Pro chondrogenic effect of mesenchymal stromal cell-based treatment of chondral defects under physioxia in a novel ex vivo organ model

Andrea Schwab 1, Alexa Buß 1, Oliver Pullig 1,2, Franziska Ehlicke 1,5

1 University Hospital Wuerzburg, Department Tissue Engineering and Regenerative Medicine,
2 Roentgenring 11, 97070 Wuerzburg, Germany
3 Fraunhofer Institute for Silicate Research ISC, Translational Center Regenerative Therapies,
4 Roentgenring 11, 97070 Wuerzburg, Germany

Andrea Schwab
- University Hospital Wuerzburg, Department Tissue Engineering and Regenerative Medicine
- Present address: AO Research Institute Davos, Clavadelerstrasse 8, 7270 Davos, Switzerland
- E-Mail: andrea.schwab@aofoundation.org

Alexa Buß
- University Hospital Wuerzburg, Department Tissue Engineering and Regenerative Medicine,
- Present address: Klinikum Würzburg Mitte gGmbH, Juliussspital, Juliuspromenade 19, 97070 Wuerzburg, Germany
- E-Mail: alexa.buss@kwm-klinikum.de

Oliver Pullig
- University Hospital Wuerzburg, Department Tissue Engineering and Regenerative Medicine,
- Fraunhofer Institute for Silicate Research ISC, Translational Center Regenerative Therapies,
- Roentgenring 11, 97070 Wuerzburg, Germany

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Abstract

Objective

High failure rates of (trauma induced) knee injuries highlight the need to improve current treatment strategies aiming to decrease the number of secondary osteoarthritis developed by patients in later stage [1-3]. In this controlled laboratory study the stimulative effect of mesenchymal stromal cells (MSC) on chondrocyte (CHON) extracellular matrix production was investigated in an *ex vivo* cartilage defect model (chondral vs. full thickness defects) cultured under normoxic (20 % O$_2$) and physioxia (2 % O$_2$) conditions.

Design

Porcine CHON or co-culture of 20 % CHON and 80 % MSC (MIX) were embedded in collagen type I hydrogel, implanted into 4 mm diameter cartilage defects of osteochondral explants and cultured with tissue specific media without addition of TGF-β under normoxia and physioxia. Chondral defects were induced automatically, while full thickness defects were created with biopsy punch. After 28 days of culture, samples were histologically processed, and treatments outcome was evaluated using international cartilage repair society (ICRS)-II scoring.

Results

Under physioxic conditions, cartilage repair scoring results of the MIX treatment (chondral 8.67 ± 2.42, full thickness 5.67 ± 1.21) were close to those of CHON treatment (chondral 8.17 ± 0.75, full thickness...
7.33 ± 1.21). Overall, scoring results were higher in physioxia compared to normoxia conditions in chondral defects, but less or no prevalent for full thickness defects.

Conclusion

Co-culture of CHON with MSC represents a promising approach to stimulate chondrogenic repair and tissue formation in our ex vivo model and reduces total amount of CHON needed for cell-based treatment.

Keywords

cartilage defect, ex vivo model, cartilage test system, chondrocytes, MSC, collagen type I
hydrogel

1 Introduction

Defects of the articulating surface are frequently occurring diseases in the field of orthopedics, traumatology and sports medicine [4]. Around 14% of patients who suffered from a trauma induced knee injury during their middle ages, including fractures of the tibia, fibula, femur, or patella, develop osteoarthritis in the same knee joint at later age (>65 years) [3]. High risk of failure of more than 20% in cartilage defect repair require to rethink and improve current cartilage treatment concepts with the aim for long-term repair of cartilage defects [1, 2].

Due to the avascular and aneural character of adult articular cartilage, large defects do not heal spontaneously [5]. Defects that are classified according to International Cartilage Repair Society (ICRS) grade III (defect depth >50% cartilage depth) and IV (defect reaches subchondral bone) need surgical intervention to repair [6]. Gold standard for defects larger than 2-3 cm² is the autologous chondrocyte implantation (ACI), resulting in similar outcome as microfracture [7, 8]. In a recent data analysis for cartilage defects, matrix-associated chondrocyte implantation (M-ACI) showed a significant lower reoperation rate than microfracture 2 years post-op [9]. M-ACI as well as ACI require two surgical
interventions; the first to take a cartilage tissue sample for subsequent chondrocyte isolation and the second one for injection of isolated in vitro expanded autologous chondrocytes into defect site.

However, (M-)ACI has two main limitations: Due to the limited number of healthy and non-degenerated chondrocytes, cells have to be expanded in vitro to achieve sufficient number of cells needed for the implantation (ACI: 1 Mio/cm², M-ACI: 20 Mio/cm³) [10-12]. Further, chondrocyte-based cartilage treatments, as well as microfracture techniques, result in formation of fibrocartilage, characterized by its inferior mechanical properties and thus lacking functional restoration compared to healthy hyaline cartilage [2, 13-17]. One approach to overcome the limitation of cell number for (M-)ACI treatment are MSC-chondrocyte co-cultures, e.g., 80 % MSC and 20 % chondrocytes, that reduces autologous chondrocyte cell number at same total cell density. Co-cultures of MSCs and chondrocytes have shown to increase cartilaginous matrix production and reduce hypertrophy, associated with MSCs during chondrogenic differentiation [18, 19].

To bring cartilage treatments to the next level, cartilage repair needs to be studied and understood in more detail, starting with basic research questions in a physiologically relevant environment.

An ex vivo cartilage defect test system based on osteochondral explants was established by Schwab et al. [23]. This test system represents a valuable ex vivo platform for biomaterial evaluation in critical size trauma-induced cartilage defects in terms of biocompatibility, biomaterial tissue integration and cartilage repair with higher throughput compared to in vivo models. Separated media compartments of the culture device allow for controlled, tissue- and cell-specific nutrient supply during ex vivo culture of osteochondral explants [20]. This model also allows direct comparison of different treatment approaches under controlled conditions and has been shown to stimulate cartilage-like tissue formation of chondrocytes or MSCs in osteochondral lesions [21, 22].

However, this defect model was limited to full thickness defects created with a biopsy punch. To date, the creation of defects that do not fully penetrate the cartilage with the help of a biopsy punch or scalpel suffer from low reproducibility and are dependent on the operator. Therefore, an automated device with
control over drilling depth to create defects with high reproducibility can overcome this limitation to study chondral wound healing. Of note, the drilling should not harm surrounding cartilage tissue and cell viability, neither by mechanical tissue disruption nor by friction induced heating. For the creation of defects in hard tissues like cartilage or even bone, there is no device available for automated defect creation that meets the above-mentioned requirements.

Following, in the present study the defect creation of the ex vivo defect model introduced by Schwab et al. was modified by implementation of a semi-automated drilling device, originally developed to mechanically induce standardized and reproducible wounds in full thickness skin equivalents [23]. Key features of this artificial tissue cutter (ARTcut®) are the sensor controlled optical barrier in combination with a moveable milling machine along x-, y- and z-axis. The light-barrier controlled drilling allows for creation of defects with defined depths in a reproducible set-up. Creation of tissue defects with ARTcut® can be performed under sterile conditions for subsequent in vitro or ex vivo culture with the possibility to adjust defect geometry and control of defect creation process.

Another fact that has been neglected in the studies by Schwab et al. is the low oxygen tension present in the articular capsule [24]. It has been shown in literature that low oxygen tension is an important stimulus in chondrogenesis and increases the chondrogenic potential of articular cartilage progenitor cells and MSCs [24-26].

Taken together, the present study aimed to investigate two cartilage treatment strategies and the influence of defect depth (full thickness defects and chondral defects) on tissue repair. Additionally, the effect of oxygen tension (normoxia 20 % vs. physioxia 2 %) was studied in both defect and treatment approaches. For the treatment strategies either chondrocytes only (CHON) or a mixture of 80 % MSC and 20 % CHON (MIX) were embedded in a collagen type I hydrogel for implantation into cartilage defects in the ex vivo osteochondral model without further supplementation of growth factors to elusively study the effect of the culture conditions on cartilage repair.
2 Methods

2.1 Osteochondral cylinders: Isolation, defect creation and ex vivo culture

Osteochondral cylinders (diameter: 8 mm, height: 5 mm) were isolated from medial femoral condyles of 6-8-month-old domestic pigs as previously described [20]. Creation of defects in osteochondral cylinders was carried out either manually or automatically, depending on the intended defect depth: Full thickness defects were manually created with a biopsy punch (diameter 4 mm; Kai Medical, BPP-40F). Chondral defects of 1 mm depth were created using custom-built ARTcut® (Figure 1A), a software-controlled device for automatic wound placement in tissues developed by Fraunhofer ISC and IGB, Wuerzburg [23]. Osteochondral cylinders were fixed in support plate (Figure 1C) at specific x- and y-positions, and onset of drilling 1 mm chondral defects (z-axis) was determined with light beam (Figure 1B). For more details on the standardized defect creation process using ARTcut®, see supplementary information.

After creation of full thickness or 1 mm chondral defects, the cartilage defects were filled with cell embedded collagen type I hydrogels (Error! Reference source not found. 2). For subsequent ex vivo culture, osteochondral explants were transferred into custom-made culture platform [20] and cultured for 28 days with tissue specific media (Table 1) changed every 3-4 days. The ex vivo culture was carried out in a humidified atmosphere at 37°C and 5 % CO₂ (BBD 6220 CO₂ Incubator, Thermo Scientific™) either under normoxic (20 %) or physioxic (2 %) conditions.

2.2 Chondrocyte and mesenchymal stromal cells (MSC): isolation and in vitro expansion

Porcine chondrocytes were isolated from lateral condyles of 6-8 month-old domestic pigs by enzymatic digestion as previously described [20]. Chondrocytes were used at passage 0 – directly after isolation - for embedding in collagen type I hydrogel.

Porcine MSC were isolated from bone marrow aspirate (iliac crest) under the approval (reference number: 55.2 2532-2-256) of the District Government of Lower Franconia and the local animal welfare committee and performed according to the German Animal Welfare Act and the EU Directive 2010/63/EU.
gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences, 17-5442-03), mononuclear cell fraction was expanded in DMEM HG medium (GlutaMax, Gibco 61965) supplemented with 1 ng/mL FGF-2 (R&D Systems, 233-FB-025), 10 % FCS (Gibco, 10270106) and 1 % Antibiotic-Antimycotic (10 U/mL penicillin, 10 µg/mL Streptomycin, 0.25 µg/mL Amphotericin, Gibco, 15240) in humidified atmosphere (37°C, 5 % CO₂). Media change was performed every 3-4 days. MSC were used at passage 2-3 for embedding in collagen type I hydrogel.

2.3 Collagen type I hydrogel: hydrogel preparation and cell embedding

Cartilage defects were treated with collagen type I hydrogel. Collagen was isolated from rat tails (Wistar IGS Rat; Charles River Laboratories), dissolved in 0.1 % (v/v) acetic acid to a concentration of 4.5 mg/mL, and mixed with half volume of gel neutralizing solution (GNS) to achieve a final concentration of 3 mg/mL. GNS consists of 2x DMEM high glucose (DMEM, PAA Laboratories, G0001,3010), 0.09 M HEPES (Sigma, H3375), 3 % FCS (Bio&Sell, FCS.ADD.0500), 0.05 mg/mL chondroitin sulphate (Sigma, C4384) with pH value adjusted to pH 8.5. Cell embedding was realized by mixing the porcine chondrocytes and/or MSCs in GNS, resulting in a final total cell concentration of 20 Mio/mL hydrogel. Gelation was performed via incubation of GNS collagen solution mixture for 20 min at 37°C under humidified atmosphere.

For implantation, two different cell laden constructs, as illustrated in Figure 2, were prepared and each cultured under normoxic and physioxic conditions. 1) CHON were prepared by embedding of chondrocytes in collagen type I hydrogel without expansion at a final density of 20 Mio/mL hydrogel volume; 2) MIX were prepared by mixing 80 % MSC with 20 % chondrocytes at same cell density. CHON and MIX implants were filled into the cartilage defect for gelation at 37°C.
2.4 Live-dead viability staining

To visualize a possible effect of defect creation process on cell viability of tissue samples, live-dead viability staining was performed (live-dead staining kit for mammalian cells, L3224, Invitrogen). Osteochondral explants were incubated with 4 µM Calcein AM and 2 µM ethidium homodimer-1 in DMEM HG and visualized with fluorescence microscope (494 nm/517 nm and 517 nm/617 nm wavelength) Keyence BZ-9000 (Biorevo). Living cells are stained by Calcein AM in green, dead cells are stained by ethidium homodimer-1 in red.

2.5 Histological evaluation

Osteochondral explants, harvested on day 0 and day 28, were washed with phosphate buffered saline, fixed for 24 h with 4 % formalin (Roti Histofix, Carl Roth P087.3) and processed with plastic embedding (Technovit T9100, Heraeus Kulzer, 66006735) as detailed described in the supplementary.

For immune-histological stainings, antigens were enzymatically retrieved depending on antibody (Table 2). Before incubation with primary antibody over night at 4°C, slides were blocked with 3 % (v/v) H2O2 (Carl Roth, 8070) and incubated with 5 % (w/v) bovine serum albumin. All stainings were visualized with Horseradish-peroxidase kit (DCS Innovative, Dako, K3468) following manufacturer’s instructions. Cell nuclei were counterstained with Mayer’s hematoxylin (Morphisto, 11895), rehydrated and mounted with Entellan (Merck, 1079610500). All washing steps between blocking or chemical incubation were performed with PBS supplemented with 0.5 % Tween-20 (VWR, 8.22184).

2.6 Histological scoring

Histological scoring for the evaluation of the immunohistological stainings was performed according to the standardized guidelines of the international cartilage repair society (ICRS-II-Score). All stainings for collagens type I, II, X and aggrecan of this study (n = 2 biological replicates) were evaluated blinded by three independent operators according to the criteria summarized in Table 3. Maximum possible score for the here reported study was 12 points.
2.7 Statistics

Statistical analysis was performed with GraphPad Prism 6.07. Scoring values obtained from two independent experiments, each evaluated by three examiners were used for statistical analysis, resulting in \( n = 6 \) for each experimental group (Figure 2). Following tests for outlier and Gaussian distribution, the data was either compared using an unpaired t-test (normally distributed values) or a Mann-Whitney test (not normally distributed values). A \( p \)-value <0.05 was considered statistically significant.

3 Results

3.1 Chondral defect creation with ARTcut®

ARTcut® (Figure 1) represents an automated device for reproducible creation of chondral defects (1 mm in depth; Figure 3A). Main advantage of using the ARTcut® is the ability to create defined chondral defects with a flat bottom to allow a tide and uniform contact with any solid pre-formed cylindrical implant, for example bioprinted constructs with flat bottom. The laser beam to detect the surface of every single osteochondral explant is the unique feature of the ARTcut® to define the onset of the drilling, thus all defects result with the same depth. Chondral cartilage defect induced with ARTcut® appeared as a well-defined rectangular defect boundary in cross section (diameter: 4 mm, height: 1 mm) with flat bottom (Figure 3B). In contrast, the manually created defect (using biopsy punch) resulted in a half-round shape and required removing of residual cartilage tissue with a second tool (e.g., a sharp spoon) shown in Figure 3C. Following, the accuracy of chondral defect geometry was much lower in manual procedure - using biopsy punch - compared to software – using automated ARTcut®.

The automated defect creation took less than 5 sec and thus heating of tissue due to friction and rotation during drilling was very unlikely. Microscopic images of live-dead stainings of osteochondral explants with
1 mm cartilage defects (diameter: 4 mm) confirmed no indications of cell death after tissue defect creation, neither with ARTcut® nor with biopsy punch (Figure 3D-E).

For the creation of full thickness cartilage defects, the ARTcut® was not required, since the bottom of the defect was given by the cartilage-bone interface. This two-tissue interface was easily separated using biopsy punch by pulling out the upper cartilage part.

3.2 Evaluation of cartilage treatments in the ex vivo model

The results of our study demonstrate the feasibility and reproducibility of the ex vivo cartilage test system as a platform to compare cartilage repair strategies: Scoring results of the co-culture (MIX) are close to the CHON treatment in chondral and full thickness defects. Results of the adapted ICRS-II scoring are shown in Figure 4.

Under physioxic conditions, scoring results of defect repair after co-culture treatment (MIX) was significantly higher ($p = 0.0218$) in chondral defect than in full thickness defects. Scoring values of CHON treatment in chondral and full thickness experimental groups did not differ much (n.s. $p = 0.1828$). At physioxic conditions the scoring value was significant higher for CHON treatment compared to MIX treatment in full thickness defects ($p = 0.0384$).

Under normoxic conditions, full thickness defects reached higher scoring values compared to the chondral defects.

Comparing the outcome of same defect depth and treatment group (cell type), but cultured under physioxic or normoxic conditions, the oxygen tension plays only a significant role in chondral defects using MIX ($p = 0.0451$, higher scoring values under physioxic conditions) and in full thickness defects treated with CHON ($p = 0.0316$, lower scoring values under physioxic conditions).

Overall, treatment of the two defect depths with MIX showed slightly reduced scoring values compared to the CHON treatment group. Only exception is MIX chondral (physioxia) with a slightly higher value as
the corresponding one for CHON. The results of the MIX treatment groups also tend to spread more than the CHON treatment group independently of defect depth and oxygen tension.

4 Discussion

The choice of optimal treatment approach for cartilage repair is dependent on the size and depth of the cartilage defect and thus the severity of pathological condition.

To mimic these different pathological defect scenarios, the implementation of the ARTcut® allowed to overcome the low reproducibility of manual induced chondral defects. So far, there is no other automated defect creation device published that has comparable features, namely possibility to select different drilling parameters within one run and the application of one device for a wide range of soft and hard tissues or tissue equivalents, as it is demonstrated with the ARTcut®. The induction of defects in osteochondral explants allows to compare different cell-based treatment approaches by implantation of cell loaded materials into the defects or to study the effect of defect depth on repair in an ex vivo model.

The software-controlled drilling parameters allow for standardized and reproducible defect creation in other soft and hard tissues including bone tissue as well. In vitro as well as ex vivo studies require sample procession at high throughput rates under a negative microbial environment. ARTcut® device addresses these demands, resulting in higher methodological accuracy and reproducibility, compared to manual procedure using biopsy punch.

Current treatments of small or large cartilage defects mainly aim on pain and symptom relief rather than on functional tissue repair [27]. From a scientific point of view, there is way for improvement of cartilage defects, especially on long term results. Material-assisted as well as cell-based therapies, including (M-)ACI, AMIC and microfracture, tend to result in formation of mechanical inferior fibrocartilage in long term follow up [27-29]. Moreover, the treatment of large defects with autologous cells are limited due to the high number of chondrocytes needed for the current techniques. Therefore, in this laboratory-controlled study, an ex vivo osteochondral defect model was modified to screen cell-based approaches to treat
chondral and full thickness cartilage defects. Different cell types, namely chondrocytes and MSCs, were embedded in collagen type I hydrogel (CHON or MIX) and implanted in trauma induced chondral and full thickness defects. A stimulative and beneficial effect of MIX treatment (20 % CHON and 80 % MSCs) was observed regarding cartilaginous matrix formation with the main advantage to reduce the overall number of CHON used for traditional ACI treatment.

Promising results regarding cartilage matrix production were obtained comparing CHON and MIX in chondral and full thickness defects in the ex vivo model. The MIX treatment reduces the amount of autologous cartilage tissue needed for chondrocyte isolation, since chondrocytes represented only 20 % of the total cell number in our model. Several in vitro studies and in silico models have shown that chondrocytes in co-culture with MSCs increase the chondrogenic differentiation potential [18, 19, 30, 31].

Scalzone et al. reported of an enhanced chondrocyte activity measured by proteoglycan and collagen type II production after co-culture with MSC compared to chondrocyte monoculture both embedded in chitosan based hydrogels [19].

One advantage of the co-culture approach – when clinically applied – would be the one-step procedure, addressing the surgical intervention of the knee joint, for the treatment of the cartilage defect: The reduced number of chondrocytes in the MIX treatment can be isolated intra-operatively without expansion and thus reduced the risk of chondrocyte dedifferentiation [32]. Further, the required MSCs, e.g., can be harvested independently form the knee surgery from bone marrow aspirate in a minimal invasive procedure with subsequent expansion prior to the cartilage repair procedure to ensure a sufficient number of MSCs.

Considering the absence of TGF-β in the culture media, which is known to drive MSC chondrogenesis [33], the here reported cartilage repair solely derives from the stimulating effect of the surrounding osteochondral tissue, cell-cell signaling of the cell laden implants and the culture conditions.
Results of a similar *ex vivo* model, based on horse and bovine osteochondral explants, suggested that the presence of the osteochondral tissue in the explants increase cartilage-like matrix deposition compared to free swelling conditions of cell seeded material only [21, 22].

Beside the cell source, the influence of oxygen tension (normoxia 20 % O$_2$, physioxia 2 % O$_2$) was studied during the *ex vivo* culture on chondrogenic matrix deposition, resulting in different outcomes between the treatment groups in chondral and full thickness defects. Based on the histological scoring, our results showed that the oxygen tension only plays a role in chondral defects - marked by elevated matrix production. In contrast, full thickness defects showed different response in cartilage-like matrix deposition related to the treatment approach: While higher scoring values were reached with CHON treatment under normoxic conditions, the pMIX treatment did not show differences comparing physioxic and normoxic conditions. Physioxic conditions resulted in an increase in cartilaginous matrix deposition in chondral defects compared to full thickness defects independently of the used cell type (CHON, MIX). Once the defect reaches the cartilage-bone interface, the cells in the implant receive stimuli from the subchondral bone that seem to counteract with the physioxic induced stimuli in the here presented model. One explanation of the differential cell response to oxygen tension in chondral and full thickness defects may originate from the variation in oxygen tension present in the human body. Chondrocytes are exposed to lower oxygen tension in avascular cartilage (2-5 %) [34], synovial fluid and the synovial capsule (6.5-9.0 %) [25, 35], with an increasing oxygen level in the bone marrow of subchondral bone (>7 %) [26, 36] and a maximum of 12 % in arterial blood [37]. Once the cartilage defect progresses to the exposure of the subchondral bone in patients, chondrocytes within the defect are exposed to higher levels of oxygen present in the bone supplied by vascular invasion [38]. It has been shown that low oxygen inhibits the degradation of hypoxia induced factors (HIF) [39]. HIFs are described to be essential for maintaining CHON homeostasis and extracellular matrix synthesis and activate the transcription of genes [40].

While the here presented results are consistent, traceable and in line with literature, there is one limitation in the current experimental setup: Due to limitations in availability of human healthy osteochondral tissue...
absence of any osteoarthritic phenotype – the authors decided to use porcine tissue instead, as introduced by Schwab et al. [20]. The performance of an extensive comparative ex vivo study comprising three parameters – namely 1) defect depth (chondral vs. full thickness), 2) different cell types for defect filling and treatment (100 % chondrocytes vs. 20 % chondrocytes and 80 % MSCs), and 3) oxygen tension (physioxia vs. normoxia) requires tissue material in the required quantity and of similar quality. Due to the well-known donor issues associated with donor variability of human tissue and the already mentioned limited availability of healthy human osteochondral tissue, this could not be achieved for human tissue [41, 42]. In contrast, the use of porcine tissue isolated from one pig population (similar age; grown up under equal conditions) minimizes the donor variation and represents a tissue source for healthy and non-degenerated tissues with higher reproducibility. Overall, the results obtained in this study strongly indicate that the surrounding tissue of the osteochondral explants plays a crucial role in the defect repair, addressing chondral or full thickness defects, and that the oxygen tension additionally stimulates the outcome in a defect depth dependent way. However, the exact mechanism controlling success or failure of a treatment approach are not fully understood and require further research. Despite the advantage of the here reported model, the mimicry of the complex 3D environment present in the knee joint with the additional ability to reproducibly create (trauma induced) defects, this model has its potential in pre-screening several biomaterials or treatment approaches, but best candidates still require further pre-clinical and clinical testing to confirm obtained results.

To conclude, in this ex vivo study two cell-based approaches (CHON vs. MIX embedded in collagen type I hydrogel) for the treatment of chondral and full thickness defects, cultured under normoxic and physioxic conditions, were compared on their cartilage repair potential. Independently of defect depth, the co-culture of chondrocytes and MSC (MIX) has proven scoring results close to those of CHON treatment. Hence, usage of MIX could be a promising approach to reduce the number of chondrocytes to one fifth and thus the amount of tissue for harvest and accompanied side morbidities for the patient. Further, in
this experimental set up the oxygen tension showed an influence in a defect depth dependent way: an increased extracellular matrix production resulted in chondral defect under physioxic conditions but was not present in full thickness defects. This finding of defect dependent stimulus of oxygen tension on cartilage repair has not been reported in literature before.

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Author contribution

Conception and design of the study: Franziska Ehlicke and Andrea Schwab; Acquisition of data: Alexa Buss and Andrea Schwab; Analysis and interpretation of data: Andrea Schwab, Alexa Buss, Franziska Ehlicke and Oliver Pullig; Manuscript drafting: Andrea Schwab and Alexa Buss; Revision of the manuscript: Franziska Ehlicke and Oliver Pullig; Final approval: Franziska Ehlicke, Andrea Schwab, Alexa Buss and Oliver Pullig; Funding: Franziska Ehlicke and Alexa Buss.

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Competing interest statement

The authors declare no conflict of interest.

Ethics approval and consent to participate
Animal experiment was approved (reference number: 55.2 2532-2-256) by the District Government of Lower Franconia and the local animal welfare committee and performed according to the German Animal Welfare Act and the EU Directive 2010/63/EU. Following heparinization of the pig, porcine MSCs were obtained by bone marrow aspiration.
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Figure Legends

Figure 1: Artificial tissue cutter (ARTcut®): A) View of the computerized numerical controlled device equipped with B) optical barrier (white arrow indicates the laser light beam) and a C) supporting plate to fix the samples and place on the bottom of the machine (marked with black star). Reprinted from Schwab 2017 [43].

Figure 2: Schematic overview of all experimental groups: Full thickness and chondral defects of osteochondral explants were treated with collagen type I hydrogel (grey) either containing chondrocytes (CHON, blue) or MIX (80 % MSC and 20 % chondrocytes), implanted in chondral or full thickness defects and cultured ex vivo under normoxic (20 % O₂, blue frame) or physioxic conditions (2 % O₂, red frame).

Figure 3: Osteochondral explant cross section with chondral defect. A) Schematic illustration of A) 4 mm chondral defect. B-E) Comparison of chondral defect created automated with ARTcut® (B) and manually with biopsy punch (C) (scale bar 500 µm). D-E) Live-dead staining of explant cross section highlighting accurate borders of defect induced with ARTcut® (D) and more irregular shaped geometry of manually induced defect (E) (scale bar 200 µm). Reprinted from Schwab 2017 [43].

Figure 4: Evaluation of defect repair after 28 days ex vivo culture. Macroscopic images of full thickness and chondral defects (diameter 4 mm) treated with A) porcine Chondrocytes (CHON) or B) co-culture of porcine MSC and CHON (MIX). Red frame indicates culture under physioxic, blue frame under normoxic oxygen tension. Macroscopically, there are no obvious differences comparing full thickness and chondral defects. C-D) Scoring evaluation of cartilage repair strategies in the ex vivo model (mean ± standard deviation with single scoring results as dots resp. listed values for 1st and 2nd biological replicate). Unpaired t-test * p < 0.05, ** p < 0.01.
Figure 2

CHON

MIX

full thickness defect

cartilage
subchondral bone

Cells
• MSC
• chondrocytes
• MIX (MSC 80%, chondrocytes 20%)

Biomaterial
• collagen type I hydrogel

Culture conditions (oxygen tension)
• normoxia 20% O₂
• physioxia 2% O₂
Figure 3

A

4 mm

chondral defect

B

ARTcut®

C

biopsy punch

D

E
Figure 4

Ex vivo culture under normoxia 20% O₂ or physioxia 2% O₂

|                  | CHON (2% O₂) | Normoxia (20% O₂) | Physioxia (2% O₂) | Normoxia (20% O₂) |
|------------------|--------------|--------------------|--------------------|--------------------|
| **Cumulative score (max 12)** | 7.33 ± 1.21   | 9.17 ± 1.33        | 5.67 ± 1.21        | 6.5 ± 1.38         |
| **1st biological replicate** | 9; 8; 8      | 9; 9; 7            | 6; 7; 4            | 8; 8; 7            |
| **2nd biological replicate** | 7; 6; 6      | 9; 11; 10          | 5; 7; 5            | 5; 5; 6            |
| **Mean ± standard deviation** | **8.17 ± 0.75** | **7.50 ± 0.84**   | **8.67 ± 2.42**    | **5.17 ± 2.86**    |
| **1st biological replicate** | 7; 8; 9      | 7; 7; 7            | 11; 9; 12          | 4; 2; 2            |
| **2nd biological replicate** | 8; 9; 8      | 9; 8; 7            | 7; 7; 6            | 8; 8; 7            |
| **Mean ± standard deviation** | **8.17 ± 0.75** | **7.50 ± 0.84**   | **8.67 ± 2.42**    | **5.17 ± 2.86**    |
Table 1: Composition of bone and cartilage media for ex vivo culture of osteochondral explants.

|                          | Cartilage media                  | Bone media                  | Supplier               |
|--------------------------|----------------------------------|-----------------------------|------------------------|
| DMEM GlutaMAX (high glucose) | 98 %                              | 89 %                        | Gibco 61965            |
| Antibiotics              | 10 U/mL penicillin,              | 10 U/mL penicillin,         | Gibco, 15240           |
|                          | 10 µg/mL Streptomycin,           | 10 µg/mL Streptomycin,      |                        |
|                          | 0.25 µg/mL Amphotericin (1 % (v/v)) | 0.25 µg/mL Amphotericin (1 % (v/v)) |            |
| Sodium-pyruvate          | 1 mM                              | -                           | Gibco, 11360           |
| L-ascorbic acid-2-phosphate | 50 µg/mL                          | 50 µg/mL                    | Wako chemicals, 013-19641 |
| L-proline                | 40 µg/mL                          | -                           | Sigma-Aldrich, P5607   |
| Dexamethasone            | 100 nM                            | 100 nM                      | Sigma-Aldrich, D4902   |
| fetal bovine serum (FBS) |                                   | 10 % (v/v)                  | Gibco, 10270106        |
| Insulin-transferrin-selenin | 1 % (v/v)                        | -                           | ITS^+/-premix, Gibco, Life Technologies, 32430 |
| β-glycerophosphate       | -                                 | 10 mM                       | Sigma-Aldrich, G9422   |
Table 2: List of antibodies for immune-histological stainings.

| Antibody          | clone   | Dilution and final antibody concentration | Antigen retrieval | Antibody supplier                |
|-------------------|---------|-------------------------------------------|-------------------|----------------------------------|
| Collagen type I   | EPR7785 | 1:1000; 0.875 µg/mL                       | pepsin & hyaluronidase | Abcam, ab138492                  |
| Collagen type II  | II-4C11 | 1:1000; 1 µg/mL                           | pepsin & hyaluronidase | MP Bio, 63171                    |
| Collagen type X   | COL-10  | 1:4000; 0.25 µg/mL                        | pronase            | Sigma-Aldrich, C7974             |
| Aggrecan          | EPR7785 | 1:2000; 0.5 µg/mL                         | pronase            | Invitrogen, AHP0012              |
Table 3: Criteria for evaluating immune histological stainings with modified ICRS-II-Score with maximum of 12 points (0-3 scores for each criteria judging the uniformity and darkness of aggrecan, collagen type I, II and X staining).

| ICRS-II-Scoring | Uniformity and darkness* of Aggrecan stain                                                                 |
|-----------------|-----------------------------------------------------------------------------------------------------------|
|                 | 0: No stain, 1: Weak staining of poorly formed matrix, 2: Moderately even staining, 3: Even dark stain   |
| Uniformity and darkness* of Collagen type II stain                                                      |
|                 | 0: No stain, 1: Weak staining of poorly formed matrix, 2: Moderately even staining, 3: Even dark stain   |
| Uniformity and darkness* of Collagen type I stain                                                     |
|                 | 3: No stain, 2: Weak staining of poorly formed matrix, 1: Moderately even staining, 0: Even dark stain    |
| Uniformity and darkness* of Collagen type X stain                                                     |
|                 | 3: No stain, 2: Weak staining of poorly formed matrix, 1: Moderately even staining, 0: Even dark stain    |
