Research Article

Ex Vivo Culture Platform for Assessment of Cartilage Repair Treatment Strategies

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Summary

There is a great need for valuable *ex vivo* models that allow for assessment of cartilage repair strategies to reduce the high number of animal experiments. In this paper we present three studies with our novel *ex vivo* osteochondral culture platform. It consists of two separated media compartments for cartilage and bone, which better represents the *in vivo* situation and enables supply of factors specific to the different needs of bone and cartilage. We investigated whether separation of the cartilage and bone compartments and/or culture media results in the maintenance of viability, structural and functional properties of cartilage tissue. Next, we evaluated for how long we can preserve cartilage matrix stability of osteochondral explants during long-term culture over 84 days. Finally, we determined the optimal defect size that does not show spontaneous self-healing in this culture system.

It was demonstrated that separated compartments for cartilage and bone in combination with tissue-specific medium allow for long-term culture of osteochondral explants while maintaining cartilage viability, matrix tissue content, structure and mechanical properties for at least 56 days. Furthermore, we could create critical size cartilage defects of different sizes in the model.

The osteochondral model represents a valuable preclinical *ex vivo* tool for studying clinically relevant cartilage therapies, such as cartilage biomaterials, for their regenerative potential, for evaluation of drug and cell therapies, or to study mechanisms of cartilage regeneration. It will undoubtedly reduce the number of animals needed for *in vivo* testing.

Keywords: *ex vivo* model, osteochondral biopsy, cartilage repair, critical size defect, replacement

1 Introduction

Cartilage defects and diseases remain major clinical issues in orthopedics. The most common cartilage disease is osteoarthritis (OA), a degenerative joint disease that results from an imbalance in cartilage matrix remodeling marked by gradual loss of cartilage on the joint surface. In a late stage of OA, cartilage thickness decreases to an extent that subchondral bone is exposed, leading to joint damage and causing severe pain (Setton et al., 1999; Lories and Luyten, 2011). Changes in loading during everyday life, after treatments of knee injuries, but also age, obesity or chronic overloading due to sports are risk factors that can lead to the onset of OA (Felson et al., 2000; Abramson and Attur, 2009; Zhang and Jordan, 2010). According to the National Health Interview Survey between 2010 and 2012 by the Centers for Disease Control and Prevention (CDC), more than 52 million people suffer from OA in the United States alone (Barbour et al., 2013).

OA is a degenerative joint disease marked by loss in articular cartilage that is believed to lead to changes in the subchondral bone (Abramson and Attur, 2009; Goldring, 2012). Due to the aneural and avascular character of cartilage tissue, OA and articular cartilage defects do not heal spontaneously and clinical intervention is required (Chu et al., 2010). Current clinical strategies focus on treatment of symptoms with the aim to diminish clinical signs of injury and to restore the defects. From a scientific point of view, the clinical treatments are limited in their ability to functionally regenerate cartilage defects, as they

Received July 11, 2016;
Accepted October 18, 2016;
Epub October 21, 2016;
https://doi.org/10.14573/altex.1607111

often result in the formation of fibrotic tissue, which consists mainly of collagen I and is therefore mechanically inferior to native cartilage (Wakitani et al., 1994; Dewan et al., 2014; Caldwell and Wang, 2015). With regard to the long-term outcome of existing strategies there is still a need to develop new, respectively improved treatment strategies that lead to proper cartilage regeneration.

The development of new treatments and the refinement of existing treatments to repair cartilage defects is typically studied in animal models, such as rodent, sheep, goat, pig and horse. They represent the complexity of the *in vivo* situation. It has to be taken into account that cartilage defect models in rodents and mature rabbits show spontaneous self-repair (Cook et al., 2014). However, this phenomenon of intrinsic repair is limited in humans and other species (Fox et al., 2009). Further disadvantages of animal models are the limited control over physiological parameters and the limited possibilities for monitoring and controlling the healing progress from a biological and biomechanical point of view. With respect to joint size, the only clinically relevant models are large animal models (horse, sheep, goat, dog and pig), whose use is accompanied by high costs for animal caring and ethical issues (Cook et al., 2014; Hurtig et al., 2011).

In order to refine and reduce the number of animal studies performed in the field of cartilage repair, standardized and representative *ex vivo* models that allow for a whole array of simultaneous tests are valuable tools (de Vries-van Melle et al., 2012; Vinardell et al., 2009; Pretzel et al., 2013). These *ex vivo* models need to fulfill the following requirements: The model has to be stable, without loss of physiological properties, viability and metabolic activity, for a relevant culture period, optimally 8 weeks, as this is the typical duration of *in vivo* animal studies on cartilage regeneration therapies (Sakata et al., 2015). Additionally, the model should provide the possibility to monitor functional parameters, such as metabolic activity, viability and, preferably, also mechanical properties. Existing models to study cartilage, to test biomaterials or therapies for cartilage repair can be classified into three groups: cell seeded biomaterials under free swelling conditions, cartilage-only models, and osteochondral models. They differ in the presence and type of

Tab. 1: Overview of *in vitro* cartilage models published in literature and their suitability

Model	Suitability
Cell-laden 3D biomaterials under free swelling conditions 1. Cartilage derived matrix scaffolds (Benders et al., 2014) 2. Natural hydrogels (Sheehy et al., 2015) 3. Synthetic scaffolds (Endres et al., 2012)	+ high reproducibility and repeatability + investigate material-cell interaction + high throughput + high controllability + evaluate effect of material properties (pore size, cross linking degree) on cartilage matrix production
	– lack of *in vivo* physiological complexity – absence of native tissue – lack of external stimuli secreted by native tissue – no oxygen & nutrient gradients present
Cartilage-only models 1. Mature human cartilage discs (ø 5 mm) from OA femoral condyles (Wardale et al., 2015) 2. Mature bovine cartilage discs (ø 6 mm) from lateral facets of trochlea/patella groove (Pretzel et al., 2013) 3. Immature porcine chondral explants (ø 6mm) from femoropatellar joints (Vinardell et al., 2009)	+ presence of cartilage tissue + high repeatability + evaluation of scaffold-tissue integration + evaluation of MSC chondrogenesis in the presence of cartilage tissue + secretion of growth factors and molecules from surrounding cartilage tissue
	– absence of subchondral bone – missing crosstalk between bone and cartilage – no oxygen and nutrient gradients from superficial to deep zone
Osteochondral models 1. Immature bovine osteochondral cylinders (8 mm) from metacarpophalangeal joints of 3-8-month-old calves (de Vries-van Melle et al., 2012, 2014a)	+ presence of bone and cartilage tissue + subchondral bone provides cells migrating into defect site and promotes cartilage homeostasis + secretion of growth factors and molecules from surrounding bone and cartilage + nutrient and oxygen gradients in cartilage tissue
	– cartilage matrix degenerates over time – decrease in bone viability – no separation of bone and cartilage media

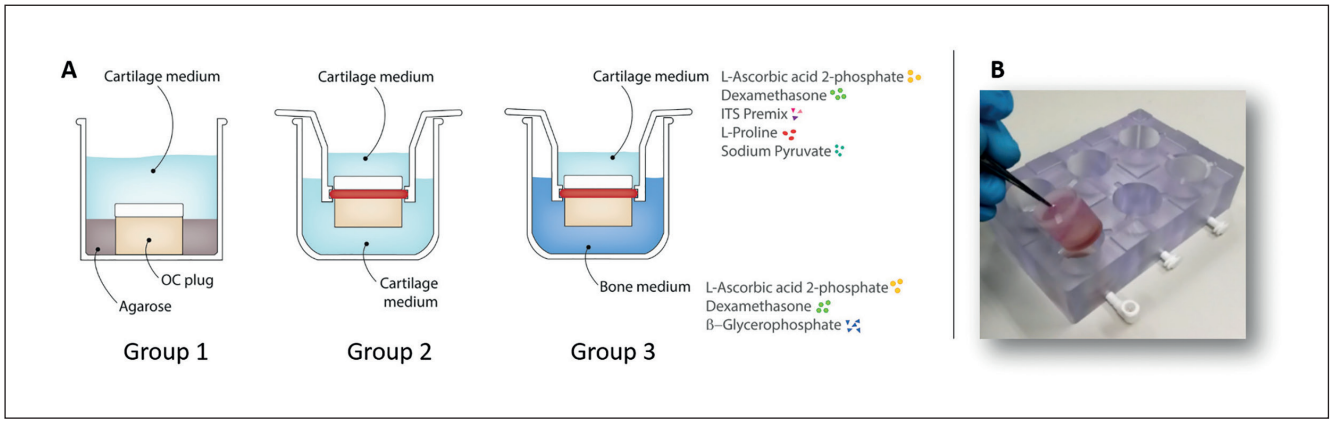

Fig. 1: Schematic illustration of the platform for *ex vivo* culture of osteochondral explants in study A
(A) Group 1 represents agarose embedding of subchondral bone of biopsies cultured with cartilage medium. In group 2 and 3 osteochondral biopsies are fixed in inserts in our osteochondral platform with either cartilage medium in both compartments (group 2) or cartilage medium in the upper compartment and bone medium in the lower compartment (group 3). ITS: insulin, transferrin and selenous acid, bovine serum albumin, linoleic acid. (B) Osteochondral culture platform in 6-well plate format.

surrounding native tissue and thus in their complexity, controllability and repeatability (Tab. 1).

Cell-seeded 3D biomaterials under free swelling conditions and cartilage-only models are useful for gaining insight into cell-material interactions and to characterize the stimulatory effect of different materials on cell phenotype and metabolic activity, but the absence of bone is an inherent limitation for testing of cartilage repair treatments. It has been reported before that the presence of subchondral bone is critical for the success of microfracture procedures (Frisbie et al., 2003, 2006). Moreover, de Vries-van Melle et al. (2012, 2014b) showed that cartilage-related genes were more highly expressed in their *ex vivo* cultured osteochondral explant compared to cartilage-only explants. Their osteochondral model is a promising approach to studying cartilage biology as it simulates an *in vivo* joint-like environment. However, the model is not suitable for long-term *ex vivo* cultures since the expression of cartilage-related genes decreases over 28 days of culture.

We hypothesized that this decrease may be caused by factors secreted from the subchondral bone that are released and concentrated in the culture medium, which also serves as nutrient supply for the cartilage of the osteochondral explants. *In vivo*, the crosstalk between the tissues is restricted to the bone cartilage interface. Factors secreted by osteoblasts isolated from sclerotic bone, but also mononucleated cells that are present in the bone marrow, have been shown to influence chondrocyte metabolism indicated by a reduction of cartilage matrix synthesis in a co-culture model (Sanchez et al., 2005a,b; Iwai et al., 2011). Therefore, we thought that if the direct communication of all factors released from the subchondral bone, including the bone marrow, with the cartilage of osteochondral explants can be restricted to crosstalk via the cartilage bone interface, cartilage matrix composition may be maintained.

To test this hypothesis, we developed a next generation *ex vivo* osteochondral culture platform. It consists of two separated compartments (Fig. 1) for cartilage and bone respectively, which is more representative of the *in vivo* situation than the models summarized in Table 1. Furthermore, this design allows adding specific media and factors to the different tissues and allows us to analyze secretion of soluble factors by bone and cartilage separately. The overall aim was to obtain an *ex vivo* osteochondral culture model that is highly suitable to, at least partly, replace animal testing by serving as a screening tool prior to animal studies. We investigated in study A whether separation of the cartilage and bone compartments and/or culture media results in the long-term preservation of viability, structural and functional properties of cartilage tissues. In study B, we evaluated for how long we can maintain matrix stability of osteochondral explants during long-term culture. Finally, in study C, we determined the optimal defect size in which self-healing does not occur during 28 days of culture.

2 Materials and methods

2.1 Isolation of osteochondral biopsies

Osteochondral biopsies (on average n = 3 per animal; n = 28 for study A; n = 28 for study B) were isolated from 10 fresh medial femoral condyles of 6-8 month old pigs per study. The femoral condyles are left-over materials of pigs slaughtered for human consumption. The biopsies were isolated using an 8.5 mm trephine drill (Smith & Nephew, Hoofddorp, NL) while cooling with sterile phosphate-buffered saline (PBS; Sigma, Zwijndrecht, NL) supplemented with 200 U/ml penicillin (Lonza, Westburg, Leusden, NL), 200 µg/ml streptomycin (Lonza, Westburg, Leusden, NL) and 5 µg/ml fungizone (LifeTechnologies, Bleiswijk, NL). Biopsies were cut to a bone length of 4 mm and incubated overnight in DMEM (42430, LifeTechnologies, Bleiswijk, NL) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone to maintain sterility. For study C, osteochondral biopsies (n = 12), 8 mm in diameter (MF dental, Mantel, GER) and

5 mm in total height (consequently 3.5-4 mm mean bone height), were isolated from 10 medial femoral condyles of 5-7-month-old landrace pigs (retrieved from a local slaughter house) as described above.

2.2 *Ex vivo* osteochondral culture platform

The *ex vivo* osteochondral culture platform is developed and owned by LifeTec Group BV. It consists of custom inserts that are suspended into an in-house developed six-well plate (Fig. 1). The osteochondral explants are mounted into the inserts using an O-ring positioned exactly at the interface between cartilage and bone, creating two isolated culture compartments: the upper compartment containing the cartilage and the lower compartment containing the bone, each with their respective media. Complete separation of the two compartments has been confirmed (data not shown).

2.3 Experimental outline

Study A: Effect of separation of the cartilage and bone compartments and of culture with tissue-specific media

Osteochondral biopsies were divided into three experimental groups, each n = 8 (Fig. 1). Group 1 consisted of biopsies that were placed in a standard six-well plate filled with 2% low-gelling agarose (Sigma, Zwijndrecht, NL) in PBS (Sigma, Zwijndrecht, NL), such that the subchondral bone was surrounded by agarose and the cartilage was above the agarose surface as previously described (de Vries-van Melle et al., 2012). Biopsies were cultured with 5.5 ml cartilage medium consisting of DMEM high glucose (42430, Gibco, LifeTechnologies, Bleiswijk, NL) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 1 mM sodium pyruvate (Gibco, LifeTechnologies, Bleiswijk, NL), 40 µg/ml L-proline (Sigma-Aldrich, Zwijndrecht, NL), 50 µg/ml L-ascorbic acid-2-phosphate (Sigma-Aldrich, Zwijndrecht, NL), 1% ITS+ Premix (Corning, Fisher Scientific, Landsmeer, NL) and 100 nM dexamethasone (Sigma-Aldrich, Zwijndrecht, NL). The biopsies of group 2 and 3 were positioned in our platform such that the cartilage was separated from the bone. The inserts with biopsies of group 2 were placed in custom 6-wells plates and the upper and lower compartment was filled with 2.5 ml and 3 ml cartilage medium respectively (Fig. 1). In group 3, biopsies were cultured in the same setup as group 2, but with cartilage medium in the upper compartment and bone medium, consisting of DMEM high glucose supplemented with 10% fetal bovine serum (FBS, Gibco, LifeTechnologies), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 50 µg/ml L-ascorbic acid-2-phosphate, 100 nM dexamethasone and 10 mM β-glycerophosphate (Sigma-Aldrich, Zwijndrecht, NL), in the lower compartment.

Biopsies were cultured for 14 or 28 days at 37°C and 5% CO_2. Medium was changed every 3-4 days. At each time point, four biopsies per group were used for analyses. Biopsies were cut in half, cartilage and bone tissue were separated, and the cartilage tissues of one set of halves was analyzed for their biochemical content. The other set of halves was used for histology (n = 2) and evaluation of cell viability (n = 2). Four further biopsies were used to create time point zero for all measurements.

Study B: Evaluation of long-term preservation of matrix stability in osteochondral explants cultured with tissue-specific medium in the osteochondral platform

Osteochondral biopsies (n = 28) were cultured for 0, 14, 28, 42, 56, 70 and 84 days at 37°C and 5% CO_2 in the platform with cartilage medium in the cartilage compartment and bone medium in the bone compartment (based on results of study A; Fig. 1). Medium was changed every 3-4 days. At each time point, n = 4 biopsies were used to determine the equilibrium compressive modulus. Afterwards, these biopsies were cut in half, cartilage and bone were separated, and the cartilage tissues of one self of halves were analyzed for the biochemical content (n = 4). The other set of halves was used for histology (n = 2) and evaluation of cell viability (n = 2).

Study C: Define critical size of chondral defects ex vivo

Full thickness cartilage defects with a diameter of 1 mm, 2 mm or 4 mm (each n = 3) were created with a biopsy punch (PFM medical AG, Cologne, GER) (Fig. 2). Residual debris on the calcified layer was removed with a sharp spoon (MF Dental, Mantel, GER). The osteochondral biopsies with inflicted defects were cultured for 28 days in the osteochondral platform with 3 ml cartilage medium in the upper compartment and 3 ml bone medium in the bone compartment (Fig. 1, group 3). Medium was changed every 3-4 days and after 28 days of culture, samples underwent plastic embedding.

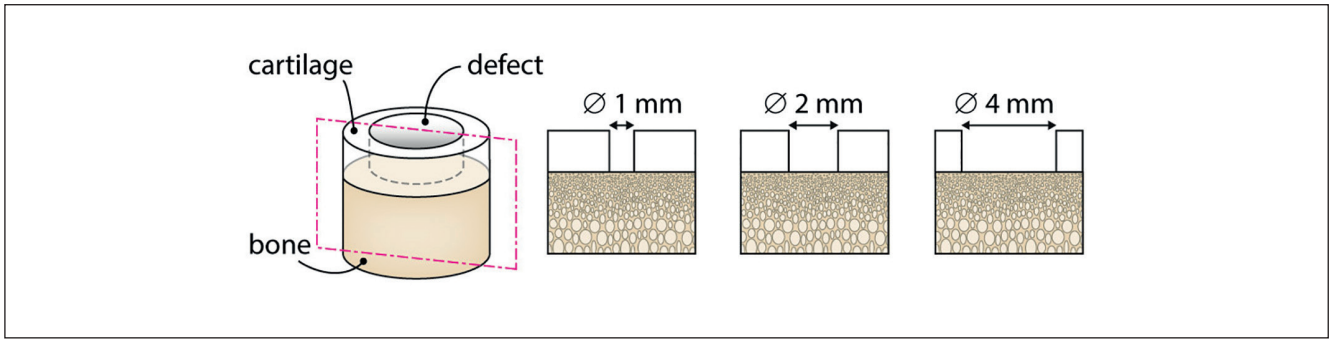

Fig. 2: Schematic illustration of the osteochondral defect model in study C

Full thickness cartilage defects in osteochondral explants with different defect diameters (⌀ 1 mm, 2 mm and 4 mm).

2.4 Analyses

Viability assay

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich, Zwijndrecht, NL) was used to evaluate viability of the cells in explants. Osteochondral biopsies were washed three times with PBS and incubated with 1 mg/ml MTT solution in DMEM for 90 min at 37°C. After washing with PBS, macroscopic pictures were taken. Freshly isolated samples served as positive control and samples that were incubated in dimethylsulfoxide (DMSO; Sigma-Aldrich, Zwijndrecht, NL) for 24 h served as negative control (data not shown).

Safranin-O/Fast Green staining

Samples from each group (study A and B) were fixed in 3.7% formalin (Sigma, Zwijndrecht, NL), decalcified in 10% EDTA (Amresco, USA) in deionized water, and processed for paraffin embedding. In study C, samples were fixed with 4.0% formalin (Roti®-Histofix, Carl Roth, Karlsruhe, GER), washed in deionized water, dehydrated and incubated in pre-infiltration solution (Heraeus Kulzer, Hanau, GER) before polymerized for plastic embedding. All samples were cut in 4 µm sections. Slides were stained with Safranin-O and Fast Green (Sigma, Zwijndrecht, NL) to visualize the distribution of proteoglycans and proteins, respectively, in the tissue. Hematoxylin (Sigma, Zwijndrecht, NL) was used as nuclear counterstaining. Slides of all three studies were imaged by light microscopy (study A: Zeiss Axio Observer Z1; study B,C: Keyence BZ-9000, Biorevo, Neu-Isenburg, GER).

Biochemical content in cartilage tissue

Cartilage samples were lyophilized and digested in a 140 µg/ml papain solution (Sigma Aldrich, Zwijndrecht, NL) for 16 h at 60°C. DNA content was determined using the Hoechst dye method with a reference curve of calf thymus DNA (Sigma, Zwijndrecht, NL). The glycosaminoglycan (GAGs) content was determined using a modification of the DMMB assay described by Farndale et al. (1982) and a shark cartilage chondroitin sulfate reference (Sigma, Zwijndrecht, NL). For determination of the collagen content (HYP), digested tissue samples were hydrolyzed in 6 M hydrochloric acid (Merck, Amsterdam, NL), and orthohydroxyproline content was assessed as described previously using an assay modified from Huszar et al. (1980), and a trans-4-hydroxyproline reference (Sigma, Zwijndrecht, NL).

Unconfined compression testing

Mechanical testing of samples of study B was performed at every time point in unconfined compression between impermeable platens using a custom-designed biomechanical testing apparatus containing a voice coil system (SMC Pneumatics, Amsterdam, NL) to determine the compressive equilibrium modulus (Eq) as previously described by Olivera et al. (2015). The cartilage thickness of each sample was measured. Then the samples were submerged in PBS at room temperature and equilibrated in creep to a tare load of 1 N for 120 s. From this offset, stress relaxation tests were performed with a ramp speed of 1 µm/s until reaching 10% strain. After equilibrium was reached, Eq was calculated from the ratio of the equilibrium stress to the applied strain.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.00. In study A, the effects of treatment and time as well as their interaction were examined for significant differences by two-way ANOVA with GAG, DNA and HYP content as dependent variables. Differences were assumed statistically significant for $p < 0.05$ of the corrected values by Tukey HSD post hoc test. For study B, the effect of culture time on Eq, GAG, DNA and HYP was analyzed by one-way ANOVA with Fisher's LSD post-hoc test.

3 Results

3.1 Study A: Effect of separation of the cartilage and bone compartments and of culture with tissue-specific media

MTT staining of explants at day 0, 14 and 28 of *ex vivo* culture revealed preserved metabolic activity over the full depth of the cartilage tissue without apparent differences between time points and groups (Fig. 3). Metabolic activity of cells residing

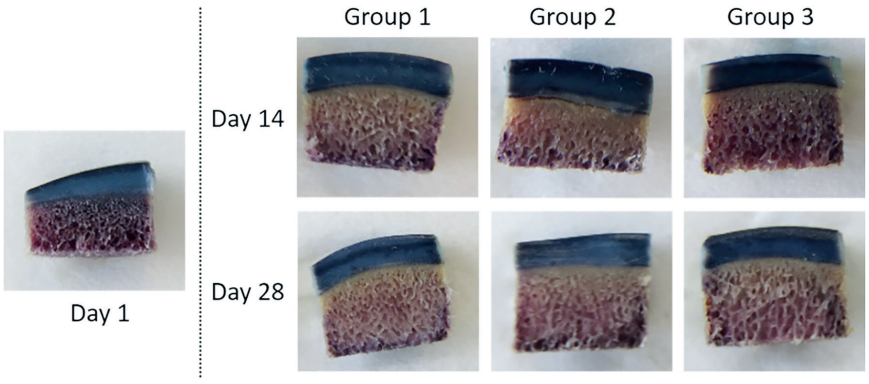

Fig. 3: Vitality staining (MTT in blue) of cross-sections of osteochondral explants cultured for 14 and 28 days *ex vivo* in study A

No differences in metabolic activity were observed in explants between groups (group 1: agarose embedding, group 2: cartilage medium in both compartments, group 3: tissue-specific medium).

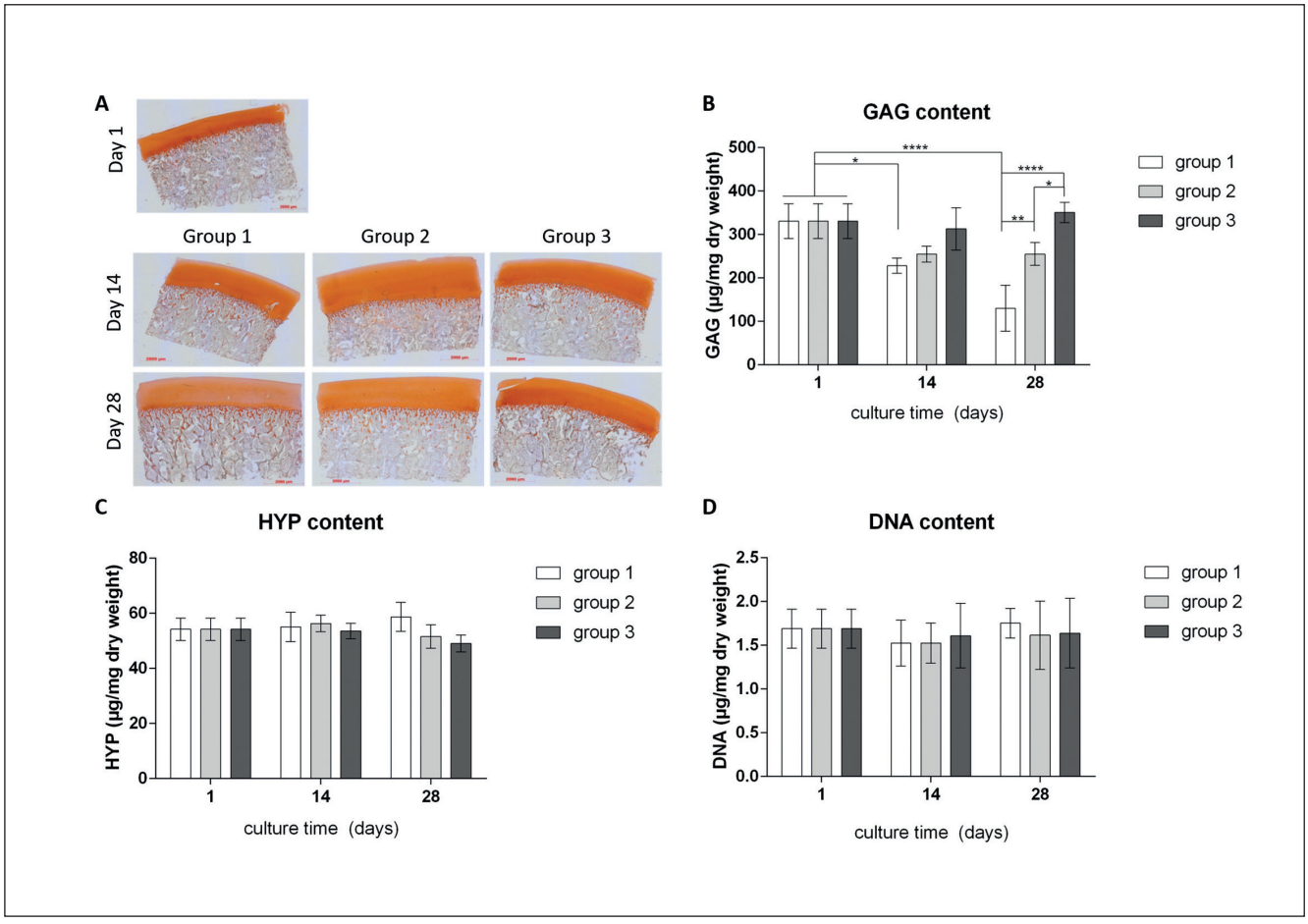

Fig. 4: Visualization and quantification of cartilage matrix composition at day 1, day 14 and day 28
(A) Safranin-O staining showing GAGs in red/orange. (B) Glycosaminoglycan (GAG) content in the cartilage, quantified as µg per mg dry weight. (C) Hydroxyproline (HYP) content in the cartilage, quantified as µg per mg dry weight. (D) DNA content in the cartilage, quantified as µg per mg dry weight (group 1: agarose embedding, group 2: cartilage medium in both compartments, group 3: tissue-specific medium). Values are displayed as mean ± SD (n = 3-4). *p < 0.05, **p < 0.005, ****p < 0.0001 compared between groups and time points.

in the subchondral bone appeared slightly decreased over time in all groups.

Osteochondral samples embedded in agarose that were cultured only in presence of cartilage medium (group 1) demonstrated a significant decrease in GAG content in culture (Fig. 4B). At day 14, GAG content of group 1 samples was significantly reduced to 68.9% of the value at day 0 ($p = 0.0231$) and GAG content decreased further to 39.3% at day 28 ($p < 0.0001$). Samples of group 2, cultured in our osteochondral platform with cartilage medium in both compartments, revealed that culturing in separated compartments reduced GAG loss (group 2, 3). After 14 days of culture 77.1% of the day 0 GAG content was still present in group 2 and no further decrease was observed between days 14 and 28. At day 28, GAG content in group 2 was significantly higher compared to group 1 at day 28 ($p = 0.0035$). Remarkably, full preservation of the native GAG content during 28 days of culture was observed in osteochondral explants cultured in separated compartments with specific medium added to the respective tissues (group 3). GAG content in group 3 at day 28 showed significantly higher GAG content compared to group 1 ($p < 0.0001$) and group 2 ($p = 0.0417$).

Safranin-O staining confirmed the quantitative findings and demonstrated that GAG loss in group 1 and 2 was most pronounced in the superficial zone of the cartilage tissue (Fig. 4A). In contrast to the GAG content, the HYP and DNA content of the samples did not show significant differences among groups or time points (Fig. 4C, D).

3.2 Study B: Long-term culture of osteochondral explants with tissue-specific culture media

No changes in MTT staining were observed over time in the cartilage tissue during 84 days of culture (Fig. 5). MTT staining in the subchondral bone seemed to decrease during the first 14 days of culture, beyond which no apparent differences were observed.

The intensity of Safranin-O staining was maintained in explants up to day 42 (Fig. 6). A slight reduction in red staining intensity was observed in the superficial zone of the cartilage

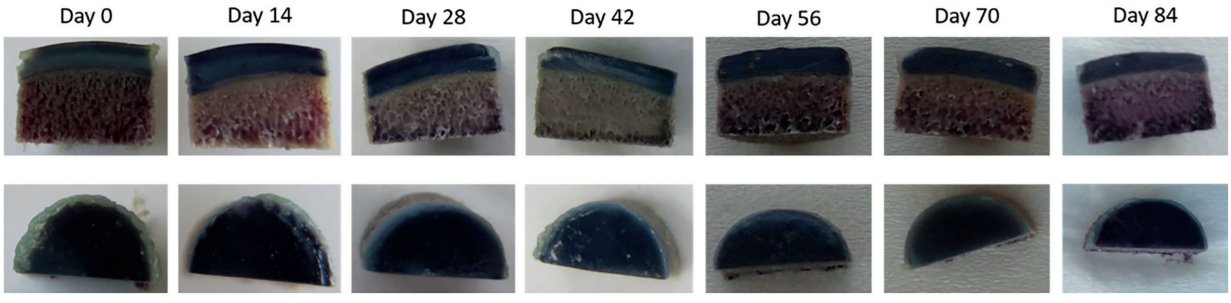

Fig. 5: Vitality staining (MTT, in blue) of osteochondral explants, with cross sections (top row) and the articular surface (bottom row)
Metabolic activity of cartilage was maintained over 84 days *ex vivo* culture in our platform with tissue-specific media.

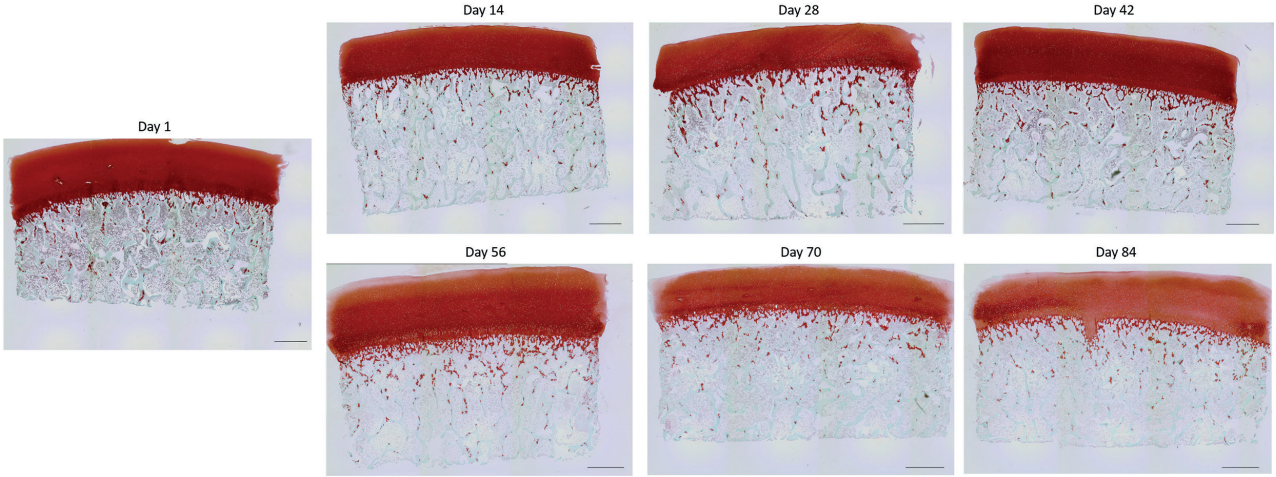

Fig. 6: Safranin-O staining of osteochondral explants during *ex vivo* culture for 84 days in our platform with tissue-specific media (Scale bar, 1,000 µm)
Preservation of Safranin-O staining up to day 42; a slight reduction in staining intensity in the superficial zone from day 56 on.

tissue at day 56. From then on, staining intensity further decreased, also becoming noticeable in the middle and deep zones of the tissue at day 70 and 84. Quantitative measurements of the amount of GAG confirmed the histological staining results (Fig. 7A). Native GAG content was preserved during 56 days of culture and then decreased (day 70 vs. day 28: $p = 0.0252$).

The collagen content in the cartilage remained stable during the entire culture period of 84 days (Fig. 7B). DNA content was higher at day 28, day 42 and day 70 compared to day 0 (Fig. 7C). The equilibrium modulus of the native cartilage tissue was preserved during 56 days of culture, similar to GAG content (Fig. 7D). After day 56, the equilibrium modulus decreased albeit not statistically significantly.

3.3 Study C: Defining *ex vivo* critical size defects

Full thickness cartilage defects with diameters of 1, 2 and 4 mm did not heal spontaneously during 28 days of *ex vivo* culture (Fig. 8B-D). Neo-tissue formation was mostly observed in defects with a diameter of 2 and 4 mm (8C'-D'). Safranin-O staining revealed deposition of GAG-containing matrix on the surface of the 2 and 4 mm cartilage defects at day 28 (indicated by arrows in Fig. 8C' and D', respectively), which was not

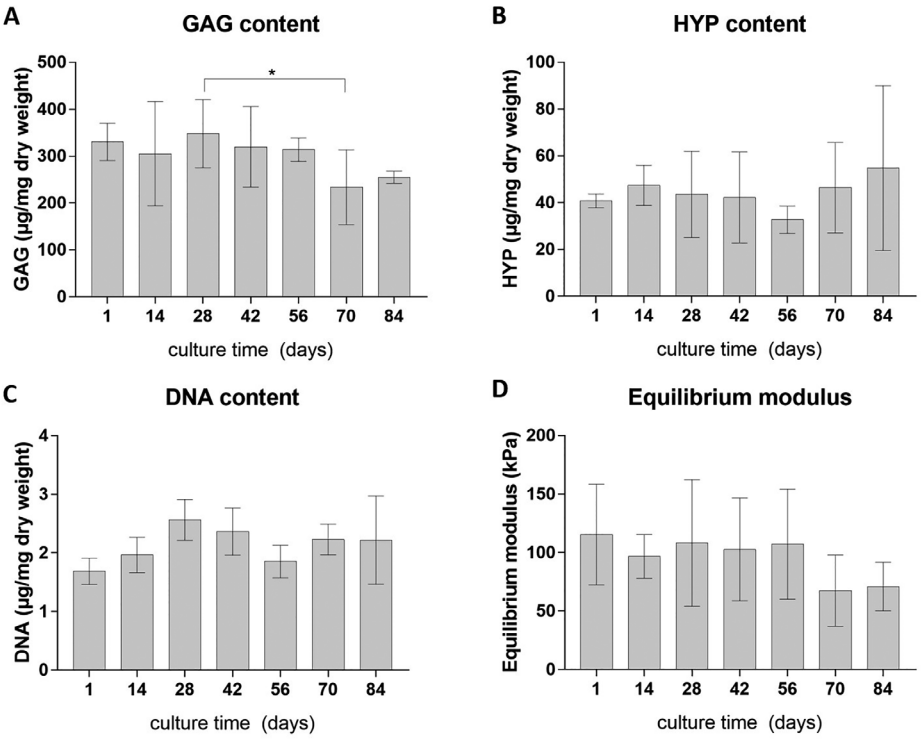

Fig. 7: Characterization of equilibrium modulus (Eq) and biochemical content (GAG, DNA, HYP) of osteochondral explants during *ex vivo* culture for 84 days
(A) Glycosaminoglycan (GAG) content normalized to cartilage dry weight. (B) Hydroxyproline (HYP) content normalized to cartilage dry weight. (C) DNA content normalized to cartilage dry weight. (D) Compressive equilibrium modulus. Values are displayed as mean ± SD (n = 3-4); *p < 0.05 compared between time points.

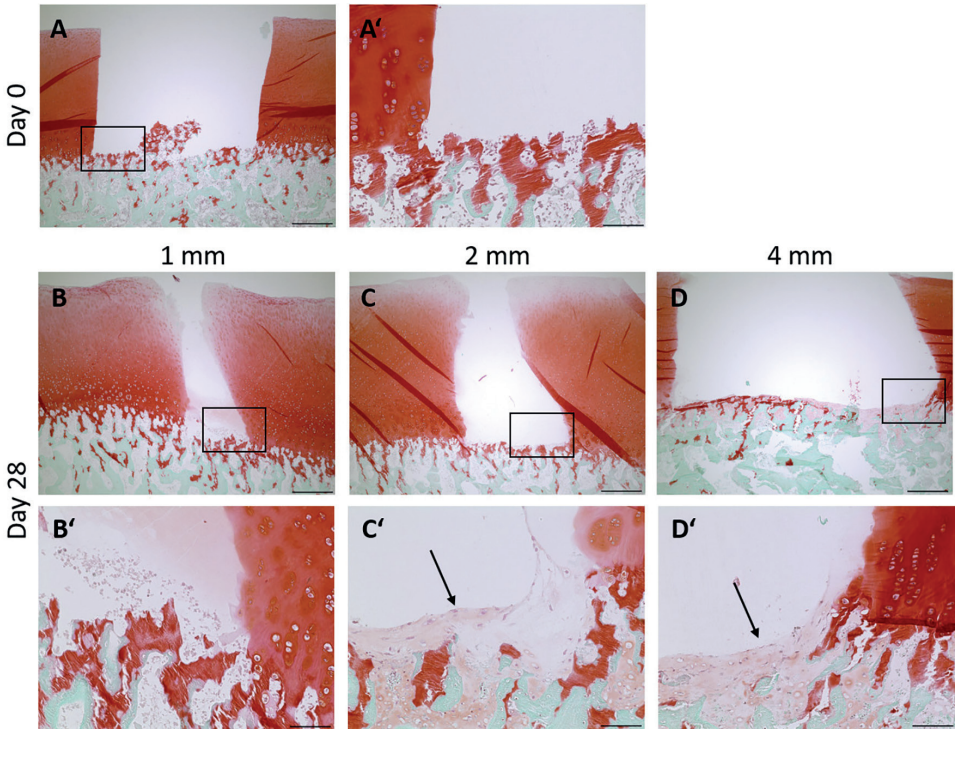

Fig. 8: Safranin-O staining of proteoglycans in osteochondral defect model after 28 days *ex vivo* culture in our platform with tissue-specific media
(A) Safranin-O staining of representative osteochondral explants with full thickness cartilage defects of 4 mm at day 0. (B-D) 1, 2 and 4 mm in diameter at day 28. (A'-D') higher magnifications of the surface of the defect. Scale bars: A-D, 500 µm; A'-D', 100 µm.

visible in samples with a 1 mm defect (Fig. 8B-B'). Migration of cells into the defect site was only observed in 2 mm and 4 mm defects. Metabolic activity was similar to results shown for study A and B (not shown).

4 Discussion

In this study, we developed a culture platform that allows *ex vivo* long term culture over at least 56 days of osteochondral biopsies under controlled conditions, while maintaining cartilage tissue content, structure and mechanical properties. It is a predictive platform suitable for preclinical assessment of cartilage repair strategies in critical size defects.

The platform is characterized by its two separated media compartments, which allows culture of cartilage and bone with tissue-specific nutrients. To our knowledge, this unique feature of two separated media compartments differs from existing *ex vivo* osteochondral culture models. The platform mimics the *in vivo* situation, in which diffusion of signaling molecules between the cartilage and bone is only possible through the subchondral bone plate at the bone-cartilage interface. In contrast to the previously described agarose model, there is no external communication via the surrounding culture medium (de Vries-van Melle et al., 2012).

We have shown that separation of compartments already partly reduced the unwanted GAG loss observed in group 1 and previously by de Vries-van Melle et al. (2014a). We postulate that this can be explained by the fact that cartilage tissue in our platform is not exposed to factors secreted by bone cells or other cells residing in the bone marrow, such as cytokines and other stress factors via the culture media. Such factors are naturally secreted by cells in the bone during apoptosis or inflammation, which can inhibit cartilage metabolism or stimulate degradation thereof. Sanchez et al. (2005a,b) have shown that sclerotic osteoblasts inhibit aggrecan production on protein and gene expression level and lead to a reduction in chondrogenic transcription factor SOX9 and collagen II on mRNA level compared to non-sclerotic osteoblasts. Furthermore, Tetlow et al. (2001) have demonstrated that the presence of inflammatory cytokines such as IL-1β and TNF-1α reduced cartilage matrix synthesis and caused proteoglycan loss.

Astonishingly, supplying the osteochondral explants with tissue-specific culture medium resulted in the complete preservation of the native ECM composition during at least 56 days of culture. It is difficult to explain which exact mechanism is responsible for maintaining cartilage tissue content and properties in case of separation of bone and cartilage tissues and using tissue-specific medium. It has been shown before that cartilage homeostasis and function strongly depend on the crosstalk between cartilage and bone (Mariani et al., 2014). It is likely that cartilage and bone cells perform optimally when they receive the specific factors they require for their metabolism, which in turn can lead to positive signaling between the two tissues. The supplementation of cartilage medium with ascorbic acid, insulin, transferrin and selenic acid is crucial to maintain the cartilaginous matrix production (Clark et al., 2002; Urban et al., 2012). In contrast to chondrocytes, bone cells require β-glycerophosphate, dexamethasone and serum, providing growth factors (Urban et al., 2012; Langenbach and Handschel, 2013).

Unlike the cartilage, MTT staining intensity of the subchondral bone decreased during culture. There are a number of cell types present in the bone, such as bone cells and blood cells. The bone cells are osteoclasts, osteoblasts and osteocytes, which are responsible for bone remodeling, but make up only a small percentage of the total cells present in the bone (Taichman, 2005). Due to the low concentration of bone cells in trabecular matrix, we believe that they are hardly visible in the MTT staining. We therefore assume that mainly dying blood cells are the cause of the decreased viability staining of the bone. Because of the interrupted blood circulation after explanting the biopsies, the blood cells and platelets no longer have a vital function and therefore likely undergo apoptosis. In future studies, we aim to gain more insight into the process of active bone remodeling in our explants by staining or quantification of alkaline phosphatase activity or by visualization of osteoclast activity by means of tartrate-resistant acid phosphatase staining.

We demonstrated that we are able to maintain native GAG content and mechanical properties for 56 days with tissue-specific medium, which, as far as we know, has not been shown in any other *ex vivo* osteochondral culture model before. Culture duration of 56 days is a relevant period for testing repair strategies and the model can therefore, at least partly, replace animal testing or serve as a screening tool before testing in animals. An array of tests with dozens of samples can be performed simultaneously to study the effects of material composition and mechanical properties, compound concentrations, drug dose responses, etc.

We hypothesize that incorporating mechanical loading into the system will allow for even longer culture periods, as we postulate that the decrease in GAGs and reduction in equilibrium modulus in cartilage after 56 days of *ex vivo* culture is caused by the absence of mechanical triggers. Numerous groups have shown the importance of mechanical loading on cartilage metabolism (Guilak et al., 2004; Vinardell et al., 2012; Kock et al., 2012, 2013). As a next step in the development of our model we are now integrating a mechanical loading module into the platform while maintaining a closed system.

In study C, all three defect sizes tested in our platform proved to be critical size defects. No explant cultured *ex vivo* over 28 days attained complete healing of full thickness cartilage defects. Since larger defect diameters are more feasible for use with implant materials, we will use 4 mm as the optimal *ex vivo* critical size defect diameter of full thickness cartilage defects. This is smaller than the typically described critical sizes of *in vivo* cartilage defects in animal studies, such as for example in adult miniature pigs in which critical defect diameters range from 4.5 mm to 6.0 mm (Harman et al., 2006; Chang et al., 2014). However, compared to animal studies, it is much easier to create large numbers of samples with cartilage defects with different diameters and depths (chondral, subchondral, osteochondral) in a reproducible and controlled manner in our model.

Therefore, the defect model represents a tool for studying clinically relevant cartilage repair therapies, namely preclinical testing of cartilage biomaterials for their regenerative potential, evaluation of drug and cell therapies, or to study basic biological mechanisms and pathways during cartilage regeneration, which will certainly reduce the number of animals needed for *in vivo* testing.

5 Conclusion

To conclude, our *ex vivo* culture platform with the unique feature of separated compartments for cartilage and bone allows for long-term culture of osteochondral explants while maintaining cartilage tissue content, structure and mechanical properties. As such, our model offers a highly relevant new platform for assessment of cartilage repair strategies in critical size defects.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

HydroZONES – The work leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 309962. The osteochondral culture platform is developed and owned by LifeTec Group BV, a company specialized in contract research.

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