PROJECT DELIVERABLE REPORT

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1.0	30/04/2019	RIMED	First draft
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1.2	11/05/2019	RIMED	Final version

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1 Summary

Osteoarthritis affects the whole joint and all tissues play a role in the disease. In particular, the subchondral bone has been reported to be critical in the pathogenesis of osteoarthritis. Although the hallmark of osteoarthritis is cartilage degeneration, all joint components are involved, and subchondral bone specifically is believed to play a key role in maintaining cartilage homeostasis. Both animal and clinical studies have highlighted an inverse relationship between bone mineral density and osteoarthritis and more generally subchondral bone plays a key role in cartilage health. Thus, a better experimental representation of the joint is to consider the whole of osteochondral complex, accounting for the interplay and communication between articular cartilage and subchondral bone in health and in the pathogenesis of osteoarthritis. A key challenge in doing so is to ensure the different culture conditions required by cartilage and bone. The osteochondral bioreactor we have developed is characterized from two chamber (upper and lower), and is optimized to allow the study of both native osteochondral plugs or engineered osteochondral constructs with separate media flow for the cartilage and bone compartments, ensuring at the same time the bone-cartilage contact and communication.

2 Introduction

The screening of *in vitro* OA osteochondral units was conducted onto tissues obtained from patients undergoing total joint replacement. The tissues were harvested from the post-surgery waste and cultured *in vitro* for up to 4 weeks within our osteochondral bioreactor that allowed to separately interrogate the cartilaginous and osseous components. Cartilage was cultured in pro-chondrogenic medium (upper chamber) and bone was be cultured in pro-osteogenic medium (lower chamber), both without any anti-inflammatory drugs. In addition, tissues from areas of macroscopically healthy or macroscopically minimally damaged cartilage were compared with tissues from areas of moderate to severe OA, and media were collected at predetermined time points. This approach allowed to investigate the presence of several factors linked to cartilage and bone tissue inflammation and prognostic for osteoarthritis. We optimized our developed in house bioreactors to guarantee an optimal fit of the osteochondral plugs harvested from human donors.

3 Markers and cellular responses in OA osteochondral units

3.1 Tissue harvesting

We harvested osteochondral plugs from tissues obtained from human donors underwent to complete knee prosthetic surgery (figure 1). More in detail, we used the surgical waste obtained from each donors according to ethical committee of University of Pittsburgh.

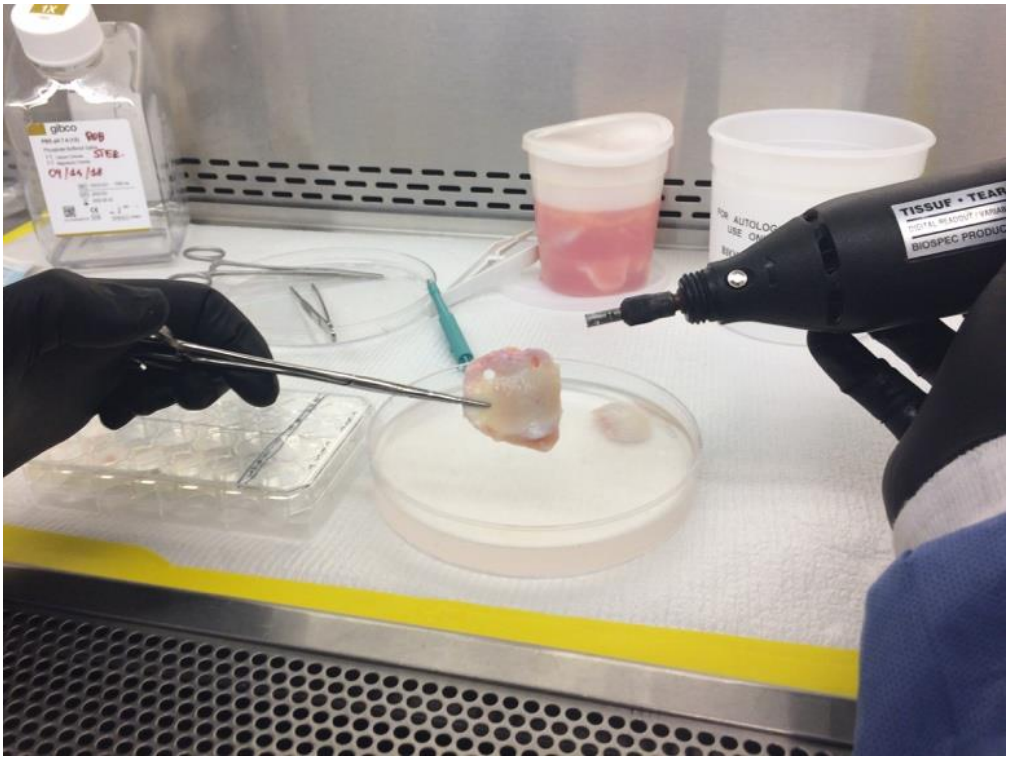

Figure 1: Harvesting procedure performed on surgical waste

The preliminary phase of our work was intended to optimize the harvesting procedures to develop a standard protocol to be employed for all tissue extractions. With this aim, we used a tissue extractor with tunable rotating speed equipped with a rotating tip (d = 4mm) (Figure 2a) and we adjusted systematically the instrumental setting to obtain whole and viable cylinder shaped plugs (Figure 2b).

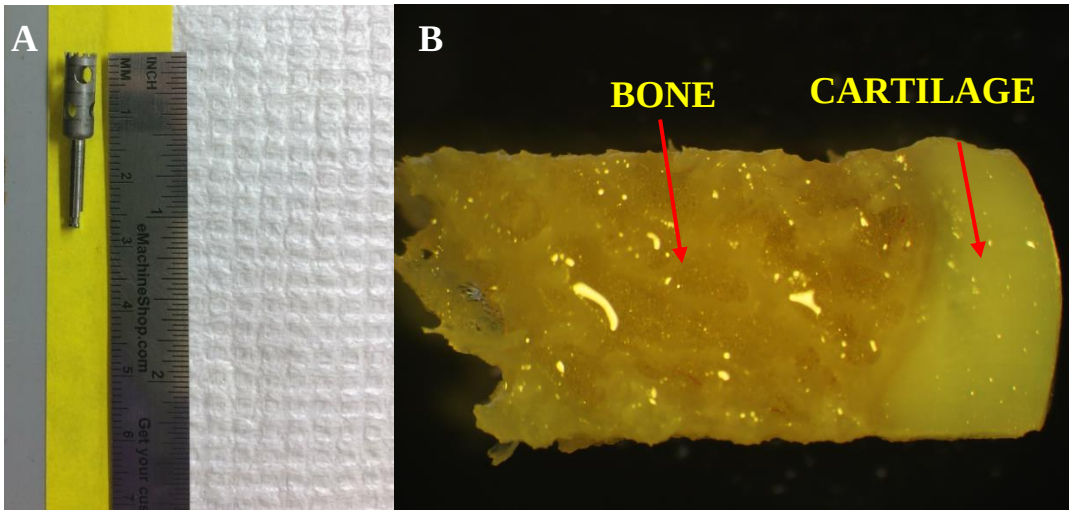

Figure 2: Tissue extractor's tip (a), example of an osteochondral plug immediately after the harvesting

In particular, rotation speed and tip-tissue contact time were accurately set to avoid tissue over-heating (and consequential damage) during harvesting procedure. Furthermore, we controlled temperature by maintaining constantly wet the tissue during harvesting. Wetting was achieved using PBS at room temperature testing two different approaches: in the first the drilling was performed maintaining the tissue under a constant flow of PBS (irrigation), in the second the tissues was soaked in a bath of PBS (submersion). We followed the tissue viability at cellular level by employing a Live/Dead assay, and the results were encouraging for both protocols (figure 3).

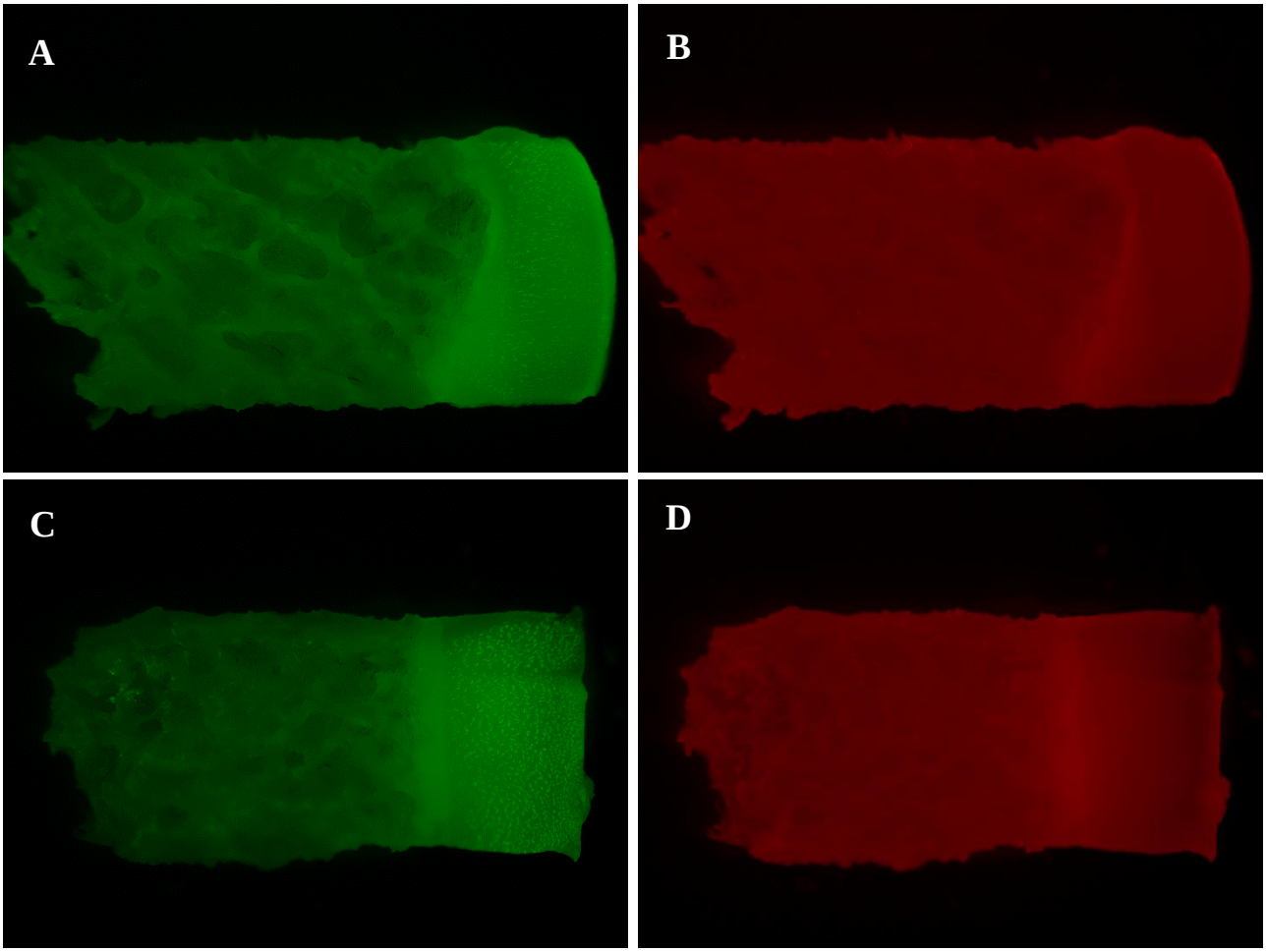

Figure 3: Live/Dead assay conducted onto osteochondral plugs after harvesting with Submersion (a, b) and Irrigation (c, d) technique. The assay is based on the use of two different molecules that selectively stain live cells (green) and dead cells (red)

After this preliminary analysis, we systematically harvested human tissues by drilling out osteochondral plugs from areas of minimally damaged cartilage and from areas of marked OA. We employed the clinical Outerbridge score to evaluate the degree of osteoarthritic damage by visual observation, prior to any destructive testing. The Outerbridge score is mainly based on a macroscopically evaluation of the tissue damage (figure 4), and is characterized by *ex ante* feasibility, essential characteristic that allows a tissue selection before the extraction.

A

	MR IMAGING	ARTHROSCOPY	MACROSCOPY	SCHEMATIC DRAWING OF THE ARTICULAR CARTILAGE
Grade 0	homogenous and smooth delineation	uniform thickness and intact surface	normal cartilage	
Grade 1	focal areas of hyperintensity with normal contour	softening or swelling of cartilage	focal thickening	
Grade 2	blister-like swelling/fraying of articular cartilage extending to the surface	fragmentation and fissuring within soft areas of articular cartilage	superficial defect(s), less than 50%	
Grade 3	partial thickness cartilage loss with focal ulceration	partial thickness cartilage loss with fibrillation ("crab-meat appearance")	Deep defect(s) more than 50%	
Grade 4	exposed subchondral bone	cartilage destruction with exposed subchondral bone	Full thickness defect(s)	

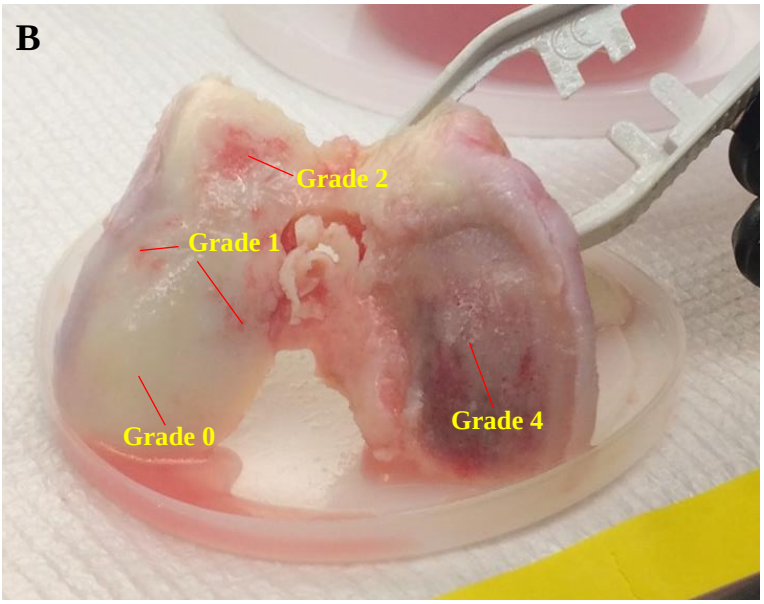

Figure 4: Schematic representation of the Outerbridge classification (a) and Osteoarthritis degree evaluation onto a human femoral condyle (b)

A more complete OA score can be achieved *ex post* by using the OARSI grading system and analyzing the histological sections.

3.2 Culturing under continuous flow and optimizing of the bioreactors

After the standardization of the harvesting procedure, we cultured the native osteochondral plugs under continuous flow within our bioreactors, in which osteochondral plugs can be cultured with separate media conditions for cartilage and bone, while maintaining intact the cartilage-bone unit. We used a chondro-specific medium in the upper chamber in contact with cartilage and an osteo-specific medium in the lower chamber in contact with bone (figure 5).

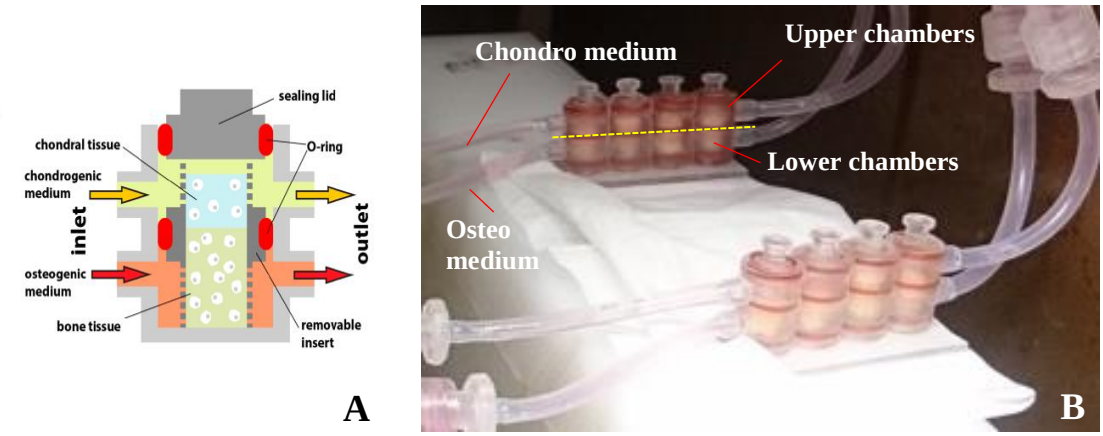

Figure 5: Schematic representation of the bioreactor architecture (a) and picture of a set of osteochondral plugs cultured under dynamic flow (b)

However, the native osteochondral plugs showed unforeseen fitting issues within the bioreactors' slots, causing leaking (figure 6) and consequential break of sterility that negatively affected the timing of our initial experiments.

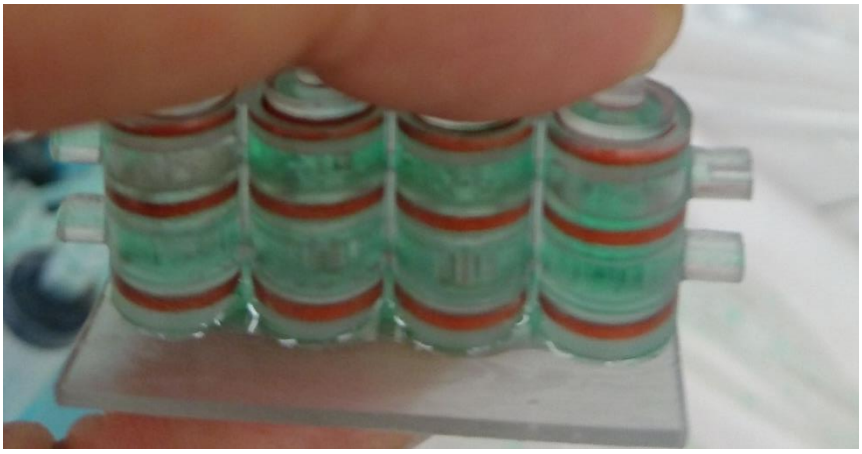

Figure 6: Leaking issue affecting a bioreactor

The origin of the issue was identified in the small changes in the parameters of the 3D printer used to create the bioreactors after the printer was serviced. The problem was fixed by implementing a systematic and iterative set of revisions of the bioreactors' structure to eliminate any leaking and made them fit using the new printer's parameters. More in detail, we increased the height of the slot and reduced the thickness of the lower part of the lid.

We then achieved the culturing of osteochondral plugs for 7 days, collecting the efflux media at defined timepoints (1, 3, 5, and 7 days) to detect the presence of specific OA-associated soluble factors. At day 7, some of the osteochondral plugs were divided into cartilage and bone and processed to study each tissue's gene expression, while replicate plugs were processed for histomorphology analyses.

3.3 ELISA assays

ELISA analyses were performed to investigate the presence of prognostic degradation/inflammation marker after culturing of native OC tissue. ELISA solid

phase sandwich assay were performed on collected efflux media to monitor the level of hyaluronate in solution, a clinical biomarker of cartilage catabolism. The results showed a higher level of hyaluronate in efflux media derived from osteoarthritic tissues, with a clear increasing trend over time. As expected, the level of hyaluronate was lower in efflux media from pristine osteochondral tissues from the same joint (figure 7). Bone effluent also exhibited some baseline levels of hyaluronan, higher for OA tissues.

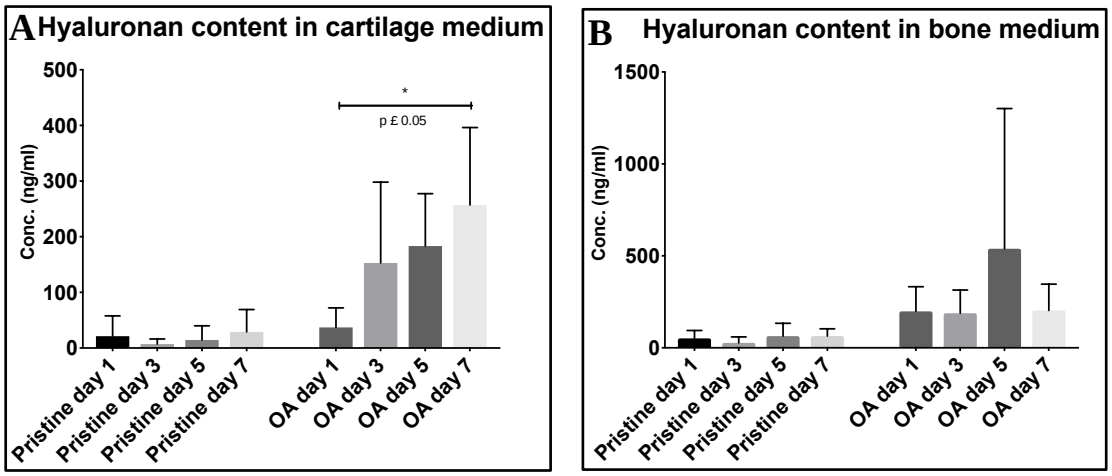

Figure 7: ELISA assay on cartilage (a) and bone (b) efflux media.

4 Conclusions

To date, we successfully developed and optimized a valid harvesting and culturing procedure able to obtain and maintain viable and functional osteochondral units from human and animal tissue. For each donor, we harvested a set of 8 osteochondral units, divided in macroscopically healthy (n = 4) and OA (n = 4) (Outerbridge score). To date, osteochondral units from 10 donors were harvested and cultivated for 7 days in our bioreactors and histomorphology analyses are still on going.