Towards In-Theatre Cartilage Tissue Engineering: Evaluation Of A Tri-Layered Collagen-Based Scaffold Seeded With A Rapidly Isolated Chondrocyte/Stromal Cell Co-Culture In A Goat Model of Osteochondral Defect Repair

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INTRODUCTION: Current cell-based tissue engineering therapies for cartilage repair, such as matrix induced autologous chondrocyte implantation (MACI), require a two-stage treatment process. The chondrocytes isolated from cartilage biopsies taken in stage one require an *in vitro* expansion phase in order to obtain a clinically relevant number of cells for their subsequent implantation in stage two. Recent studies, however, have demonstrated that smaller numbers of chondrocytes are needed if used in co-cultures with stromal cells, as the trophic factors released by stromal cells can drive the proliferation of chondrocytes leading to the generation of phenotypically stable cartilaginous tissues [1]. The infrapatellar fat pad (IFP) is one such source of stromal cells and the anatomical site of this tissue allows for its biopsy during arthroscopy. Furthermore, stromal cells can be rapidly isolated from IFP tissue [2], which raises the possibility of circumventing the *in vitro* expansion step associated with cell-based therapies by utilizing a rapid cell isolation procedure to obtain a clinically relevant number of cells from tissue biopsies taken while a patient is in theatre. We have previously fabricated a tri-layered collagen-based scaffold using an iterative freeze-drying process which, when implanted cell-free into osteochondral defects in goats, was shown to enhance cartilage repair compared to defects left empty [3]. Herein, we propose to move towards an 'in-theatre' approach to cartilage tissue engineering, by seeding tri-layered collagen-based scaffolds with rapidly isolated chondrocyte/stromal cell co-cultures prior to their implantation in goat osteochondral defects. We hypothesized that the seeding of scaffolds with chondrocyte/stromal cell co-cultures would improve their regenerative capacity compared to cell-free scaffolds.

METHODS: Multi-layered collagen-based scaffolds consisting of a type I collagen/hydroxyapatite bone layer, a type I collagen/hyaluronic acid intermediate layer, and a type I collagen/type II collagen/hyaluronic acid cartilage layer were fabricated as previously described [3]. Chondrocytes and stromal cells were isolated from allogeneic goat cartilage and IFP tissue using a novel rapid isolation technique [2]. Briefly, cartilage pieces (< 1 mm) were rotated in collagenase (350 U/mL, 8 mL/g cartilage) for 2 hours at 37 °C before being sieved (40 µm strainer) and placed in fresh collagenase for 1 hour after which bovine foetal serum (FBS) was added prior to counting. Minced IFP tissue was rotated in collagenase (750 U/mL, 4 mL/g IFP) at 37 °C for 3-4 hours after which FBS was added and the solution was sieved (150 µm strainer), centrifuged to allow removal of floating fat, and sieved once more (40 µm strainer) prior to counting. A co-culture of 500,000 chondrocytes/IFP stromal cells at a ratio of 1:3 were seeded onto the cartilage layer of tri-layered scaffolds and implanted into Ø 6 x 6 mm cylindrical load-bearing osteochondral defects created in the medial femoral condyles of goats. Animals were euthanized at 3 months, 6 months and 12 months post-implantation. Safranin-O histological staining was performed and Photoshop CS6 was used to quantify areas of cartilage in the articular cartilage and subchondral bone regions of the repair tissue. Micro-computed tomography (µCT) scans were carried out to assess bone regeneration. Animal experiments were approved by the Research Ethics Committee of University College Dublin, Ireland. Statistical comparisons were performed using two-way ANOVA (n=6-8 samples per group) using Graphpad Prism. Significance was accepted at $p<0.05$. Results are presented as mean + standard deviation from mean.

RESULTS: Scanning electron microscopy (SEM) of tri-layered scaffolds demonstrated a porous interconnected structure with seamlessly integrated layers (Fig. 1A). Safranin-O histological staining indicated the formation of cartilaginous repair tissue in both cell-free and cell-seeded scaffold groups 3 months post-implantation (Fig. 1B). No significant differences in cartilage repair tissue were observed between cell-free and cell-seeded groups in the articular cartilage layer at 3 months, although the cartilage repair tissue in cell-free group was found to decrease significantly over the subsequent 6- and 12-month time points (Fig. 1C). Interestingly, this feature was not observed in the cell-seeded group, suggesting that the addition of the chondrocyte/IFP stromal cell co-culture may have had a beneficial role in maintaining cartilage levels over the experimental period. In the subchondral bone region, both scaffold groups demonstrated high levels of cartilage at 3 months which were found to decrease noticeably by 12 months (Fig. 1D). Allied to this, µCT quantification demonstrated trends towards significant increases in bone volume in both groups at 12 months compared to 6 months (Fig. 1E), suggesting that the bone in the subchondral region was forming through an endochondral pathway whereby a decrease in cartilaginous matrix was being mirrored by an increase in bone matrix deposition.

DISCUSSION: This work has resulted in a step towards an in-theatre approach to cartilage tissue engineering by utilizing a rapidly isolated chondrocyte/stromal cell co-culture seeded onto a tri-layered scaffold which was shown to better maintain cartilage matrix formation over 12 months *in vivo*. Due to limitations in equipment and on-site facilities, this study used allogeneic tissues as cell sources. The approach could be adapted, however, for autologous cell transplantation, as the tissue sources used (cartilage and infrapatellar fat pad) can be biopsied during arthroscopy and digested rapidly (3-4 hours) to achieve sufficient cell-yields. Furthermore, the porous tri-layered scaffolds used allow for the immediate seeding of cells which can migrate easily through the pores of the scaffold. The combination of these technologies could, therefore, lead to the elimination of the *in vitro* cell expansion phase associated with procedures such as MACI, as the biopsy, cell isolation and implantation of cell-seeded scaffolds could all potentially take place while a patient is in theatre. Further work in this regard should investigate whether the procedures used in this study could be applied to autologous tissues, which would strengthen the case for their inclusion in current clinically approved protocols.

CLINICAL RELEVANCE: Herein, we provide a framework for an in-theatre approach to cartilage tissue engineering which leverages rapid cell isolation techniques and porous collagen-based scaffolds to circumvent the requirement for an *in vitro* cell expansion phase currently used clinically. If successful, this approach would reduce the hospital times and additional costs associated with multi-stage cartilage tissue engineering treatments.

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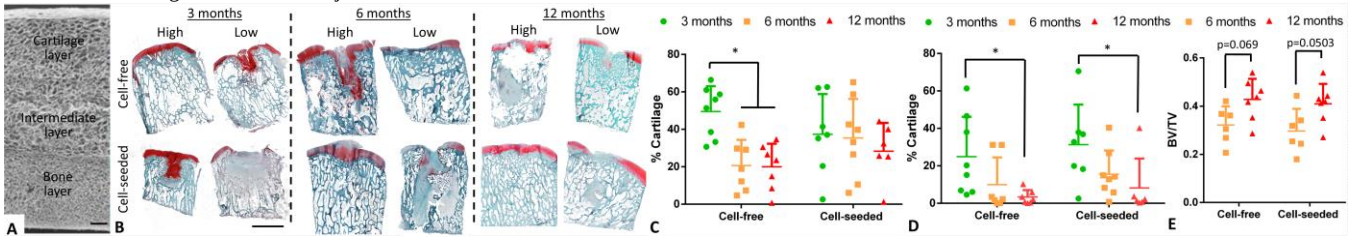

Fig. 1. A) SEM of tri-layered scaffold. Scale-500 µm. B) Safranin-O staining of cell-free and cell-seeded scaffolds. High and low-ranked samples are presented for each group. Scale-5 mm. C) Quantification of cartilage tissue within the articular cartilage layer of osteochondral defects. D) Quantification of cartilage tissue within the subchondral bone layer of osteochondral defects. E) Bone volume (BV)/Total volume (TV) quantified by µCT. Significance *; $p<0.05$.