

Chondrocyte-based Intraoperative Processing Strategies for the Biological Augmentation of a Polyurethane Meniscus Replacement

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Abstract

Purpose/Aim of Study: Menisectomies account for over 1.5 million surgical interventions in Europe annually and there is a growing interest in regenerative strategies to improve outcomes in meniscal replacement. The overall objective of this study was to evaluate the role of intraoperatively applied fresh chondrocyte isolates compared to minced cartilage fragments, used without cell isolation, to improve bioactivity and tissue integration when combined with a polyurethane replacement.

Materials and Methods: Firstly, to optimise the intraoperative cell isolation protocol, caprine articular cartilage biopsies were digested with 750 U/ml or 3000 U/ml collagenase type II (ratio of 10 ml per g of tissue) for 30 min, 1 h or 12 hrs with constant agitation and compared to culture expanded chondrocytes in terms of matrix deposition when cultured on polyurethane scaffolds. Finally, fresh chondrocytes and minced cartilage augmented polyurethane scaffolds were evaluated in a caprine meniscal explant model to assess the potential enhancements on tissue integration strength.

Results: Adequate numbers of fresh chondrocytes were harvested using a 30 min chondrocyte isolation protocol and demonstrated improved matrix deposition compared to standard culture expanded cells *in vitro*. Upon evaluation in a meniscus explant defect model, both fresh chondrocytes and minced cartilage showed improved matrix deposition at the tissue scaffold interface and enhanced push-out strength, 4 fold and 2.5 fold respectively compared with the acellular implant.

Conclusions: Herein, we have demonstrated a novel approach that could be applied intraoperatively using fresh chondrocytes or minced cartilage for improved tissue integration with a polyurethane meniscal replacement.

Key words: chondrocytes, minced cartilage, polyurethane scaffold, meniscal replacement, intraoperative

Introduction

Meniscal injury poses a significant health burden with an estimated 1.5 million surgical interventions performed in Europe annually (1). The meniscus plays a pivotal role in load transfer and knee stabilisation during normal joint motion. Damage to the meniscus exposes articular surfaces to excessive peak stresses and predisposes them to cartilage degeneration and progressive osteoarthritis (2, 3). In pursuit of a feasible clinical substitute for damaged meniscus, clinical evidence supports the use of fresh frozen allografts (4-6) or as an alternative, synthetic meniscus replacements such as the Collagen Meniscus Implant (Ivy Sports Medicine) (7-9) or Actifit® (a polyurethane meniscus implant; Orteq) (10). This polyurethane scaffold, through its unique combination of a porous structure and polymer strength, promotes ingrowth of new tissue (11) and has been shown to relieve pain and symptoms. However, even under ideal conditions, failure rates remain as high as 30% (12). These approaches lack universal clinical efficacy with limitations surrounding graft shrinkage, inadequate attachment, lack of host tissue integration and subsequent myxoid degeneration (13). To improve outcomes in surgical meniscal replacement and to maintain joint health, there is a growing interest in regenerative strategies.

Delivery of a regenerative solution may be possible through biological augmentation of existing synthetic acellular replacements. The incorporation of a cellular component providing extracellular matrix (ECM) elaboration can improve host tissue integration with enhanced bioactivity (14). In applying a biomimetic approach, cells should reconstitute similar matrix components to maximise the amount of matrix deposited at the tissue-replacement interface to provide biological strength (15). In assessing appropriate cell sources, Marsano et al (16) demonstrated articular chondrocytes to be effective in tissue reconstitution with higher levels of matrix components, sulphated glycosaminoglycans (sGAG) and collagen type II when compared to cells derived from human inner meniscal, fat pad and synovial membrane. Previous work has shown that differentiated primary cell populations can adapt to

microenvironmental cues, retain phenotypic expression and regenerative potential in vivo without the need for exogenous growth factor supplementation (17). Interestingly, Mumme et al recently demonstrated the use of autologous nasal chondrocyte constructs to repair cartilage defects in human studies (18). Equally, regenerative ventures using articular chondrocytes have also been successfully demonstrated with Matrix-induced Autologous Chondrocyte Implantation (MACI) and DeNovo® ET cartilage derived tissue implants for cartilage regeneration (19, 20).

Given the need for large populations of viable chondrocytes and low cell yields from cartilage biopsies, laboratory based monolayer expansion had been the standard in the use of autologous chondrocytes to date. However, high costs and technical expertise required for GMP biomanufacturing facilities and concerns with respect to cellular dedifferentiation (21), constrains or restricts the clinical application and efficacy of such approaches to specialist centres (22). In addition, there is a need for two surgical procedures; one for tissue harvest and a second procedure for subsequent implantation of a cell-seeded or tissue engineered construct. This increases the associated costs, risk of infection and other complications for patients. As such, incorporation of fresh biologics amenable to intraoperative processing and single step interventions could be cost effective and facilitate favourable logistics for widespread application (23).

The development of an intraoperative cell processing protocol and devices to optimize cell yield for fresh delivery and regeneration, may hold potential in this regard. There remains significant variation in the literature, proposing altering combinations of enzyme regimes, multistep isolations, enzyme concentrations and incubation times to improve cell yields (24). We have recently developed a protocol for rapid isolation of nasal chondrocytes, whereby manipulating time and concentration of collagenase exposure in combination with intermittent physical agitation, we could obtain clinically relevant cell yields for tissue regeneration

strategies (25). Similarly, the success of such protocols has previously been demonstrated for cartilage regeneration (26). These protocols could potentially be adopted to yield an intraoperative strategy for articular cartilage processing and application to improve integration of a meniscal replacement. For example, Numpaisal et al explored the use of rapidly dissociated meniscal cartilage with 0.2% collagenase in a 3D fibrin hydrogel to repair meniscal defects (27). However, minced meniscal tissue was subject to enzymatic digestion to yield the rapidly dissociated tissues in this study. While this allows for single stage application, the regulatory landscape for the intraoperative use of enzymes may limit widespread clinical adoption.

Alternatively, to overcome the need for enzymatic digestion and processing, there is increasing evidence to support the use of minced autologous donor cartilage in tissue transplantation. As demonstrated in 3rd generation Autologous Chondrocyte Implantation (ACI) with the use of DeNovo NT (ISTO tech.) (20) and cartilage autograft implantation system (CAIS – Depuy™) (28), utilising minced cartilage makes for a chondroinductive implant with good regenerative outcomes. While the feasibility of these approaches have been demonstrated both *in vitro* and *in vivo* for cartilage regeneration (26, 29), there are no studies evaluating their use to biologically enhance integration of polyurethane scaffolds for meniscal replacement. Thus, the overall objective of this study was to evaluate the augmentation of a polyurethane implant with fresh biologics amenable to intraoperative processing, and to improve tissue integration with a meniscal replacement. Phase 1 involved evaluating a rapid isolation protocol for the extraction of fresh chondrocytes (FC) from a biopsy of caprine articular cartilage. Phase two evaluated both FCs and culture expanded chondrocytes (EC) in terms of matrix deposition in the Actifit® polyurethane scaffold. Finally, phase 3 assessed FC and minced cartilage (MC) augmented polyurethane scaffolds compared to acellular controls in a caprine meniscal explant model to assess the potential enhancements on tissue integration strength.

Materials and Methods

1. Rapid isolation of articular chondrocytes from caprine knee joint tissue

Caprine knee joints were obtained from a local farm within 6 h of sacrifice. Biopsies of articular cartilage were harvested from both condyles at random, washed with phosphate buffered saline (PBS) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (P/S) (GIBCO, Invitrogen, Dublin, Ireland) and minced. These biopsies were minced to approximately 1mm size pieces. Half of these were subject to enzymatic digestion with physical agitation as outlined previously to harvest FC while the other half were used as minced cartilage in this study. Phase 1: Tissues for enzymatic digestion were subjected to either 750 units of activity/ml (U/ml) or 3000 U/ml collagenase type II (Gibco, Dublin, Ireland) at a ratio of 10 ml per g of tissue for 30 min, 1 h and 12 h under constant rotation at 37 °C in serum free low-glucose Dulbecco's modified eagles medium (LG-DMEM, 1 mg/ml D Glucose, 200 mM L - Glutamine;) containing antibiotic/antimycotic (100 U/ml penicillin, 100 mg/ml streptomycin) (all GIBCO, Invitrogen, Dublin, Ireland) and amphotericin B (0.25 mg/ml, Sigma-Aldrich, Dublin, Ireland). A schematic representation of the applied protocols is shown in Fig. 1, Digested tissue/cell suspensions were passed through a 40 µm cell strainer to remove tissue debris and washed three times by repeated centrifugation at 650 g for 5 min. Cell yield and viability was determined with a hemocytometer and trypan blue exclusion. For experiments utilising expanded chondrocytes (EC), cells were seeded at an initial density of 5×10^3 cells/cm² in T-175 flasks in LG-DMEM supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/ml)–streptomycin (100 mg/ml) and amphotericin B (0.25 mg/ml, Sigma-Aldrich, Dublin, Ireland) (Fig.1, Phase II). Cultures were expanded to passage one (P1) (7 days from initial isolation) in a humidified atmosphere at 37 °C, 5% O₂ and 5% CO₂.

2. Determination of proliferation kinetics

When subconfluent (~80%), ECs were detached by treatment with 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) (all Sigma-Aldrich, Dublin, Ireland) and counted using trypan blue exclusion. The number of cell doublings during the expansion phase was determined as the logarithm (base 2) of the fold increase in the number of cells during expansion. The population doubling time was defined as the culture expansion time divided by the number of doublings during the expansion phase (30).

3. Cell seeding onto polyurethane scaffolds

Cylindrical constructs (2 x 2 mm) were obtained by coring of entire Actifit® implants using a standard 2mm biopsy punch. Cells were seeded onto polyurethane scaffolds to assess matrix deposition by rapidly isolated cells, as FC or EC (Fig. 1, Phase II). FC and EC were resuspended in 20 µl of media at a concentration of 5×10^6 cells/ml (100,000 cells/scaffold) and loaded onto scaffolds. Culture media used for seeding consisted of high glucose (HG) Dulbecco's Modified Eagle Medium (Gibco, Invitrogen, Dublin, Ireland) with 10% fetal bovine serum (Gibco, Invitrogen, Dublin, Ireland), 50 µg/ml L-ascorbic acid-2-phosphate, penicillin (100 U/ml)–streptomycin (100 mg/ml) and amphotericin B (0.25 mg/ml, Sigma-Aldrich, Dublin, Ireland) at 37 °C and 5% CO₂. Scaffold constructs were incubated for 30 min to facilitate cellular attachment and subsequently a further 2 mL of the same media was added to each well. Scaffolds were incubated at 37 °C, 5% O₂ and 5% CO₂ for up to 28 days.

4. Isolation and culture of meniscal explants from caprine knee joints

A previously described meniscal replacement model system (31-33) was employed to assess *ex vivo* integrative meniscal replacement (n = 3 per treatment group, all from different animals). A schematic representation of the experimental setting is shown in Fig.1, Phase III. Explants (5 mm diameter) were cored from caprine menisci. To simulate a full-thickness tear, a 2 mm concentric core defect was introduced in the intermediate zone using a custom made double punch. The central core was removed and discarded. Explants were cut parallel to the meniscal

surface with a scalpel to a uniform thickness of 2 mm using a custom-made cutting block. Immediately after harvest, samples were incubated in 12- well plates in 4 mL of culture media consisting of high glucose (HG) Dulbecco's Modified Eagle Medium (Gibco, Invitrogen) with 10% fetal bovine serum (Gibco, Invitrogen), 0.1 mM nonessential amino acids (Gibco), 400 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, penicillin (100 U/ml)–streptomycin (100 mg/ml) and amphotericin B (0.25 mg/ml, Sigma-Aldrich) at 37 °C and 5% CO₂. Samples were cultured for 48 h to allow equilibration of cell metabolic activity in culture. Subsequently, 20 µl of either FC (100,000 cells/scaffold) or MC (50 mg/scaffold) were loaded onto scaffolds, incubated for 30 min as described earlier, press-fit into meniscal explants and subsequently transferred to culture media (n=6/group) with acellular scaffolds serving as controls (n=6/group). Media changes were performed every 3-4 days, with explants cultured for a total of 28 days at 37 °C and 5% O₂.

5. Assessment of cell viability

Cell viability was assessed using a viability/cytotoxicity assay kit (LIVE/DEAD®, Invitrogen, Dublin, Ireland) in accordance with the manufacturer's protocol. Briefly, constructs were cut in half, washed in PBS followed by incubation in PBS containing 2 µM calcein AM (live, intact cell membrane) and 4 µM ethidium homodimer-1 (dead, disrupted cell membrane). Sections were again washed in PBS, imaged with a Leica SP8 Confocal Microscope, Wetzlar, Germany with a Z stack encompassing the depth of the scaffold at 515 and 615 nm channels and analysed using Leica LAS X Core Software, Wetzlar, Germany.

6. Quantitative biochemical analysis

On removal from culture, wet weight of the scaffolds was recorded and constructs were frozen at -85 °C for further analysis. Samples were digested with 125 µg/mL papain in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich, Dublin, Ireland) at 60 °C under constant rotation for 18 h. DNA content was determined using the

Hoescht 33258 dye-based assay (DNA QF Kit, Sigma-Aldrich, Dublin, Ireland) with a calf thymus DNA standard. Proteoglycan (sulphated glycosaminoglycan, sGAG) content was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Dublin, Ireland), with a chondroitin sulphate standard. Total collagen was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 °C for 18 h with an equal volume of concentrated (38%) HCl and assayed using a chloramine-T assay (34) with a hydroxyproline:collagen ratio of 1:7.69 (35). Each biochemical constituent was normalised to the tissue wet weight (% w/w).

7. Histological Analysis

Constructs were removed from culture, washed in PBS and fixed with 4% paraformaldehyde solution overnight at room temperature. After removing fixative, samples were rinsed with PBS, and embedded using optimum cutting temperature (OCT) cryoembedding compound. Routine cryosectioning was performed on frozen blocks (8 µm cross-sections along the longitudinal plane) using a cryostat (Leica CM 1850, Wetzlar, Germany) at -21 °C. sGAG deposition was evaluated using 1% alcian blue 8GX in 0.1 M HCL, collagen distribution was assessed using picro-sirius red (all from Sigma-Aldrich, Dublin, Ireland). Collagen type II was evaluated through immunohistochemistry. Briefly, sections were treated with peroxidase, followed by treatment with chondroitinase ABC (Sigma –Aldrich, Dublin, Ireland) in a humidified environment at 37°C to enhance permeability of the ECM. Sections were incubated with goat serum to block nonspecific sites and primary antibody (mouse monoclonal, Abcam, Cambridge, UK) were applied for 18h at 4°C. Next, the secondary antibody (Anti-Mouse IgG biotin conjugate 1:200; 2.1mg/ml) (Sigma Aldrich, Dublin, Ireland) was added for 1h followed by incubation with ABC reagent (Vectastain PK400, Vector Labs, Peterborough, UK) for 45min. Colour was developed using the Vectastain ABC reagent followed by exposure to peroxidase DAB substrate kit (Vector Labs, Peterborough, UK).

Positive and negative controls of ligament and cartilage were incubated for each batch (Supplementary Fig. 1). Sections were imaged with an Olympus IX51 inverted microscope fitted with an Olympus DP70 camera (all Olympus, Waltham, MA, USA).

8. Biomechanical testing

After 28 days of culture, the integration strength between the outer ring and inner core of meniscal explants was quantified using a push-out test as previously described (31-33). Briefly, explants were centered in a custom-made holder, such that the 2 mm inner core was centered over a 2.5 mm concentric hole in the bottom of the dish. Using a standard materials testing machine (Zwick Z005, Roell, Germany), a flat round shaped indenter with a diameter of 1.8 mm attached to a 5N load cell displaced the inner core at a rate of 2% strain per minute until the inner core was dislodged from the outer ring and a sharp fall in force was observed. The force required for displacement was recorded over time. Integration strength (in kPa) was calculated by dividing the peak force required to push the inner core through the outer annulus measured during the push out test by the surface area of the interface.

9. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5) software with 3-4 samples analysed for each experimental group. One-way or two-way ANOVA was used for analysis of variance with Bonferroni's post-tests to compare between groups. Graphical results are displayed as mean \pm standard deviation from three independent donors. Significance was accepted at a level of $P < 0.05$.

Results

1. *Rapid chondrocyte isolation and characterisation*

The relationship between biopsy size and weight was determined to assess the clinical validity and relevance of obtaining a cartilage biopsy from a non-load bearing region of the knee similar to that performed for ACI. For typical ACI procedures, approximately 300-500 mg of tissue is harvested for cell isolation (17), implying that a minimum biopsy size of 3mm is required (Fig. 2A). Cell yield was optimized for articular cartilage incorporating physical agitation to investigate the effect of enzyme exposure for three incubation times of 30 min, 1 h and 12 h, with a low (750 U/ml) and high (3000 U/ml) collagenase type II concentration as described previously by our laboratory (25). With 3000U/ml of enzyme, the cell yield (Fig. 2B) was found to improve 5 fold in 30 min and 1 h digest times compared with 750 U/ml ($P<0.0001$). Cell yield was increased 2 fold ($\sim 2.5 \times 10^6$ to 5×10^6 per g of cartilage) when increasing the incubation time from 30 min to 1 h at 3000 U/ml with a minor (4.74%) reduction in cell viability ($P<0.05$) (Fig. 2C). Increased temporal exposure had more of an effect on cell yield than enzyme concentration after 12 h (Fig. 2B), although cell viability was significantly reduced for both enzyme concentrations (Fig. 2C) ($P<0.0001$). Further, when assessing proliferation kinetics in terms of population doubling time (Fig. 2D), the rapidly isolated group (30 min 3000U/ml) was found to display significantly faster doubling kinetics (~ 2 fold) when compared with the standard isolation group (12 h 750U/ml) ($P<0.001$). Taken together, results suggest that the number of cells that can be isolated from biopsies of joint cartilage ranges from 0.75×10^6 (3mm biopsy) to 1.25×10^6 (4mm biopsy) which can be used for subsequent tissue regeneration approaches.

2. *Cell proliferation, viability and matrix forming capacity of freshly isolated and culture expanded cells*

Having demonstrated the feasibility of fresh cell isolation, we next sought to compare FC (30 min isolation protocol, 3000U/ml collagenase concentration) and EC in terms of

proliferation and matrix deposition. FC seeded constructs group exhibited higher (1.5 fold) DNA content (Fig. 3A) compared to EC seeded constructs ($P < 0.001$). LIVE-DEAD imaging revealed a higher density of cells in FC constructs compared to the EC (Fig. 3D). ECM matrix deposition was subsequently evaluated in terms of sGAG and collagen accumulation. Higher sGAG accumulation (~55%) was observed for FC compared to EC seeded constructs ($P < 0.001$) in terms of sGAG (% w/w) and sGAG/DNA (Fig. 3B). This was also confirmed through histology staining with more intense alcian blue staining in FC compared to EC constructs (Fig. 3E). Similar trends were also observed for collagen with increased accumulation for FC compared to EC constructs in terms of Collagen (% w/w) and Collagen/DNA ($P < 0.05$) (Fig. 3C), which was also evident upon histological evaluation (Fig. 3F).

3. Cell viability of freshly isolated chondrocytes and minced cartilage in a meniscal explant culture model

Having demonstrated FC isolates to be superior to standard EC in terms of matrix elaboration, we next sought to directly compare the use of FC to MC, which is an equally feasible strategy for intraoperative use obviating the need for enzymatic cell isolation. Both MC and FC were loaded onto polyurethane scaffolds (2x2mm) and cultured in an explant defect model, with acellular scaffolds serving as controls. Following explant culture for 28 days, some cell migration into the acellular implant was observed, although lower cell numbers and diminished viability were observed at the tissue interface (Fig. 4A, top). With increasing distance from the scaffold-tissue interface, higher cell numbers were evident within both MC (Fig. 4A, middle) and FC (Fig. 4A bottom). Specifically, FC constructs exhibited the highest cell density, which was further reflected in the DNA biochemical quantification (Fig. 4B).

4. Matrix forming capacity in explant culture of acellular, minced cartilage and rapidly isolated chondrocytes

Increased amounts of sGAG was observed in MC and FC constructs, with FC exhibiting ~3 fold higher levels of FC and ~2 fold higher for MC compared to acellular controls

($P < 0.0001$) (Fig. 5A) in terms of sGAG (% w/w) and sGAG/DNA. Based on histological evaluation of the interface, more intense staining was observed for FC when compared with MC groups, with negligible deposition observed at the interface for acellular constructs (Fig. 5B).

In evaluating collagen accumulation, collagen deposition was 25% higher for FC constructs ($P < 0.0001$) compared with MC which was 50% higher ($P < 0.0001$) compared to acellular controls (Fig. 5C) in terms of both collagen (%w/w) and collagen/DNA. Similarly, intense collagen staining was observed at the tissue-construct interface for both FC and MC constructs with negligible deposition evident at the interface for acellular controls (Fig. 5D). Additionally, on evaluation of collagen type II deposition through immunohistochemistry, positive staining was observed at the tissue construct interface and was more intense for FC compared to MC, with minimal staining present at the interface of acellular controls (Fig 5E).

5. Interface integration strength in explant culture of fresh, rapidly isolated chondrocytes and minced cartilage

Finally, to assess functional improvements at the replacement-tissue interface, the push-out strength was determined by means of a pushout test as shown in Fig. 6A. The integration strength of the interface was found to be 4 fold higher for FC constructs (81.9 ± 19.4 kPa) and 2.5 fold greater for MC groups (58.0 ± 15.2 kPa) compared to the acellular implant (21.6 ± 13.3 kPa) only (Fig. 6B).

Discussion

The burden of meniscal pathology in active and aging populations extends to chronic repercussions in joint degeneration and osteoarthritis. Acellular scaffold replacements have been shown to improve pain, functional and radiological outcomes, but there remain limitations

with delamination and failure. The potential of combining biological augmentation with commercially available polyurethane scaffolds is highly attractive to overcome some of the associated limitations such as graft shrinkage, inadequate attachment and host tissue integration (14, 36). Therefore, there is an ever-growing need for low cost and viable interventions to improve tissue integration and replacement of defects. Current approaches in regenerative joint therapeutics utilising chondrocytes have not been widely adopted perhaps due to the time, costs and expertise required for cell expansion (37). In this context, intraoperative point of care (POC) biologics amenable to single step interventions appear attractive and may provide an alternative and viable strategy (38).

The overall aim of this study was to assess the role of intraoperative cartilage derived biologics with the objective of improving integration of a commercially available polyurethane replacement in a meniscus explant defect model. Having demonstrated the feasibility of fresh cell isolation, improved matrix accumulation was observed with FC isolates when compared with laboratory cultured EC on a polyurethane scaffold, strengthening their rationale for use. These FC were then compared with MC and both showed improved replacement-tissue matrix deposition and consequent push-out strength with a 4-fold and 2.5-fold improvement when compared with the acellular implant. These observations highlight the potential role and significance of intraoperative transplantation of articular cartilage derived biologics for meniscus replacement.

While there are a number of studies advocating biological augmentation to improve surgical outcomes, there are only three studies recorded in the literature that are in the clinical phase (39-41), highlighting a fall-off in translation. These involve the use of platelet rich plasma or isolated mesenchymal stem cell (MSC) population to augment healing and the delivery of cells within a fibrin clot delivery system and some further trials in this area are underway. The use of chondrocytes for meniscal applications has not been addressed in the

clinical setting. Articular chondrocytes have been shown to promote chemotaxis, cellular proliferation, and matrix deposition at the interface site, without the need for exogenous growth factor supplementation (42, 43). In addition, clinical parameters and economic feasibility considerations will be vital to the widespread adoption of regenerative approaches to benefit patients. Incorporating such considerations from the outset in the *in vitro* conceptualisation of approaches can be beneficial (23). Biological augmentation strategies attempt to overcome the inherent limitations in healing related to poor vascularity and heterogeneous cellularity. Thus, single step intraoperative cartilage based biologics applied to existing commercial synthetic implants such as the polyurethane scaffold (10, 11, 44, 45) can improve bioactivity and render favourable logistics to improve meniscal repair or replacement.

However, low cells yields from cartilage digests remains a key limitation. The heterogeneity of chondrocyte isolation and poor consistency in reporting enzyme units in the literature limit the delivery of optimized protocols for intraoperative processing (24, 25). To address this, a protocol previously developed in our laboratory for rapid cell isolation from nasal cartilage (25) was applied to articular cartilage. Improved cell yields were observed for articular cartilage with a cell yield of $2\text{-}2.5 \times 10^6$ cells/g from a clinically relevant biopsy (~300 mg) (17) using a reduced isolation time of 30 min. This is comparable to previous work by Bekkers et al. who report similar cell yields $0.56\text{-}1.06 \times 10^6$ cells/g (26). Exposure time was found to be more detrimental to cell viability than concentration as previously demonstrated by Bos et al (46). Previous work in our laboratory has also shown that higher exposure time (12 hrs) of tissue to enzymes is also detrimental to subsequent chondrogenesis of nasal chondrocytes, while short incubation times using high enzyme concentrations (3000U/ml) for 1 hr combined with physical dissociation results in robust matrix formation (25). In the current study, we demonstrate further improvements in cell viability using a 30 min isolation regime, when compared with a 1 h protocol at 3000 U/ml or 750 U exposure for 12 h. Reduced

population doubling time was observed for chondrocytes isolated using short term, high enzyme concentration exposure compared with conventional chondrocyte isolation protocols. This data suggests that rapidly isolated cells, which may be available in lower numbers for implantation are capable of superior proliferation (47). Improved cell vitality and matrix forming capacity were confirmed with higher levels of sGAG and collagen accumulation of freshly derived isolates (i.e. FC) on a polyurethane compared with tissue culture expanded chondrocytes (i.e. EC). In addition to improved proliferation of FC over MC, higher sGAG/DNA and Collagen/DNA ratios demonstrate that increased cell metabolism and higher matrix production of individual cells is also contributing to the higher overall amounts of matrix accumulation. This is in keeping with our previous observations with rapidly isolated fresh nasal chondrocytes whereby greater attrition of pericellular matrix (PCM) with prolonged digest times is seen to result in higher metabolism of cells (25). Herein, we observe both diminished proliferation and reduced matrix turnover with minced cartilage in comparison to isolated cells. This would favour the use of fresh chondrocytes to achieve higher levels of matrix reconstitution.

As proposed by Numpaisal et al (27), an advantage of utilising articular chondrocytes is the similar matrix composition between the inner meniscus and hyaline cartilage. Similarly, in this study, we observed collagen accumulation for both FC and MC, and importantly this was predominantly Collagen Type II, which is the desired collagen type for meniscus regeneration. In this manner, the application of fresh chondrocyte based biologics demonstrates the additional critical advantage of improved matrix reconstitution. The application of such intraoperative processing with chondrocytes has been limited to the commercially available Carti-One™ (Orteq® Ltd., United Kingdom), facilitating a single stage procedure for cartilage defect replacement. Where this service is limited by the need for a trained technician and the time involved in tissue mincing, the consolidation of such with physical agitation to maximise

surface area exposure to collagenase enzymes in a contained unit can potentially permit future advances in the application of fresh chondrocytes to meniscal replacement.

Where the use of enzymatic isolation protocols intraoperatively is largely reliant on the evolving regulatory landscapes, the efficacy of tissue transplantation with minced cartilage without the need for enzymatic cell isolation has been demonstrated in cartilage approaches (26, 29). However, to the best of the authors' knowledge, the application of such has not been applied to meniscal replacement procedures. In comparing both FC to MC in a meniscal explant defect model, improved proliferation, sGAG and collagen production was observed compared with the acellular implant.

Importantly, upon histological evaluation of the interface, improved cell viability, sGAG and collagen accumulation was observed at tissue implant interface for both FC and MC compared with the acellular implant. Where acellular interface strength values were comparable to Hennerbichler et al (31), who demonstrated healing of the native meniscus, improved push out strengths were observed (4 fold higher for FC and 2.5 fold for MC) compared with the acellular implant. Wherein native healing is not possible with large defect sizes requiring meniscal replacement, biological augmentation was found to improve tissue integration. The push out strength values for FC and MC obtained in this study were also found to be superior to those achieved with platelet rich plasma (PRP) and growth factor (GF) delivery in similar studies in the literature (48, 49). Both MSCs and chondrocytes have been proposed as potential cell sources for meniscal regeneration. In particular, animal models using MSCs have demonstrated increased healing and integration of meniscal lesions (50, 51). However, direct comparisons on the effectiveness of different cell types is difficult as many of these studies employ histological grading and do not provide biomechanical data in terms of integration strength. However, Schwartz et al previously demonstrated superior chondrogenic potential of meniscal

cells compared with adipose or synovium derived cells in a nude mouse model (52). Of note, no exogenous growth factors were employed in this study, which are normally required to initiate differentiation when using MSC populations and may as such limit their clinical translation and application.

This work was carried out in an *ex-vivo* caprine meniscal explant defect model allowing the examination of the interface, facilitating both qualitative and quantitative assessments of the role of biologics (31, 53). To facilitate the investigative groups employed in this study, lower cell numbers were used in seeding 2 mm polyurethane implants for defect replacement in a 5 mm explant with a 2 mm defect. Where a full biopsy (300-500mg) can be applied, this approach would require further evaluation to determine adequate cell dosing in critical meniscal defects in terms of the ideal cell number for optimal regeneration. Whether the findings of this work directly translate when applied with human tissues also warrants further investigation and validation. In addition, further studies addressing cellular response in the context of the biophysical environment of the knee joint and *in vivo* animal assessments of these approaches would be required to permit translatable clinical advancements for meniscal replacement.

Conclusion

The application of regenerative orthobiologics in the clinical setting may offer significant potential in augmenting synthetic replacements to address the demands of meniscal surgery. Fresh chondrocytes from a clinically relevant sized biopsy were successfully isolated within 30 min, to yield numbers suitable for augmentation of meniscal replacements. Improved matrix formation was demonstrated in the absence of growth factors in a polyurethane scaffold using fresh cells compared to standard laboratory culture expanded populations. Herein, we

demonstrated the benefits of using enzymatically isolated cells (FC) or minced cartilage in an *ex-vivo* caprine meniscal explant model in terms of improved interface-matrix and integration strength. Taken together, these findings provide the rationale for pursuing single step, POC ventures which may provide exciting new horizons and opportunities for meniscal repair surgery.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figures & Legends:

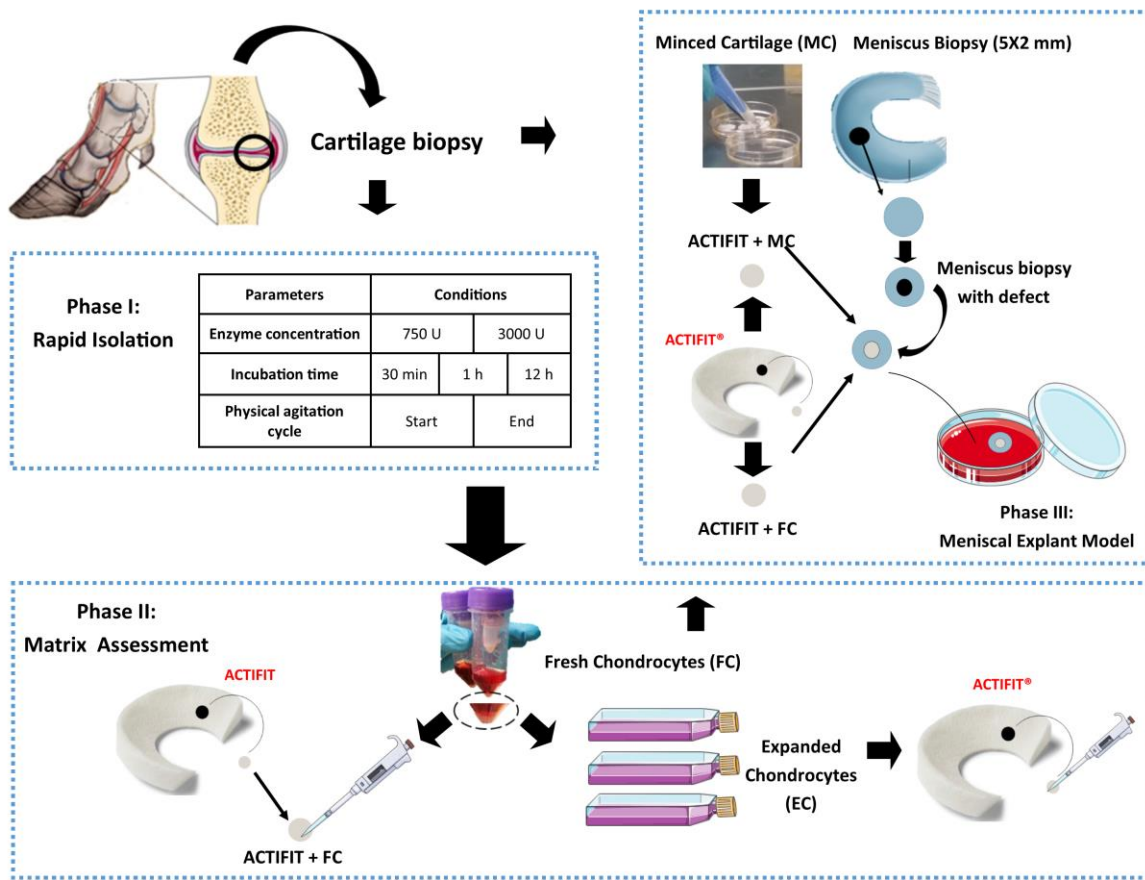


Figure 1: Experimental outline. Phase 1 evaluated a rapid isolation protocol (enzyme concentration, incubation time, physical agitation cycle) for the extraction of fresh chondrocytes (FC) from a biopsy of articular cartilage. Phase 2 evaluated both FC and expanded chondrocytes (EC) in terms of matrix deposition in Actifit® polyurethane scaffolds. Phase 3 assessed FC and MC augmented polyurethane scaffolds compared to acellular controls in a caprine meniscal explant model.

Phase I: Rapid Isolation

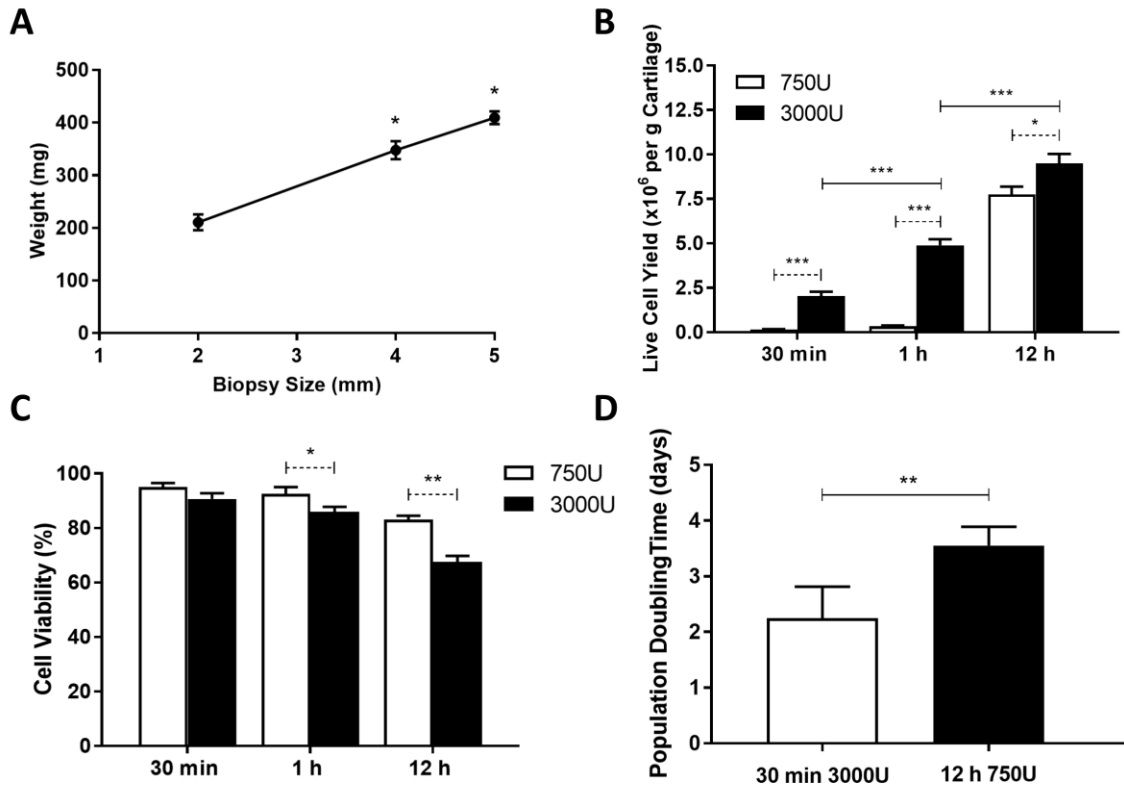


Figure 2: Phase I: Rapid Isolation; Comparison of rapid and conventional cell isolation methods from cartilage tissue. (A) Weight (mg) of 2 mm thick cartilage biopsies obtained using 2, 4 and 5mm cores. (B) Cell yield ($\times 10^6$) normalised to weight per gram of cartilage for digest times of 30 min, 1 h, 12 h at 750 U/ml or 3000 U/ml of collagenase with tissue dissociator cycles at the start and end of the digest time (N=6). (C) Cell viability (%) as determined by trypan blue staining and haemocytometer count. (D) Population doubling time (days) for chondrocytes seeded at an initial density of 5×10^3 cells/cm² (N=3). This is shown for rapid isolation (30 min at 3000 U/ml with physical agitation) and conventional protocol (12 h at 750 U/ml without physical agitation). Statistical significance: * P<0.05, ** P<0.001 and *** P<0.0001

Phase II: Matrix Assessment

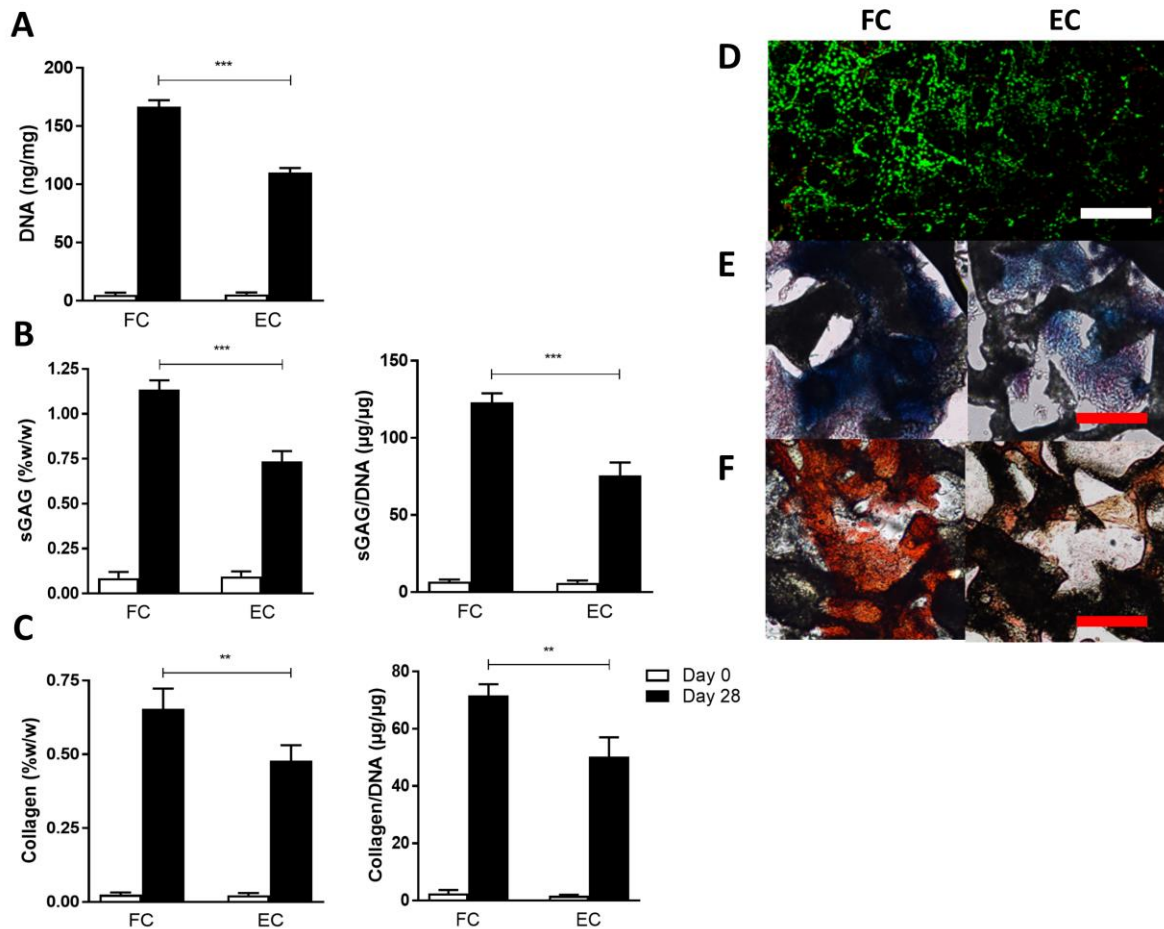


Figure 3: Phase II: Matrix Assessment; Cell proliferation, viability and matrix forming capacity of freshly isolated and culture expanded cells on polyurethane scaffolds at day 28 (N=3). (A) DNA content (ng/mg). (B) sGAG % w/w and sGAG/DNA (C) Collagen (% w/w) and Collagen/DNA (D) Cell viability for populations of fresh chondrocytes (FC) and expanded chondrocytes (EC). (E) Histological evaluation with alcian blue to identify sGAG at day 28; blue staining indicates sGAG accumulation with pink nuclear staining. (F) Histological evaluation with picro-sirius red to identify collagen at day 28. Red staining indicates collagen deposits. All scale bars: 1 mm. Statistical significance: ** P<0.001 and *** P<0.0001.

Phase III: Meniscal Explant Model

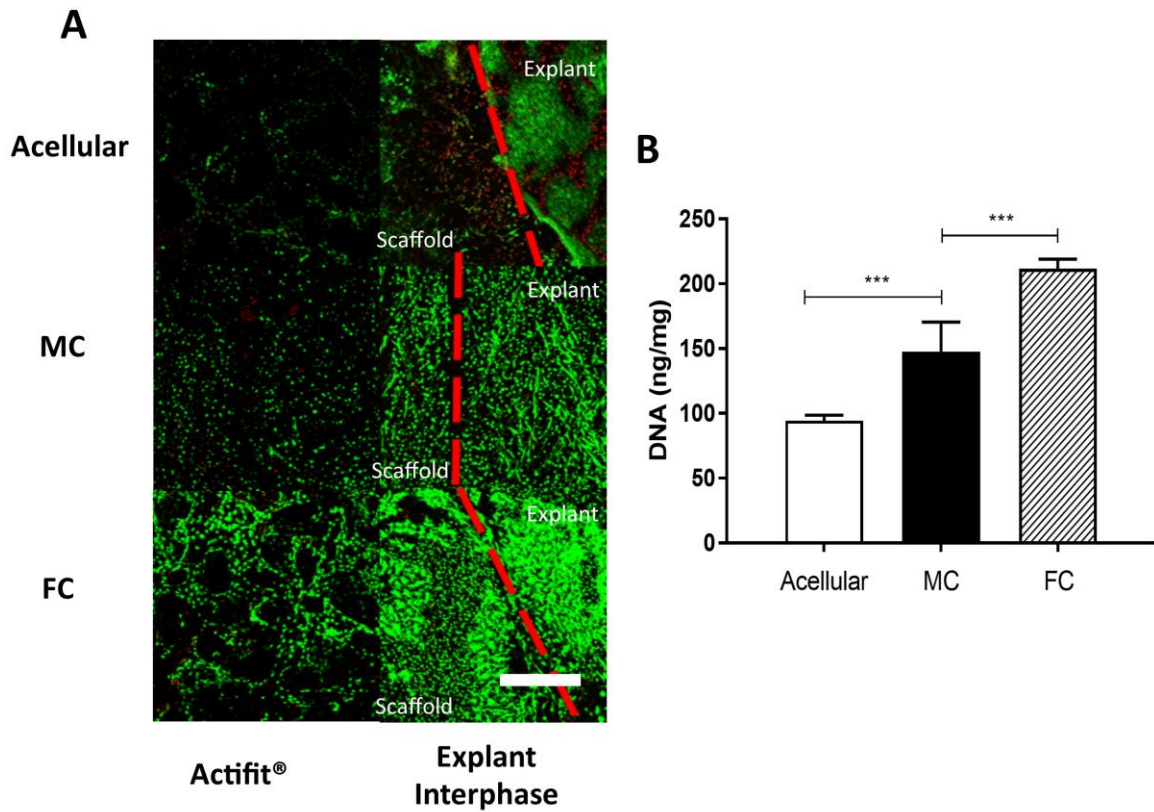


Figure 4: Phase III: Meniscal Explant Model; Cell viability of freshly isolated chondrocyte-seeded, minced cartilage-loaded and acellular polyurethane implants at day 28. (A) Cell viability at implant-explant interface at day 28. Scale bar: 1mm (B) DNA content (ng/mg) (N=3). Statistical significance: * P<0.0001.**

Phase III: Meniscal Explant Model

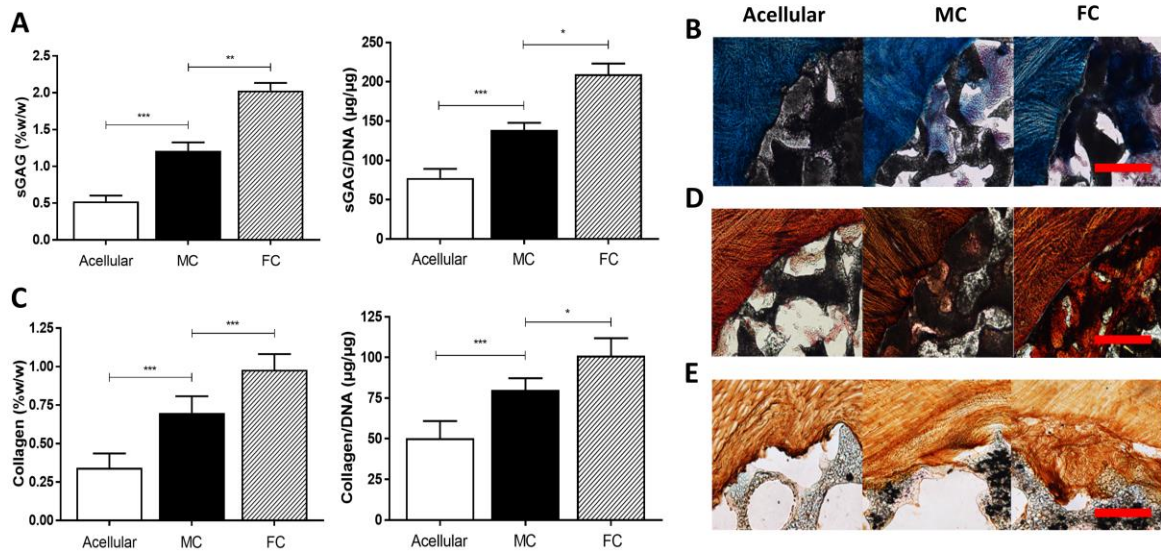


Figure 5: Phase III: Meniscal Explant Model; Matrix forming capacity in explant culture of acellular, minced cartilage and rapidly isolated chondrocyte loaded polyurethane implants at day 28 (N=3). (A) sGAG (%w/w) and sGAG/DNA (B) Histological evaluation with alcian blue to identify sGAG; blue staining indicates sGAG accumulation with pink nuclear staining. (C) Collagen (%w/w) and Collagen/DNA (D) Histological evaluation with picro-sirius red to identify collagen. Red staining indicates collagen deposits. (E) Collagen type II evaluated with immunohistochemistry staining. All scale bars: 1mm. Statistical significance: * P<0.05, ** P<0.001 and *** P<0.0001.

Phase III: Meniscal Explant Model

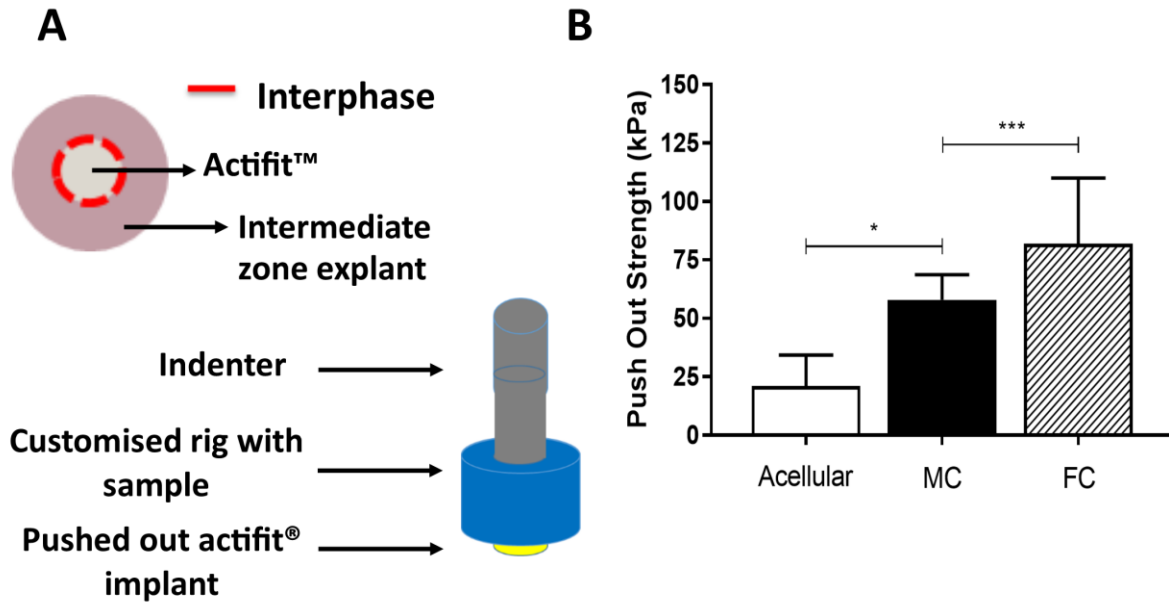


Figure 6: Phase III: Meniscal Explant Model; Push out strength in explant culture of fresh, rapidly isolated chondrocytes and minced cartilage loaded polyurethane implants at day 28 (N=3). (A) Polyurethane implants in meniscus explant defect model indicating the tissue-construct interface (red line). Integration strength was assessed with a biomechanical push out test (bottom). (B) Push out strength (kPa) (N=3 for each donor). Statistical significance: * P<0.05, and *** P<0.0001.

	Col II	
Cartilage		Positive Control
Ligament		Negative Control

Supplementary Figure 1: Positive and Negative controls for Collagen Type II immunohistochemistry. Positive control cartilage and negative control ligament is shown for Collagen type II. Scale bar: 1mm.

References

1. Parenteau N, Hardin-Young J, Shannon W, Cantini P, Russell A. Meeting the need for regenerative therapies I: target-based incidence and its relationship to U.S. spending, productivity, and innovation. *Tissue Eng Part B Rev.* 2012;18(2):139-54.
2. Salata MJ, Gibbs AE, Sekiya JK. A systematic review of clinical outcomes in patients undergoing meniscectomy. *Am J Sports Med.* 2010;38(9):1907-16.
3. Englund M, Lohmander LS. Risk factors for symptomatic knee osteoarthritis fifteen to twenty-two years after meniscectomy. *Arthritis Rheum.* 2004;50(9):2811-9.
4. Peters G, Wirth CJ. The current state of meniscal allograft transplantation and replacement. *Knee.* 2003;10(1):19-31.
5. Rodeo SA. Meniscal allografts--where do we stand? *Am J Sports Med.* 2001;29(2):246-61.
6. McCormick F, Harris JD, Abrams GD, Hussey KE, Wilson H, Frank R, et al. Survival and reoperation rates after meniscal allograft transplantation: analysis of failures for 172 consecutive transplants at a minimum 2-year follow-up. *Am J Sports Med.* 2014;42(4):892-7.
7. Steadman JR, Rodkey WG. Tissue-engineered collagen meniscus implants: 5- to 6-year feasibility study results. *Arthroscopy.* 2005;21(5):515-25.
8. Zaffagnini S, Giordano G, Vascellari A, Bruni D, Neri MP, Iacono F, et al. Arthroscopic collagen meniscus implant results at 6 to 8 years follow up. *Knee Surg Sports Traumatol Arthrosc.* 2007;15(2):175-83.
9. Rodkey WG, DeHaven KE, Montgomery WH, 3rd, Baker CL, Jr., Beck CL, Jr., Hormel SE, et al. Comparison of the collagen meniscus implant with partial meniscectomy. A prospective randomized trial. *J Bone Joint Surg Am.* 2008;90(7):1413-26.
10. Verdonk P, Beaufils P, Bellemans J, Djian P, Heinrichs EL, Huysse W, et al. Successful treatment of painful irreparable partial meniscal defects with a polyurethane scaffold: two-year safety and clinical outcomes. *Am J Sports Med.* 2012;40(4):844-53.
11. Verdonk R, Verdonk P, Huysse W, Forsyth R, Heinrichs EL. Tissue ingrowth after implantation of a novel, biodegradable polyurethane scaffold for treatment of partial meniscal lesions. *Am J Sports Med.* 2011;39(4):774-82.
12. Maak TG, Fabricant PD, Wickiewicz TL. Indications for meniscus repair. *Clin Sports Med.* 2012;31(1):1-14.
13. Lee CH, Rodeo SA, Fortier LA, Lu C, Erisken C, Mao JJ. Protein-releasing polymeric scaffolds induce fibrochondrocytic differentiation of endogenous cells for knee meniscus regeneration in sheep. *Sci Transl Med.* 2014;6(266):171.
14. Moran CJ, Busilacchi A, Lee CA, Athanasiou KA, Verdonk PC. Biological Augmentation and Tissue Engineering Approaches in Meniscus Surgery. *Arthroscopy.* 2015;31(5):944-55.
15. Marsano A, Vunjak-Novakovic G, I. M. Towards tissue engineering of meniscus substitutes: selection of cell source and culture environment. *Conf Proc IEEE Eng Med Biol Soc* 2006; 1:3656–3658.
16. Marsano A, Millward-Sadler SJ, Salter DM, Adesida A, Hardingham T, Tognana E, et al. Differential cartilaginous tissue formation by human synovial membrane, fat pad, meniscus cells and articular chondrocytes. *Osteoarthritis Cartilage.* 2007;15(1):48-58.
17. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *The New England journal of medicine.* 1994;331(14):889-95.
18. Mumme M, Barbero A, Miot S, Wixmerten A, Feliciano S, Wolf F, et al. Nasal chondrocyte-based engineered autologous cartilage tissue for repair of articular cartilage defects: an observational first-in-human trial. *Lancet.* 2016;388(10055):1985-94.

19. Brittberg M. Cell carriers as the next generation of cell therapy for cartilage repair: a review of the matrix-induced autologous chondrocyte implantation procedure. *Am J Sports Med.* 2010;38(6):1259-71.
20. Hettrich CM, Crawford D, Rodeo SA. Cartilage repair: third-generation cell-based technologies--basic science, surgical techniques, clinical outcomes. *Sports Med Arthrosc.* 2008;16(4):230-5.
21. Gunja NJ, Athanasiou KA. Passage and reversal effects on gene expression of bovine meniscal fibrochondrocytes. *Arthritis Res Ther.* 2007;9(5):R93.
22. Kotlarz H, Gunnarsson CL, Fang H, Rizzo JA. Insurer and out-of-pocket costs of osteoarthritis in the US: evidence from national survey data. *Arthritis Rheum.* 2009;60(12):3546-53.
23. Vedicherla S, Buckley CT. Cell-based therapies for intervertebral disc and cartilage regeneration- Current concepts, parallels, and perspectives. *J Orthop Res.* 2017;35(1):8-22.
24. Oseni AO, Butler PE, Seifalian AM. Optimization of chondrocyte isolation and characterization for large-scale cartilage tissue engineering. *J Surg Res.* 2013;181(1):41-8.
25. Vedicherla S, Buckley CT. Rapid Chondrocyte Isolation for Tissue Engineering Applications: The Effect of Enzyme Concentration and Temporal Exposure on the Matrix Forming Capacity of Nasal Derived Chondrocytes. *BioMed Research International.* 2017;2017:2395138. doi:10.1155/2017/2395138.
26. Bekkers JE, Tsuchida AI, van Rijen MH, Vonk LA, Dhert WJ, Creemers LB, et al. Single-stage cell-based cartilage regeneration using a combination of chondrons and mesenchymal stromal cells: comparison with microfracture. *Am J Sports Med.* 2013;41(9):2158-66.
27. Numpaisal PO, Rothrauff BB, Gottardi R, Chien CL, Tuan RS. Rapidly dissociated autologous meniscus tissue enhances meniscus healing: An in vitro study. *Connect Tissue Res.* 2017;58(3-4):355-65.
28. Farr J, Cole BJ, Sherman S, Karas V. Particulated articular cartilage: CAIS and DeNovo NT. *J Knee Surg.* 2012;25(1):23-9.
29. Bekkers JE, Creemers LB, Tsuchida AI, van Rijen MH, Custers RJ, Dhert WJ, et al. One-stage focal cartilage defect treatment with bone marrow mononuclear cells and chondrocytes leads to better macroscopic cartilage regeneration compared to microfracture in goats. *Osteoarthritis Cartilage.* 2013;21(7):950-6.
30. Buckley CT, Kelly DJ. Expansion in the presence of FGF-2 enhances the functional development of cartilaginous tissues engineered using infrapatellar fat pad derived MSCs. *J Mech Behav Biomed Mater.* 2012;11:102-11.
31. Hennerbichler A, Moutos FT, Hennerbichler D, Weinberg JB, Guilak F. Interleukin-1 and tumor necrosis factor alpha inhibit repair of the porcine meniscus in vitro. *Osteoarthritis Cartilage.* 2007;15(9):1053-60.
32. McNulty AL, Guilak F. Integrative repair of the meniscus: lessons from in vitro studies. *Biorheology.* 2008;45(3-4):487-500.
33. McNulty AL, Moutos FT, Weinberg JB, Guilak F. Enhanced integrative repair of the porcine meniscus in vitro by inhibition of interleukin-1 or tumor necrosis factor alpha. *Arthritis Rheum.* 2007;56(9):3033-42.
34. Kafienah W, Sims TJ. Biochemical methods for the analysis of tissue-engineered cartilage. *Methods Mol Biol.* 2004;238:217-30.
35. Ignat'eva NY, Danilov N, Averkiev S, Obrezkova M, Lunin V, Sobol, E. Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. *Journal of Analytical Chemistry.* 2007;62(1):51-7.

36. Anz AW, Hackel JG, Nilssen EC, Andrews JR. Application of biologics in the treatment of the rotator cuff, meniscus, cartilage, and osteoarthritis. *J Am Acad Orthop Surg.* 2014;22(2):68-79.
37. Makris EA, Hadidi P, Athanasiou KA. The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration. *Biomaterials.* 2011;32(30):7411-31.
38. Coelho MB, Cabral JM, Karp JM. Intraoperative stem cell therapy. *Annual review of biomedical engineering.* 2012;14:325-49.
39. Jang SH, Ha JK, Lee DW, Kim JG. Fibrin clot delivery system for meniscal repair. *Knee Surg Relat Res.* 2011;23(3):180-3.
40. Vangsnest CT, Jr., Farr J, 2nd, Boyd J, Dellaero DT, Mills CR, LeRoux-Williams M. Adult human mesenchymal stem cells delivered via intra-articular injection to the knee following partial medial meniscectomy: a randomized, double-blind, controlled study. *J Bone Joint Surg Am.* 2014;96(2):90-8.
41. Ra HJ, Ha JK, Jang SH, Lee DW, Kim JG. Arthroscopic inside-out repair of complete radial tears of the meniscus with a fibrin clot. *Knee Surg Sports Traumatol Arthrosc.* 2013;21(9):2126-30.
42. Arnoczky SP, Cook JL, Carter T, Turner AS. Translational models for studying meniscal repair and replacement: what they can and cannot tell us. *Tissue Eng Part B Rev.* 2010;16(1):31-9.
43. Acosta FL, Jr., Metz L, Adkisson HD, Liu J, Carruthers-Liebenberg E, Milliman C, et al. Porcine intervertebral disc repair using allogeneic juvenile articular chondrocytes or mesenchymal stem cells. *Tissue Eng Part A.* 2011;17(23-24):3045-55.
44. Bouyarmane H, Beaufils P, Pujol N, Bellemans J, Roberts S, Spalding T, et al. Polyurethane scaffold in lateral meniscus segmental defects: clinical outcomes at 24 months follow-up. *Orthop Traumatol Surg Res.* 2014;100(1):153-7.
45. De Coninck T, Huysse W, Willemot L, Verdonk R, Verstraete K, Verdonk P. Two-year follow-up study on clinical and radiological outcomes of polyurethane meniscal scaffolds. *Am J Sports Med.* 2013;41(1):64-72.
46. 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.
47. Malda J, Frondoza CG. Microcarriers in the engineering of cartilage and bone. *Trends Biotechnol.* 2006;24(7):299-304.
48. Kisiday JD, McIlwraith CW, Rodkey WG, Frisbie DD, Steadman JR. Effects of Platelet-Rich Plasma Composition on Anabolic and Catabolic Activities in Equine Cartilage and Meniscal Explants. *Cartilage.* 2012;3(3):245-54.
49. Collier S, Ghosh P. Effects of transforming growth factor beta on proteoglycan synthesis by cell and explant cultures derived from the knee joint meniscus. *Osteoarthritis Cartilage.* 1995;3(2):127-38.
50. Zellner J, Mueller M, Berner A, Dienstknecht T, Kujat R, Nerlich M, et al. Role of mesenchymal stem cells in tissue engineering of meniscus. *J Biomed Mater Res A.* 2010;94(4):1150-61.
51. Horie M, Driscoll MD, Sampson HW, Sekiya I, Caroom CT, Prockop DJ, et al. Implantation of allogenic synovial stem cells promotes meniscal regeneration in a rabbit meniscal defect model. *J Bone Joint Surg Am.* 2012;94(8):701-12.
52. Schwartz JA, Wang W, Goldstein T, Grande DA. Tissue Engineered Meniscus Repair: Influence of Cell Passage Number, Tissue Origin, and Biomaterial Carrier. *Cartilage.* 2014;5(3):165-71.

53. Kobayashi K, Fujimoto E, Deie M, Sumen Y, Ikuta Y, Ochi M. Regional differences in the healing potential of the meniscus-an organ culture model to eliminate the influence of microvasculature and the synovium. *Knee*. 2004;11(4):271-8.