way. In the next session, the result of clinical trials using microfracture augmentation techniques as well as the current strategies used by our group to improve cartilage healing will be discussed.

REFERENCES

1. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. Sports Health. 2009;1(6):461-8.

2. Mankin HJ. The response of articular cartilage to mechanical injury. J Bone Joint Surg Am. 1982;64(3):460-6.

3. Grande DA, Breitbart AS, Mason J, Paulino C, Laser J, Schwartz RE. Cartilage tissue engineering: current limitations and solutions. Clin Orthop Relat Res. 1999(367 Suppl):S176-85.

4. Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthritis Cartilage. 2002;10(6):432-63.

5. Minas T. A primer in cartilage repair. J Bone Joint Surg Br. 2012;94(11 Suppl A):141-6.

6. Merkely G AJ, Lattermann C Articular Cartilage Defects: Incidence, Diagnosis and Natural History. Oper Tech Sport Med. 2018;26:156-61.

7. Farr J, Cole B, Dhawan A, Kercher J, Sherman S. Clinical cartilage restoration: evolution and overview. Clin Orthop Relat Res. 2011;469(10):2696-705.

8. Curl WW, Krome J, Gordon ES, Rushing J, Smith BP, Poehling GG. Cartilage injuries: a review of 31,516 knee arthroscopies. Arthroscopy. 1997;13(4):456-60.

9. Hjelle K, Solheim E, Strand T, Muri R, Brittberg M. Articular cartilage defects in 1,000 knee arthroscopies. Arthroscopy. 2002;18(7):730-4.

10. Widuchowski W, Widuchowski J, Trzaska T. Articular cartilage defects: study of 25,124 knee arthroscopies. Knee. 2007;14(3):177-82.

11. Shelbourne KD, Gray T. Results of anterior cruciate ligament reconstruction based on meniscus and articular cartilage status at the time of surgery. Five- to fifteen-year evaluations. Am J Sports Med. 2000;28(4):446-52.

12. Ichiba A, Kishimoto I. Effects of articular cartilage and meniscus injuries at the time of surgery on osteoarthritic changes after anterior cruciate ligament reconstruction in patients under 40 years old. Arch Orthop Trauma Surg. 2009;129(3):409-15.

13. McCormick F, Harris JD, Abrams GD, Frank R, Gupta A, Hussey K, et al. Trends in the surgical treatment of articular cartilage lesions in the United States: an analysis of a large private-payer database over a period of 8 years. Arthroscopy. 2014;30(2):222-6.

14. Biant LC, McNicholas MJ, Sprowson AP, Spalding T. The surgical management of symptomatic articular cartilage defects of the knee: Consensus statements from United Kingdom knee surgeons. Knee. 2015;22(5):446-9.

15. Gowd AK, Cvetanovich GL, Liu JN, Christian DR, Cabarcas BC, Redondo ML, et al. Management of Chondral Lesions of the Knee: Analysis of Trends and Short-Term Complications Using the National Surgical Quality Improvement Program Database. Arthroscopy. 2019;35(1):138-46.

16. Montgomery SR, Foster BD, Ngo SS, Terrell RD, Wang JC, Petrigliano FA, et al. Trends in the surgical treatment of articular cartilage defects of the knee in the United States. Knee Surg Sports Traumatol Arthrosc. 2014;22(9):2070-5.

17. Steadman JR, Rodkey WG, Briggs KK. Microfracture to treat full-thickness chondral defects: surgical technique, rehabilitation, and outcomes. J Knee Surg. 2002;15(3):170-6.

18. Steadman JR, Rodkey WG, Briggs KK. Microfracture: Its History and Experience of the Developing Surgeon. Cartilage. 2010;1(2):78-86.

19. Frisbie DD, Trotter GW, Powers BE, Rodkey WG, Steadman JR, Howard RD, et al. Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. Vet Surg. 1999;28(4):242-55.

20. Frisbie DD, Oxford JT, Southwood L, Trotter GW, Rodkey WG, Steadman JR, et al. Early events in cartilage repair after subchondral bone microfracture. Clin Orthop Relat Res. 2003(407):215-27.

21. Mienaltowski MJ, Huang L, Frisbie DD, McIlwraith CW, Stromberg AJ, Bathke AC, et al. Transcriptional profiling differences for articular cartilage and repair tissue in equine joint surface lesions. BMC Med Genomics. 2009;2:60.

22. Frisbie DD, Morisset S, Ho CP, Rodkey WG, Steadman JR, McIlwraith CW. Effects of calcified cartilage on healing of chondral defects treated with microfracture in horses. Am J Sports Med. 2006;34(11):1824-31.

23. Theodoropoulos J, Dwyer T, Whelan D, Marks P, Hurtig M, Sharma P. Microfracture for knee chondral defects: a survey of surgical practice among Canadian orthopedic surgeons. Knee Surg Sports Traumatol Arthrosc. 2012;20(12):2430-7.

24. Kroell A, Marks P, Chahal J, Hurtig M, Dwyer T, Whelan D, et al. Microfracture for chondral defects: assessment of the variability of surgical technique in cadavers. Knee Surg Sports Traumatol Arthrosc. 2016;24(7):2374-9.

25. Orth P, Duffner J, Zurakowski D, Cucchiarini M, Madry H. Small-Diameter Awls Improve Articular Cartilage Repair After Microfracture Treatment in a Translational Animal Model. Am J Sports Med. 2016;44(1):209-19.

26. Chen H, Sun J, Hoemann CD, Lascau-Coman V, Ouyang W, McKee MD, et al. Drilling and microfracture lead to different bone structure and necrosis during bone-marrow stimulation for cartilage repair. J Orthop Res. 2009;27(11):1432-8.

27. Chen H, Hoemann CD, Sun J, Chevrier A, McKee MD, Shive MS, et al. Depth of subchondral perforation influences the outcome of bone marrow stimulation cartilage repair. J Orthop Res. 2011;29(8):1178-84.

28. Hoemann CD, Gosselin Y, Chen H, Sun J, Hurtig MB, Carli A, et al. Characterization of initial microfracture defects in human condyles. J Knee Surg. 2013;26(5):347-55.

29. Min BH, Truong MD, Song HK, Cho JH, Park DY, Kweon HJ, et al. Development and Efficacy Testing of a "Hollow Awl" That Leads to Patent Bone Marrow Channels and Greater Mesenchymal Stem Cell Mobilization During Bone Marrow Stimulation Cartilage Repair Surgery. Arthroscopy. 2017;33(11):2045-51.

30. Steadman JR, Briggs KK, Rodrigo JJ, Kocher MS, Gill TJ, Rodkey WG. Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up. Arthroscopy. 2003;19(5):477-84.

31. Miller BS, Steadman JR, Briggs KK, Rodrigo JJ, Rodkey WG. Patient satisfaction and outcome after microfracture of the degenerative knee. J Knee Surg. 2004;17(1):13-7.

32. Bae DK, Yoon KH, Song SJ. Cartilage healing after microfracture in osteoarthritic knees. Arthroscopy. 2006;22(4):367-74.

33. Mithoefer K, Williams RJ, 3rd, Warren RF, Potter HG, Spock CR, Jones EC, et al. The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study. J Bone Joint Surg Am. 2005;87(9):1911-20.

34. Kreuz PC, Steinwachs MR, Erggelet C, Krause SJ, Konrad G, Uhl M, et al. Results after microfracture of full-thickness chondral defects in different compartments in the knee. Osteoarthritis Cartilage. 2006;14(11):1119-25.

35. Asik M, Ciftci F, Sen C, Erdil M, Atalar A. The microfracture technique for the treatment of full-thickness articular cartilage lesions of the knee: midterm results. Arthroscopy. 2008;24(11):1214-20.

36. Miller BS, Briggs KK, Downie B, Steadman JR. Clinical Outcomes following the Microfracture Procedure for Chondral Defects of the Knee: A Longitudinal Data Analysis. Cartilage. 2010;1(2):108-12.

37. Solheim E, Hegna J, Inderhaug E, Oyen J, Harlem T, Strand T. Results at 10-14 years after microfracture treatment of articular cartilage defects in the knee. Knee Surg Sports Traumatol Arthrosc. 2016;24(5):1587-93.

38. Von Keudell A, Atzwanger J, Forstner R, Resch H, Hoffelner T, Mayer M. Radiological evaluation of cartilage after microfracture treatment: a long-term follow-up study. Eur J Radiol. 2012;81(7):1618-24.

39. Blevins FT, Steadman JR, Rodrigo JJ, Silliman J. Treatment of articular cartilage defects in athletes: an analysis of functional outcome and lesion appearance. Orthopedics. 1998;21(7):761-7; discussion 7-8.

40. Steadman JR, Miller BS, Karas SG, Schlegel TF, Briggs KK, Hawkins RJ. The microfracture technique in the treatment of full-thickness chondral lesions of the knee in National Football League players. J Knee Surg. 2003;16(2):83-6.

41. Mithoefer K, Williams RJ, 3rd, Warren RF, Wickiewicz TL, Marx RG. High-impact athletics after knee articular cartilage repair: a prospective evaluation of the microfracture technique. Am J Sports Med. 2006;34(9):1413-8.

42. Cerynik DL, Lewullis GE, Joves BC, Palmer MP, Tom JA. Outcomes of microfracture in professional basketball players. Knee Surg Sports Traumatol Arthrosc. 2009;17(9):1135-9.

43. Namdari S, Baldwin K, Anakwenze O, Park MJ, Huffman GR, Sennett BJ. Results and performance after microfracture in National Basketball Association athletes. Am J Sports Med. 2009;37(5):943-8.

44. Gobbi A, Karnatzikos G, Kumar A. Long-term results after microfracture treatment for full-thickness knee chondral lesions in athletes. Knee Surg Sports Traumatol Arthrosc. 2014;22(9):1986-96.

45. Van Assche D, Staes F, Van Caspel D, Vanlauwe J, Bellemans J, Saris DB, et al. Autologous chondrocyte implantation versus microfracture for knee cartilage injury: a prospective randomized trial, with 2-year follow-up. Knee Surg Sports Traumatol Arthrosc. 2010;18(4):486-95.

46. Knutsen G, Drogset JO, Engebretsen L, Grontvedt T, Isaksen V, Ludvigsen TC, et al. A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. J Bone Joint Surg Am. 2007;89(10):2105-12.

47. Saris DB, Vanlauwe J, Victor J, Haspl M, Bohnsack M, Fortems Y, et al. Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. Am J Sports Med. 2008;36(2):235-46.

48. Vanlauwe J, Saris DB, Victor J, Almqvist KF, Bellemans J, Luyten FP, et al. Five-year outcome of characterized chondrocyte implantation versus microfracture for symptomatic cartilage defects of the knee: early treatment matters. Am J Sports Med. 2011;39(12):2566-74.

49. Kon E, Gobbi A, Filardo G, Delcogliano M, Zaffagnini S, Marcacci M. Arthroscopic second-generation autologous chondrocyte implantation compared with microfracture for chondral lesions of the knee: prospective nonrandomized study at 5 years. Am J Sports Med. 2009;37(1):33-41.

50. Kon E, Filardo G, Berruto M, Benazzo F, Zanon G, Della Villa S, et al. Articular cartilage treatment in high-level male soccer players: a prospective comparative study of arthroscopic second-generation autologous chondrocyte implantation versus microfracture. Am J Sports Med. 2011;39(12):2549-57.

51. Basad E, Ishaque B, Bachmann G, Sturz H, Steinmeyer J. Matrix-induced autologous chondrocyte implantation versus microfracture in the treatment of cartilage defects of the knee: a 2-year randomised study. Knee Surg Sports Traumatol Arthrosc. 2010;18(4):519-27.

52. Saris D, Price A, Widuchowski W, Bertrand-Marchand M, Caron J, Drogset JO, et al. Matrix-Applied Characterized Autologous Cultured Chondrocytes Versus Microfracture: Two-Year Follow-up of a Prospective Randomized Trial. Am J Sports Med. 2014;42(6):1384-94.

53. Brittberg M, Recker D, Ilgenfritz J, Saris DBF, Group SES. Matrix-Applied Characterized Autologous Cultured Chondrocytes Versus Microfracture: Five-Year Follow-up of a Prospective Randomized Trial. Am J Sports Med. 2018;46(6):1343-51.

54. Crawford DC, DeBerardino TM, Williams RJ, 3rd. NeoCart, an autologous cartilage tissue implant, compared with microfracture for treatment of distal femoral cartilage lesions: an FDA

phase-II prospective, randomized clinical trial after two years. J Bone Joint Surg Am. 2012;94(11):979-89.

55. Petri M, Broese M, Simon A, Liodakis E, Ettinger M, Guenther D, et al. CaReS (MACT) versus microfracture in treating symptomatic patellofemoral cartilage defects: a retrospective matched-pair analysis. J Orthop Sci. 2013;18(1):38-44.

56. Gudas R, Kalesinskas RJ, Kimtys V, Stankevicius E, Toliusis V, Bernotavicius G, et al. A prospective randomized clinical study of mosaic osteochondral autologous transplantation versus microfracture for the treatment of osteochondral defects in the knee joint in young athletes. Arthroscopy. 2005;21(9):1066-75.

57. Gudas R, Gudaite A, Mickevicius T, Masiulis N, Simonaityte R, Cekanauskas E, et al. Comparison of osteochondral autologous transplantation, microfracture, or debridement techniques in articular cartilage lesions associated with anterior cruciate ligament injury: a prospective study with a 3-year follow-up. Arthroscopy. 2013;29(1):89-97.

58. Ulstein S, Aroen A, Rotterud JH, Loken S, Engebretsen L, Heir S. Microfracture technique versus osteochondral autologous transplantation mosaicplasty in patients with articular chondral lesions of the knee: a prospective randomized trial with long-term follow-up. Knee Surg Sports Traumatol Arthrosc. 2014;22(6):1207-15.

59. Solheim E, Hegna J, Inderhaug E. Long-term clinical follow-up of microfracture versus mosaicplasty in articular cartilage defects of medial femoral condyle. Knee. 2017;24(6):1402-7.

60. Lim HC, Bae JH, Song SH, Park YE, Kim SJ. Current treatments of isolated articular cartilage lesions of the knee achieve similar outcomes. Clin Orthop Relat Res. 2012;470(8):2261-7.

61. Orth P, Gao L, Madry H. Microfracture for cartilage repair in the knee: a systematic review of the contemporary literature. Knee Surg Sports Traumatol Arthrosc. 2019.

62. Negrin LL, Vecsei V. Do meta-analyses reveal time-dependent differences between the clinical outcomes achieved by microfracture and autologous chondrocyte implantation in the treatment of cartilage defects of the knee? J Orthop Sci. 2013;18(6):940-8.

63. Oussedik S, Tsitskaris K, Parker D. Treatment of articular cartilage lesions of the knee by microfracture or autologous chondrocyte implantation: a systematic review. Arthroscopy. 2015;31(4):732-44.

64. Mithoefer K, McAdams T, Williams RJ, Kreuz PC, Mandelbaum BR. Clinical efficacy of the microfracture technique for articular cartilage repair in the knee: an evidence-based systematic analysis. Am J Sports Med. 2009;37(10):2053-63.

65. Krych AJ, Pareek A, King AH, Johnson NR, Stuart MJ, Williams RJ, 3rd. Return to sport after the surgical management of articular cartilage lesions in the knee: a meta-analysis. Knee Surg Sports Traumatol Arthrosc. 2017;25(10):3186-96.

66. Chalmers PN, Vigneswaran H, Harris JD, Cole BJ. Activity-Related Outcomes of Articular Cartilage Surgery: A Systematic Review. Cartilage. 2013;4(3):193-203.

67. Devitt BM, Bell SW, Webster KE, Feller JA, Whitehead TS. Surgical treatments of cartilage defects of the knee: Systematic review of randomised controlled trials. Knee. 2017;24(3):508-17.

68. DiBartola AC, Everhart JS, Magnussen RA, Carey JL, Brophy RH, Schmitt LC, et al. Correlation between histological outcome and surgical cartilage repair technique in the knee: A meta-analysis. Knee. 2016;23(3):344-9.

69. Miller DJ, Smith MV, Matava MJ, Wright RW, Brophy RH. Microfracture and osteochondral autograft transplantation are cost-effective treatments for articular cartilage lesions of the distal femur. Am J Sports Med. 2015;43(9):2175-81.

70. Aae TF, Randsborg PH, Luras H, Aroen A, Lian OB. Microfracture is more cost-effective than autologous chondrocyte implantation: a review of level 1 and level 2 studies with 5 year follow-up. Knee Surg Sports Traumatol Arthrosc. 2018;26(4):1044-52.

71. Schrock JB, Kraeutler MJ, Houck DA, McQueen MB, McCarty EC. A Cost-Effectiveness Analysis of Surgical Treatment Modalities for Chondral Lesions of the Knee: Microfracture, Osteochondral Autograft Transplantation, and Autologous Chondrocyte Implantation. Orthop J Sports Med. 2017;5(5):2325967117704634.

CHAPTER 2:

MICROFRACTURE AUGMENTATION AND CARTILAGE REPAIR USING SELF-ASSEMBLING PEPTIDE HYDROGEL

Microfracture Augmentation

Microfracture augmentation, or "microfracture plus", refers to the use of tissue engineering concepts (scaffold, cells and growth-factors) to improve microfracture(1). Microfracture augmentation has the ultimate goal of providing a cost-effective long-term solution for patients with cartilage defects (2). In 2003, Steadman and coworkers published a long-term follow up of microfracture treatment for traumatic chondral defects. The authors mentioned, despite the satisfactory results, that improving cartilage healing using cytokines, cell therapy, gene therapy and other newly developed techniques should be the scope for future work(3). A literature review compiling results from experimental and clinical studies using adjuvant therapies to improve microfracture was published in 2010. In this review, the authors demonstrated researchers' growing interest in actively developing and evaluating what they called "new second-generation" therapies to improve microfracture outcome. The results obtained by these earlier studies showed exciting potential options to improve the quality of cartilage repair in clinical settings(4). In 2014, Dr. Adam Yanke and Dr. Brian Cole expressed that microfracture augmentation is the most conducive area for innovation in cartilage regeneration(1). Since then, several clinical reports have been published using scaffolds and biological therapies for microfracture augmentation. In the next section, we will focus on controlled clinical trials comparing microfracture augmentation to standard microfracture for the treatment of chondral defects in the knee.

Autologous matrix-induced chondrogenesis (AMIC) was the first term used to describe a solid collagen type I/III scaffold covering microfracture treated defects. This term is a registered trademark of the company that provides the ChondroGide® membrane, but has been generally used to describe any technique that uses a solid scaffold to cover microfracture treated defects(5, 6). Data from studies including ChondroGide® (collagen type I/III), Chondrotissue® (polyglycolic acid) and Hyalofast® (hyaluronic acid) scaffolds in addition to microfracture were evaluated in a systematic review. Overall, results from 10 studies (219 patients) were available for analysis. After reviewing the papers, the authors concluded that autologous matrix-induced chondrogenesis is a promising microfracture augmentation technique, which demonstrated comparable clinical and MRI outcomes to other cell-based cartilage methods(5).

In a 2013 study, ChondroGide® (AMIC), a solid collagen type I/III scaffold, was used to cover microfracture treated defects and compared to microfracture alone. Thirty-eight patients were enrolled and divided in 3 groups: microfracture only, sutured AMIC and fibrin glued AMIC. Patients were evaluated for clinical, functional and MRI outcomes at 1 and 2 years, however only 27 patients were available for the 2 year time-point evaluation. All techniques showed significant improvement in clinical and functional outcomes, however there was no statistically significant difference amongst groups(7). A five year follow up of this same set of patients, in addition to newly enrolled patients, was later published. The authors were able to enroll a total of 48 patients, with 37 available at the final follow up. Modified Cincinnati score decreased between the 2 and 5 year time points in the microfracture group, while AMIC group showed stable results. This allowed for statistically significant differences between groups at the 5-year follow up. Also, 66% of the patients in the microfracture group classified themselves as having abnormal or severely abnormal knee function, compared to only 7% in the AMIC group. Furthermore, there was a tendency for

patients in the microfracture group to have increased pain from 2 to 5 years, while the AMIC group demonstrated very low pain levels during that time period. Defect filling on MRI was the lower in the microfracture group compared to AMIC group. The authors concluded that AMIC is an effective treatment for cartilage repair, resulting in stable and significantly better outcomes than microfracture at 5 years (8).

In a 2014 study, A porcine decellurized biomembrane (Arthifilm®) was used to cover microfracture treated defects in 45 patients. Clinical, MRI and functional outcomes were compared to patients treated with microfracture only(12) at 6, 12 and 24 months post surgery. Average patient age (47.4 year-old) and average defect size (1.3 cm^2) in the treated group did not differ statistically from the control group. At 6 and 24 months, magnetic resonance imaging outcomes showed improved defect repair filling for the microfracture plus biomembrane group compared to microfracture only. Additionally, surface morphology and peripheral gap were improved in the treatment group at 6 months, but no statistical difference was found at 24 months. Clinical and functional outcomes were not different between groups at any time-point(9).

More recently, a meta-analysis study compared clinical and functional outcomes of patients treated with AMIC. Data from 28 papers (767 patients) with cartilage defects in the knee, ankle and acetabulum were analyzed. Only three of these papers compared microfracture and AMIC for cartilage defects in the knee. Superior results for AMIC treated patients were appreciated at the 5 year follow up, however the authors concluded that no recommendation could be made due to the lack of high-quality, randomized controlled studies(6).

BST-Cargel® is a chitosan/glycerol phosphate thermo-sensitive hydrogel scaffold. This product has been used for microfracture augmentation and results of several studies are summarized here. First, a randomized clinical trial was published in 2013, where clinical and functional outcomes

were compared in 41 patients treated with BST-Cargel® plus microfracture or microfracture alone, for a one year follow up period. On MRI, there was an 8% improvement in defect filling and a 17% decrease in T2 relaxation time for the BST-Cargel® treated group compared to microfracture alone. However, clinical and functional outcomes were only statistically significantly different for a subset of younger and more active patients (10). Data from the 5-year follow up in the same set of patients was later published. Overall, the clinical findings observed in the first year were sustained at the 5-year follow up(11). Despite the good study design used in this randomized clinical trial, results should be interpreted with caution. First, during patient randomization, the microfracture group had a duration of clinical signs that was almost two times longer than BST-CarGel® group. Also, the 8% superiority in cartilage filling on MRI of the proposed treatment is of questionable clinical relevance. Additionally, adverse events were reported in higher numbers for the BST-CarGel® group compared to the microfracture group. Further, BST-CarGel® treatment is mixed with patient's whole blood, which makes difficult to understand if the benefit comes from the treatment itself, of from the presence of increased blood clots covering the defect. Finally, Piramal Healthcare, proprietary of the BST-CarGel® brand, employed two authors, which creates a significant conflict of interest in this study.

These results led to an economic evaluation of the BST-CarGel® as adjunctive therapy with microfracture. This economic study used data from the randomized clinical trial published by Stanish and coworkers (2013)(10). The authors concluded that in a 20 year period, BST-CarGel® used as adjunctive therapy with microfracture would result in cumulative total incremental saving of approximately $7,330.00 dollars per patient, compared to microfracture alone(12).

Another injectable scaffold (CartiFill®), made from soluble collagen peptides, was combined with microfracture to treat cartilage defects in a 2017 study. Twenty-eight patients (14 CartiFill® vs.

14 microfracture) who underwent a high tibial osteotomy to correct angular deformity were evaluated for clinical and functional outcomes for a period of 1 year. MRI was used to evaluate defect healing and second-look arthroscopy was performed at the final follow up. One osteochondral biopsy per defect was harvested at the second-look arthroscopy and histological analysis was performed. The authors reported that CartiFill® combined with microfracture resulted in improved gross appearance (ICRS) of the defect in 65% of samples and improved total histological (ICRSII) score in 18%. Histological parameters that were improved by the treatment were tissue morphology, cell morphology, surface architecture, mid/deep zone and overall appearance. However, no statistically significant differences were observed for clinical or functional outcomes at 1-year post surgery. Also, hypertrophy of the repaired cartilage was found in 5 patients in the treatment group. The authors concluded that CartiFill® combined with microfracture resulted in significant improvement of the cartilage repair quality over microfracture alone(13).

Later, a thermo-sensitive injectable hydrogel produced by the combination of polyglucosamine/glucosamine was evaluated for improvement of microfracture technique in 46 patients treated for OCD lesions. Twenty-three patients treated with microfracture alone were used as a control. Clinical outcomes were recorded using WOMAC questionnaire at 6, 12 and 24 months. The treatment resulted in 75% improvement in total WOMAC scores at 6 months, 90% improvement at 12 months and 94% improvement at 24 months compared to microfracture alone. Compared to pre-surgical evaluation, pain was improved by 96.4% in the treatment group compared to only 1.6% in the microfracture group at the final follow up. The authors concluded that microfracture combined with a thermo-sensitive injectable hydrogel resulted in significantly improved clinical outcomes compared to microfracture alone(14). However, it is important to note

that in this report, microfracture performed significantly worse than in previous reports. One of the reasons could be that the standardized post-operative protocol used for both groups allowed patients to weightbear as much as tolerable immediately after surgery. This was justified based on the mechanical strength of the tested hydrogel, however regular microfracture patients are usually not allowed to weightbear until 6 to 8 weeks after surgery(3).

In a study investigating the use of biological therapy for microfracture augmentation, adipose-derived mesenchymal stem cells secured in place with fibrin glue were used with microfracture and compared with microfracture alone. Eighty patients (40 in each group) with symptomatic focal chondral lesions were enrolled and followed up for 2 years. Average patient age was 38 years old and average defect size was 4.8cm^2. Second-look arthroscopy was performed in 57 patients. Adipose-derived stem cells combined with microfracture resulted in 20% improvement in pain and 18% improvement in clinical symptoms compared to microfracture alone. Also, histological scores for ICRSII in the treated group showed improvement in matrix staining (30%), cell morphology (16%), mid/deep zone (25%) and overall evaluation (24%) compared to microfracture alone. The authors concluded that adipose-derived stem cells combined with microfracture resulted in significantly better clinical and histological outcomes compared to microfracture alone(15).

Three studies have been published comparing platelet-rich plasma (PRP) injection to improve microfracture outcomes. First, three PRP injections were performed at one week, three weeks and seven weeks after microfracture surgery in 10 patients. Functional outcome (IKDC) and pain level (VAS) of these patients were compared with 10 patients that underwent microfracture without PRP injections. Functional outcome (IKDC) was 25% improved at 6 months post-surgery in patients treated with microfracture and PRP. The improvement in functional outcome declined to 15% at

12 months and was no longer statistically significant(16). In the second study, PRP was injected intra-operatively in microfracture treated patients. In this study the authors failed to demonstrate a clear benefit of PRP injection concomitant to microfracture surgery on functional (IKDC, SF36) and pain (VAS) outcomes in any time point, up to 24 months after surgery(17). These findings are somewhat similar to those published by Lee and coworkers (2013), where PRP injections were used following microfracture treatment of focal chondral lesions in osteoarthritic knees. After microfracture was performed, PRP was injected in 24 randomly selected patients, while 25 patients receive microfracture treatment only and served as control. No significant statistical difference was noted for clinical or functional outcomes. However, more patients in the PRP plus microfracture group had normal or nearly normal IKDC activity scores compared to the microfracture alone group(18).

Recently, Arshi and coworkers (2018) published a systematic review evaluating the effect of biological augmentation on the clinical outcome of microfracture treated patients. Eighteen published papers met the inclusion criteria, but only seven were considered to have clinical evidence levels of I or II. Ten of these studies used scaffolds, and eight used injectable adjuvants to improve cartilage repair after microfracture. Data from 625 patients in total were included in this analysis. The authors concluded that microfracture augmentation is an emerging concept, and no definitive conclusion on is benefit could be made due to the mixed results amongst individual studies(19).

Cartilage Repair Using Self-Assembling Peptide Scaffolds

Despite good short-term results, microfracture has a tendency to fail in the mid to long-term follow up periods. The reasons are not totally elucidated, however poor reparative tissue quality (fibrocartilage), decreased defect filling, and incomplete neo-tissue integration to the surrounding native cartilage (lateral integration) have been implicated as likely causes. More recently, microfracture augmentation strategies have utilized tissue engineering concepts to improve microfracture outcomes in a cost-effective way. Our group proposed studying microfracture augmentation using a self-assembling peptide hydrogel scaffold, functionalized with growth factors (HB-IGF-1 and PDGF-BB) and combined with trypsin enzymatic pretreatment of the cartilage defect surface. In the next section, the papers most relevant to this approach will be reviewed.

Self-assembling kld hydrogel and its ability to encapsulate chondrocytes and progenitor cells

The use of scaffolds to augment microfracture has been shown to improve clinical outcomes, reparative tissue histology, and defect filling(8, 9, 11, 13). Biodegradable, *in situ* forming hydrogels are considered promising scaffolds for cartilage resurfacing techniques, as they can be injected arthroscopically to fill different anatomical defects(20).

These self-assembling peptides are dissolved in deionized water and are composed of alternating hydrophobic and hydrophilic amino acid sequences to form a stable β-sheet. When the ionic strength of the solution exceeds a certain threshold, the self-assembling process occurs rapidly. Another advantage of using amino acids as building blocks that it gives the ability to control

hydrogel degradation and cell interaction and to deliver tethered growth factors. Self-assembling KLD hydrogel is composed of alternating hydrophilic positively charged lysine (K) and negatively charged aspartic acid (D) on the lower side of the β-sheet, and hydrophobic leucine (L) on the upper side(21). Our group has extensively studied this hydrogel for cartilage tissue engineering proposes (21-33).

Initially, the ability of self-assembling KLD hydrogel to encapsulate chondrocytes was tested *in vitro*. This study showed that chondrocytes in self-assembling KLD peptide hydrogel had a rounded morphology and were fully encapsulated within a continuous GAG-rich matrix. Self-assembling KLD hydrogel favored cell division and maintained high cell viability (>80%). Synthesis of extracellular matrix (ECM) components (proteoglycans and total protein) was similar to what is observed in chondrocytes seeded in agarose gel (control). Increase in ECM content resulted in improvement of the biomechanical properties after 4 weeks of incubation. Equilibrium and dynamic stiffness reached 10% and 5% of the normal cartilage biomechanical properties, respectively. These findings showed that self-assembling KLD hydrogel maintains chondrocyte differentiation and stimulates extra-cellular matrix deposition and accumulation(21).

Further, self-assembling KLD hydrogel proved to be a suitable way to accelerate deposition of mature proteoglycan-rich extracellular matrix when stimulated by long-term intermittent dynamic compression(23). Additionally, self-assembling peptide hydrogel seems to prevent catabolic degradation of the newly synthetized glycosaminoglycan (22).

Later, the chondrogenic capacity of adult equine bone marrow and adipose-derived progenitor cells was compared in hydrogel cultures. There was approximately 4-6 times more extracellular matrix synthesis in bone marrow stromal cells (BMSC) seeded in self-assembling KLD hydrogel compared to agarose gels. Also, BMSC seeded in peptide hydrogel had similar capacity to

synthetize proteoglycan and protein to that of adult chondrocytes; even at a seeding density 3 times lower(24). With regards to the cell type, BMSC had a clearly superior chondrogenic capacity compared to adipose-derived progenitor cells(24).

A direct comparison between adult bone marrow mesenchymal stromal cells (BM-MSC) and age-matched adult chondrocytes, both encapsulated in self-assembling peptide hydrogel, showed significantly superior cartilage extracellular matrix production by the BM-MSC(22). In addition, aggrecan produced by BM-MSC is biomechanically superior to aggrecan produced by adult cartilage (25).

Strategic delivery of growth factors for cartilage resurfacing techniques using self-assembling peptide hydrogel

Several growth factors have been shown to promote cellular migration, induce *in vitro* chondrogenic differentiation of progenitor cells, and stimulate extracellular matrix deposition. However, in order to make applicable for clinical use, a sustained delivery strategy must be developed.

Transform growth factor-β1 (TGF-β1) has shown strong *in vitro* chondrogenic differential potential in adult equine bone marrow stromal cells cultured in self-assembling peptide hydrogel(24). Moreover, TGF-β1 adsorbed in self-assembling KLD hydrogel resulted in 39% more GAG content in the gel than with continuous supplementation of TGF-β1 in the media. Also, only a transient phosphorylation of the Smad2/3 receptors was observed when TGF-β1 was adsorbed in the hydrogel, compared to persistent phosphorylation with continuous TGF-β1

supplementation(27). Later, self-assembling peptide hydrogel was shown to retain 80% of the pre-mixed TGF-β1 for up 21 days in culture, stimulating chondrogenesis of encapsulated BMSC(28). Insulin-like growth factor 1 (IGF-1) has strong anabolic and anti-catabolic activity on articular cartilage metabolism(34). An experimental study using an equine model compared cartilage repair using fibrin/chondrocyte grafts supplemented with IGF-1 to fibrin/chondrocyte grafts alone. The results showed that IGF-1 improved reparative tissue appearance macroscopically with enhanced integration to surrounding cartilage, and increased collagen type II content(35). Another study showed that chondrocytes genetically modified to express IGF-1 allowed for improved defect filling, collagen type II content, and reparative tissue containing more hyaline-type cartilage(36). These collective findings make IGF-1 a strong candidate to be applied to cartilage resurfacing techniques. Unfortunately, local delivery of IGF-1 using self-assembling peptide hydrogel proves to be slightly more problematic than with TGF-β1. Unlike with TGF-β1, the adsorption of IGF-1 in self-assembling peptide hydrogel was not as rewarding. By tethering IGF-1 to a peptide hydrogel using a streptavidin-biotin binding system, longer-term retention is accomplished, however unfortunately its bioactivity is affected(29). Knowing these limitations, a different approach was developed to improve IGF-1 retention in the hydrogel while maintaining its bioactivity. The heparin-binding domain of epidermal growth factor was fused to the IGF-1 protein, creating what is called heparin-binding insulin-like growth factor (HB-IGF-1). With this approach, HB-IGF-1 is successfully attached to proteoglycan and the cell surface while maintaining similar bioactivity to IGF-1(31). *In vitro*, HB-IGF-1 is able to stimulate proteoglycan synthesis several days after wash out from the culture. Further, HB-IGF-1 can selectively bind to cartilage, and in small quantity to the meniscus, after intra-articular injections in rats(26). Pre-mixing HB-IGF-1 with the self-assembling peptide hydrogel resulted in sustained stimulus of the

surrounding cartilage explants, proving that this approach could serve as a growth factor delivery system for cartilage resurfacing techniques(32).

Combining IGF-1 with platelet-derived growth factor-BB (PDGF-BB) demonstrated additive chemotactic effects in one study(37). PDGF-BB also plays a major role in mesenchymal stem cell migration and has been shown to induce chondrocyte proliferation and prevent terminal endochondral differentiation of chondrocytes(38). Entrapment efficiency of PDGF-BB in self-assembling peptide hydrogel is reported to be approximately 80%, with 58% of the loaded PDGF-BB remaining in the gel after 4 days. Further, combination of PDGF-BB with TGF-β1 resulted in strong progenitor cell migration into the self-assembling peptide hydrogel(39).

Improving Cartilage to Cartilage Integration with Trypsin Enzymatic Pretreatment

Integration of reparative cartilage into surrounding cartilage and underlining subchondral bone is vital for long-term articular cartilage repair. Incomplete integration of the reparative tissue to the surrounding cartilage or subchondral bone can result in micromotion, leading to tissue degeneration over time(40). While cartilage-to-bone integration can be significantly improved by removing the calcified cartilage during microfracture(41), achieving adequate cartilage-to-cartilage integration remains challenging.

Several factors have been reported to affect cartilage integration to surrounding native tissue(42). *In vitro* studies suggest that chondrocyte necrosis and apoptosis occurs up to 400 μm from the edge of the debrided defect. Although debridement triggers cellular mitotic activity at the wound edge, a study demonstrated that young and adult chondrocytes have a low capacity to divide and they showed no attempt to migrate toward the wound edge(43). Huzinker and Quinn (2003) showed

similar findings *in vivo* as cartilage debridement resulted in significant and long-term loss of cells near the wound edge (44). The implication of cellular death at the wound edge affecting cartilage integration was further supported by the fact that adding anti-apoptotic agents to the cultured media significantly improve cartilage integration (42).

DiMicco and Sah (2001) studied adhesive strength between two blocks of cartilage. Interestingly, cartilage integration between a live and devitalized cartilage explant was possible due to a newly synthetized collagen network produced by the live chondrocytes. The practical implication of these findings is that even if chondrocyte death occurs during cartilage debridement, live cells present at the interface may supply the necessary metabolic activity to induce cartilage integration (45). Further, the group studied cartilage integration at different developmental stages and noticed significantly higher integration in young cartilage explants than in fetal or adult cartilage explants. The cartilage integration seemed to be partially associated with collagen metabolism, since collagen biosynthesis was four to five times higher in young explants compared to adult explants. But interestingly, collagen metabolism in young and fetal cartilage explants was similar. The authors found that the formation of collagen cross-links could explain this difference. The authors concluded that cartilage integration is dependent on the developmental stage and collagen metabolism(46). Another study showed that the treatment of cartilage explants with highly purified collagenase resulted in increased chondrocyte density at the wound edges, leading to improved cartilage integration(47). However, the clinical use of collagenase can be difficult, since prolonged exposure can induce permanent damage to the collagen network, similar to that which is observed in early stages of osteoarthritis(48).

Enzymatic proteoglycan depletion from the cartilage defect surface was directly correlated with fibrin matrix attachment and improved cartilage integration in a 1996 study(49). Additionally,

another study showed that enzymatic digestion of the proteoglycans present on cartilage defect surfaces resulted in enhanced reparative cell adhesion, with proteoglycan loss compensated by the newly synthetized proteoglycan from chondrocytes near the interface(50). These findings suggested enzymatic treatment of the cartilage defect surface have a promising role in integrative cartilage repair, as supported by several strategies and enzymes studied over the years(51).

Obradovic and coworkers (2001) showed that cartilage integration between engineered constructs and native cartilage was dependent on the construct's developmental stage, as well as on the removal of proteoglycan from the adjacent cartilage. Immature constructs showed better cartilage integration due to increased presence of proliferating cells and the formation of a cartilaginous tissue bond at the interface. Furthermore, this study showed that trypsin enzymatic pretreatment partially removed proteoglycans without significantly affect collagen network(52).

Recently, our group tested if trypsin enzymatic pretreatment of the cartilage defect surface combined with a single dose of HB-IGF1 premixed in self-assembling KLD hydrogel would improve cartilage-to-cartilage integration. This study was performed using cartilage explant annuli created from immature bovine calves. First, we investigated the ability of trypsin to induce GAG depletion. Different concentrations and exposure periods were tested, and ultimately we found that 50μg/mL trypsin for a period of 2 minutes resulted in consistent GAG depletion up to 200μm into the cartilage annuli. Additionally, we found that chondrocyte migration from the hydrogel into surrounding cartilage was achieved after trypsin enzymatic pretreatment. Further, the addition of the HB-IGF-1 premixed in the hydrogel was able to overcome the GAG depletion by trypsin at the interface. Histological evaluation of constructs cultured for 4 weeks showed the highest amount of trans-interfacial proteoglycan content when trypsin and HB-IGF-1 were combined. Similar to what has been previously reported(52), trypsin preserved the native cartilage collagen network. Finally,

biomechanical tests demonstrated that HB-IGF-1 significantly improved cartilage integration strength. These results supported our hypothesis that a single, premixed dose of HB-IGF-1 in chondrocyte seeded KLD hydrogel combined with trypsin enzymatic pretreatment results in enhanced integration between neo-tissue and native surrounding cartilage(33).

Self-assembling KLD hydrogel for cartilage repair: in vivo studies

Initially, the effect of self-assembling peptide hydrogel as well as the effect of chondrogenic factors and bone marrow stromal cells on cartilage repair was investigated using a rabbit model. Twenty skeletally mature female New Zealand rabbits were used in this study. Bilateral 3x2 mm osteochondral defects were created in the central portion of the femoral trochlear groove. One leg was randomly selected to receive one of the three experimental treatments: self-assembling KLD hydrogel, self-assembling KLD hydrogel plus chondrogenic factors (TGF-B1, dexamethasone, and IGF-1), and self-assembling KLD hydrogel plus chondrogenic factors plus bone marrow stromal cells. The contra-lateral limb defect was left untreated and served as control. In this study, TGF-B1 and dexamethasone were adsorbed in the hydrogel, while IGF-1 was tethered to it with a streptavidin-biotin binding system. Radiographic, gross pathologic, histologic and immunohistochemistry evaluation were performed. It was observed that filling the defect with self-assembling KLD hydrogel significantly improved the total cumulative histology score, matrix-staining (SOFG) and collagen type II immunostaining compared to untreated contralateral limb (control). The addition of chondrogenic factors did not result in any additional benefits. Furthermore, the combination of self-assembling peptide hydrogel, chondrogenic factor and BMSC resulted in poorer repair than hydrogel or hydrogel plus chondrogenic factor groups. These

results supported the used of self-assembling KLD hydrogel as a scaffold material to improve cartilage resurfacing techniques, while emphasizing the need to further optimize chondrogenic factors and progenitor cells usage in cartilage repair(26).

The significant improvement in cartilage healing observed with the use of self-assembling KLD hydrogel motivated further investigation of its use in microfracture augmentation in a subsequent study. Bilateral, critically sized (15mm diameter), chondral defects were created on the medial trochlear ridge of 16 horses. Each defect was randomly assigned to receive one of the four treatments: no treatment, microfracture only, self-assembling KLD hydrogel only or the combination of self-assembling KLD hydrogel with microfracture. Lameness and radiographic exams were performed at several time points during the study. Second-look arthroscopy was performed at 6 months and 12 months. At the end of the study period (12 months) all horses were euthanized and MRI, gross pathological evaluation of the joint, histology, immunohistochemistry and biomechanical analysis were performed. Clinical improvement was observed in the microfracture only group (16% on lameness and 13% on flexion test) and in the KLD only group (13% improvement on lameness and 41% improvement on range of motion), but the combination of hydrogel and microfracture showed no additional benefits. Surprisingly, the combination of self-assembling peptide hydrogel and microfracture resulted in the smallest amount of defect filling and decreased amount of collagen type II immunostaining. While some benefits were observed with the use of hydrogel alone, the combination of the hydrogel with microfracture was not beneficial in this study. It is possible that the poor results were due to insufficient cellular and growth factor infiltration when these two treatments were combined(30).

Conclusion

Microfracture augmentation is a promising strategy to improve long-term outcomes of the microfracture technique in a cost-effective way. The use of cell-free scaffolds has been extensively studied in experimental and clinical settings. While most of the controlled clinical trials have shown initial encouraging results, long-term efficacy still unknown. Self-assembling peptide hydrogel has shown promise *in vitro* and *in vivo*, however, like any other technique, complete cartilage regeneration has not yet been achieved. Our group has been testing different tissue engineering strategies to improve cartilage repair and integration to surrounding native cartilage. In the next section, we will be presenting the results of small (rabbit) and large (equine) animal models of cartilage repair, where we tested the combination of trypsin enzymatic pretreatment with self-assembling KLD hydrogel functionalized with growth factors (HB-IGF-1 and PDGF-BB).

REFERENCES

1. Yanke AB, Cole BJ. Microfracture: dead or the future? Orthopedics. 2014;37(12):798-800.

2. Albright JC, Daoud AK. Microfracture and Microfracture Plus. Clin Sports Med. 2017;36(3):501-7.

3. Steadman JR, Briggs KK, Rodrigo JJ, Kocher MS, Gill TJ, Rodkey WG. Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up. Arthroscopy. 2003;19(5):477-84.

4. Strauss EJ, Barker JU, Kercher JS, Cole BJ, Mithoefer K. Augmentation Strategies following the Microfracture Technique for Repair of Focal Chondral Defects. Cartilage. 2010;1(2):145-52.

5. Lee YH, Suzer F, Thermann H. Autologous Matrix-Induced Chondrogenesis in the Knee: A Review. Cartilage. 2014;5(3):145-53.

6. Gao L, Orth P, Cucchiarini M, Madry H. Autologous Matrix-Induced Chondrogenesis: A Systematic Review of the Clinical Evidence. Am J Sports Med. 2019;47(1):222-31.

7. Anders S, Volz M, Frick H, Gellissen J. A Randomized, Controlled Trial Comparing Autologous Matrix-Induced Chondrogenesis (AMIC(R)) to Microfracture: Analysis of 1- and 2-Year Follow-Up Data of 2 Centers. Open Orthop J. 2013;7:133-43.

8. Volz M, Schaumburger J, Frick H, Grifka J, Anders S. A randomized controlled trial demonstrating sustained benefit of Autologous Matrix-Induced Chondrogenesis over microfracture at five years. Int Orthop. 2017;41(4):797-804.

9. Chung JY, Lee DH, Kim TH, Kwack KS, Yoon KH, Min BH. Cartilage extra-cellular matrix biomembrane for the enhancement of microfractured defects. Knee Surg Sports Traumatol Arthrosc. 2014;22(6):1249-59.

10. Stanish WD, McCormack R, Forriol F, Mohtadi N, Pelet S, Desnoyers J, et al. Novel scaffold-based BST-CarGel treatment results in superior cartilage repair compared with microfracture in a randomized controlled trial. J Bone Joint Surg Am. 2013;95(18):1640-50.

11. Shive MS, Stanish WD, McCormack R, Forriol F, Mohtadi N, Pelet S, et al. BST-CarGel(R) Treatment Maintains Cartilage Repair Superiority over Microfracture at 5 Years in a Multicenter Randomized Controlled Trial. Cartilage. 2015;6(2):62-72.

12. Frappier J, Stanish W, Brittberg M, Steinwachs M, Crowe L, Castelo D, et al. Economic evaluation of BST-CarGel as an adjunct to microfracture vs microfracture alone in knee cartilage surgery. J Med Econ. 2014;17(4):266-78.

13. Kim MS, Koh IJ, Choi YJ, Pak KH, In Y. Collagen Augmentation Improves the Quality of Cartilage Repair After Microfracture in Patients Undergoing High Tibial Osteotomy: A Randomized Controlled Trial. Am J Sports Med. 2017;45(8):1845-55.

14. Pipino G, Risitano S, Alviano F, Wu EJ, Bonsi L, Vaccarisi DC, et al. Microfractures and hydrogel scaffolds in the treatment of osteochondral knee defects: A clinical and histological evaluation. J Clin Orthop Trauma. 2019;10(1):67-75.

15. Koh YG, Kwon OR, Kim YS, Choi YJ, Tak DH. Adipose-Derived Mesenchymal Stem Cells With Microfracture Versus Microfracture Alone: 2-Year Follow-up of a Prospective Randomized Trial. Arthroscopy. 2016;32(1):97-109.

16. Manunta AF, Manconi A. The treatment of chondral lesions of the knee with the microfracture technique and platelet-rich plasma. Joints. 2013;1(4):167-70.

17. Manco A, Goderecci R, Rughetti A, S DEG, Necozione S, Bernardi A, et al. Microfracture versus microfracture and platelet-rich plasma: arthroscopic treatment of knee chondral lesions. A two-year follow-up study. Joints. 2016;4(3):142-7.

18. Lee GW, Son JH, Kim JD, Jung GH. Is platelet-rich plasma able to enhance the results of arthroscopic microfracture in early osteoarthritis and cartilage lesion over 40 years of age? Eur J Orthop Surg Traumatol. 2013;23(5):581-7.

19. Arshi A, Fabricant PD, Go DE, Williams RJ, McAllister DR, Jones KJ. Can Biologic Augmentation Improve Clinical Outcomes Following Microfracture for Symptomatic Cartilage Defects of the Knee? A Systematic Review. Cartilage. 2018;9(2):146-55.

20. Pascual-Garrido C, Rodriguez-Fontan F, Aisenbrey EA, Payne KA, Chahla J, Goodrich LR, et al. Current and novel injectable hydrogels to treat focal chondral lesions: Properties and applicability. J Orthop Res. 2018;36(1):64-75.

21. Kisiday J, Jin M, Kurz B, Hung H, Semino C, Zhang S, et al. Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair. Proc Natl Acad Sci U S A. 2002;99(15):9996-10001.

22. Kopesky PW, Vanderploeg EJ, Sandy JS, Kurz B, Grodzinsky AJ. Self-assembling peptide hydrogels modulate in vitro chondrogenesis of bovine bone marrow stromal cells. Tissue Eng Part A. 2010;16(2):465-77.

23. Kisiday JD, Jin M, DiMicco MA, Kurz B, Grodzinsky AJ. Effects of dynamic compressive loading on chondrocyte biosynthesis in self-assembling peptide scaffolds. J Biomech. 2004;37(5):595-604.

24. Kisiday JD, Kopesky PW, Evans CH, Grodzinsky AJ, McIlwraith CW, Frisbie DD. Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. J Orthop Res. 2008;26(3):322-31.

25. Lee HY, Kopesky PW, Plaas A, Sandy J, Kisiday J, Frisbie D, et al. Adult bone marrow stromal cell-based tissue-engineered aggrecan exhibits ultrastructure and nanomechanical properties superior to native cartilage. Osteoarthritis Cartilage. 2010;18(11):1477-86.

26. Miller RE, Grodzinsky AJ, Cummings K, Plaas AH, Cole AA, Lee RT, et al. Intraarticular injection of heparin-binding insulin-like growth factor 1 sustains delivery of insulin-like growth factor 1 to cartilage through binding to chondroitin sulfate. Arthritis Rheum. 2010;62(12):3686-94.

27. Kopesky PW, Vanderploeg EJ, Kisiday JD, Frisbie DD, Sandy JD, Grodzinsky AJ. Controlled delivery of transforming growth factor beta1 by self-assembling peptide hydrogels induces chondrogenesis of bone marrow stromal cells and modulates Smad2/3 signaling. Tissue Eng Part A. 2011;17(1-2):83-92.

28. Kopesky PW, Byun S, Vanderploeg EJ, Kisiday JD, Frisbie DD, Grodzinsky AJ. Sustained delivery of bioactive TGF-beta1 from self-assembling peptide hydrogels induces chondrogenesis of encapsulated bone marrow stromal cells. J Biomed Mater Res A. 2014;102(5):1275-85.

29. Miller RE, Kopesky PW, Grodzinsky AJ. Growth factor delivery through self-assembling peptide scaffolds. Clin Orthop Relat Res. 2011;469(10):2716-24.

30. Miller RE, Grodzinsky AJ, Barrett MF, Hung HH, Frank EH, Werpy NM, et al. Effects of the combination of microfracture and self-assembling Peptide filling on the repair of a

clinically relevant trochlear defect in an equine model. J Bone Joint Surg Am. 2014;96(19):1601-9.

31. Tokunou T, Miller R, Patwari P, Davis ME, Segers VF, Grodzinsky AJ, et al. Engineering insulin-like growth factor-1 for local delivery. FASEB J. 2008;22(6):1886-93.

32. Florine EM, Miller RE, Liebesny PH, Mroszczyk KA, Lee RT, Patwari P, et al. Delivering heparin-binding insulin-like growth factor 1 with self-assembling peptide hydrogels. Tissue Eng Part A. 2015;21(3-4):637-46.

33. Liebesny PH, Mroszczyk, K., Zlotnick, H., Hung, H., Eliot, F., Kurz, B., Zanotto, G., Frisbie, D., Grodzinsky, A. Enzyme Pretreatment Plus Locally Delivered HB-IGF-1 Stimulates Integrative cartilage Repair In Vitro. Tissue Eng. 2019.

34. Schmidt MB, Chen EH, Lynch SE. A review of the effects of insulin-like growth factor and platelet derived growth factor on in vivo cartilage healing and repair. Osteoarthritis Cartilage. 2006;14(5):403-12.

35. Fortier LA, Mohammed HO, Lust G, Nixon AJ. Insulin-like growth factor-I enhances cell-based repair of articular cartilage. J Bone Joint Surg Br. 2002;84(2):276-88.

36. Goodrich LR, Hidaka C, Robbins PD, Evans CH, Nixon AJ. Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. J Bone Joint Surg Br. 2007;89(5):672-85.

37. Ozaki Y, Nishimura M, Sekiya K, Suehiro F, Kanawa M, Nikawa H, et al. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. Stem Cells Dev. 2007;16(1):119-29.

38. Kieswetter K, Schwartz Z, Alderete M, Dean DD, Boyan BD. Platelet derived growth factor stimulates chondrocyte proliferation but prevents endochondral maturation. Endocrine. 1997;6(3):257-64.

39. Liebesny PH, Byun S, Hung HH, Pancoast JR, Mroszczyk KA, Young WT, et al. Growth Factor-Mediated Migration of Bone Marrow Progenitor Cells for Accelerated Scaffold Recruitment. Tissue Eng Part A. 2016;22(13-14):917-27.

40. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am. 1993;75(4):532-53.

41. Frisbie DD, Morisset S, Ho CP, Rodkey WG, Steadman JR, McIlwraith CW. Effects of calcified cartilage on healing of chondral defects treated with microfracture in horses. Am J Sports Med. 2006;34(11):1824-31.

42. Khan IM, Gilbert SJ, Singhrao SK, Duance VC, Archer CW. Cartilage integration: evaluation of the reasons for failure of integration during cartilage repair. A review. Eur Cell Mater. 2008;16:26-39.

43. Tew SR, Kwan AP, Hann A, Thomson BM, Archer CW. The reactions of articular cartilage to experimental wounding: role of apoptosis. Arthritis Rheum. 2000;43(1):215-25.

44. Hunziker EB, Quinn TM. Surgical removal of articular cartilage leads to loss of chondrocytes from cartilage bordering the wound edge. J Bone Joint Surg Am. 2003;85-A Suppl 2:85-92.

45. DiMicco MA, Sah RL. Integrative cartilage repair: adhesive strength is correlated with collagen deposition. J Orthop Res. 2001;19(6):1105-12.

46. DiMicco MA, Waters SN, Akeson WH, Sah RL. Integrative articular cartilage repair: dependence on developmental stage and collagen metabolism. Osteoarthritis Cartilage. 2002;10(3):218-25.

47. Bos PK, DeGroot J, Budde M, Verhaar JA, van Osch GJ. Specific enzymatic treatment of bovine and human articular cartilage: implications for integrative cartilage repair. Arthritis Rheum. 2002;46(4):976-85.

48. Grenier S, Bhargava MM, Torzilli PA. An in vitro model for the pathological degradation of articular cartilage in osteoarthritis. J Biomech. 2014;47(3):645-52.

49. Hunziker EB, Rosenberg LC. Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane. J Bone Joint Surg Am. 1996;78(5):721-33.

50. Hunziker EB, Kapfinger E. Removal of proteoglycans from the surface of defects in articular cartilage transiently enhances coverage by repair cells. J Bone Joint Surg Br. 1998;80(1):144-50.

51. Boushell MK, Hung CT, Hunziker EB, Strauss EJ, Lu HH. Current strategies for integrative cartilage repair. Connect Tissue Res. 2017;58(5):393-406.

52. Obradovic B, Martin I, Padera RF, Treppo S, Freed LE, Vunjak-Novakovic G. Integration of engineered cartilage. J Orthop Res. 2001;19(6):1089-97.

CHAPTER 3:

TRYPSIN PRE-TREATMENT COMBINED WITH GROWTH-FACTOR FUNCTIONALIZED SELF-ASSEMBLING PEPTIDE HYDROGEL IMPROVES CARTILAGE REPAIR IN RABBIT MODEL

Introduction

Clinicians are still seeking a technique that returns damaged cartilage to its original state, although many consider subchondral bone microfracture the standard of care. However, after microfracture, the repaired tissue is composed of fibrous cartilage[1, 2] coupled with poor horizontal integration, which may account for the higher re-operation rate long-term (10 years) when compared to more involved or aggressive procedures of cartilage repair such as osteochondral autograft/allograft and second-generation autologous chondrocyte transplantation.[3] While these advanced techniques have an improved long-term outcome, they are not without significant challenges. In this study, we explored ways to improve the repair tissue characteristics as well as the horizontal integration following microfracture.

Self-assembling peptide hydrogel scaffold has shown promise in augmenting microfracture.[4] The self-assembling capacity allows this product to be injected arthroscopically, and its high water content allows cells to migrate and deposit extracellular matrix. Self-assembling KLD hydrogel has proven to be biocompatible and stimulate cartilage repair by improving defect filling and increasing in tissue properties.[5] It can also act as a carrier of other molecules if desired. Safety, feasibility and clinical improvement in cartilage healing have

previously been observed with self-assembling KLD hydrogel using critically sized chondral defects in a long-term equine model with strenuous exercise.[6]

It has been demonstrated that the chondrocytes present in the native cartilage have a limited capacity to proliferate and migrate. For that reason, undifferentiated mesenchymal stem cells (MSCs) present in the marrow cavity are often targeted as a cell population to augment cartilage damage.[7] Several growth factors have been studied to stimulate MSC proliferation and migration into the defect.[8] Recently, platelet-derived growth factor (PDGF) and transforming growth factor B1 (TGF-B1) have been shown to increase MSC migration into the self-assembling KLD hydrogel. In this same study, heparin-binding insulin like growth factor 1 (HB-IGF-1) stimulated extracellular matrix (ECM) deposition within the scaffold and in surrounding cartilage.[4] We sought to assess the improved healing with these growth factors through delivery with self-assembling KLD hydrogel.

The lack of the neo synthetized cartilage integration with the surrounding cartilage is believed, by some, to result in instability of the repair tissue, leading to tissue degeneration.[7] The lack of cartilage-to-cartilage or horizontal integration is not completely understood; however, several factors seem to play an important role, including low chondrocyte viability in the surrounding cartilage, and dense surrounding ECM.[9] Horizontal integration could be improved by utilizing chemotactic agents to attract more viable chondrocytes to this area, or by enzymatic digestion of the surrounding cartilage. Horizontal integration has been shown to be highly dependent on collagen deposition and cross-linking[10, 11] as well as cellular repopulation.[12] Several proteolytic enzymes have been studied to improve cartilage integration.[13] Recently the authors' demonstrated that enzymatic pretreatment of cartilage explants with trypsin for 2 minutes was able to promote proteoglycan depletion on the surrounding cartilage up to 200 μm,

which resulted in increased cellular migration to this region.[14] This enzymatic pretreatment was then followed by a fetal bovine serum (FBS) rinse to neutralize and remaining trypsin.

The present study evaluated cartilage repair and integration using self-assembling KLD hydrogel functionalized with PDGF and HB-IGF-1 associated enzymatic pre-treatment of the native cartilage using trypsin in a rabbit model. We hypothesize that self-assembling KLD hydrogel functionalized with PDGF and HB-IGF-1 associated enzymatic trypsin pre-treatment will result in improved cartilage repair and horizontal (cartilage-to-cartilage) integration with the surrounding native tissue.

Methods

Study design

The Institutional Animal Care and Use Committees of Colorado State University, protocol # 15-5769A, approved all aspects of this study. White, female, New Zealand rabbits (Western Oregon Rabbit Company, USA) were used in the present study (N=48). Forty-four skeletally mature/aged (14 months) rabbit were used in this study, and to compare the effect of rabbit age, four 7-month-old rabbits were also utilized but not included in the statistical analysis. Bilateral critically sized defects were created (3 mm diameter x 2 mm deep) in the central portion of the trochlear groove using a drill bit and customized guide. One leg was randomly selected to receive the treatment and the contralateral served as a control. Details about animal management, anesthesia protocol and surgery can be found in supplemental material (Table 5, 6, and Figure 14).

Treatments

The study occurred in two temporal blocks due to the number of animals used. All trypsin-treated defects were followed with subsequent inactivation with FBS for 2 minutes. In total, five treatment combinations and 2 different controls were used (Table 1): 1) KLD+Trypsin group (24 limbs): Defect exposed to trypsin for 2 minutes, followed by filling the defect with KLD hydrogel; 2) KLD+HB-IGF-1 group (8 limbs): Defect filled with KLD premixed with 615nM HB-IGF-1 before assembly; 3) KLD+Trypsin+HB-IGF-1 group (8 limbs): Trypsin treatment, followed by filling the defect with KLD premixed with 615nM HB-IGF-1; 4) KLD+Trypsin+PDGF group (12 limbs): Trypsin treatment, followed by filling the defect with 100ng/ml of PDGF-BB premixed in KLD and 5) KLD+Trypsin+HB-IGF-1+PDGF group (12 limbs): Trypsin treatment, followed by filling the defect with 100ng/ml of PDGF-BB plus 615nM of HB-IGF-1 premixed in KLD. Control defects were treated with KLD only (24 limbs), which consisted of filling the defect with KLD hydrogel, gently flushing with saline solution and allowing 5 minutes for hydrogel assembly, or defects were left empty (8 limbs), or defects received the trypsin treatment and no hydrogel.

Table 1: Study design, treatment and control groups.

Treated limbs	Contra-lateral control limbs	Number of animals
KLD + Trypsin	KLD	8
KLD + HB-IGF1	KLD	8
KLD + Trypsin + HB-IGF1	KLD	8
KLD + Trypsin + PDGF-BB	KLD + Trypsin	8
KLD+ Trypsin + HB-IGF1+ PDGF-BB	KLD + Trypsin	8
KLD + Trypsin + PDGF-BB	Trypsin	4
KLD+ Trypsin + HB-IGF1+ PDGF-BB	Trypsin	4

Post-operative/ Endpoint procedures

Three months after surgery, all animals were euthanized (Table S-4). The circumferential measurement of each leg at one centimeter proximal to the patella was recorded as a measure of muscle mass. Joints were placed in 50 mL conical tube and kept at 4 C° for MRI analyses.

Magnetic resonance imaging

Images were acquired using a 2.4 Tesla nuclear magnetic resonance machine (Bruker Biospec Avance /30 cm). The imaging protocol consisted of transverse and sagittal fat-saturated proton density (PDFS) and spoiled gradient echo (SPGR) sequences; both with 1mm slice thickness and 256 x 256 μm in plane resolution. A board-certified radiologist read the images and classified subchondral bone reconstitution in a 0 to 100% grading scale, where 0 was no subchondral bone reconstitution and 100% was complete subchondral bone reconstitution (at the level of the surrounding tissue). Defect filling was scored using a 0 to 4 grading scale with 0= 100%, 1= 99 – 75%, 2= 74-50%, 3=49-25% and 4= 24-0% of defect filled. Surface regularity, subchondral bone sclerosis and integrity were scored using a 0 to 4 ordinal grading scale with 0 = normal, 1= slight, 2 = mild, 3 = moderate and 4 being severely abnormal.

Radiography

The femorotibial joint was radiographed acquiring cranio-caudal and latero-medial images, which were scored for osteophyte formation, defect visualization and sclerosis around the defect

using a 0 to 4 ordinal grading scale with 0 = normal, 1= slight, 2 = mild, 3 = moderate and 4 being severely abnormal.

Gross Pathologic Evaluation of the Joint

Gross evaluation of the repaired tissue was performed by two blinded evaluators using both previously reported[15] and ICRS criteria. Joint appearance was classified as a global score including inflammation of the synovial membrane and presence of osteophytes.

Histology and Immunohistochemistry

After the macroscopic evaluation, synovial membrane was harvested and fixed for 48 hours in 10% formol. A 1.5cm long by 1cm wide and 1cm deep block including the defect and surrounding normal tissue was harvested from the distal femur with an Exakt bandsaw and fixed for 72 hours in 10% formol 1% zinc solution (Z-fix, Anathec Ltd., Battle Creek, MI), followed by decalcification with 10% formic acid (Immunocal, Statlab, Mckinney, TX) for 5 days at room temperature. After complete decalcification, the samples were placed in PBS solution at 4°C until processed.

The osteochondral defect was equally divided transversely and the distal portions dehydrated and paraffin embedded for routine histology/immunohistochemistry, while the proximal portion was embedded in optimal cutting temperature compound (TissueTek OCT Sakura Finetek USA, Torrance, CA) for immunohistochemistry.

For routine histology, 5 μm thick sections were created from paraffin embedded blocks of each sample and transferred to slides followed by staining with hematoxylin/eosin and safranin–O/fast green. Two blinded evaluators scored each section using ICRSII[16] microscopic score system for cartilage repair. Synovial membrane samples were evaluated for cellular infiltration, vascularity, intimal hyperplasia, subintimal edema and fibrosis using a previously described 0-4 score system (0= normal, 1, slight, 2= mild, 3 = moderate, 4 severe).[6]

Immunohistochemistry for collagen type II and aggrecan was performed using paraffin embedded samples, while frozen samples embedded in OCT were used for pro-collagen type I immunostaining. Paraffin blocks were cut in 5 μm thick sections and mounted on regular histology slides. An antigen retrieval step was performed with protein kinase (37°C for 17 minutes) for collagen type II and protein kinase (37°C for 15 minutes) plus chondroitinase ABC (37°C for 30 minutes) (Sigma-Aldrich, Saint Louis, MO) for aggrecan immunostaining. After the antigen retrieval step, sections were incubated with primary antibody solution for collagen type II at neat concentration (Collagen II #II-II6B3, Hybridoma Bank) or aggrecan at 1:100 (Alexis Biochemicals, San Diego, CA). Pro-collagen type I immunostaining was performed using 8 μm thick sections created from frozen samples embedded in OCT. The tissues were transferred and mounted on slides using CryoJane Tape-Transfer System (Leica Biosystems, Buffalo Grove, IL). Slides were incubated in acetone for 10 minutes at -20°C for tissue fixation. Sections were incubated in mouse anti pro-collagen type I antibody at neat concentration (Hybridoma Bank Cat#M-38). For all immunostaining, the endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol. Sections were incubated with goat anti-mouse HRP secondary antibody solution at 1:500 (collagen type II and procollagen type I) or 1:250 (aggrecan) (Jackson Immunoresearch, Westgrove, PA) and stained with Vector Nova RED (Vector Laboratories, Burlingame, CA).

Control sections were incubated with protein matched negative control solution and gave no signal. Non-calcified tissues were evaluated blindly by two evaluators for the percentage of repair tissue stained positive (0=no stain, 1=1–25%, 2=26–50%, 3=51–75%, 4=76–100%).

Statistical Analyses

Data analyses were performed using SAS version 9.4 (Cary, NC). The effect of *Trypsin* was analyzed as a main effect independently or averaging over all other effects. The effect of Growth Factor (HB-IGF1 and/or PDGF-BB) was analyzed as a main effect as well as their interaction in animals that had KLD and Trypsin in the contralateral limb.

The main effect of KLD hydrogel was analyzed for animals that had trypsin as contralateral limbs. For the three previously described analyses (Trypsin, Growth Factor and KLD hydrogel) the model included subject as a random effect. Further, when a main or interaction effect had p-value < 0.05, individual comparisons were performed using Least Square Means (LSMeans) procedure and differences with p-value <0.05 were considered significant. LSMeans and 95% confidence limits are reported.

Results

Magnetic Resonance Imaging

Trypsin pre-treatment was associated with improved (13%) subchondral bone reconstitution (trypsin= 46.11 (38.70 – 53.52, LSMeans (95% CI)) and control= 33.20 (22.76 – 43.65); p=0.047) (Figure 1). MRI defect filling was on average between 50 to 75% category with trypsin

resulting in slightly more defect filling compared to controls (trypsin= 1.9 (1.6 – 2.1) and control= 2.5 (2.1 – 2.9); p=0.008). Overall, reparative tissue had a mildly irregular surface with trypsin treated defects having slightly smoother surface than control (trypsin= 2.0 (1.7 – 2.2) and control= 2.5 (2.1 – 2.9); p=0.014). No significant growth factor main effect or interactions were observed (Supplemental Material Table 3).

Radiography

Trypsin pre-treatment did not result in statistically significant differences for any parameters evaluated by radiography. However, PDGF-BB mildly increased subchondral bone healing, defined as decreased defect visualization on radiography (PDGF-BB= 1.43 (1.01 – 1.84) and control= 2.05 (1.64 – 2.45); p= 0.0170). Osteophytes were not radiographically observed in defects treated with self-assembling KLD hydrogel, however some were present on control defects (KLD hydrogel= 0.08 (-0.13 – 0.30) and control= 0.71 (0.32 – 1.10); p=0.008) (Supplement Material Table 3).

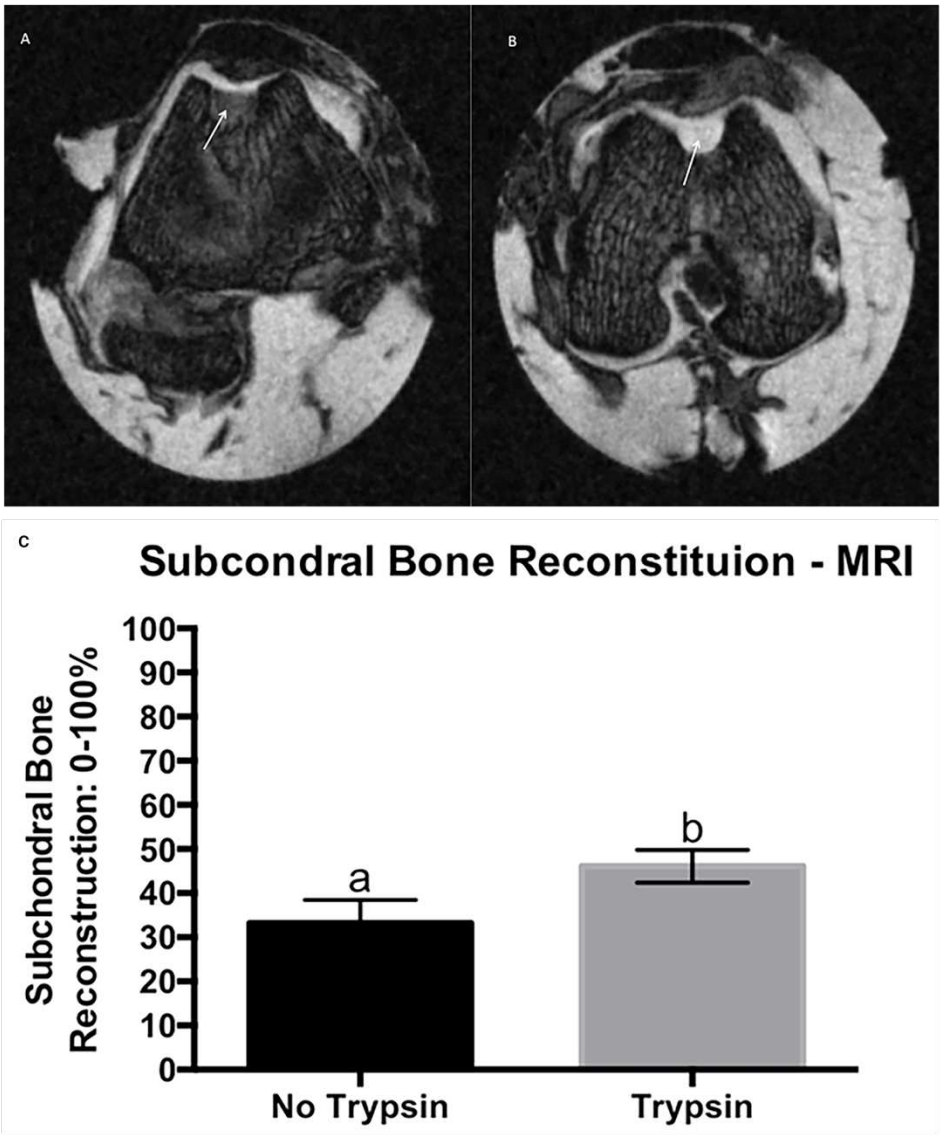

Figure 1. Trasnverse PDFS MRI image showing improved subchondral bone reconstitution (arrows) of the reparative tissue treated with trypsin (B), compared to its contralateral control (A), MRI score for main effect of trysin on subchondral bone reconstitution, grading scale 0-100%, where 0% is no subchrondral bone reconstitution and 100% is subchondral bone reconstituted to the level of surrounding tissue. Error bar represents standard error. Different letters mean statistical siginificant difference (C).

Gross Pathologic Evaluation of the Joint

Pre-treatment with trypsin demonstrated a slight but significant reduction in muscle atrophy

(based on the circumferential measurement of each leg at one centimeter proximal to the patella)

(trypsin= 13.33 (13.20 – 13.47) and control= 13.17 (13.03 – 13.31); $p< 0.05$), mild improvement

of cartilage firmness (trypsin= 0.86 (0.68 – 1.03) and control= 1.17 (0.93 – 1.41); p= 0.0322) and a shift from yellow/white to white repair tissue color (trypsin= 5.20 (5.08 – 5.32) and control= 5.84 (5.66 – 6.01); p= 0.0001), as well as slightly improvement on total cumulative macroscopic score (trypsin= 17.32 (16.42 – 18.21) and control= 18.79 (17.59 – 19.99); p= 0.0391). Even though the defects treated with trypsin resulted in slightly higher values of joint inflammation (trypsin= 0.41 (0.24 – 0.58) and control= 0.07 (-0.13 – 0.28); p= 0.0027) and synovial membrane proliferation (trypsin= 0.86 (0.64 – 1.07) and control= 0.48 (0.19 – 0.77); p= 0.032), these values were still under the "slightly affected" category. PDGF-BB treated joints showed slightly more joint inflammation compared to joints that did not received PDGF-BB (PDGF-BB= 0.60 (0.32 – 0.87) and control= 0.24 (-0.02 – 0.51); p=0.0314). Joints which had the defects filled with self-assembling KLD hydrogel were closer to normal in appearance while defects not filled with KLD evidenced slight inflammation (KLD hydrogel= 0.16 (-0.08 – 0.42) and control= 1.0 (0.55 – 1.44); p=0.002) (Supplemental Material Table 4).

Histology

*Synovial membrane***:** There is a main effect for trypsin enzymatic pre-treatment in reducing the grade of the synovial membrane intimal hyperplasia by about half (Trypsin = 0.55 (0.31 – 0.78) and control = 0.96 (0.62 – 1.30); p= 0.05). All the other parameters were not statistically significantly different (Supplemental Material Table 5).

Osteochondral histology: Trypsin pre-treatment improved cell morphology in 20% (trypsin= 79.83 (73.48 – 86.17) and control= 59.83 (50.86 – 68.8); p= 0.0005) (Figure 2) and slightly decreased (less than 10%) the number of cell clusters observed (trypsin= 88.41 (85.04 – 91.78)

and control= 82.00 (77.23 – 86.76); p= 0.0317). A trend (p=0.059) for trypsin treatment to improve tissue morphology was also observed, (trypsin= 51.49 (45.38 – 57.61) and control= 41.47 (32.90 – 50.05). However, trypsin pre-treatment slightly increased (less than 10%) repaired tissue vascularization (trypsin= 92.08 (87.74 – 96.42) and control= 99.58 (93.67 – 105.5); p= 0.035).

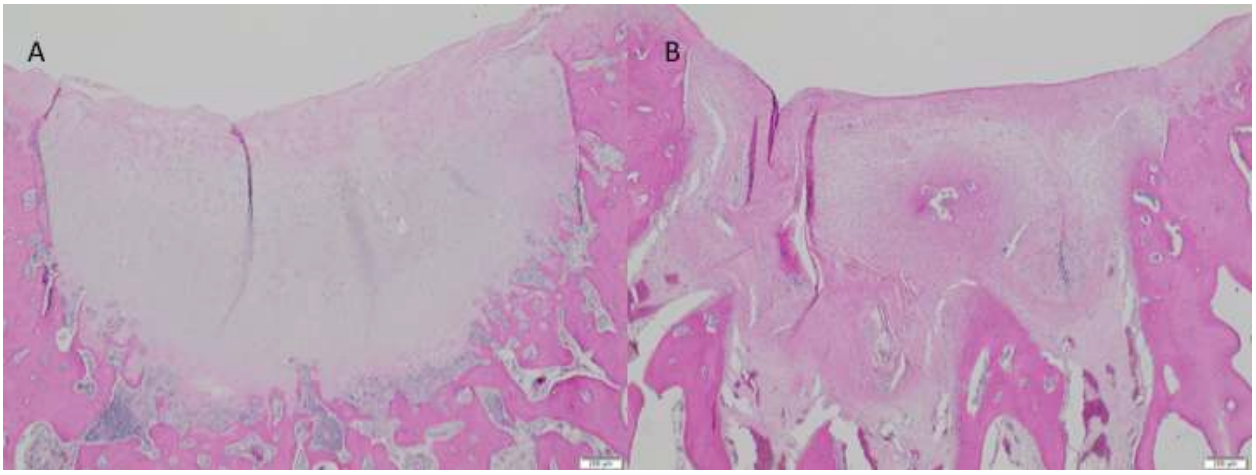

Figure 2. Improved cell morphology of a reparative tissue treated with trypsin (A) compared to its control not treated with trypsin (B). H&E stainning, 20 x magnification. Scale bar 500μm.

PDGF-BB resulted in 23% improvement on chondrocyte cell morphology (PDGF-BB= 88.62 (76.97 – 100.28) and control= 65.21 (55.13 – 75.29); p= 0.0037) and 16% increase in basal integration of the repair tissue with the underlying bone (Figure 3) compared to defects not treated with PDGF-BB (PDGF-BB= 86.12 (75.39 – 96.85) and control= 70.69 (61.40 – 79.97); p=0.0337).

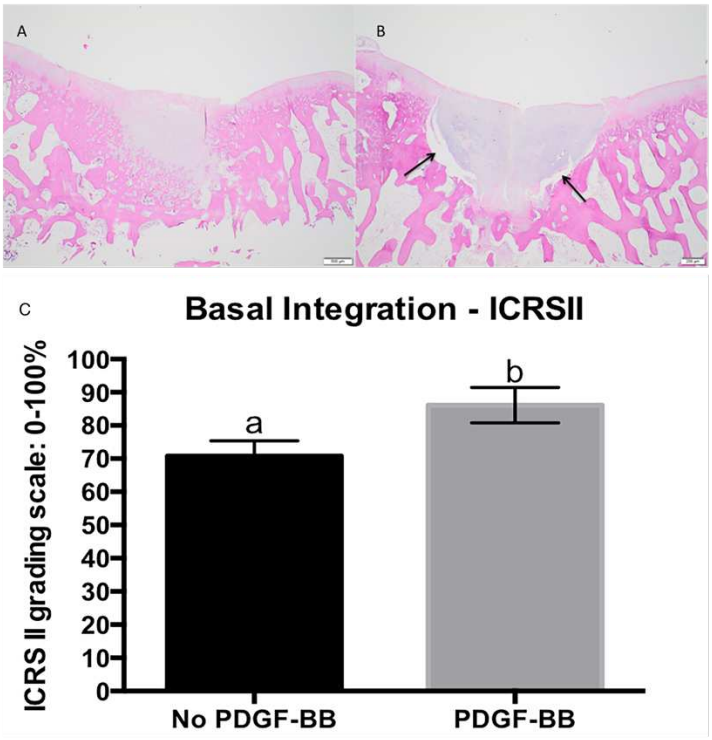

Figure 3. Improved basal integration on defect exposed to PDGF-BB (A) compared to its control not treat with PDGF-BB (B). Lack of basal integration is demosntrated by the space between subchondral bone and repairative tissue (arrows). H&E stainning, 20 x magnification. Scale bar 500μm, histology score for main effect of PDGF-BB on basal integration. ICRSII grading scale: 0-100%, where 0 is completely abnormal and 100% is completely normal. Error bar represents standard error. Different letters mean statistical siginificant difference (C).

The addition of PDGF-BB and HB-IGF-1 resulted in a significant effect over HB-IGF-1 alone (p=0.0100). Specifically, with respect to improvement in tissue morphology (HB-IGF-1 + PDGF-BB= 66.25 (50.07 – 82.42) and HB-IGF-1= 36.25 (20.07 – 52.42); p=0.01) (Figure 4) as well as chondrocyte morphology (Figure 5): (HB-IGF-1 + PDGF-BB= 96.25 (78.88 – 113.62) and HB-IGF-1= 50.00 (32.63 – 67.36); p=0.0046). Also, the combination of HB-IGF-1 and PDGF-BB resulted in about 20% less subchondral bone abnormalities/marrow fibrosis compared to HB-IGF-1 alone (HB-IGF-1 + PDGF-BB= 92.23 (80.75 – 103.71) and HB-IGF-1= 72.50 (61.01 – 83.98); p= 0.0174).

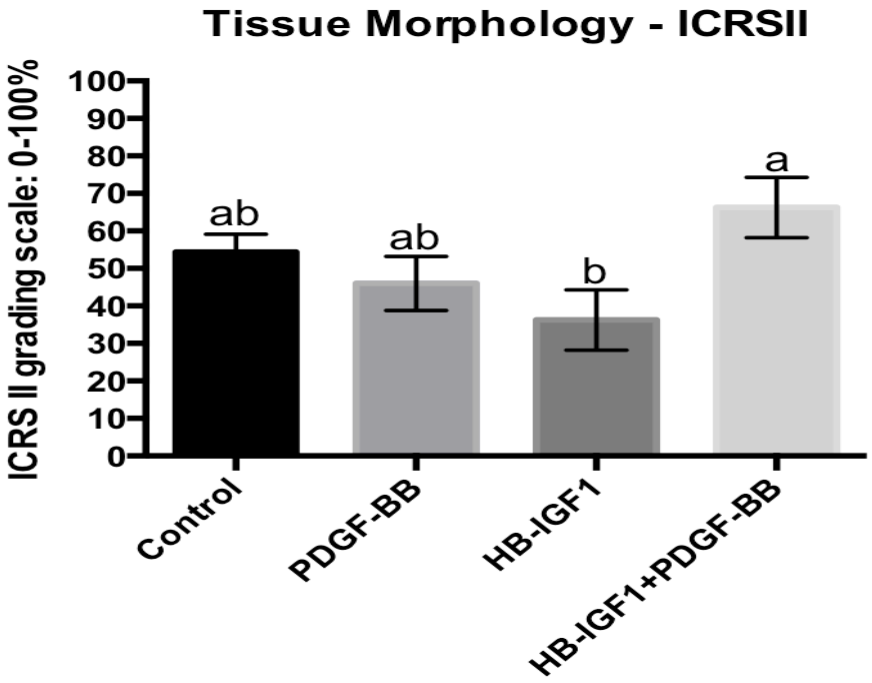

Figure 4. Histology score for main effect and interaction of HB-IGF1 and PDGF-BB on tissue morphology. ICRSII grading scale: 0-100%, where 0 is completely abnormal and 100% is completely normal. Error bar represents standard error. Different letters mean statistical siginificant difference.

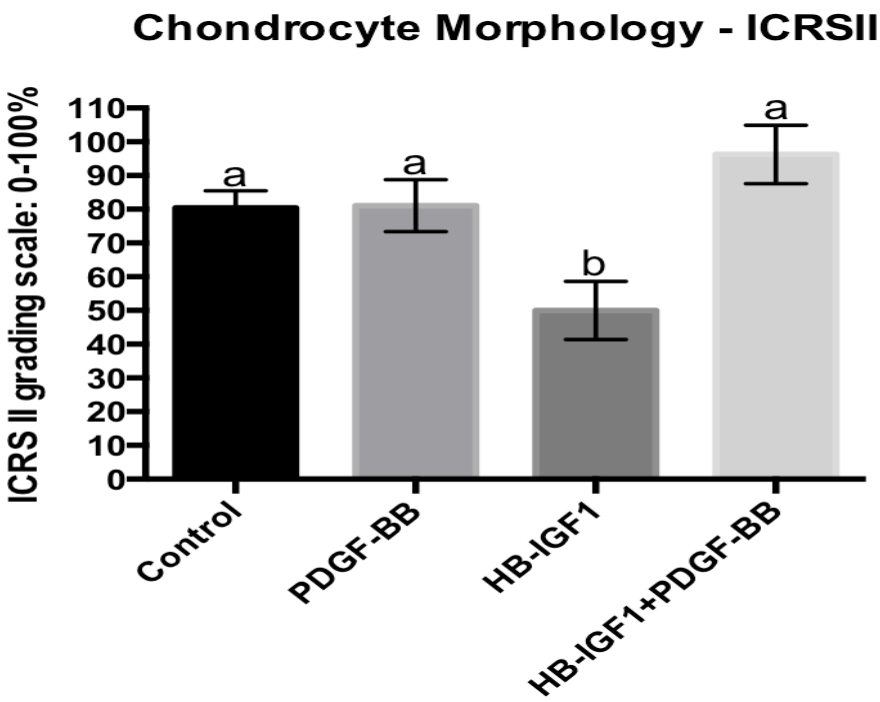

Figure 5. Histology score for main effect and interaction of HB-IGF1 and PDGF-BB on chondrocyte morphology. ICRSII grading scale: 0-100%, where 0 is completely abnormal and 100% is completely normal. Error bar represents standard error. Different letters mean statistical siginificant difference

Chondrocyte clustering was observed to be significantly greater when the defects were treated with HB-IGF-1 or PDGF-BB alone compared to control defects. When these growth factors were combined there was no statistical difference when compared to controls (control= 91.14 (85.74 – 96.54), HB-IGF-1= 80.00 (70.89 – 89.10), PDGF-BB= 80.90 (72.52 – 89.27) and HB-IGF1 + PDGF-BB= 88.01 (78.60 – 97.42); p= 0.032) (Supplemental Material Table 5).

Immunohistochemistry

There is a main effect of trypsin or PDGF-BB to induce more pro-collagen type I immunostaining compared to control (trypsin= 2.19 (1.83 – 2.55) and control= 1.43 (0.92 – 1.95); p= 0.0187) (PDGF-BB= 2.56 (1.92 – 3.20) and control= 1.71 (1.12 – 2.30); p=0.055). Defects treated with self-assembling KLD hydrogel had significantly more aggrecan than those

left empty (KLD hydrogel= 3.72 (3.40 – 4.04) and controls= 2.87 (2.34 – 3.40); p= 0.0092). Aggrecan and collagen type II immunostaining did not demonstrate any statistically significant differences based on presence of growth factors (Supplemental Material Table 5).

Discussion

Some of the reasons for cartilage repair failure are related to the poor quality of the neo-synthetized tissue, which usually consists of more fibrocartilage than hyaline cartilage, and the poor integration of the neo cartilage to the surrounding tissue.[7] In the present study, trypsin pre-treatment of the osteochondral defect resulted in a significant improvement of the defect filling, subchondral bone reconstitution and surface regularity observed on MRI, as well as cartilage firmness and color on gross appearance; and cell morphology and cluster formation histologically. Considering all of these findings together, along with the reduced muscle atrophy observed with trypsin treated defects, the authors' believe trypsin pre-treatment induced an overall improvement in cartilage repair which led to better use (evidence by decreased muscle atrophy) of the treated limb compared to the contralateral limb.

Recently our group demonstrated that enzymatic pre-treatment of cartilage explants with trypsin for 2 minutes were able to promote proteoglycan depletion extending from the center of the cartilage annuli in about 200 μm thick, dramatically influencing cellular migration and promoting better cartilage horizontal integration in an *in vitro* model.[14] Trypsin enzymatic pre-treatment of the cartilage results in proteoglycan depletion in a time dependent manner, not affecting collagen network[17] and results in increased cell volume and total proteoglycan

deposition on partial thickness cartilage defects in rabbits.[18] Furthermore, chondrocyte proliferation was shown to increase post intra-articular injection of trypsin in rabbits.[19] The trypsin pretreatment mildly increased synovial membrane proliferation and slightly increased macroscopic joint inflammation (scale: normal, slight, mild, moderate or severe categories). These findings were in contrast to previous reports where trypsin was injected intra-articularly at a much higher concentration (100x more concentrated than in this study).[18-20] In these reports, trypsin did not result irritation of the synovial membrane macroscopically. In the present study, our macroscopic results differ from our histologic findings where trypsin resulted in less histologic synovial membrane intimal hyperplasia, suggesting lack of active inflammation. Without histologic abnormalities the level of macroscopic change most likely is not of clinical significance.

In the current study the combination of HB-IGF-1 and PDGF-BB resulted in improved histologic tissue and cell morphology (30% and 46%) when compared to HB-IGF-1 treated defects. The authors consider an effect size greater than 10% to have clinical relevance. The authors chose this combination of growth factors because PDGF and IGF-1 have been reported to be the most potent agents capable of induction of bone marrow-MSC migration when 16 chemokines where assessed.[21] Similarly, in an *in vitro* rabbit model this combination also showed superior chemotactic effects on MSC's compared to either growth factor alone.[22] The authors believe this is the first example of an in vivo application of these growth factors in a cartilage repair model, which supports our in vitro work.[4]

In the current study PDGF-BB alone was noted to have a significant effect improving radiographic subchondral bone healing (30%) and histologic basal integration (16%) of the repair tissue when compared to controls. PDGF has been shown to induce bone repair[23], but also inhibit

endochondral differentiation.[24] Subchondral bone reconstitution is mainly derived by two different mechanism of bone production: endochondral and direct intramembranous ossification.[7] Thus, given PDGF's capacity to improve intramembranous ossification and our observed improved subchondral bone healing and increased basal integration the authors credit these observations to PDGF.

Ultimately, we would expect reformation of the tidemark with improved bone healing, which was not observed in the current study. However, the current study period of 12 weeks was most likely too short to have expected significant tidemark reformation as this has been reported to take 24 weeks[7] in other work. A longer-term study would be needed to better address this effect.

In the current study, a significant increase in osteophyte formation was observed with PDGF-BB treatment when compared to control, but its noteworthy this difference was slightly and may be of questionable clinical significance. Such findings of increased osteogenic activity on the subchondral bone have been previously reported.[25] In this way, it is possible that the slight increase in osteophyte formation may be related to a higher subchondral bone activity, since no other signals of active inflammation or instability were noticed in the current study outcome parameters (radiographic and histologic).

Surprisingly, no significant main effects of HB-IGF-1 were observed. Insulin Growth Factor-1 has been studied in cartilage repair and has been reported both in vitro and in vivo to significantly improve chondrogenesis and cartilage integration.[26-29] Our group has reported that HB-IGF-1 is strongly retained in articular cartilage post intra-articular injection[30] and leads to sustained proteoglycan biosynthesis in cartilage explants.[31] Interestingly, an in vitro kinetics study showed that 84% of the HB-IGF-1 that was premixed into a KLD hydrogel was released

within the first four days of culture[4]; however, this HB-IGF-1 seems to be bound by surrounding cartilage annuli instead of being released in the media (unpublished data). Further studies are necessary to elucidate the lack of effect in the present study.

When assessing the effect of KLD hydrogel we observed a 32% increase (3.7 vs 2.8) in aggrecan deposition and a 90% improvement (0.1 vs 1) in macroscopic joint observation when compared to defects not treated with KLD hydrogel. These findings are consistent with our previous studies.[5, 6]

We used MRI, as it can be potentially used as a non-invasive imaging modality in future studies, to quantitatively describe the healing process in hopes that this technique may be used in future human patients. MRI outcomes demonstrate a 39% improvement of subchondral bone healing with trypsin treatment compared to control (46 vs 33, respectively). Similarly, we also observed a 24% improvement (1.9 vs 2.5) in defect filling and 20% improvement (2.0 vs 2.5) in surface regularity with trypsin treatment in a similar comparison. These results suggest an improvement with trypsin treatment as well as usefulness of the MRI outcomes.

The authors have been utilizing older rabbits, at least 12 months old, to assess cartilage healing techniques as we believed that younger rabbits have a greater propensity to heal and maybe provide overly optimistic outcomes. In the current work we included four (N=4) 7-month-old rabbits. Due to the low numbers statistical analyses were not attempted but rather qualitative data assessed. Young adult (7-month-old) rabbits tended to have improved scores when compared to the older rabbits. Some of the most striking improvements were in cartilage integration (macroscopically) (80%, 0.25 vs 1.25), and histologic matrix metachromasia (20%, 65 vs 50) as well as vascularization (ICRSII) (15%, 92 vs 80). Previous reports support our subjective findings that seven to eight-month-old New Zealand white rabbits have a decreased healing of

full thickness cartilage defect, compared to 5 (adolescent) and 3 (immature) month-old.[32] The authors are unaware of other publications comparing 7 (skeletally mature) to 14-month-old (aged) rabbits as done in the current study.

The authors utilized the current rabbit model to help ascertain the best treatment group(s) to assess in a large (equine) animal model. It was accepted that using the rabbit as a test species included inherent limitations as highlighted by a robust intrinsic healing capacity (90% of the defect area and 80% of defect volume were reconstituted across all treatment/control groups). Despite limitations in the current study, it did offer insight to treatments resulting in significant improvements to guide further studies.

CONCLUSION

Self-assembling KLD hydrogel functionalized with PDGF-BB and HB-IGF-1 with associated trypsin pretreatment of the native cartilage resulted in an overall improvement on the cartilage repair process compared to other control and treatment combination. The trypsin pretreatment improved numerous outcome parameters (muscle atrophy, defect filling, subchondral bone reconstitution, surface regularity, cartilage firmness and color, cell morphology and cluster formation). While the PDGF-BB had some improved outcomes alone, when outcomes were taken in aggregate, the best results were seen with the combination of both growth factors in the presence of trypsin. Further studies in a long-term, large animal model are warranted based on the current study.

REFERENCES

1. Frisbie DD, Trotter GW, Powers BE, et al. 1999. Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. Vet Surg 28: 242-255.
2. Erggelet C, Vavken P. 2016. Microfracture for the treatment of cartilage defects in the knee joint - A golden standard? J Clin Orthop Trauma 7:145-152.
3. Riboh JC, Cvetanovich GL, Cole BJ, Yanke AB. 2017. Comparative efficacy of cartilage repair procedures in the knee: a network meta-analysis. Knee Surg Sports Traumatol Arthrosc 25: 3786-3799.
4. Liebesny PH, Byun S, Hung HH, et al. 2016. Growth Factor-Mediated Migration of Bone Marrow Progenitor Cells for Accelerated Scaffold Recruitment. Tissue Eng Part A 22: 917-927.
5. Miller RE, Grodzinsky AJ, Vanderploeg EJ, et al. 2010. Effect of self-assembling peptide, chondrogenic factors, and bone marrow-derived stromal cells on osteochondral repair. Osteoarthritis Cartilage 18:1608-1619.
6. Miller RE, Grodzinsky AJ, Barrett MF, et al. 2014. Effects of the combination of microfracture and self-assembling Peptide filling on the repair of a clinically relevant trochlear defect in an equine model. J Bone Joint Surg Am 96: 1601-1609.
7. Shapiro F, Koide S, Glimcher MJ. 1993. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am 75: 532-553.
8. Fortier LA, Barker JU, Strauss EJ, et al. 2011. The role of growth factors in cartilage repair. Clin Orthop Relat Res 469: 2706-2715.

9. Khan IM, Gilbert SJ, Singhrao SK, et. al. 2008. Cartilage integration: evaluation of the reasons for failure of integration during cartilage repair. A review. Eur Cell Mater 16: 26-39.
10. DiMicco MA, Sah RL. 2001. Integrative cartilage repair: adhesive strength is correlated with collagen deposition. J Orthop Res19: 1105-1112.
11. DiMicco MA, Waters SN, Akeson WH, Sah RL. 2002. Integrative articular cartilage repair: dependence on developmental stage and collagen metabolism. Osteoarthritis Cartilage 10: 218-225.
12. Bos PK, DeGroot J, Budde M, et al. 2002. Specific enzymatic treatment of bovine and human articular cartilage: implications for integrative cartilage repair. Arthritis Rheum 46: 976-985.
13. Boushell MK, Hung CT, Hunziker EB, et al. 2017. Current strategies for integrative cartilage repair. Connect Tissue Res 58: 393-406.
14. Zlotnick HM AA, Chen HJ, Liebesny PH, et al. 2019. Tissue Engineering. Enzyme Pretreatment Plus Locally Delivered HB-IGF-1 Stimulates Integrative Cartilage Repair In Vitro. Manuscript submitted for publicaiton.
15. Frisbie DD, Bowman SM, Colhoun HA, et al. 2008. Evaluation of autologous chondrocyte transplantation via a collagen membrane in equine articular defects: results at 12 and 18 months. Osteoarthritis Cartilage 16: 667-679.
16. Mainil-Varlet P, Van Damme B, Nesic D, et al. 2010. A new histology scoring system for the assessment of the quality of human cartilage repair: ICRS II. Am J Sports Med 38: 880-890.
17. Griffin DJ, Vicari J, Buckley MR, et. al. 2014. Effects of enzymatic treatments on the depth-dependent viscoelastic shear properties of articular cartilage. J Orthop Res. 32: 1652-1657.

18. Quinn TM, Hunziker EB. 2002. Controlled enzymatic matrix degradation for integrative cartilage repair: effects on viable cell density and proteoglycan deposition. Tissue Eng. 8: 799-806.
19. Havdrup T. 1979. Trypsin-induced mitosis in the articular cartilage of adult rabbits. Acta Orthop Scand 50:15-19.
20. Lack W, Bosch P, Lintner F. 1986. Influence of trypsin on the regeneration of hyaline articular cartilage. Acta Orthop Scand 57: 123-125.
21. Ponte AL, Marais E, Gallay N, et al. 2007. The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. Stem Cells. 25: 1737-1745.
22. Ozaki Y, Nishimura M, Sekiya K, et al. 2007. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. Stem Cells Dev 16: 119-129.
23. Chung R, Foster BK, Zannettino AC, Xian CJ. 2009. Potential roles of growth factor PDGF-BB in the bony repair of injured growth plate. Bone. 44: 878-885.
24. Kieswetter K, Schwartz Z, Alderete M, et al. 1997. Platelet derived growth factor stimulates chondrocyte proliferation but prevents endochondral maturation. Endocrine 19: 257-264.
25. Hulth A, Johnell O, Miyazono K, et al. 1996. Effect of transforming growth factor-beta and platelet-derived growth factor-BB on articular cartilage in rats. J Orthop Res 14: 547-553.
26. Fortier LA, Mohammed HO, Lust G, Nixon AJ. 2002. Insulin-like growth factor-I enhances cell-based repair of articular cartilage. J Bone Joint Surg Br 84: 276-288.
27. Zhang Z, Li L, Yang W, et al. 2017. The effects of different doses of IGF-1 on cartilage and subchondral bone during the repair of full-thickness articular cartilage defects in rabbits. Osteoarthritis Cartilage 25: 309-320.

28. Worster AA, Brower-Toland BD, Fortier LA, et al. Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J Orthop Res 19: 738-749.
29. Zhou Q, Li B, Zhao J, et al. 2016. IGF-I induces adipose derived mesenchymal cell chondrogenic differentiation in vitro and enhances chondrogenesis in vivo. In Vitro Cell Dev Biol Anim. 52: 356-364.
30. Miller RE, Grodzinsky AJ, Cummings K, et al. Intraarticular injection of heparin-binding insulin-like growth factor 1 sustains delivery of insulin-like growth factor 1 to cartilage through binding to chondroitin sulfate. Arthritis Rheum 62:3686-3694.
31. Tokunou T, Miller R, Patwari P, et al. 2008 Engineering insulin-like growth factor-1 for local delivery. FASEB J 22: 1886-1893.
32. Wei X, Messner K. 1999. Maturation-dependent durability of spontaneous cartilage repair in rabbit knee joint. J Biomed Mater Res 46: 539-548.

CHAPTER 4:

MICROFRACTURE AUGMENTATION WITH TRYPSIN PRETREATMENT AND GROWTH FACTOR FUNCTIONALIZED SELF-ASSEMBLING PEPTIDE HYDROGEL SCAFFOLD IN AN EQUINE MODEL

Introduction

Microfracture is one of the most common marrow stimulation techniques for cartilage resurfacing and is frequently considered as the first line of treatment. Microfracture is a single step, simple, and cost-effective technique to improve cartilage healing in a biologic fashion[11]. However, microfracture-treated chondral lesions result in various degrees of defect filling, with the reparative tissue being composed mostly of fibrocartilage, which leads to decreased biomechanical properties[1, 3, 11, 12].

Recently, there has been an increasing interest in improving microfracture techniques using tissue engineering principles[26, 28]. Scaffolds, cells, and growth factors are used in combination with microfracture to improve clot stabilization and create a favorable environment for cells to migrate and deposit extracellular matrix [6, 26].

Our group has been studying self-assembling KLD peptide hydrogel as the scaffold for tissue engineering proposes [14-18, 21, 22, 29] . This hydrogel can be injected arthroscopically, allows cells to migrate and deposit extracellular matrix, and can be functionalized with growth factors [17]. Osteochondral defects filled with this hydrogel showed an increase in reparative tissue filling, as well as aggrecan (SOFG) and collagen type II (IHC) content, resulting in overall improved cartilage healing compared to empty controls[22]. Also, improved clinical outcomes were observed

for defects treated with the hydrogel compared to empty controls in an equine model of cartilage defect [21].

Self-assembling KLD peptide hydrogel has an interesting ability to serve as a growth factor delivery system. Studies have shown that platelet-derived growth factor (PDGF-BB) and transforming growth factor β1 (TGF-β1) remained significantly retained in the gel for up 4 days, resulting in effective migration of bone marrow progenitor cells to the hydrogel [15, 17]. Insufficient integration between neo-synthesized and surrounding native cartilage can cause tissue instability, leading to tissue degeneration over time[25]. Cartilage integration is highly dependent on cellular repopulation, collagen deposition and cross-linking at the interface between neo-synthesized and surrounding native cartilage[2, 4, 5]. Recently, we used a clinically relevant controlled enzymatic digestion of the surrounding native cartilage with trypsin, combined with locally delivered heparin-binding insulin-like growth factor-1 (HB-IGF-1) to improve cartilage integration *in vitro*. This approach allowed progenitor cells to migrate from the hydrogel into the surrounding native cartilage, resulting in improved cartilage integration[18]. Further, we observed that the trypsin treatment combined with a functionalized hydrogel resulted in improved defect filling, subchondral bone reconstitution, surface regularity, cartilage firmness, cell morphology and chondrocyte cluster formation in a small animal model of cartilage repair[29]. Thus, the present study aim is to evaluate the effects of trypsin pretreatment combined with a self-assembling KLD peptide hydrogel functionalized with two growth factors, PDGF-BB and HB-IGF-1, ("functionalized hydrogel") in augmenting microfracture healing of a critically-sized cartilage defect in horses subjected to strenuous exercise. Based on our previous studies, we believe this combination of treatments will result in overall improved cartilage healing and better

integration of the newly-synthetized cartilage to native surrounding cartilage when compared to microfracture alone.

Methods

Study Designing

The Institutional Animal Care and Use Committees approved all aspects of this study (protocol # 15-5769A). Bilateral, 15 mm diameter, cartilage defects were created on the medial trochlear ridge of the femur in eight adult horses (16 defects total). One defect was randomly assigned to receive microfracture plus treatment, while the other served as the control and received microfracture only. All horses were submitted to a standardized exercise protocol on a high-speed treadmill during the post-operative period. Clinical exam and radiographic evaluation were performed on multiples time points. At six months after defect creation, a second-look arthroscopy was performed to evaluate cartilage healing and to obtain a 4mm osteochondral biopsy for histology. At 12 months after defect creation, all animals were euthanized with an overdose of pentobarbital. Magnetic resonance imaging (MRI), arthroscopy, gross pathologic joint evaluation, histology, immunohistochemistry, and biomechanical testes were also performed. Evaluators were blinded to the treatments until the end of the study.

Animals

Eight clinically health adult (2-5 year-old) horses without any evidence of musculoskeletal problems were used in this study. Animals were housed in individual 15 m^2 stalls, with water *ad libitum* and hay provided twice a day.

Surgery

Under general anesthesia, bilateral (left and right) femoropatelar arthrotomy was carried out in each horse. A critically sized (15mm diameter) cartilage defect was created over the medial trochlear ridge using a round, custom designed instrument and hand curette. Calcified cartilage was carefully removed leaving the subchondral bone intact. Standard microfracture technique was applied to both defects as previously described[12]. One defect was randomly selected to receive the treatment (trypsin pretreatment combined with functionalized hydrogel) and the contralateral defect served as the control (microfracture only). The joint capsule, subcutaneous tissue and skin incision were routinely sutured in three distinct planes (Figure 6).

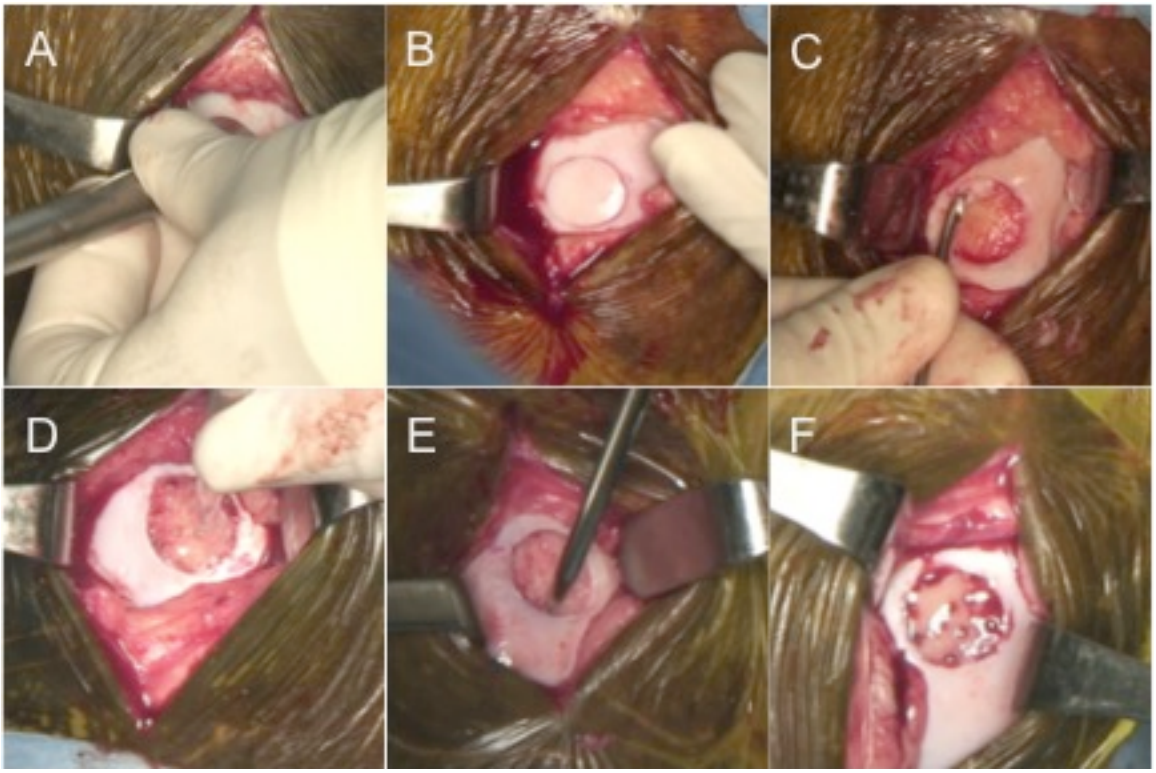

Figure 6: Surgical approach of the treated defects. Defect creation (A, B) and calcified cartilage removal using hand curette (C). Trypsin treatment application (D). Performance of standard microfracture (E). Defect filling with assembled hydrogel (F).

Treatment

Defects assigned to the treatment group were filled with 50 μg/ml trypsin solution for 2 minutes followed by inactivation with FBS for 2 minutes. The defect was then carefully dried and microfracture was performed. Subsequently, the defect was filled with hydrogel premixed with 100ng/ml of PDGF-BB and 615nM of HB-IGF-1. The hydrogel assembling process was initiated by gently flowing sterile saline solution over the hydrogel.

Perioperative care

Non-steroidal anti-inflammatories and antibiotics were given IV prior and up to 5 days post-surgery. Routine physical exam and wound care were performed daily until suture removal, or as necessary.

Exercises

All horses were kept in stall rest for 5 weeks post-operatively followed by a gradual increase in exercise intensity as part of the standard rehabilitation protocol. The exercise program was composed of controlled walking (4.5 Km/h), trotting (12 Km/h), canter (29 Km/h) and gallop (45 Km/h) exercises on a high-speed treadmill.

Lameness evaluation

Subjective and objective lameness evaluation, joint flexion test, passive range of motion (ROM) and effusion were assessed prior surgery and monthly, until the end of this study. Lameness was subjectively scored using a 0 to 5-grade scale (0= no evidence of lameness, 1= slight, 2= mild,

3= moderate, 4= severe and 5= non-weight bearing lameness). The joint flexion test was graded by assessing lameness exacerbation induced after full flexion (0= no change in lameness, 1= slightly, 2= mildly, 3= moderately and 4= severely exacerbated lameness). Passive joint range of motion (ROM) and synovial effusion were also subjectively graded as 0 to 4 (0= no effusion/normal ROM, 1= slight, 2= mild, 3= moderate and 4= severe effusion/limited ROM). Objective lameness evaluation was performed using commercially available inertial sensors system (Lameness Locator®, Equinosis, MO - USA). This system uses built-in accelerometers to detect asymmetry on the maximum and minimum pelvic excursion between right and left pelvic limb. Data is presented as maximum and minimum mean difference, in millimeters, between both pelvic limbs.

Radiographic Evaluation

A board-certified radiologist (MB) evaluated both stifles (equivalent to human knee) joints of each horse using four standard radiographic views. The presence of osteophytes, subchondral bone sclerosis, and lysis adjacent to the defect site were graded from 0 to 4 (0 = normal, 1 = slight, 2 = mild, 3 = moderate, and 4 = severe). Radiographs were evaluated at baseline, 2, 16, 24, 32, 40 and 52 weeks post defect creation.

Second-Look Arthroscopy

Bilateral arthroscopy of the femoropatelar joints was performed under general anesthesia 6 months post defect creation, as well as post-mortem at the end of the study (12 months). An

experienced surgeon (DF) evaluated both defects using the previously described scoring system[21]. For each horse, the joint containing the overall better reparative tissue was identified. At the end of the 6-month arthroscopy recheck only, a 4 mm diameter osteochondral biopsy (encompassing 2 mm of repair tissue and 2 mm of surrounding cartilage) was harvested at the dorsolateral aspect of each defect.

Magnetic Resonance Imaging (MRI)

Images were obtained with a 1.5-T GE Signa scanner (GE Medical Systems, Waukesha, Wisconsin), using the following sequences: proton density sequences with and without fat suppression and T2-weighted fast-spin-echo performed in sagittal, frontal, and transverse planes with 3-mm-thick slices. A board-certified radiologist (MB) evaluated all images for joint effusion, synovial proliferation, joint capsule fibrosis, subchondral bone sclerosis, subchondral edema, subchondral bone lysis and defect filling. Changes were scored from 0 to 4 (0= normal, 1= slightly, 2= mildly, 3= moderately and 4= severely abnormal). Also, reparative tissue signal intensity on T1 and T2, were compared to surrounding cartilage and classified as isointense (1), moderately hypo/hyperintense (2) and severely hypo/hyperintense (3).

Gross Pathologic Evaluation of the Joint

Gross pathologic evaluation of the femoropatelar joint was performed with the joints completely exposed during the post-mortem exam (12 months post-defect creation). Multiple parameters, including overall joint condition, defect integrity, repaired tissue attachment to surrounding

cartilage, total defect area, and volume filled by reparative tissue were scored by two evaluators in agreement using a previously described scoring system[21].

Tissue harvest and processing

Following gross pathologic evaluation of the joint, osteochondral samples for histology, immunohistochemistry, and for biomechanical tests were obtained by transversally sectioning the defect using an exact saw, as illustrated in figure 7. Osteochondral samples from the proximal and distal aspects of the defect (including the surrounding tissue) were processed routinely by fixing in buffered aqueous zinc formalin for 48 hours (Z-fix, Anatech, MI - USA), decalcifying with 12% formic acid solution for 72 hours (Immunocal™, Stat Lab Medical Products, TX, USA) followed by paraffin embedding. Osteochondral samples used for biomechanical tests were wrapped in gauze soaked with phosphate-buffered saline solution (PBS) and frozen until analysis was performed. Synovial membrane samples were consistently collected from the patellar apex region, fixed in 10% formalin and processed for histology.

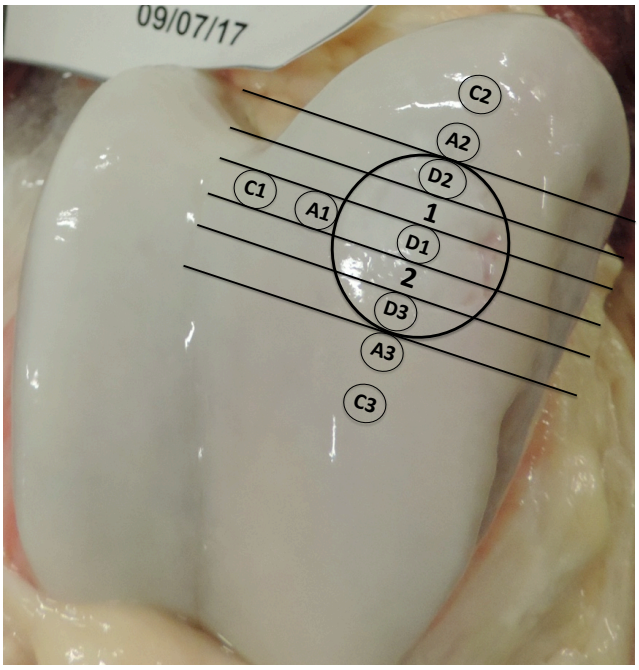

Figure 7: Photo representation of the tissue harvesting. Proximal (1) and distal (2) osteochondral samples were harvested for histology/immunohistochemistry. Three cartilage plugs from the defect site (D1, D2, D3), adjacent cartilage (A1, A2, A3) and remote surrounding cartilage (C1, C2, C3) were subjected to biomechanical tests.

Histologic Evaluation

Paraffin embedded blocks of the osteochondral and synovial membrane samples were sectioned (5 μm thick) and mounted on histology slides. Osteochondral sections were stained with hematoxylin/eosin and safranin-O fast green (SOFG), while synovial membrane sections were stained with hematoxylin/eosin. Two evaluators (DF and GZ) graded each histological osteochondral section in consensus using ICRSII score system[19]. Synovial membrane sections were evaluated for cellular infiltration, intimal hyperplasia, subintimal edema, subintimal fibrosis, and vascularity with a 0 to 4 grading scale (0= normal, 1= slight, 2= mild, 3= moderate and 4= severe).

Immunohistochemistry

Immunostaining for collagen type I, collagen type II, and aggrecan were performed by the following described technique. Paraffin embedded osteochondral samples from the proximal and distal aspect of the defect were sectioned (5μm thick) and mounted on histology slides. An antigen retrieval step was performed with protein kinase and sections were then incubated with the primary antibody solution for collagen type I at 1:100 (Accurate Chemical and Scientific, NY - USA), collagen type II at neat concentration (Developmental Studies Hybridoma Bank, IA - USA) or aggrecan at 1:100 (Acris antibodies, MD - USA) Alexis Biochemicals). Endogenous peroxidase was blocked using 0.3% H_2O_2 diluted in methanol. Sections were then incubated with donkey anti-mouse HPR secondary antibody solution at 1:500 (Jackson Immunoresearch, Westgrove, PA - USA), stained with Vector Nova RED (Vector Laboratories, Burlingame, CA - USA) and counter-stained with fast-green. Controls sections were incubated with protein matched negative control solution and gave no signal. Slides were graded for the percentage of defect stained (0 = no staining, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76% to 100%) by two evaluators (DF and GZ) in agreement.

Biomechanical Tests

Osteochondral samples were thawed in phosphate buffered saline solution with protease inhibitors. Cartilage plugs from the defect, adjacent native cartilage and remote cartilage (as far as possible from the defect) were harvest from the proximal, central and distal aspect of the medial trochlear ridge (Figure 7). When multiple plugs were harvested from the same region, the measurements were averaged to give only one value per region. Cartilage plugs measured

7.07mm^2 in cross-sectional area and were at least 0.5 mm in height. Unconfined dynamic and equilibrium compressive stiffness, and dynamic shear forces were measured using a Dynastat mechanical spectrometer (IMASS, Hingham, Massachusetts) [21]. A total of 15% offset strain was created by applying three 5% ramp-and-hold steps. This was followed by a frequency sweep with 0.5% amplitude sinusoidal strains at 1.0 Hz. Dynamic compressive stiffness was calculated using the stress strain curves, and equilibrium compressive stiffness was calculated from linear regression of equilibrium stress versus strain. For dynamic shear modules, the plugs underwent 15% and 30% axial compression, followed by a frequency sweep of 1.5% amplitude sinusoidal shear strains at 1 Hz.

Statistical Analysis

Sample size calculation based on previous studies using the same model and similar outcomes[11, 21] determined that 8 defects per treatment group would provide >80% power at $\alpha = 0.05$[23]. Statistical analysis was performed using generalized linear mixed models in SAS software (version 9.2; SAS Institute, Cary, North Carolina), with horse as a random variable. Treatment, week, location (distal or proximal), or tissue type (native or repair) was classified as main effects. When the main effect or an interaction had a p-value that was considered to be significant (<0.05) or indicated a trend (0.05 to <0.10), least square means were used for individual comparisons. All data is presented as the mean and confidence interval.

Results

Lameness

Over the period of the study, mild lameness was observed in the early post-operative period (4 to 16 weeks) followed by a decrease in severity up to 36 weeks. However, at 40 weeks, a gradual increase in lameness was observed and persisted to the end of the study (52 weeks). No statistically significant difference was observed between treatment groups. Lameness exacerbation after joint flexion tests varied from slight to mild and had a tendency (p=0.0572) to be less severe on treatment limbs compared to control (treatment= 1.18 (0.98 – 1.37) and control= 1.35 (1.15 – 1.55) p= 0.0572). No statistical differences in joint effusion or passive ROM were observed between groups.

On objective lameness evaluation (inertial sensor system), the treatment group had an overall improvement in impact (50%) and push off (60%) lameness compared to control group (Figure 8).

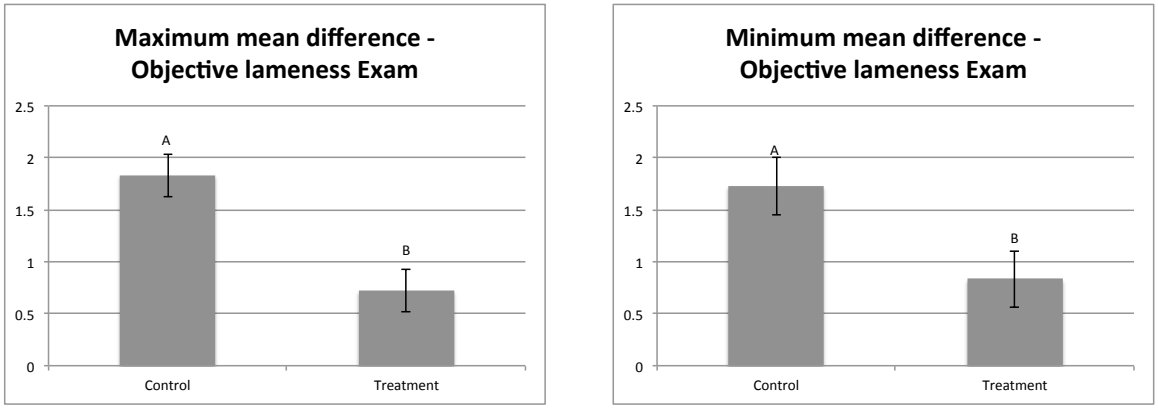

Figure 8: Improvement in push off (maximum mean difference) and impact (minimum mean difference) in treatment group compared to control. Values are presented as mean difference and standard error (bars) between maximum and minimum hip excursion in millimeters. Different letters indicate statistically significant differences

Radiographic evaluation

Slight subchondral bone sclerosis and subchondral bone lysis was noted on radiographs after defect creation compared to radiographs taken before surgery. The treatment group had 27% more subchondral bone sclerosis compared to the control (treatment= 1.2 (0.96 – 1.43) and control= 0.94 (0.70 – 1.17), p= 0.0062), however both were graded as "slight". There were no other statistical differences found between groups for any other radiographic parameters.

Magnetic Resonance Image Evaluation

Magnetic resonance imaging evaluation showed slight to mild joint effusion, with treated joints having 39% more effusion than control. Subchondral bone sclerosis was classified as mild to moderate in treated defects, which was significantly increased (31%) compared to the control defects. T1 signal intensity in treated defects was considered moderately hypointense compared to surrounding cartilage, while control defects were considered isointense (Table 2).

Table 2: Magnetic resonance imaging scores showing mean, least-square mean and confidence interval for each parameter evaluated. Significant p-values are marked in bold.

	Control		Treatment			
	Mean	CI	Mean	CI	LS Means	CI	p- value
Defect Filling	3.25	(2.56 3.93)	3.38	(2.69 4.05)	-0.13	(-0.95 0.70)	0.7318
Joint Effusion	1.37	(0.96 1.78)	2.25	(1.83 2.66)	-0.88	(-1.41 -0.33)	**0.0062**
Joint Capsule Fibrosis	0.88	(0.20 1.54)	1.00	(0.33 1.66)	-0.13	(-0.95 0.70)	0.7318
Synovial Membrane Proliferation	0.87	(0.15 1.59)	1.25	(0.53 1.96)	-0.37	(-1.14 0.39)	0.2849
Subchondral Bone Edema	0.25	(-0.04 0.54)	0.00	(-0.31 0.31)	0.25	(-0.18 0.68)	0.2049
Subchondral bone Lysis	2.87	(1.96 3.78)	2.62	(1.71 3.53)	0.25	(-0.72 1.22)	0.5630
Sunchondral Bone Sclerosis	1.63	(0.85 2.39)	2.37	(1.60 3.14)	-0.75	(-1.49 -0.01)	**0.0479**
T1 Signal	1.25	(0.88 1.61)	1.84	(1.45 2.24)	-0.59	(-1.07 0.12)	**0.0213**
T2 Signal	1.25	(0.86 1.63)	1.25	(0.86 1.63)	0.00	(-0.54 0.54)	1.0000

Second-Look Arthroscopy

On arthroscopic evaluation, tissue degeneration beyond the defect was mild at 6 months, but not apparent at 12 months, with no difference between treatment groups. (6 months= 0.93 (0.84 – 1.02) and 12 months= 0 (-0.09 – 0.09), $p< 0.0001$). Six out of eight treated defects were considered to have overall better reparative tissue than the control (contral-lateral limb) at 12 months. This difference was not observed at the earlier time point (6 months) (Figure 9). No other statistically significant effects were observed in the other parameters.

Gross Pathologic Evaluation of the Joint

Overall, reparative tissue was present in more than 95% of the defect area and accounted for 66% (control) to 75% (treatment) of the defect volume. Reparative tissue was classified as slightly soft and mildly to moderately attached to surrounding native cartilage. No statistically significant differences between groups were noted in any parameter on gross pathologic evaluation of the joint (Supplemental Material Table 7).

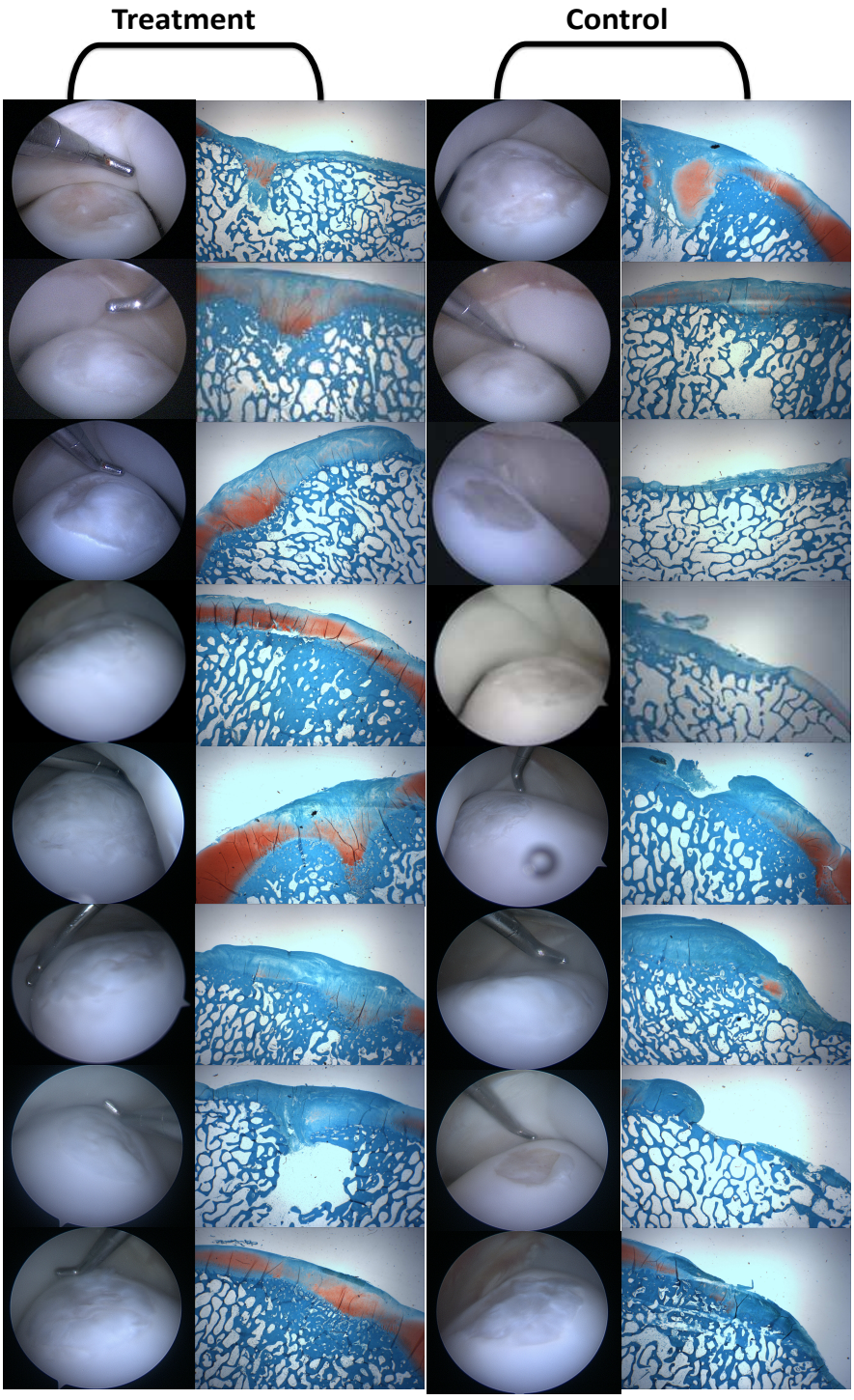

Figure 9: Arthroscopic view of the reparative tissue at 12 months of all the 16 defects. Proximal osteochondral histological slides of the reparative tissue are provided for comparative purposes (SOFG 10x).

Histology

Osteochondral samples: Interaction between treatment and location was statistically significant for various histological parameters. When evaluating the proximal osteochondral samples, treated defects had 53% improved tissue morphology, 44% smoother surface architecture and 62% improvement in the defect mid/deep zone compared to control defects (Figure 10-A, B, C). Furthermore, there was an overall 50% improvement of the repaired tissue on histology at the proximal aspect of treated defects compared to controls at same location (Figure 10-D).

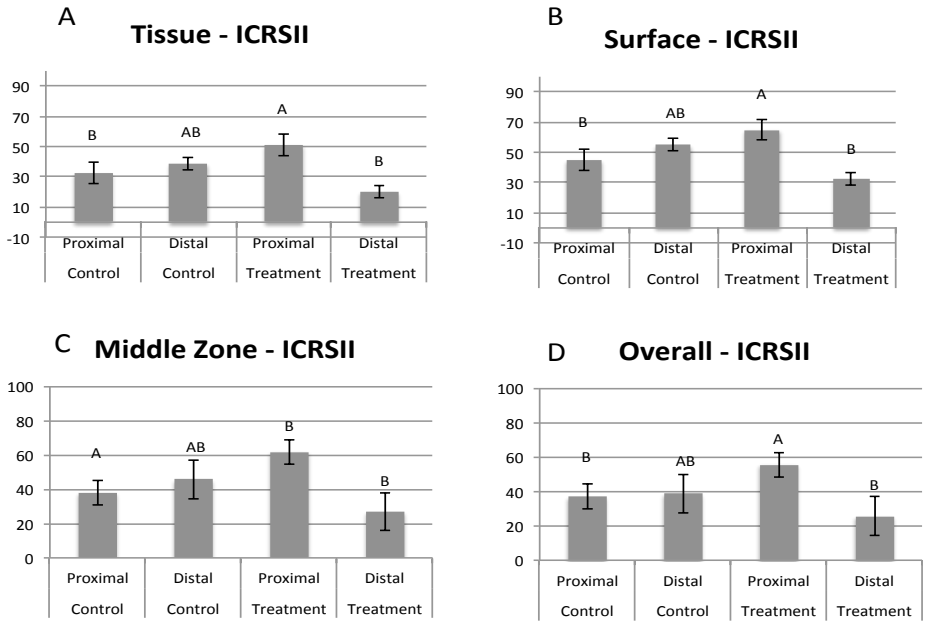

Figure 10: Microscopic ICRSII evaluation of tissue morphology (A), surface regularity (B), middle/deep zone (C) and overall quality of the repaired tissue (D). Values are presented as mean and standard error (bars). Different letters indicate statistically significant differences

Matrix staining was present in about 23% of the reparative tissue in proximal osteochondral samples of treated defects compared to only 10% in the proximal osteochondral samples of control defects. Meanwhile, for the distal osteochondral samples, treated defects had virtually no matrix staining compared to 6% of matrix staining in control defects (Figure 11-A) (Supplemental Material Table 8).

Overall, proximal osteochondral samples had 43% improvement in tissue morphology (proximal= 41.71 (35.35 – 48.08) and distal 29.53 (19.36 – 39.69), p= 0.039)) and 41% improvement in the overall ICRSII score (proximal= 46.25 (36.67 – 55.82) and distal= 32.18 (19.13 – 45.23), p= 0.029) compared to distal osteochondral samples.

A statistically significant interaction between treatment and time was observed in evaluation of matrix staining and abnormal calcification. In defects receiving the treatment, matrix staining was not observed on osteochondral biopsy taken at 6 months, but at 12 months 23% of the defect was positively stained. In the control defects, minimal (<5%) matrix staining was present at 6 months and remained statistically unchanged by 12 months. Furthermore, matrix staining was higher in the treatment group compared to the control at 12 months, nearly reaching significance (p=0.0582) (Figure 11-B). In evaluating calcification, treated defects had 20% more abnormal calcification than control defects at 6 months, however this difference was not observed at 12 months, where both defect were nearly free of abnormal calcification (Figure 12)(Supplemental Material Table 9).

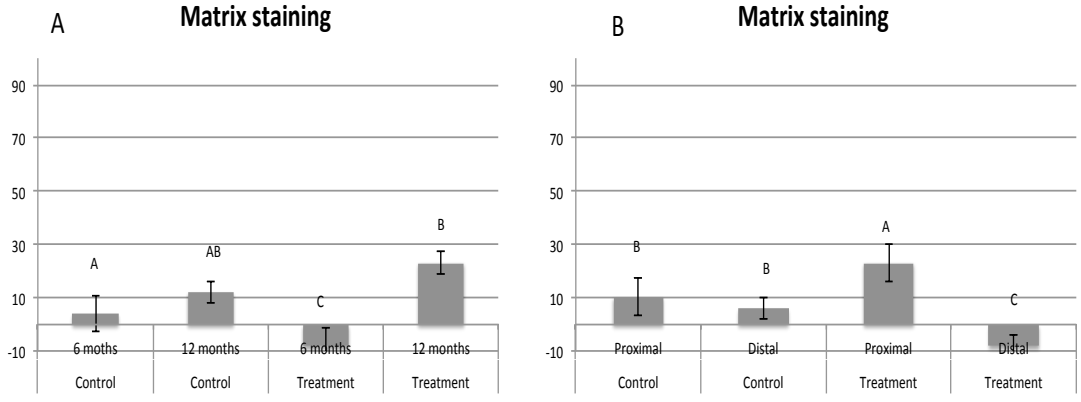

Figure 11: ICRSII score for percentage of the reparative tissue positively staining with SOFG. Treatment affected matrix staining differently depending on time point (A) or location (B). Values are presented as mean and standard error. Different letters indicate statistically significant differences.

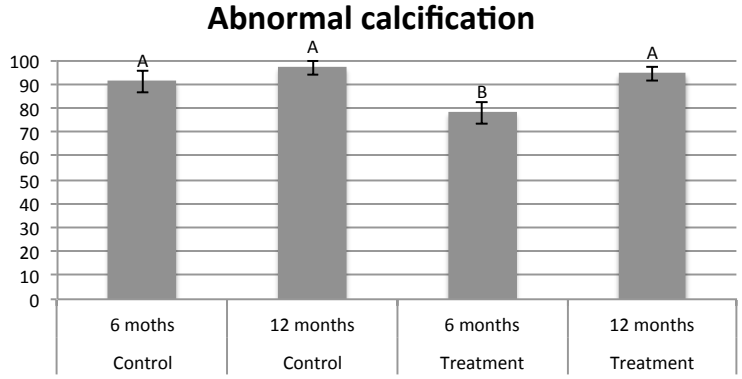

Figure 12: ICRSII score for the presence of abnormal calcification based on time point and treatment group. 100% means cartilage is completely free of abnormal calcification. Values are presented as mean and standard error. Different letters indicate statistically significant differences.

Synovial membrane: Synovial membrane evaluation showed slight cellular infiltration and intimal hyperplasia, and slight to mild subintimal fibrosis and vascularity. Subintimal edema was virtually not present. There was no statistically significant difference between groups.

Immunohistochemistry

No statistically significant difference in collagen type II, collagen type I and aggrecan immunostaining was observed between treatment groups or locations. Positive staining for collagen type II was observed in approximately 50 to 61% of the repaired tissue, while collage type I immunostaining was present in about 41 to 45%. Aggrecan immunostaining was present in about 42 to 55% of the repaired tissue.

Biomechanical Test

The treated defects had 25% improvement in dynamic shear stiffness compared to controls when considering the average values over all cartilage plug locations (repair, adjacent cartilage or remote cartilage) and regions (central, proximal and distal) (treatment= 0.45 (0.37 – 0.54) and control= 0.36 (0.28 – 0.44), p= 0.0419). Additionally, there was a tendency for treated defects to demonstrate 24% improved dynamic compressive stiffness and 38% improved equilibrium stiffness compared to controls (p=0.0822 and p= 0.1006, respectively).

When evaluating only the osteochondral samples from the central aspect of the defect, the treatment improved dynamic compressive stiffness by 76% (treatment= 3.14 (2.54 – 3.74) and control= 1.78 (1.20 – 2.36), p= 0.0004) and shear stiffness by 21% (treatment= 0.40 (0.27 – 0.52) and control= 0.33 (0.23 – 0.42). When the individual comparison is made averaged across the different aspects of the defect (central, proximal and distal), adjacent cartilage from treated defects had 3.5 times improved dynamic compressive stiffness and 3 times improved dynamic shear stiffness compared to controls, but this comparison was not supported by a protected F-test (Figure 13).

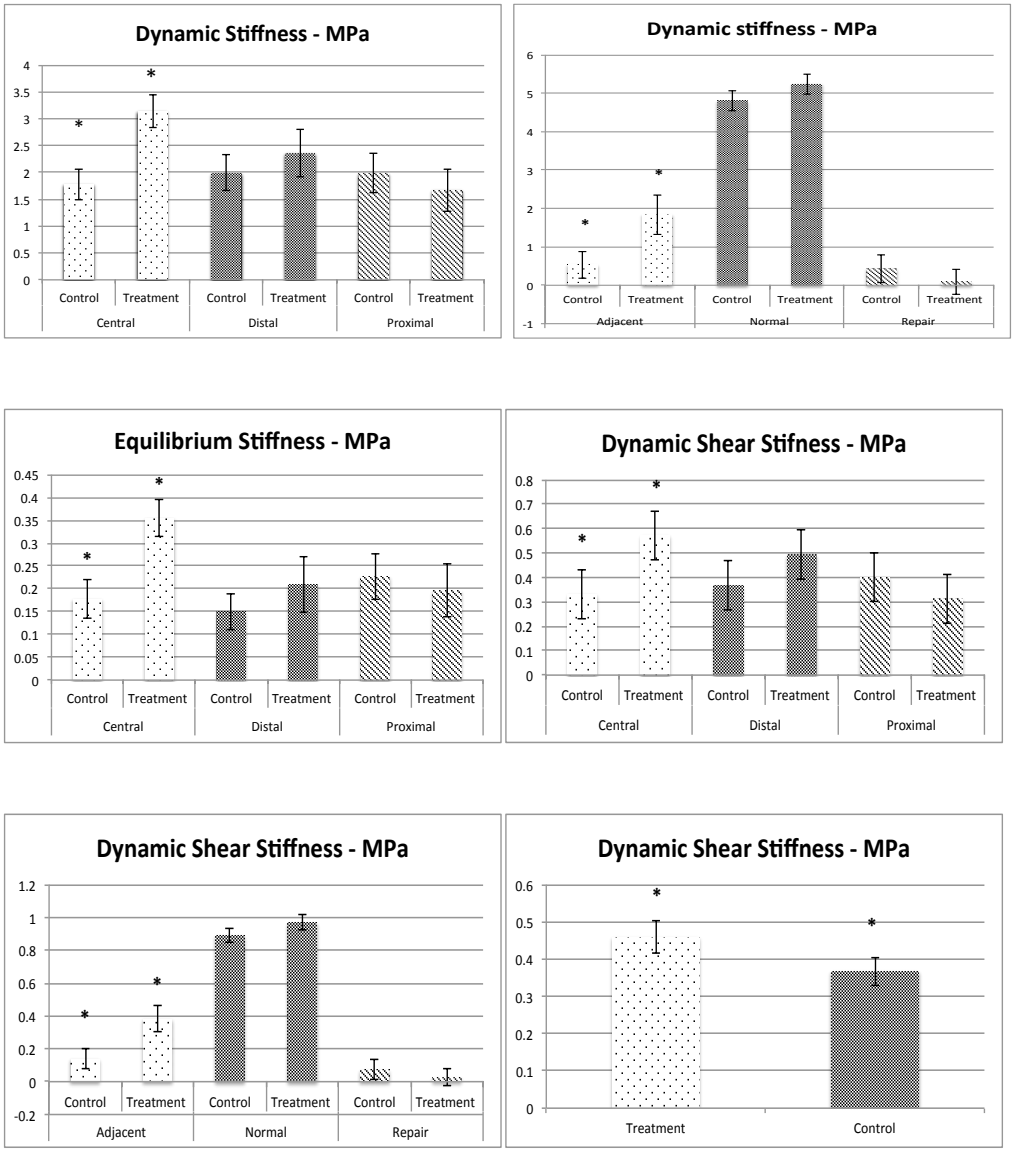

Figure 13: Effect of treatment on dynamic stiffness (A, B), equilibrium stiffness (C) and dynamic shear stiffness (D, E, F) by region (A, C, D) or by tissue type (B, E). Main effect of treatment on dynamic shear stiffness (F). Values are represented in mega-pascal (MPa). Asterisks represent statistically significant differences $p < 0.05$.

Overall, the dynamic compressive and shear stiffness forces of the reparative tissue measured less than 1% of those in the remote cartilage and approximately 20% of those in the adjacent cartilage. Also, adjacent cartilage dynamic compressive and shear stiffness forces were 23 to 27% respectively of those in remote cartilage. Equilibrium stiffness of the reparative tissue and adjacent cartilage was only 2% and 21% respectively of that of the remote cartilage, but no

statistically significant difference was observed between repaired tissue and adjacent cartilage equilibrium stiffness.

Discussion

In the present study we tested the hypothesis that trypsin pretreatment combined with functionalized hydrogel would be a suitable way to augment microfracture techniques in an equine model involving strenuous exercise. The proposed technique resulted in overall improvement of functional outcomes, subjectively improved reparative tissue at the 12 month second-look arthroscopy, enhanced histological healing of the proximal aspect of the reparative tissue, and improved biomechanical properties of the cartilage. Previously we demonstrated self-assembling KLD hydrogel improved functional outcome and defect filling, however it failed to promote additional benefits over microfracture only. Insufficient cellular and growth factor infiltration into the hydrogel was hypothesized as a cause[21]. Based on that hypothesis, we started to develop alternative approaches to improve cellular migration by functionalizing the hydrogel with pro-anabolic growth factors. The findings of the present study are further supported by our previous studies where trypsin pretreatment combined with functionalized hydrogel resulted in improved cellular migration and better horizontal integration of cartilage *in vitro* (Liebesny et al., 2019) and overall improvement in cartilage repair *in vivo* (Zanotto et al., 2019).

In the current study, objective gait analysis and flexion tests showed statistically significant improvement in joint function outcomes for the treatment group, despite the fact that no differences were appreciated on subjective lameness evaluation. Objective gait analysis is more sensitive in detecting gait asymmetries [20], facilitating the detection of small differences in

bilateral lameness, such as were created in this model. This suggests lameness improvement in the treatment group was modest and its long-term clinical significance unknown.

A mild increase in subchondral bone sclerosis (MRI and radiography) and joint effusion (MRI) was noted in treated limbs in the current study. Some degree of subchondral bone sclerosis was expected after microfracture [8, 21], but the combination of microfracture and hydrogel should have partially mitigated this effect, as previously observed [21]. This contradictory finding in this study can be attributed to the presence of growth factors mixed into the hydrogel. PDGF has been shown to increase osteogenic activity on the subchondral bone [13], while IGF-1 has been shown to induce subchondral bone remodeling [30]. Alternatively, improved clinical outcome of the treated limbs could have resulted in higher load applied to the subchondral bone during strenuous exercise, inducing adaptive remodeling. However, this hypothesis could not be tested in the current design.

Despite the significant relationship between synovitis/joint effusion and knee pain that is reported in humans[27], the findings in the present study demonstrated better functional outcomes in joints with slight effusion noted on MRI. Altogether, we believe the slight increase in joint effusion without signs of active synovitis (MRI/histology) probably has minimal clinical significance in this model and was not supported by the objective pain data.

Overall differences in cartilage healing was observed between treatment and control groups on second-look arthroscopy at 6 and 12 months after defect creation. Six out of the eight treated defects were considered to have overall better reparative tissue when compared to contralateral control defects at 12 months. However, this difference was not evident at the 6-month recheck arthroscopy, where four defects in each group (treatment and control) were considered to have

better healing. Little progression in cartilage healing after 4 months post-surgery has been documented after subchondral bone microfracture techniques [11, 12]. In contrast, defect filling is significantly increased up to 12 months following cartilage autograph implantation systems and autologous chondrocyte implantation techniques[9]. This indicates the proposed treatment resulted in improvement of the cartilage healing over time (similar to what is observed with other techniques) and is better than microfracture alone.

Overall, reparative tissue had poor (less than 25%) extracellular matrix staining. However, significantly increased matrix staining was observed between 6 and 12 months in the treatment group. Also, treated defects had a tendency to have more matrix staining than control defects at the end of the study. It has been shown that microfracture techniques do not influence aggrecan content (matrix staining) in the reparative tissue[11], while contradictory findings are reported with self-assembling KLD hydrogel[21, 22, 29]. Further, insulin growth factor-1 induces proteoglycan synthesis *in vitro*[18, 24], however *in vivo* effects are less consistent[7, 29]. Even though it still unclear which factor or combination resulted in improvement in matrix staining, the proposed treatment resulted in the highest amount of proteoglycan staining at 12 months.

In general, the proximal aspect of the defect showed better healing compared to distal aspect of the defect, microscopically. Moreover, beneficial effects of the treatment on tissue morphology, surface architecture, mid/deep zone, as well as increase in matrix staining were only observed in the proximal osteochondral samples. Differences in cartilage healing dependent on defect location have been reported in previous studies using similar models with two defects per joint (one proximal and one distal)[6, 9]. This difference in cartilage healing was also observed within the proximal and distal region of the same defect[21]. In horses, the trochlea does not receive any load when in the stationary full weight-bearing position, however at early stages of the joint

flexion, the proximal aspect of the trochlea is subjected to increased load compared to the distal aspect, creating a different biomechanical environment between these two regions[10]. We believe the biomechanical stimulus received during earlier stages of reparative process worked synergistically with the proposed treatment resulting in overall better cartilage repair in the proximal aspect of the defect suggesting earlier loading of these treated defects resulted in an improved outcome.

The treated defect had more abnormal calcification at 6 months post defect creation, however at 12 months, the treatment defect was nearly free of abnormal calcification and was not statistically significant different from the control group. Additionally, mild degenerative changes beyond the defect were observed at 6 months but not at 12 months post defect creation, with no difference between groups. This finding is in contrast with the previous study where degenerative changes in the defect were most observed at 12 months[21]. These findings indicate that significant tissue remodeling occurs between 6 to 12 months, and the remodeling is more pronounced in the treatment group.

Conclusion

In conclusion, trypsin pretreatment combined with functionalized hydrogel can be considered a suitable strategy for microfracture augmentation. Similar to other currently available cartilage resurfacing techniques, complete regeneration of the hyaline cartilage was not achieved with the proposed technique. However, compared to microfracture treatment alone, the observed technique improved functional outcomes, reparative healing on second-look arthroscopy at 12

months, as well as improved histological outcomes and biomechanical properties of the cartilage, which may potentially result in superior long-term clinical benefits.

REFERENCES

1. Bae DK, Yoon KH, Song SJ. Cartilage healing after microfracture in osteoarthritic knees. *Arthroscopy.* 2006;22(4):367-374.

2. Bos PK, DeGroot J, Budde M, Verhaar JA, van Osch GJ. Specific enzymatic treatment of bovine and human articular cartilage: implications for integrative cartilage repair. *Arthritis Rheum.* 2002;46(4):976-985.

3. Carter AH, Guttierez N, Subhawong TK, et al. MR imaging of BioCartilage augmented microfracture surgery utilizing 2D MOCART and KOOS scores. *J Clin Orthop Trauma.* 2018;9(2):146-152.

4. DiMicco MA, Sah RL. Integrative cartilage repair: adhesive strength is correlated with collagen deposition. *J Orthop Res.* 2001;19(6):1105-1112.

5. DiMicco MA, Waters SN, Akeson WH, Sah RL. Integrative articular cartilage repair: dependence on developmental stage and collagen metabolism. *Osteoarthritis Cartilage.* 2002;10(3):218-225.

6. Fortier LA, Chapman HS, Pownder SL, et al. BioCartilage Improves Cartilage Repair Compared With Microfracture Alone in an Equine Model of Full-Thickness Cartilage Loss. *Am J Sports Med.* 2016;44(9):2366-2374.

7. Fortier LA, Mohammed HO, Lust G, Nixon AJ. Insulin-like growth factor-I enhances cell-based repair of articular cartilage. *J Bone Joint Surg Br.* 2002;84(2):276-288.

8. Fortier LA, Potter HG, Rickey EJ, et al. Concentrated bone marrow aspirate improves full-thickness cartilage repair compared with microfracture in the equine model. *J Bone Joint Surg Am.* 2010;92(10):1927-1937.

9. Frisbie DD, Lu Y, Kawcak CE, DiCarlo EF, Binette F, McIlwraith CW. In vivo evaluation of autologous cartilage fragment-loaded scaffolds implanted into equine articular defects and compared with autologous chondrocyte implantation. *Am J Sports Med.* 2009;37 Suppl 1:71S-80S.

10. Frisbie DD, McCarthy HE, Archer CW, Barrett MF, McIlwraith CW. Evaluation of articular cartilage progenitor cells for the repair of articular defects in an equine model. *J Bone Joint Surg Am.* 2015;97(6):484-493.

11. Frisbie DD, Oxford JT, Southwood L, et al. Early events in cartilage repair after subchondral bone microfracture. *Clin Orthop Relat Res.* 2003(407):215-227.

12. Frisbie DD, Trotter GW, Powers BE, et al. Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. *Vet Surg.* 1999;28(4):242-255.

13. Hulth A, Johnell O, Miyazono K, Lindberg L, Heinegard D, Heldin CH. Effect of transforming growth factor-beta and platelet-derived growth factor-BB on articular cartilage in rats. *J Orthop Res.* 1996;14(4):547-553.

14. Kisiday JD, Kopesky PW, Evans CH, Grodzinsky AJ, McIlwraith CW, Frisbie DD. Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. *J Orthop Res.* 2008;26(3):322-331.

15. Kopesky PW, Byun S, Vanderploeg EJ, Kisiday JD, Frisbie DD, Grodzinsky AJ. Sustained delivery of bioactive TGF-beta1 from self-assembling peptide hydrogels

induces chondrogenesis of encapsulated bone marrow stromal cells. *J Biomed Mater Res A.* 2014;102(5):1275-1285.

16. Kopesky PW, Vanderploeg EJ, Kisiday JD, Frisbie DD, Sandy JD, Grodzinsky AJ. Controlled delivery of transforming growth factor beta1 by self-assembling peptide hydrogels induces chondrogenesis of bone marrow stromal cells and modulates Smad2/3 signaling. *Tissue Eng Part A.* 2011;17(1-2):83-92.

17. Liebesny PH, Byun S, Hung HH, et al. Growth Factor-Mediated Migration of Bone Marrow Progenitor Cells for Accelerated Scaffold Recruitment. *Tissue Eng Part A.* 2016;22(13-14):917-927.

18. Liebesny PH, Mroszczyk, K., Zlotnick, H., Hung, H., Eliot, F., Kurz, B., Zanotto, G., Frisbie, D., Grodzinsky, A. Enzyme Pretreatment Plus Locally Delivered HB-IGF-1 Stimulates Integrative cartilage Repair In Vitro. *Tissue Eng.* 2019.

19. Mainil-Varlet P, Van Damme B, Nesic D, Knutsen G, Kandel R, Roberts S. A new histology scoring system for the assessment of the quality of human cartilage repair: ICRS II. *Am J Sports Med.* 2010;38(5):880-890.

20. McCracken MJ, Kramer J, Keegan KG, et al. Comparison of an inertial sensor system of lameness quantification with subjective lameness evaluation. *Equine Vet J.* 2012;44(6):652-656.

21. Miller RE, Grodzinsky AJ, Barrett MF, et al. Effects of the combination of microfracture and self-assembling Peptide filling on the repair of a clinically relevant trochlear defect in an equine model. *J Bone Joint Surg Am.* 2014;96(19):1601-1609.

22. Miller RE, Grodzinsky AJ, Vanderploeg EJ, et al. Effect of self-assembling peptide, chondrogenic factors, and bone marrow-derived stromal cells on osteochondral repair. *Osteoarthritis Cartilage.* 2010;18(12):1608-1619.

23. Pearce GL, Frisbie DD. Statistical evaluation of biomedical studies. *Osteoarthritis Cartilage.* 2010;18 Suppl 3:S117-122.

24. Sah RL, Chen AC, Grodzinsky AJ, Trippel SB. Differential effects of bFGF and IGF-I on matrix metabolism in calf and adult bovine cartilage explants. *Arch Biochem Biophys.* 1994;308(1):137-147.

25. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J Bone Joint Surg Am.* 1993;75(4):532-553.

26. Strauss EJ, Barker JU, Kercher JS, Cole BJ, Mithoefer K. Augmentation Strategies following the Microfracture Technique for Repair of Focal Chondral Defects. *Cartilage.* 2010;1(2):145-152.

27. Torres L, Dunlop DD, Peterfy C, et al. The relationship between specific tissue lesions and pain severity in persons with knee osteoarthritis. *Osteoarthritis Cartilage.* 2006;14(10):1033-1040.

28. Yanke AB, Cole BJ. Microfracture: dead or the future? *Orthopedics.* 2014;37(12):798-800.

29. Zanotto G, Liebesny, P., Barrett, M., Zlotnick, H., Grodzinsky, A., Frisbie, D. Trypsin Pre-Treatment Combined With Growth-Factor Functionalized Self-Assembling Peptide Hydrogel Improve Cartilage Repair in Rabbit Model. *Journal of Orthopedic Research.* 2019.

30. Zhang Z, Li L, Yang W, et al. The effects of different doses of IGF-1 on cartilage and subchondral bone during the repair of full-thickness articular cartilage defects in rabbits. *Osteoarthritis Cartilage.* 2017;25(2):309-320.

CHAPTER 5:

CONCLUSION AND FUTURE DIRECTIONS

Microfracture remains widely used as the first line of treatment for small, focal chondral defects, as it is considered minimally invasive, simple, relatively low cost and results in satisfactory clinical outcomes in the short term. However unfortunately, studies have demonstrated deterioration of the clinical improvement on mid-range (5 years) and long-term (10 years) follow-up. Unfortunately, when microfracture treated patients require ACI as a follow up intervention, decreased success is reported compared to when ACI is used as the first line therapy. For these reasons, cartilage restoration techniques such as ACI and OAT are becoming more commonly used as first line therapies. These techniques have a higher degree of technical complexity and require multiple surgical steps and cellular culture, which results in higher costs. Furthermore, the superiority of these techniques over microfracture has not been clearly defined, and microfracture may be more cost-effective in some cases.

For that reason, augmenting microfracture with tissue engineering principles may result in a more cost-effective way to treat cartilage defects. Several approaches using tissue engineering principles have been proposed, with some already in clinical use. Our group has been extensively studying the use of self-assembling peptide (KLD) hydrogel as a scaffold for tissue engineering cartilage repair. More recently, we developed a clinically applicable technique utilizing trypsin enzymatic pretreatment combined with local delivery of IGF-1 to the surrounding cartilage to improve cartilage integration. This approach was tested *in vitro*, and the promising results supported the rationale behind the experiments presented here.

Our first experiment aimed to evaluate the effect of trypsin enzymatic pretreatment and to optimize the combinations of growth factors used to functionalize the hydrogel *in vivo.* This study found that the greatest improvement in defect filling and reparative tissue quality on the macroscopic and microscopic levels was observed when combining trypsin enzymatic pretreatment with HB-IGF-1 functionalized self-assembling peptide hydrogel. In the second study, this same combination was added to the subchondral bone microfracture technique in an equine model. In this study, a significant improvement in clinical and functional outcomes was observed, along with better cartilage healing and improved biomechanical properties of the adjacent cartilage . The encouraging results observed in the equine model suggested that a phase I clinical trial could be considered a logical next step, but further optimization of this technique may result in more robust results.

In general, despite the improvement in cartilage healing observed in these studies, unfortunately the reparative tissue is still mainly composed of fibrocartilage. Interestingly, a great amount of variation in healing was observed amongst animals within the study (data not shown), which highlighted the importance of contralateral controls in each animal on experimental studies. Similar findings showing different levels of hyaline and fibrocartilage composition in reparative tissue after subchondral bone microfracture have been reported in horses and humans. Understanding the genetic basis of these differences in reparative tissue composition could provide insight regarding future therapeutic approaches. One possibility is that the modulation of the TGF-B1 pathways plays major role in this process. With modulation of this pathway, a decrease in fibrocartilage formation may result and increases in hyaline cartilage content may consequentially occur. However, this strategy could result in a decrease in overall defect filling,

which is known to be directly related to poorer clinical outcomes. Regardless, this is likely a field that will be of great interest in the next few years.

In vitro studies have shown that HB-IGF-1 is rapidly released from the hydrogel and binds to the surrounding cartilage. This could explain why HB-IGF-1 by itself did not result in improvement of the cartilage healing process when tested in the rabbit model. Also, in the equine model, treatment resulted in improvement of biomechanical properties of the surrounding cartilage, but not in the reparative tissue, which would further support this hypothesis. In this way, optimizing the fixation of the HB-IGF-1 in the defect may offer potential benefits in cartilage healing. Some future strategies that could be considered to optimize the fixation of HB-IGF-1 are the use of extracellular matrix components mixed with the self-assembling peptide hydrogel. Two possible clinically applicable techniques include the use of autologous/juvenile allogenic cartilage fragments or chondrons, since both have been shown to improve cartilage healing. The extracellular matrix in the case of cartilage fragment, or the pericellular matrix in the case of the chondrons, could potentially serve as a target to bind HB-IGF-1. While these possibilities are exciting and logical, further proof of concept studies are necessary to draw more conclusions.

SUPPLEMENTAL MATERIAL

Table 3: MRI scores of the main and interactions effects for trypsin, HB-IGF1 and PDGF-BB. Score ranges from 0 to 4, where 0 is normal and 4 is severely abnormal. Radiographic evaluation of the main and interactions effects for trypsin, HB-IGF1 and PDGF-BB. Score ranges from 0 to 4, where 0 is normal and 4 is severely abnormal.

	Trypsin		HB-IGF1		PDGF-BB		HB-IGF1*PDGF-BB		KLD	
	treated	untreated	treated	untreated	treated	untreated	treated	untreated	treated	untreated
MRI										
SB esclerosis	1.256 ± 0.11	1.237 ± 0.15	1.523 ± 0.20	1.189 ± 0.16	1.376 ± 0.18	1.335 ± 0.17	1.617 ± 0.25	1.241 ± 0.16	1.250 ± 0.17	0.714 ± 0.31
	p=0.9137		p=0.1836		p=0.8572		p=0.5559		p=0.1433	
SB integrety	0.953 ± 0.15	1.156 ± 0.21	1.257 ± 0.29	0.929 ± 0.22	0.850 ± 0.26	1.336 ± 0.25	0.800 ± 0.37	0.958 ± 0.24	0.958 ± 0.20	0.714 ± 0.37
	p=0.4403		p=0.3651		p=0.1816		p=0.2387		p=0.5702	
SB reconstitution%	46.117 ± 3.70	33.205 ± 5.24	48.572 ± 7.33	44.145 ± 5.66	54.249 ± 6.67	38.468 ± 6.32	60.002 ± 9.57	39.793 ± 6.04	39.792 ± 6.93	53.571 ± 12.83
	p=0.0476		p=0.6373		p=0.0934		p=0.4519		=0.3524	
Surface regularity	2.001 ± 0.13	2.567 ± 0.18	2.011 ± 0.25	2.160 ± 0.19	1.921 ± 0.22	2.250 ± 0.22	1.689 ± 0.32	2.166 ± 0.21	2.143 ± 0.22	1.500 ± 0.42
	p=0.0141		p=0.6361		p=0.2819		p=0.3166		p=0.1860	
Defect fill	1.917 ± 0.13	2.533 ± 0.19	1.993 ± 0.27	1.904 ± 0.21	1.650 ± 0.24	2.247 ± 0.23	1.700 ± 0.34	2.208 ± 0.22	2.208 ± 0.23	1.429 ± 0.43
	p=0.0084		p=0.7942		=0.0839		p=0.9735		p=0.1221	
Radiographic evaluation										
Defect visualization	1.777 ± 0.13	1.470 ± 0.18	1.844 ± 0.22	1.636 ± 0.20	1.430 ± 0.21	2.050 ± 0.20	1.438 ± 0.28	1.851 ± 0.20	1.870 ± 0.22	1.571 ± 0.41
	p=0.1523		p=0.4500		**p=0.0170**		p=0.4869		p=0.5256	
Osteophyte	0.347 ± 0.10	0.189 ± 0.14	0.360 ± 0.16	0.268 ± 0.14	0.343 ± 0.15	0.285 ± 0.14	0.220 ± 0.22	0.070 ± 0.14	0.087 ± 0.11	0.714 ± 0.19
	p=0.3194		p=0.6612		p=0.7688		p=0.1129		**p=0.008**	
Sclerosis around defect	1.417 ± 0.12	1.729 ± 0.17	1.395 ± 0.19	1.454 ± 0.16	1.424 ± 0.18	1.424 ± 0.17	1.414 ± 0.26	1.474 ± 0.17	1.478 ± 0.17	1.143 ± 0.31
	p=0.1250		p=8148		p=0.9996		p=0.8757		p=0.3485	

Table 4: Macroscopic scores for main and interactions effects for trypsin, HB-IGF1 and PDGF-BB. Score ranges from 0 to 4, where 0 is normal and 4 is severely abnormal.

	Trypsin		HB-IGF1		PDGF-BB		HB-IGF1*PDGF-BB		KLD	
	treated	untreated	treated	untreated	treated	untreated	treated	untreated	treated	untreated
Macroscopic evaluation										
Articular Surface	0.195 ± 0.05	0.078 ± 0.07	0.163 ± 0.09	0.133 ± 0.07	0.150 ± 0.08	0.146 ± 0.08	0.200 ± 0.12	0.167 ± 0.08	0.167 ± 0.09	0.375 ± 0.15
	p=0.1539		p=0.7977		p=0.9708		p=0.5344		p=0.2302	
Bone attachment	0.661 ± 0.10	0.662 ± 0.13	0.579 ± 0.18	0.720 ± 0.15	0.814 ± 0.16	0.485 ± 0.16	0.872 ± 0.22	0.683 ± 0.15	0.667 ± 0.15	0.875 ± 0.26
	p=0.9965		p=0.5144		p=0.1054		p=0.2391		p=0.4929	
Cartilage attachment	0.647 ± 0.10	0.659 ± 0.14	0.578 ± 0.18	0.731 ± 0.15	0.805 ± 0.17	0.504 ± 0.16	0.871 ± 0.23	0.722 ± 0.15	0.708 ± 0.16	0.750 ± 0.28
	p=0.9391		p=0.5111		p=0.1690		p=0.2229		p=0.8973	
Color	5.203 ± 0.06	5.842 ± 0.09	5.440 ± 0.11	5.259 ± 0.10	5.230 ± 0.11	5.468 ± 0.11	5.130 ± 0.14	5.187 ± 0.10	5.217 ± 0.10	4.875 ± 0.17
	p<0.0001		p=0.1829		p=0.0599		**p=0.0066**		p=0.0953	
Firmness	0.863 ± 0.09	1.176 ± 0.12	0.910 ± 0.17	0.914 ± 0.14	0.903 ± 0.15	0.920 ± 0.15	0.820 ± 0.21	0.841 ± 0.14	0.833 ± 0.16	0.875 ± 0.28
	p=0.0.0322		p=0.9855		p=0.9306		p=0.4447		p=0.8966	
Grade	2.704 ± 0.12	2.549 ± 0.17	2.787 ± 0.24	2.687 ± 0.19	2.733 ± 0.21	2.741 ± 0.21	2.859 ± 0.30	2.767 ± 0.20	2.792 ± 0.18	2.500 ± 0.32
	p=4411		p=0.7386		p=0.9773		p=0.6101		p=0.4303	
Inflammation	0.195 ± 0.08	0.142 ± 0.09	0.247 ± 0.13	0.220 ± 0.12	0.312 ± 0.12	0.155 ± 0.12	0.370 ± 0.15	0.185 ± 0.12	0.125 ± 0.13	0.375 ± 0.23
	p=0.5462		p=0.8573		p=0.2745		p=0.5706		p=0.3504	
Apposing cartilage	0.078 ± 0.03	0.031 ± 0.04	0.000 ± 0.06	0.142 ± 0.05	0.100 ± 0.06	0.042 ± 0.05	0.000 ± 0.08	0.083 ± 0.05	0.083 ± 0.05	0.000 ± 0.09
	p=0.3764		p=0.0866		p=0.4748		p=0.4748		p=0.4155	
Joint observation	0.415 ± 0.08	0.075 ± 0.11	0.339 ± 0.15	0.510 ± 0.13	0.601 ± 0.14	0.249 ± 0.13	0.428 ± 0.17	0.247 ± 0.13	0.167 ± 0.13	1.000 ± 0.22
	p=0.0027		p=3248		**p=0.0314**		p=0.3175		**p=0.0024**	
Level	2.402 ± 0.12	2.456 ± 0.18	2.167 ± 0.26	2.461 ± 0.22	2.309 ± 0.24	2.319 ± 0.22	2.333 ± 0.35	2.638 ± 0.23	2.652 ± 0.23	2.250 ± 0.40
	p=0.8068		p=0.3827		p=0.9742		p=0.3098		p=0.3910	
Osteoarthirtis	0.831 ± 0.05	0.807 ± 0.07	0.843 ± 0.10	0.778 ± 0.08	0.867 ± 0.09	0.754 ± 0.09	0.936 ± 0.13	0.758 ± 0.09	0.750 ± 0.08	1.000 ± 0.14
	p=0.7792		p=0.6036		p=0.3437		p=0.5607		p=0.1243	
Surface	1.490 ± 0.14	1.801 ± 0.19	1.808 ± 0.23	1.408 ± 0.20	1.452 ± 0.22	1.763 ± 0.20	1.365 ± 0.30	1.277 ± 0.20	1.250 ± 0.21	1.250 ± 0.37
	p=0.1426		p=0.1727		p=0.2485		p=0.0530		p=1.0	
Synovial Membrane	0.863 ± 0.11	0.485 ± 0.15	0.689 ± 0.22	0.938 ± 0.18	0.958 ± 0.20	0.669 ± 0.19	0.950 ± 0.29	0.909 ± 0.18	0.917 ± 0.17	0.875 ± 0.30
	p=0.0321		p=0.3811		p=0.2831		p=0.4132		p=0.9031	
Total score	17.323 ± 0.45	18.791 ± 0.60	17.983 ± 0.81	17.263 ± 0.71	17.539 ± 0.76	17.707 ± 0.70	17.591 ± 1.06	17.038 ± 0.70	17.000 ± 0.80	17.000 ± 1.39
	p=0.0391		p=0.4826		p=0.8585		p=0.5471		p=1.0	
Area	95.469 ± 1.57	94.844 ± 2.22	94.375 ± 3.44	94.979 ± 2.73	95.500 ± 3.25	93.854 ± 2.96	95.000 ± 4.59	93.958 ± 2.96	99.375 ± 3.02	93.958 ± .
	p=0.8186		p=0.8913		p=0.7099		p=0.9287		p<0.0001	
Volume	87.188 ± 2.11	87.188 ± 2.98	85.343 ± 4.23	87.251 ± 3.66	87.882 ± 3.98	84.713 ± 3.67	87.561 ± 5.56	86.300 ± 3.65	86.125 ± 3.79	88.125 ± 6.56
	p=1		p=0.7229		p=0.5260		p=0.8138		p=0.8701	
Muscle atrophy	13.339 ± 0.07	13.173 ± 0.07	13.251 ± 0.10	13.427 ± 0.10	13.438 ± 0.10	13.240 ± 0.10	13.440 ± 0.10	13.417 ± 0.10	13.390 ± 0.10	13.325 ± 0.17
	p=0.0002		p=0.0982		p=0.0595		p=0.0895		p=0.7492	

Table 5: IRCSII, immunohistochemistry and synovial membrane scores for main and interactions effects for trypsin, HB-IGF1 and PDGF-BB. ICRSII scores raging from 0 to 100% where 0% is completely abnormal and 100% is completely normal. Immunohistochemistry were graded a percentage of the defect staining positively. Synovial membrane were graded in a 0 to 4 score system.

	Trypsin		HB-IGF1		PDGF-BB		HB-IGF1*PDGF-BB		KLD	
	treated	untreated	treated	untreated	treated	untreated	treated	untreated	treated	untreated
ICRSII score										
Tissue morphology (viewed under polarized light)	51.496 ± 3.05	41.477 ± 4.30	51.250 ± 5.68	50.174 ± 4.30	56.125 ± 5.39	45.299 ± 4.66	66.250 ± 8.03	54.348 ± 4.74	54.348 ± 5.77	50.000 ± 9.79
	p=0.0595		p=0.8806		p=0.1357		**p=0.0100**		p=0.7047	
Matrix staining (metachromasia)	56.750 ± 4.15	55.862 ± 5.96	55.625 ± 8.36	53.120 ± 6.33	55.375 ± 7.93	53.370 ± 6.86	66.250 ± 11.83	61.739 ± 6.97	61.739 ± 7.08	57.500 ± 12.01
	p=0.9030		p=0.8123		p=0.8493		p=0.0732		p=0.7633	
Cell morphology	79.833 ± 3.19	59.833 ± 4.52	73.125 ± 6.10	80.717 ± 4.62	88.625 ± 5.79	65.217 ± 5.01	96.250 ± 8.62	80.435 ± 5.09	80.435 ± 5.58	88.750 ± 9.46
	p=0.0005		p=0.3263		**p=0.0037**		**p=0.0046**		p=0.4553	
Chondrocyte clustering (4 or more grouped cells)	88.417 ± 1.70	82.000 ± 2.40	84.008 ± 3.25	86.023 ± 2.55	84.459 ± 3.08	85.573 ± 2.63	88.016 ± 4.67	91.145 ± 2.68	91.087 ± 2.06	95.625 ± 3.49
	p=0.0317		p=0.6265		p=0.7797		**p=0.0320**		p=0.2714	
Surface architeture	67.555 ± 3.51	59.669 ± 4.86	68.068 ± 5.39	70.022 ± 4.58	73.351 ± 5.11	64.739 ± 4.54	77.386 ± 7.39	70.729 ± 4.58	70.870 ± 62.05	67.500 ± 77.77
	p=0.1775		p=0.7689		p=0.1651		p=0.1405		p=0.9787	
Basal integration	75.417 ± 3.39	67.667 ± 4.79	74.688 ± 5.62	82.130 ± 4.25	86.125 ± 5.33	70.693 ± 4.61	76.250 ± 7.94	68.261 ± 4.68	68.261 ± 5.93	71.250 ± 10.05
	p=0.1897		p=0.2964		**p=0.0337**		p=0.0875		p=0.7998	
Formation of a tidemark	3.083 ± 1.47	0.000 ± 2.07	0.000 ± 0.71	1.000 ± 0.54	1.000 ± 0.67	0.000 ± 0.58	0.000 ± 1.00	0.000 ± 0.59	0.000 ± 10.47	18.750 ± .
	p=0.2280		p=0.2656		p=0.2656		p=0.2656		p=0.1512	
Subchondral bone abnormalities/marrow fibrosis	86.228 ± 2.28	83.678 ± 3.10	82.366 ± 4.01	87.901 ± 3.35	88.734 ± 3.79	81.532 ± 3.29	92.232 ± 5.66	90.565 ± 3.34	90.217 ± 3.15	88.750 ± 5.34
	p=0.4819		p=0.2756		p=1246		**p=0.0174**		p=0.8145	
Subchondral bone calcification	24.749 ± 3.24	29.041 ± 4.58	28.767 ± 6.06	23.620 ± 4.63	27.896 ± 5.74	24.491 ± 4.85	23.783 ± 8.79	15.231 ± 4.96	15.217 ± 5.09	33.750 ± 8.63
	p=0.4452		p=0.5086		p=0.6531		p=0.0903		p=0.0744	
Vascularization (within the repaired tissue)	92.087 ± 2.17	99.585 ± 2.97	92.586 ± 4.26	94.687 ± 3.63	94.405 ± 4.04	92.867 ± 3.62	90.172 ± 5.77	90.735 ± 3.64	90.435 ± 4.88	86.250 ± 8.27
	p=0.0350		p=0.6872		p=0.7506		p=0.2301		p=0.6663	
Surface/superficial assessment	62.337 ± 3.32	60.296 ± 4.57	63.441 ± 5.30	61.604 ± 4.35	63.778 ± 5.01	61.267 ± 4.19	70.631 ± 7.78	66.284 ± 4.30	65.870 ± 5.14	65.000 ± 8.72
	p=0.7080		p=0.7881		p=0.6840		p=0.0909		p=0.9321	
Mid/deep zone assessment	60.411 ± 3.11	54.182 ± 4.39	63.125 ± 5.99	58.641 ± 4.54	63.750 ± 5.68	58.016 ± 4.92	70.000 ± 8.47	59.783 ± 5.00	59.783 ± .	58.750 ± 69.12
	p=0.2474		p=0.5537		p=0.4494		p=0.2917		p=0.9919	
Overall assessment	58.995 ± 2.51	55.896 ± 3.52	59.688 ± 4.84	58.967 ± 3.66	62.813 ± 4.59	55.842 ± 3.97	68.125 ± 6.84	60.435 ± 4.03	60.435 ± .	56.250 ± .
	p=0.4708		p=0.9061		p=0.2567		p=0.1096		p<0.0001	
Total Score	952.510 ± 31.15	887.470 ± 40.56	980.310 ± 46.62	952.480 ± 37.35	1019.560 ± 47.08	913.230 ± 40.52	1078.130 ± 70.18	943.960 ± 40.52	943.960 ± 47.45	1023.120 ± 82.18
	p=0.1589		p=0.6562		p=0.0937		p=0.1573		p=0.4107	
IHC										
Aggrecan	3.452 ± 0.12	3.323 ± 0.17	3.308 ± 0.20	3.577 ± 0.17	3.479 ± 0.18	3.407 ± 0.17	3.479 ± 0.18	3.689 ± 0.17	3.727 ± 0.16	2.875 ± 0.26
	p=0.5202		p=0.2867		p=0.7530		p=0.2428		**p=0.0092**	
Collagent type II	3.064 ± 0.14	2.968 ± 0.20	2.887 ± 0.28	3.135 ± 0.22	2.984 ± 0.25	3.038 ± 0.25	2.774 ± 0.35	3.076 ± 0.22	3.083 ± 0.24	3.000 ± 0.42
	p=0.6954		p=0.4891		p=0.8739		p=0.6304		p=0.8639	
Pro-Coll type I	2.196 ± 0.18	1.439 ± 0.26	1.777 ± 0.34	2.500 ± 0.27	2.563 ± 0.32	1.714 ± 0.29	2.125 ± 0.46	2.000 ± 0.31	2.000 ± 0.29	2.000 ± 0.47
	p=0.0187		p=0.1006		p=0.0557		p=0.7259		p=1	
Synovial Membrane										
Cellular infiltration	0.000 ± 0.01	0.038 ± 0.02	. ± .	. ± .	. ± .	. ± .	. ± .	. ± .	. ± .	. ± .
	p=0.1518		.		.		.		.	
Intimal Hyperplasia	0.554 ± 0.12	0.963 ± 0.17	0.292 ± 0.22	0.819 ± 0.18	0.611 ± 0.21	0.500 ± 0.19	0.333 ± 0.30	0.750 ± 0.20	0.750 ± 0.19	0.143 ± 0.33
	p=0.0506		p=0.0702		p=0.6977		p=0.9226		p=0.1216	
Subintimal Fibrosis	1.286 ± 0.17	1.296 ± 0.25	0.694 ± 0.31	1.350 ± 0.26	0.944 ± 0.30	1.100 ± 0.27	0.889 ± 0.43	1.700 ± 0.27	1.700 ± 0.28	1.571 ± 0.47
	p=0.9724		p=0.1123		p=0.7023		p=0.1852		p=0.8140	
Vascularity	0.272 ± 0.11	0.575 ± 0.16	0.063 ± 0.16	0.342 ± 0.13	0.167 ± 0.15	0.238 ± 0.13	0.000 ± 0.21	0.350 ± 0.14	0.350 ± 0.18	0.571 ± 0.30
	p=0.1130		p=0.1763		p=0.7289		p=0.7909		p=0.5376	

Table 5: Anesthetic, pain management and euthanasia protocol, including drugs, dose, frequency and route of administration.

	Dose	**Route**
Pre-anesthetic Drug		
Morphine	0.5 mg/Kg	SQ
Glycopyrrolate	0.005 mg/Kg	SQ
Morphine	0.1 mg/Kg	Epidural
Induction and Maintenance		
Isoflurane	5%	IH
Post-operative		
Enrofloxacin	5 mg/Kg BID 5d	SQ
Meloxicam	0.1 mg/Kg SID 3d	SQ
Buprenorphine	0.03 mg/Kg BID 3d	SQ
NaCl 0.9%	100 ml BID as needed	SQ
Euthanasia		
Xylazine	5 mg/Kg	SQ
Ketamine	35 mg/Kg	SQ
Beuthanasia (Phynitoin/Pentobarbital)	90 mg/Kg	IV

Table 6 Animal care and management.

Location	CSU Laboratory of Animals Resource
Cage type	Singly housed in standard Allentown rabbit 6-bank
Bedding material	Raised floor grates – no contact with bedding material
Light cycle	12 hours light/dark
Room temperature	61 – 72 F
Feed/water	*Ad libitum* hard pellets (Envigo Teklad global high fiber rabbit diet #2031), and grass hay. Free access to water. All animals are checked for food/water at least once a day.
Post-operative care	For post-op, the rabbits were checked at least twice a day for a week. Pain scores were done twice a day while on medications (5 days)

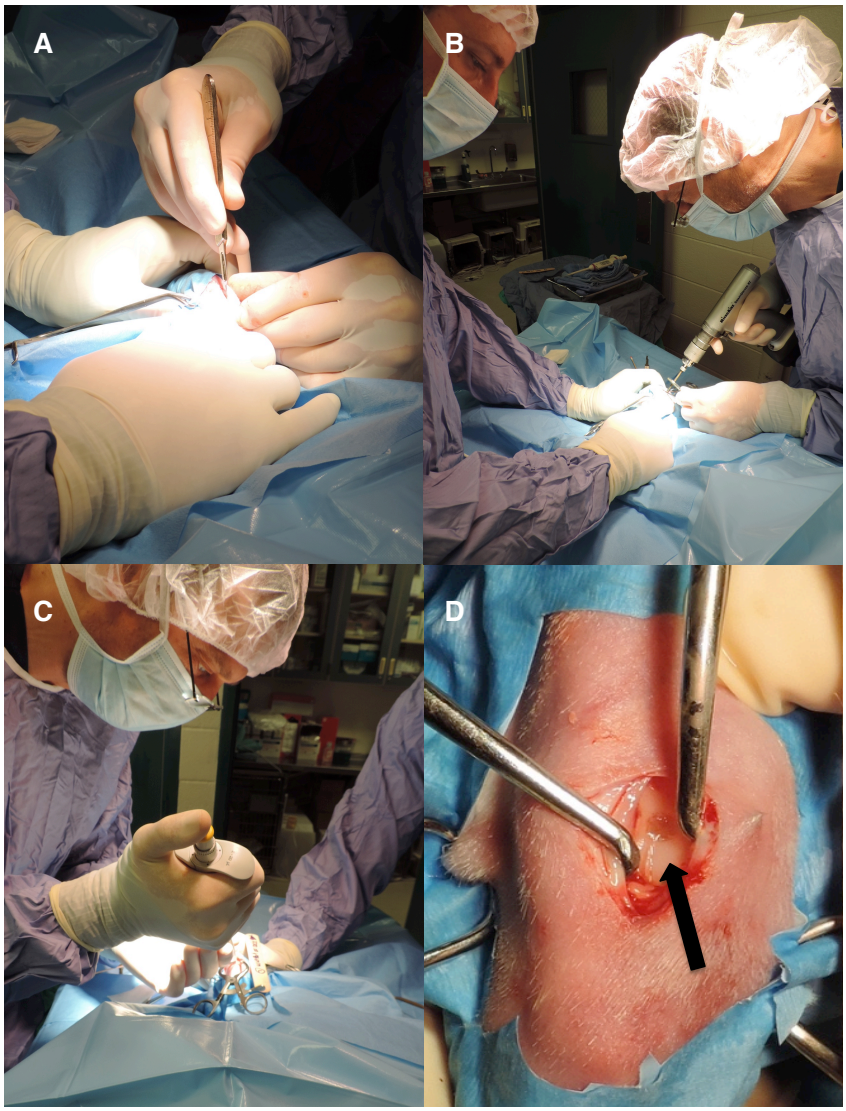

Figure 14: Surgery Procedure. The femoropatellar joint was accessed by medial parapatellar arthrotomy (A). The central portion of the trochlear groove was identified and a 3 mm diameter and 2 mm deep ostechondral defect created using a drill bit and customized guide (B). The defect was cleaned, dried and the bleeding controlled applying direct pressure with a surgical gaze. Defect was filled with treatment using a sterile 20 μL pipette (C). After the complete assembling process of the hydrogel was confirmed (D) the joint capsule and the skin were closed using a monofilament absorbable suture material, in two different layers.

Table 7: Gross pathologic examination of the joint comparing treated defects to control. Score ranges from 0 to 4, where 0 is normal and 4 is severely abnormal Values are presented as means and 95% confidence interval. Bold p-values represent statistical significance.

	Control		Treatment			
	Mean	CI	Mean	CI	LS Means	CI	p- value
Injury to Apposing Cartilage	0.12	(-0.08 - 0.33)	0.00	(-0.20 - 0.20)	0.12	(-0.17 - 0.42)	0.3506
Bone Attachment	1.62	(0.76 - 2.48)	1.12	(0.26 - 1.98)	0.50	(-0.68 - 1.68)	0.3506
Cartilage Attachment	1.50	(0.62 - 2.37)	1.25	(0.37 - 2.12)	0.25	(-0.82 - 1.32)	0.5983
Color	5.87	(5.66 - 6.08)	5.87	(5.66 - 6.08)	0.00	(0.00 - 0.00)	1.0000
Cartilage Firmness	1.75	(0.82 - 2.67)	1.25	(0.32 - 2.17)	0.50	(-0.76 - 1.76)	0.3807
Repair Grade (Tissue Quality)	2.00	(1.29 - 2.70)	2.00	(1.29 - 2.70)	0.00	(-0.99 - 0.99)	1.0000
Joint Inflammation	0.12	(-0.21 - 0.46)	0.25	(-0.09 - 0.59)	-0.12	(-0.61 - 0.36)	0.5630
Level of Repair In Defect	1.25	(0.24 - 2.25)	1.12	(0.11 - 2.13)	0.12	(-1.24 - 1.49)	0.8357
Level Comparing Io Surrounding Tissue	2.25	(1.60 - 2.89)	2.12	(1.47 - 2.77)	0.12	(-0.78 - 1.03)	0.7560
Degeneration of the tissue beyond original defect margins	0.00	(-0.27 - 0.27)	0.25	(-0.02 - 0.52)	-0.25	(-0.63 - 0.13)	0.1705
Surface	3.00	(2.29 - 3.70)	2.50	(1.79 - 3.20)	0.50	(-0.27 - 1.27)	0.1705
Synovial Membrane	0.12	(-0.21 - 0.46)	0.25	(0.09 - 0.59)	-0.12	(-0.61 - 0.36)	0.5630
Repair filling Area %	95.62	(88.49 - 102.76)	96.87	(89.74 - 104.01)	-1.25	(-9.22 - 6.72)	0.7220
Repair filling Volume %	66.87	(39.31 - 94.43)	75.62	(48.06 - 103.19)	-8.75	(-46.58 - 29.08)	0.6015

Table 8: Histological and immunochemistry evaluation of the osteochondral samples harvest at the proximal or distal aspect of the defect. ICRSII scores raging from 0 to 100% where 0% is completely abnormal and 100% is completely normal. Immunohistochemistry was score based on the percentage of the defect positively stained for aggrecan, collagen type I and II. Values are presented as means and 95% confidence interval. Bold p-values represent statistical significance.

	Proximal						Distal						
	Control			Treatment			Control			Treament			p- value
	Mean	CI		Mean	CI		Mean	CI		Mean	CI		
Tissue Morphology	32.50	(24.01	40.98)	50.93	(42.45	59.41)	38.75	(24.69	52.80)	20.31	(6.25	34.36)	**0.0020**
Matrix Staining (SOFG)	10.31	(1.91	18.71)	23.12	(14.72	31.52)	5.93	(-8.17	20.05)	-8.12	(-22.23	5.98)	**0.0216**
Cell Morphology	32.50	(22.43	42.56)	56.56	(46.50	66.62)	42.50	(25.72	59.27)	21.56	(4.78	38.33)	**0.0017**
Chondrocyte Clustering	81.25	(70.59	91.90)	80.62	(69.96	91.28)	82.50	(64.03	100.96)	84.37	(65.91	102.84)	0.8673
Surface Architeture	46.25	(29.96	65.53)	63.75	(47.46	80.03)	56.25	(31.76	80.73)	45.00	(20.51	69.48)	0.1194
Basal Integration	86.25	(74.17	98.32)	91.87	(79.79	103.95)	84.37	(63.45	105.29)	91.87	(70.95	112.73)	0.9119
Formation of a Tidemark	19.06	(4.88	33.23)	25.00	(10.82	39.17)	29.68	(5.13	54.24)	15.00	(-9.55	39.55)	0.3036
Sunchondral Bone Abnormalities	75.62	(67.03	84.21)	72.50	(63.90	81.09)	74.37	(59.49	89.25)	87.50	(72.61	102.38)	0.1833
Inflammation	96.87	(91.47	102.28)	88.12	(82.72	93.52)	91.25	(82.29	100.21)	93.12	(84.16	102.08)	0.1402
Abnormal Calcification	98.12	(92.18	104.06)	89.37	(83.43	95.31)	96.87	(87.33	106.42)	83.12	(73.58	92.66)	0.5014
Vascularization	75.62	(63.17	88.07)	87.18	(74.73	99.63)	80.62	(60.28	100.97)	89.68	(69.34	110.03)	0.8755
Surface/ Superficial Assemssment	45.00	(30.33	59.66)	65.00	(50.33	79.66)	55.00	(33.62	76.37)	32.50	(11.12	53.87)	**0.0088**
Mid/deep Zone	38.12	(23.31	52.93)	61.87	(47.06	76.68)	45.62	(22.75	68.94)	26.87	(4.00	49.74)	**0.0182**
Overal Assessment	36.87	(25.42	48.32)	55.62	(44.17	67.07)	38.75	(21.76	55.73)	25.62	(8.64	42.60)	**0.0143**
Lateral Integration	90.93	(80.88	100.99)	87.18	(77.13	97.24)	92.18	(74.91	109.46)	77.81	(60.54	95.08)	0.4475
Subchondral Bone Reconstitution	96.87	(94.24	99.50)	99.37	(96.74	102.01)	96.87	(92.46	101.28)	99.37	(94.96	103.78)	1.0000
IHC													
Aggrecan	49.37	(33.21	65.53)	55.00	(38.84	71.15)	42.50	(26.34	58.65)	47.50	(31.34	63.65)	0.9592
Collagen type I	43.75	(22.74	64.75)	41.25	(20.24	62.25)	43.75	(22.74	64.75)	45.00	(23.99	66.00)	0.8545
Collagen type II	50.00	(33.34	66.65)	61.25	(44.59	77.90)	41.25	(24.59	57.90)	50.00	33.34	66.65)	0.8775

Table 9: Histological evaluation of the osteochondral samples harvest at 6 and 12 months post defect creation. ICRSII scores raging from 0 to 100% where 0% is completely abnormal and 100% is completely normal. Values are presented as means and 95% confidence interval. Bold p-values represent statistical significance.

	6 month						12 month						
	Control			Treatment			Control			Treamtent			p-values
	Mean	CI		Mean	CI		Mean	CI		Mean	CI		
Tissue Morphology	36.87	(22.82	50.92)	30.93	(16.88	44.99)	34.37	(25.89	42.85)	40.31	(31.83	48.79)	0.2894
Matrix Staining (SOFG)	4.06	(-10.05	18.17)	-8.12	(-22.23	5.98)	12.18	(3.78	20.59)	23.12	(14.72	31.52)	**0.0459**
Cell Morphology	42.50	(25.72	59.27)	32.50	(15.72	49.27)	32.50	(22.43	42.56)	45.62	(35.56	55.68)	0.0891
Chondrocyte Clustering	83.12	(64.66	101.59)	81.87	(63.41	100.34)	80.62	(69.96	91.28)	83.12	(72.46	93.78)	0.8021
Surface Architeture	53.75	(29.26	78.23)	59.37	(34.89	83.85)	48.75	(32.46	65.03)	49.37	(33.09	65.65)	0.7829
Basal Integration	86.56	(65.64	107.48)	93.75	(72.83	114.67)	84.06	(71.98	96.14)	90.00	(77.92	102.08)	0.9412
Formation of a Tidemark	17.81	(-6.74	42.36)	3.75	(-20.8	28.3)	30.93	(16.76	45.11)	36.25	(22.07	50.42)	0.3334
Sunchondral Bone Abnormalities	75.62	(60.74	90.50)	83.75	(68.86	98.63)	74.37	(65.78	82.96)	76.25	(67.65	84.84)	0.6049
Inflammation	92.18	(83.23	101.14)	90.00	(81.04	98.95)	95.93	(90.53	101.34)	91.25	(85.84	96.65)	0.7246
Abnormal Calcification	98.12	(88.58	107.67)	78.12	(68.58	87.66)	96.87	(90.93	102.81)	94.37	(88.43	100.31)	**0.0230**
Vascularization	73.75	(53.40	94.09)	91.87	(71.53	112.22)	82.50	(70.04	94.95)	85.00	(72.54	97.45)	0.3309
Surface/ Superficial Assemssment	52.50	(31.12	73.87)	43.75	(22.37	65.12)	47.50	(32.83	62.16)	53.75	(39.08	68.41)	0.3343
Mid/deep Zone	45.00	(22.13	67.86)	35.00	(12.13	57.86)	38.75	(23.93	53.56)	53.75	(38.93	68.56)	0.1541
Overal Assessment	42.18	(25.20	59.17)	36.25	(19.26	53.23)	33.43	(21.99	44.88)	45.00	(33.55	56.44)	0.1656
Lateral Integration	96.87	(79.60	114.14)	80.31	(63.04	97.58)	86.25	(76.19	96.30)	84.68	(74.63	94.74)	0.2855
Subchondral Bone Reconstitution	93.75	(89.34	98.15)	98.75	(94.34	103.16)	100.00	(97.36	102.63)	100.00	(97.36	102.63)	0.1602