European Cells and Materials Vol. 38 2019 (pages 51-62) DOI: 10.22203/eCM.v038a05 ISSN 1473-2262

MENISCUS REGENERATION COMBINING MENISCUS AND MESENCHYMAL STROMAL CELLS IN A DEGRADABLE MENISCUS IMPLANT: AN *IN VITRO* STUDY

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Abstract

Meniscus regeneration is an unmet clinical need as damage to the meniscus is common and causes early osteoarthritis. The aim of the present study was to investigate the feasibility of a one-stage cell-based treatment for meniscus regeneration by augmenting a resorbable collagen-based implant with a combination of recycled meniscus cells and mesenchymal stromal cells (MSCs).

Cell communication and fate of the different cell types over time in co-culture were evaluated by connexin 43 staining for gap junctions and polymerase chain reaction (PCR) to discriminate between meniscus cells and MSCs, based on a Y-chromosome gene. To define optimal ratios, human meniscus cells and bone-marrow-derived MSCs were cultured in different ratios in cell pellets and type I collagen hydrogels. In addition, cells were seeded on the implant in fibrin glue by static seeding or injection.

Cellular communication by gap junctions was shown in co-culture and a decrease in the amount of MSCs over time was demonstrated by PCR. 20 : 80 and 10 : 90 ratios showed significantly highest glycosaminoglycan and collagen content in collagen hydrogels. The same statistical trend was found in pellet cultures. Significantly more cells were present in the injected implant and cell distribution was more homogenous as compared to the statically seeded implant.

The study demonstrated the feasibility of a new one-stage cell-based procedure for meniscus regeneration, using 20 % meniscus cells and 80 % MSCs seeded statically on the implant. In addition, the stimulatory effect of MSCs towards meniscus cells was demonstrated by communication through gap junctions.

Keywords: Meniscus injury, meniscus regeneration, bone marrow mesenchymal stromal cells, meniscus cells, meniscus scaffold, collagen meniscus implant.

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Introduction

Meniscus tissue is characterised by low cell density and a dense extracellular matrix (ECM), which mainly consists of water, type I collagen, glycosaminoglycans (GAGs) and elastin (Fox *et al.*, 2015a). With their semilunar wedge-shaped structure, the menisci play an important role in shock absorption, load transmission and stability of the knee (Fox *et al.*, 2015; Masouros *et al.*, 2008). Damage to the meniscus is a very common injury, which leads to loss of its chondroprotective role in the knee. Especially in young patients with high activity levels (Mitchell *et al.*, 2016; Verdonk *et al.*, 2016), loss of meniscus function can lead to an increased risk of developing early osteoarthritis (OA) (Englund *et al.*, 2009; Masouros *et al.*, 2008). (Partial) meniscectomy used to be the first choice of treatment for meniscus tears; however, due to the high risk of developing post-meniscectomy OA secondary to increased contact pressure on cartilage (Englund *et al.*, 2009; Verdonk *et al.*, 2016), meniscus repair is becoming more popular. Meniscus repair is not suitable for all types of tears. Therefore, meniscus restorations using allograft transplantation or biodegradable meniscus scaffolds are of interest (Dangelmajer *et al.*, 2017; Filardo *et al.*, 2015).

Currently, the clinically-available acellular meniscus implant is the collagen meniscus implant (CMI®) (Stryker, Kalamazoo, MI, USA). This implant has a porous structure providing an environment for cell ingrowth. Clinical results after implantation

of the CMI®, evaluated by patient-reported outcome measures (PROMs), are promising, with a post-operative increase of the Lysholm score and Tegner activity scale and a decrease in visual analogue scale (VAS) pain for up to ten years (Filardo *et al.*, 2015; Grassi *et al.*, 2014; Zaffagnini *et al.*, 2015). However, limited engraftment and neo-tissue formation by invading cells can lead to size reduction of the regenerated meniscus, consequently allowing the opportunity for improvement of this treatment (Pabbruwe *et al.*, 2010). The present study proposed that replacing the deficient segment of a meniscus with a cell-seeded meniscus implant led to improved, more consistent and better-distributed functional new meniscus-like tissue formation.

The numbers of meniscus cells recovered from the resected meniscus, even during an overnight digestion, are relatively low and not suitable for engraftment [$\pm 1.5 \times 10^3$ cells/mg meniscus (Hagmeijer *et al.*, 2018)]. It would be cost effective, causing lower patient burden and being logistically attractive to use these cells in a one-stage procedure for meniscus regeneration. Recently, a clinical study has shown the safety and feasibility of using a combination of recycled autologous chondrons with allogeneic mesenchymal stromal cells (MSCs) for cartilage repair (de Windt *et al.*, 2017a; de Windt *et al.*, 2017b). This and other studies have suggested that allogeneic MSCs provide stimulatory and immunomodulatory factors for tissue repair and are able to positively stimulate a smaller number of meniscus cells, as an alternative to engraftment and differentiation (Caplan and Correa, 2011; Prockop and Youn Oh, 2012; Schepers and Fibbe, 2016). For these reasons, allogeneic MSCs have even outperformed autologous MSCs in a comparative human study for the treatment of non-ischemic dilated cardiomyopathy (Hare *et al.*, 2017).

The goal of the present *in vitro* study was to assess the conditions for a new one-stage treatment of meniscus damage. To achieve this goal, three main questions were analysed:

1) Do MSCs and meniscus cells communicate?

2) What ratio of MSCs to meniscus cells is optimal for the production of native-like meniscus tissue?

3) What is the optimal method for delivering the cells uniformly into a clinically applicable scaffold?

Materials and Methods

Donors and cell isolation

Tissue from whole meniscus was obtained from the redundant material of 11 patients that had undergone total knee replacement [mean age 65.9 (range 55-73) years, 4 male and 7 female]. Collection of this patient material was performed according to the Medical Ethical regulations of the University Medical Centre Utrecht and the guideline "good use of redundant tissue for research" of the Dutch Federation of Medical Research Societies (van Diest, 2002; FEDERA, 2011). Meniscus tissue was rinsed in phosphate buffered saline (PBS) with 100 U/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco) (1 % pen/strep), cut into pieces of 2 mm^3 and digested overnight at 37 °C in 0.15 % collagenase type 2 (CLS-2, Worthingtom, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco) and 1 % pen/strep. Meniscus cells were expanded for one passage in DMEM supplemented with 10 % foetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1 % pen/strep (Verdonk *et al.*, 2016) and used as passage 1 in all experiments.

Human MSCs (hMSCs) were isolated from bone marrow biopsies from the iliac crest during total hip replacement from 6 patients after written informed consent was obtained (Medical Ethical Committee, University Medical Centre Utrecht) as described previously (Gawlitta *et al.*, 2012). Cells were expanded in α-MEM (minimal essential medium, Gibco) supplemented with 10 % FBS, 1 % 20 mM l-ascorbic acid-2-phospate (1 % ASAP; Sigma-Aldrich) and 1 % pen/strep to be used at passage 3. Meniscus cells and MSCs from different donors were not pooled.

Fluorescent dye transfer

To assess gap-junction-mediated communication between hMSCs and meniscus cells, fluorescent dye transfer was used (Asklund *et al.*, 2003). 10 µM Vybrant CM-DiI (Molecular Probes) and 10 µM calcein-AM (Molecular Probes) were diluted in PBS and incubated with either meniscus cells or hMSCs for 1 h at 37 °C. Afterwards, cells were washed with PBS and co-cultured in a 50 : 50 ratio for 36 h as a monolayer in a 96-well plate. Gap junctions were assessed by fluorescence microscopy (EVOS Cell Imaging System, ThermoFisher Scientific) after 24 and 36 h of culture through transfer of calcein-AM. 3 meniscus donors and 3 MSC donors were used for the experiment; all were combined and 3 technical replicates per condition were performed.

Cell pellet formation

Cells were counted with an automated cell counter (TC20TM, Bio-Rad) at 1 : 1 dilution in trypan blue (Bio-Rad). Cell suspensions were prepared at the concentrations of 0 %, 10 %, 20 %, 25 %, 50 %, 75 % and 100 % meniscus cells combined with hMSCs. In a U-bottom 96-well plate (Greiner Bio-One, CELLSTAR®), a total of 250,000 cells per well and 200 µL of differentiation medium [DMEM, supplemented with 1 % pen/strep, 2 % 20 mM ASAP, 2 % insulin-transferrin-selenium-X (ITSX, Invitrogen) and 2 % human serum albumin (HSA; Sanquin, Utrecht, the Netherlands)] were centrifuged for 5 min at 300 ×*g* to form pellets. Cell pellets were cultured for 28 d at 37 °C with 5 % CO_2; medium was changed 3 times per week and conditioned medium was stored at − 20 °C for biochemical analysis.

Type I collagen hydrogel preparation

Cell concentrations with 0 %, 10 %, 20 %, 50 % and 100 % meniscus cells were prepared in suspension

Table 1. Primers' sequences used for PCR.

Target gene		Oligonucleotide sequence	Annealing temperature (°C)
18S	Forward	5′ GTAACCCGTTGAACCCCATT 3′	58
	Reverse	5′ CCATCCAATCGGTAGTAGCG 3′	
KDM5D	Forward	5′ TAACACACACCCGTTTGACAA 3′	60
	Reverse	5′ GCTGCTGAACTTTGAAGGCTG 3′	
UTY	Forward	5′ CACAAAGAAGTTGCTCAGGTACG 3′	60
	Reverse	5′ TGTGGTTGTCGATTAGAGACAGA 3′	

with hMSCs, using the same concentrations as for the cell pellets. Collagen gels were prepared from rat tail type I collagen (Corning) with a final collagen concentration of 2 mg/mL per hydrogel; 2.5 μL of 5 M NaOH were mixed with 800 μL of collagen solution (2.5 mg/mL in 0.02 N acetic acid). Cell suspensions were added, 100 μL of the combined solution was transferred to different wells of a 12-well plate with a cell concentration of 250,000 cells in 200 μL and incubated for 60 min at 37 °C. Subsequently, 2 mL of differentiation medium were added. Hydrogels were cultured for 28 d, 1 mL of medium was changed 3 times per week and stored at − 20 °C for future biochemical analysis.

Polymerase chain reaction (PCR)

Cell pellets, fibrin glue constructs and collagen type I gel constructs of i) monoculture meniscus cells and hMSCs and ii) co-culture of 20 % meniscus cells and 80 % hMSCs were harvested at t = 0 d (4 constructs per condition), t = 14 d (4 constructs per condition) and t = 28 d (4 constructs per condition) for PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen), as described by the manufacturer. Total RNA (500 ng) was reverse transcribed using the high-capacity cDNA Synthesis Kit (Applied Biosystems). PCR was performed on 5×-diluted cDNA using iTaq Universal SYBR Green Supermix (Bio-Rad) in a LightCycler 96 (Roche Diagnostics) according to the manufacturer's instructions.

In the co-cultures used for PCR, all meniscus donors were female and all MSC donors were male; therefore, during the PCR, using primers for the genes on the Y chromosome, a distinction could be made between the different cell types. The housekeeping gene *18S* was used and primers for lysine demethylase 5D (*KDM5D*) and ubiquitously transcribed tetratricopeptide-repeat-containing, Y-linked (*UTY*) were used to amplify the Y chromosome (Table 1) and, therefore, the MSCs in the co-cultures.

Biochemical analysis

After an overnight digestion of the samples in papain buffer [250 μg/mL papain (Sigma-Aldrich), 0.2 M NaH_2PO_4, 0.1 M EDTA, 0.01 M cysteine] at 60 °C, GAG content was determined by dimethylmethylene blue (DMMB) assay. Absorption ratio was set at 525 and 595 nm using chondroitin sulphate (Sigma-Aldrich) as a standard for calculating GAG content. DNA content was determined by Picogreen DNA assay (Invitrogen), according to the manufacturer's instructions. Excitation and emission were set at 480 and 520 nm, respectively, and λDNA was used as a standard reference to calculate DNA content. Freeze-dried papain samples were used to determine collagen content of the constructs by hydroxyproline assay. 100 μL of 1.4 M citric acid (27490; Fluka) was added following overnight hydrolysis of the samples in 100 μL of 4 M NaOH (6498; Merck) at 108 °C. Choramine-T reagent (2426; Merck) and dimethylaminobenzoaldehyde (3058; Merck) were added to the samples and hydroxyproline standard (104506.0010; Merck) was used to measure the absorption at 570 nm. As 13.5 % of collagen is composed of hydroxyproline, the amount of collagen was calculated from the hydroxyproline content (Neuman and Logan, 1950).

Histology and immunohistochemistry

Samples were fixed in 4 % buffered formaldehyde, dehydrated in graded ethanol series, immersed in xylene, embedded in paraffin wax, cut in 5 μm-thick sections and stained. Before staining and immunohistochemistry, sections were deparaffinised in xylene and rehydrated in ethanol. To determine cell distribution throughout the construct, sections were stained with Mayer's haematoxylin (Merck) and counterstained with eosin (Merck) (H&E staining). To evaluate proteoglycan content, 0.125 % safranin O (Merck) counterstained with Weigert's haematoxylin (Klinipath, Duiven, the Netherlands) and 0.4 % fast green (Merck) was used. Picrosirius red (Klinipath, Leuven, Belgium)/alcian blue (Sigma-Aldrich) staining was used to visualise collagen fibre orientation by polarised light microscopy.

After rehydration, sections for connexin 43, type I and II collagen immunohistochemistry were blocked for 10 min with 0.3 % H_2O_2 solution and washed with PBS-0.1 % Tween 20 (Sigma-Aldrich). Antigen retrieval was performed using 1 mg/mL pronase (Roche) in PBS and 10 mg/mL hyaluronidase (Sigma-Aldrich) in PBS, both for 30 min at 37 °C. Sections were blocked with 5 % PBS/bovine serum albumin (BSA) for 30 min at room temperature, followed by incubation with primary antibodies for either connexin 43 (GJA1, rabbit polyclonal antibody, 1 : 50 in PBS/5 %BSA, Abcam), type I collagen (Col1, rabbit monoclonal antibody, 1 : 400 in PBS/5 % BSA, Abcam) or type II collagen (II-II6B3, mouse monoclonal antibody,

1 : 100 in PBS/5 % BSA, DSHB, Merck). As negative controls, rabbit IgG (Dako) was used for connexin 43 (1 : 2,000 in PBS/5 %BSA) and type I collagen (1 : 10,000 in PBS/5 %BSA) and mouse IgG (1 : 100 in PBS/5 % BSA, Dako) for type II collagen. Antibodies were incubated overnight at 4 °C and, subsequently, washed in 0.1 % PBS-Tween 20 and incubated with the secondary antibody for connexin 43 [goat anti-rabbit-horseradish peroxidase (HRP) 1 : 100 in PBS/5 % BSA; 3117332001; Roche], type I (EnVision + System-HRP, goat anti-rabbit; K4003, Dako) and type II (goat anti-mouse IgG HRP, 1 : 100 PBS/5 % BSA; P0447, Dako) collagen for 60, 30 and 60 min, respectively, at room temperature. Immunoreactivity, visualised with 3-diaminobenzidine (DAB, Sigma-Aldrich), was stopped using MilliQ water (Merck). Sections were counterstained with Mayer's haematoxylin diluted 1 : 1 in distilled water, dehydrated in different gradients of ethanol and mounted in Depex (Merck).

Seeding methods

CMI® pieces (with a size of approximately 150 mm^3) were seeded with 10 % meniscus cells and 90 % hMSCs, based on successful results using chondrons and MSCs in the same ratio (Bekkers *et al.*, 2013). Before seeding, the CMI® was washed for 10 d in 100 mL PBS with 1 % pen/strep. The fibrin glue (Beriplast, CSL Behring) used was diluted as described by Abbadessa *et al.* (2016) and all cells were mixed in the fibrinogen component of the fibrin glue. After seeding and incubation, scaffolds were moved to a new 24-well plate (to exclude cells not attached to the scaffold) for subsequent calculation of matrix production and cell-count. Seeded constructs were cultured for 26 d in 1 mL of differentiation medium, which was changed 3 times per week and stored for biochemical analysis.

To mimic the clinical circumstances of *ex vivo* and *in vivo* seeding during arthroscopy, two different seeding techniques were used. Static surface seeding was performed on dry CMI®, resembling *ex vivo* seeding. 75 µL of cell suspension in fibrinogen, containing a total of 5.0×10^5 cells (5.0×10^4 meniscus cells and 4.5×10^5 hMSCs), were loaded on top of the CMI®, immediately followed by 75 µL of thrombin and incubation for 15 min at 37 °C. Seeding by injection was executed on wet CMI®, immersed in 1 mL of PBS in a 24-well plate, resembling *in vivo* seeding after

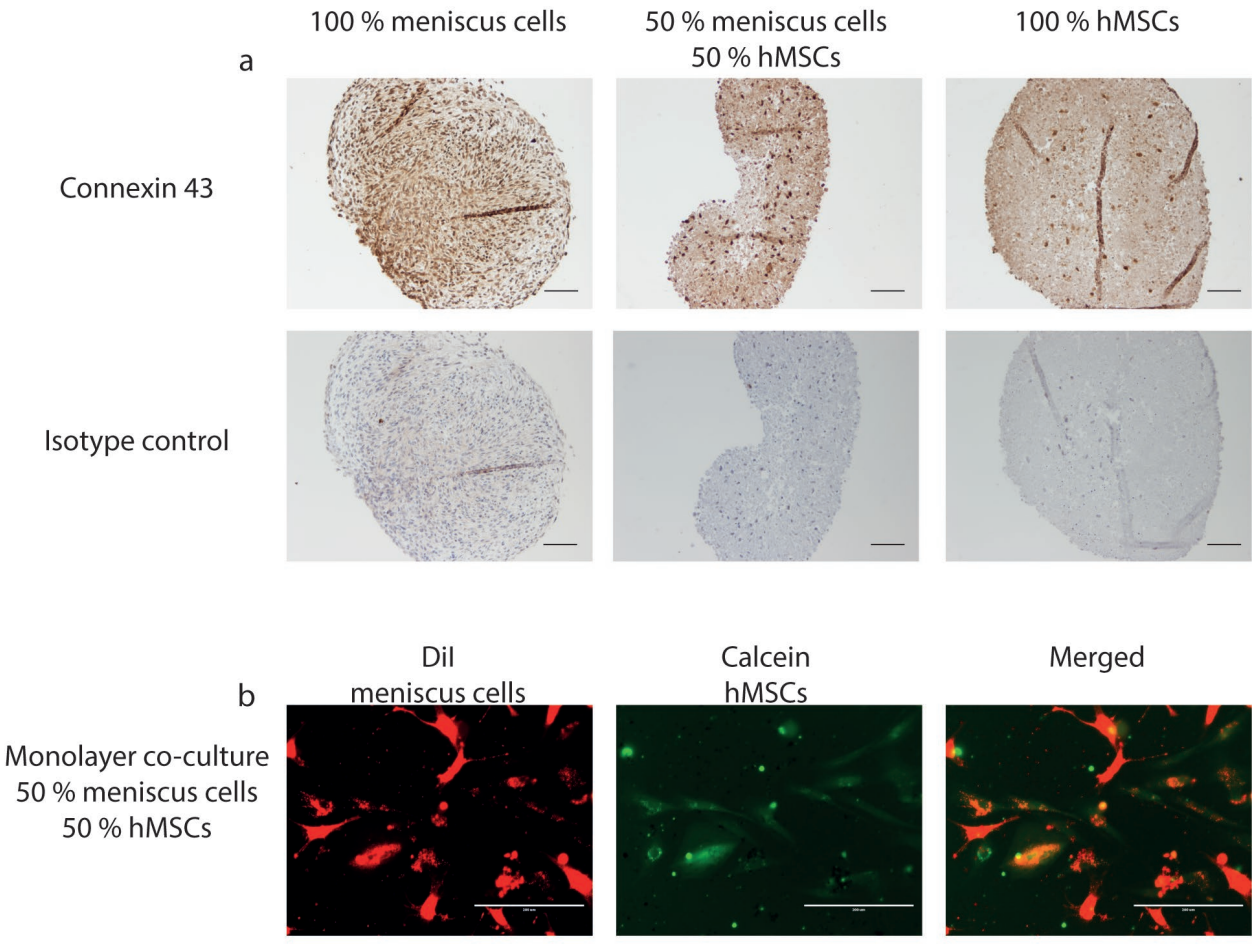

Fig. 1. Cell-cell communication. Cell-cell communication by gap junctions between hMSCs and meniscus cells was determined by (**a**) the presence of connexin 43 in mono- and co-cultures in pellets after 28 d and (**b**) dye transfer : Vybrant CM-DiI (red), calcein (green) and an overlay of Vybrant CM-DiI and calcein (merged), where transfer of the calcein stained hMSCs to the meniscus cells stained with Vybrant CM-DiI is shown after 24 h. Scale bar: 200 µm.

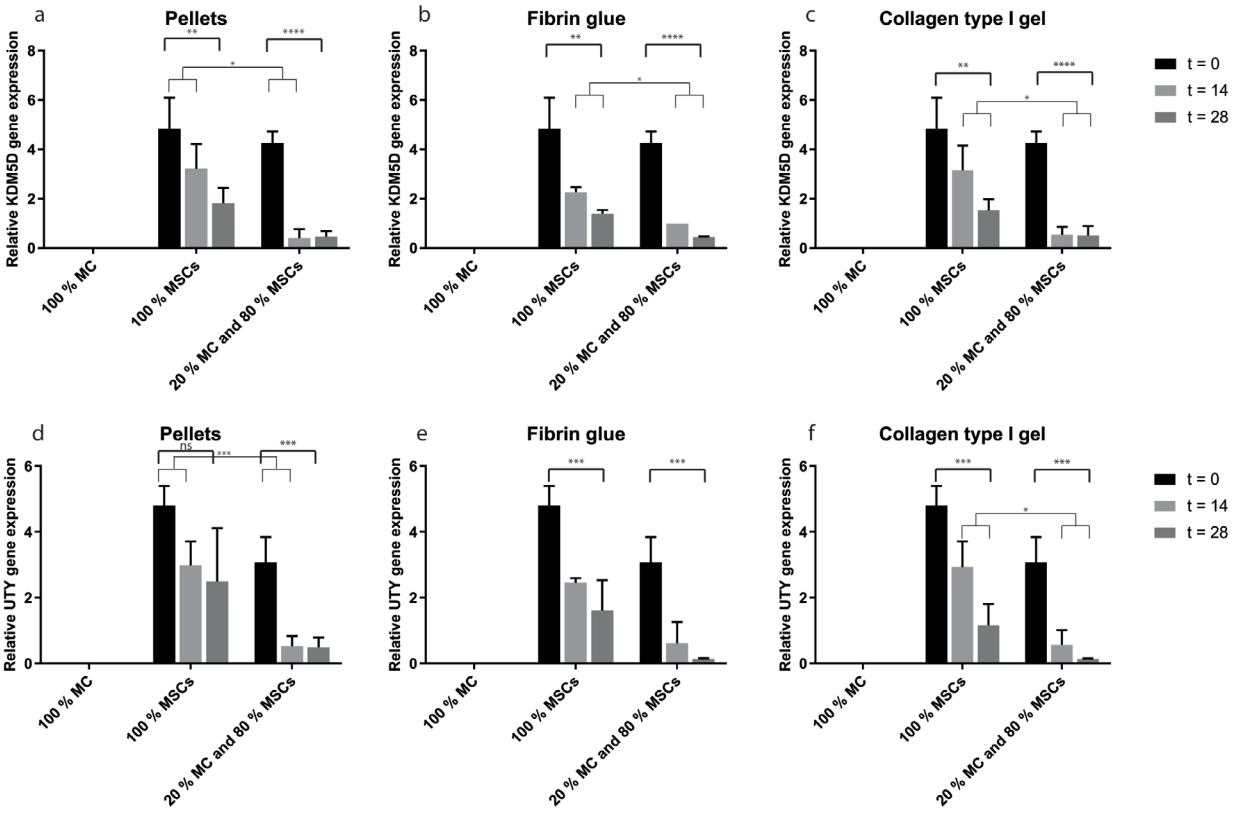

Fig. 2. PCR data after (co)culture of meniscus cells and MSCs. (**a-c**) PCR data for both *KDM5D* and (**d-f**) *UTY*, representing the Y-chromosome genes in the male MSCs, showed a decrease in the amount of MSCs over time. Absolute difference between t = 0 d and t = 28 d was calculated for both monocultures of MSCs and co-culture of MSCs and meniscus cells (ratio 80 : 20). The delta of the mean decrease per culture condition was calculated and significant differences between monocultures of MSCs and co-cultures of MSCs with meniscus cells are marked with brackets. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. MC: meniscus cells. ns: not significant.

arthroscopic implantation of the scaffold. Using a 1.0 mL syringe and a 23-gauge needle, 75 µL of cell suspension (5.0×10^5 cells, similar cell combination to static surface seeding) were injected into the CMI® and incubated at 37 °C for 15 min after injection of 75 µL of thrombin using a 23-gauge needle.

Cell distribution assessment using confocal microscopy

Accessing cell distribution throughout the CMI® after 26 d of culture using the different seeding methods was performed by creating three-dimensional (3D) images acquired by a Leica SP8 confocal microscope. Two pieces of CMI® per seeding method were stained for 30 min with 0.5 µL/mL calcein AM (Molecular Probes) at room temperature and for 4 min with 100 ng/mL 4',6-diamidino-2-phenylindole (DAPI) followed by washing with PBS. A tile scan with z-stack was performed and the 3D images were merged using Image J.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA, USA). Differences in GAG and collagen per DNA for the different ratios and seeding methods were calculated by a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. The decrease in *KDM5D* and *UTY* per culture condition at the different time points were calculated using a student's *t*-test. To determine whether there was a significant difference in relative decrease in the amount of MSCs between monoculture of MSCs and co-culture of MSCs and meniscus cells over time, the delta of the mean decrease per condition was calculated and student's *t*-tests were performed. $p < 0.05$ was considered statistically significant.

Results

Communication and cell survival in co-cultures

Immunohistochemistry for connexin 43 in the pellet co-cultures showed staining for the monocultures of meniscus cells and hMSCs as well as for the different ratios of the co-cultures (Fig. 1**a**), indicating formation of gap junctions in both mono- and co-cultures. When hMSCs were stained with calcein and meniscus

cells with Vybrant CM-DiI, the dye transfer was shown most prominently by the yellow staining of the red meniscus cells, which also stained for the calcein transferred from the hMSCs (Fig. 1**b**). When hMSCs were incubated with Vybrant CM-DiI and the meniscus cells with calcein, the dye transfer was less prominent. This suggested that there was active gap-junction-mediated communication, which was more active from hMSCs to meniscus cells than from meniscus cells to hMSCs.

PCR data of monoculture hMSCs and co-culture with meniscus cells, in either pellet, fibrin glue and type I collagen gel, showed a significant decrease in *KDM5D* and *UTY* over time for both mono and co-cultures and, therefore, a decrease in the amount of hMSCs over time (Fig. 2). The decrease in co-cultures was higher as compared to the decrease in hMSCs monocultures. In addition, in pellet culture, the decrease of hMSCs was significantly lower (*KDM5D*, $p = 0.013$; *UTY*, $p = 0.0006$) between t = 0 d and t = 14 d for monoculture of hMSCs as compared to co-culture. Whereas between t = 14 d and t = 28 d, the mean decrease in hMSCs was higher in monoculture for cultures in fibrin glue and type I collagen (fibrin glue: *KDM5D* $p = 0.0427$, *UTY* $p = 0.4762$; type I collagen: *KDM5D* $p = 0.0448$, *UTY* $p = 0.0193$) (Fig. 2).

Production of ECM in pellet co-cultures

Biochemical analysis showed a significant decrease in DNA content of cell pellets ($n = 5$ for biological replicates and $n = 3$ for technical replicates) after 4 weeks of culture for the ratios containing a percentage of hMSCs (Fig. 3**a**). The larger the proportion of hMSCs, the fewer cells were present after 28 d of culture. The ratios with more than 50 % hMSCs produced significantly more GAG content per DNA as compared to 100 % meniscus cells (Fig. 3**b**), which indicated a stimulatory effect of hMSCs on meniscus cell GAG, followed by meniscus cell apoptosis. The same assumption was demonstrated by PCR. In assessment of total GAG content of the samples combined with GAG in the medium, there were no differences observed for total GAG production. However, co-cultures with hMSCs seemed to perform better than monoculture of meniscus cells (Fig. 3**c**). Also, a trend for a higher collagen content in the cell pellets was suggested when the proportion of hMSCs was larger. However, results were not statistically significant (Fig. 3**d**).

In H&E staining, pellets containing 50 %, 80 % and 100 % meniscus cells had a higher cell density (Fig. 4**a**), which was similar to the results for DNA quantification (Fig. 3). None of the cell ratios stained for GAG, indicating that the amount of GAG was too low to be detected histologically (data not shown). Immunohistochemistry showed a more intense DAB staining for type I collagen as compared to type II collagen. These findings, *i.e.* a low amount of GAG and higher presence of type I as compared to type II collagen, were characteristic of native meniscus tissue (Fig. 4**b**,**c**).

Production of ECM in collagen type I hydrogels

After 4 weeks of co-culturing meniscus cells and hMSCs in type I collagen hydrogels ($n = 3$ for both biological and technical replicates), DNA content was not statistically significantly different among the different conditions (Fig. 3**e**). GAG content and total GAG production, both normalised to DNA content, were the highest ($p < 0.001$ and $p < 0.05$, respectively) in 20 % meniscus cells and 80 % hMSCs as compared to the other ratios (Fig. 3**f**,**g**). A trend of more GAG production was observed in the hydrogels containing > 50 % hMSCs as compared to > 50 % meniscus cells, although not all results were statistically significant. Collagen content, corrected for DNA, showed a significantly higher concentration in the conditions with 90 % and 100 % hMSCs (Fig. 3**h**).

Histology showed an even distribution of cells throughout the different constructs; however, no proteoglycan content was detected. Immunohistochemistry showed a larger presence of type I collagen as compared to type II collagen (data not shown), similar to the pellet culture.

Optimal *in vitro* seeding method

Immediately after seeding, the wet-injected CMI® contained significantly fewer cells than the total number of seeded cells ($p = 0.0070$) and the dry-statically-seeded CMI® ($p = 0.0096$). The number of cells in the dry-and statically-seeded CMI® were not statistically different from the total number of seeded cells ($p = 0.6899$) (Fig. 5**a**). After 26 d of culture ($n = 3$ for both biological and technical replicates), the CMI® seeded statically in a dry environment showed a significantly higher DNA content as compared to the CMI® injected in a wet environment ($p = 0.0491$) (Fig. 5**b**). GAG content appeared to be slightly higher in the first group although the data were not statistically significant ($p = 0.7249$) (Fig. 5**c**). GAG release into the medium was significantly higher in the dry- and statically-seeded CMI® ($p = 0.0306$) (data not shown). Because the CMI® is composed of bovine collagen, the produced collagen content was determined using the ratio of collagen before and after culture corrected for an empty CMI®. This resulted in no significant differences among the different seeding methods ($p = 0.3426$). Histological analyses showed a better cell distribution within the scaffold for the dry-seeded CMI® as compared to the wet-seeded scaffolds. Fig. 6 shows histology of the dry-seeded CMI®, with a good cell distribution shown by H&E staining in Fig. 6**a**. However, no proteoglycans were detected by histology (Fig. 6**b**). Immunohistochemistry showed a high production of type I collagen and only minimal deposition of type II collagen (Fig. 6**c**,**d**), which was similar to native meniscus tissue. 3D confocal images confirmed the homogenous distribution of cells throughout the whole CMI® when the scaffold was seeded dry and statically (Fig. 7**a**), whereas for the wet-injected CMI®, there were only pockets of cells visible (Fig. 7**b**).

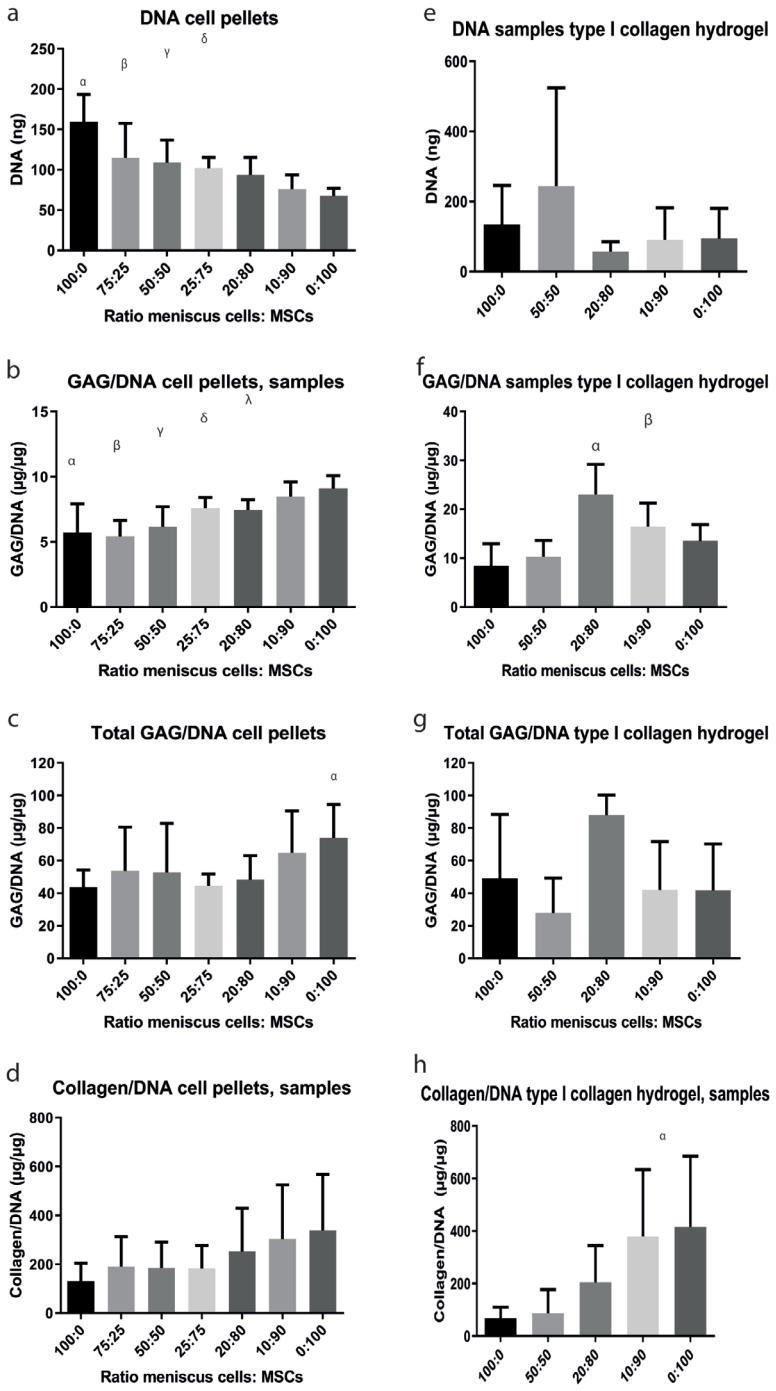

Fig. 3. Biochemical analysis after co-culture of meniscus cells and MSCs in different ratios. (**a**,**e**) DNA content, (**b**,**f**) GAG content, (**c**,**g**) total GAG production and (**d**,**h**) collagen content, all corrected for DNA content, are shown for (**a**-**d**) cell pellets and (**e**-**h**) co-cultures in type I collagen hydrogel for the different ratios of meniscus cells and hMSCs after 28 d of culture. Data are shown as mean ± standard deviation; $p < 0.05$. (**a**) 100 % meniscus cells was statistically higher in DNA content than all the other conditions (α), 75 : 25 and 50 : 50 were significantly higher than 10 : 90 and 0 : 100 (β and γ) and 25 : 75 was higher than 0 : 100 (δ). (**b**) GAG/DNA in cell pellets was significantly higher in 100 : 0 (α) as compared to 75 : 25, but significantly lower as compared to 25 : 75, 10 : 90 and 100 : 0. 75 : 25 (β) was significantly lower than 25 : 75, 20 : 80, 10 : 90 and 0 : 100. 50 : 50 (γ) was significantly lower as compared to 25 : 75, 10 : 90 and 0 : 100. 100 : 0 was significantly higher as compared to 25 : 75 and 20 : 80 (δ and λ). (**c**) Total GAG/DNA in cell pellets was significantly higher in 0 : 100 (α) as compared to 100 : 0, 25 : 75 and 20 : 80. In both (**d**) collagen/DNA in cell pellets and (**e**) DNA content in type I collagen hydrogels, no significant differences were detected. (**f**) In the samples cultured in type I collagen hydrogels, 20 : 80 was significantly higher in GAG/DNA as compared to 100 : 0, 50 : 50 and 0 : 100 (α) and 10 : 90 was significantly higher than 100 : 0 (β). (**g**) No significant differences were found for total GAG/DNA in the co-cultures using collagen type I hydrogels. (**h**) Collagen content corrected for DNA in 10 : 90 and 0 : 100 was significantly higher as compared to 100 : 0 and 50 : 50 (α).

Discussion

The goal of the present *in vitro* study was to assess the conditions for a new one-stage cell-based procedure for meniscus regeneration. The study examined the interaction through gap junctions between hMSCs and meniscus cells and demonstrated a short survival period of hMSCs in co-cultures, indicating a stimulating effect of hMSCs on meniscus cells. The optimal ratio for co-culture of MSCs and meniscus cells was reported to be 80 % hMSCs and 20 % meniscus cells, where native-like meniscus tissue, type I collagen and a minimal amount of GAG were produced. Contiguously, the best seeding method for this cell combination into a clinically applicable scaffold was shown to be dry seeding. All these findings suggested that this new treatment method for meniscus regeneration was clinically applicable.

Immunohistochemistry for connexin 43, dye transfer experiments and PCR results demonstrated transfer of information from hMSCs to meniscus cells by gap junctions and a decrease in the number of hMSCs in time. The low amount of male DNA after 4 weeks of culture, shown by PCR, indicated that MSCs disappeared after stimulating or transferring information to meniscus cells. Liu (2019) has shown that hMSCs can transfer their functional mitochondria into injured endothelial cells after ischemic stroke in mice, protecting the endothelial cells from going into apoptosis. After stimulating meniscus cells, hMSCs seemed to disappear. Xu *et al.* (2004) have described the function of hMSCs by differentiation into the required cell type in *e.g.* isolated cartilage defects, osteoarthrosis or after a myocardial infarction. However, de Windt *et al.* (2015) have shown that the DNA of the newly formed cartilage tissue, in patients treated with a combination of allogenic hMSCs and autologous chondrocytes, does not contain any DNA from the hMSC donor, only from the patient itself. The present study showed that the decrease in co-

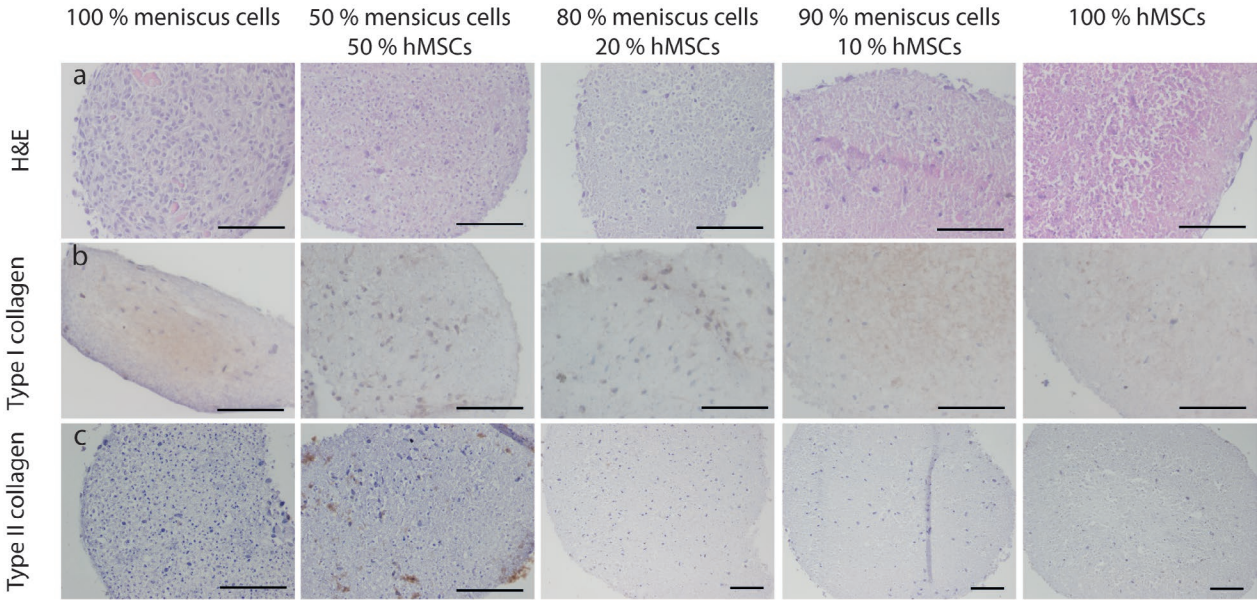

Fig. 4. Histological stainings of pellet cultures of different ratios of meniscus cells and MSCs, 20× magnification. (**a**) H&E showed cell concentration in the different ratios. (**b**) Immunohistochemistry showed staining for type I collagen and (**c**) almost no staining for type II collagen in all ratios. Scale bar: 100 μm.

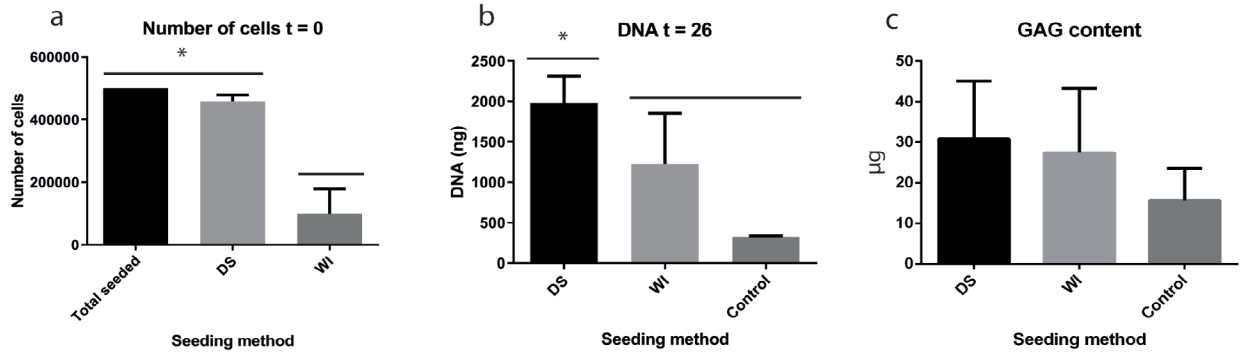

Fig. 5. Number of cells after two different seeding methods. (**a**) Total number of cells at t = 0 d, (**b**) DNA content at t = 26 d and (**c**) GAG content for co-cultures of meniscus cells and hMSCs in a 10 : 90 ratio inside the CMI® for 26 d using different seeding methods (dry static and wet-injected, respectively). A CMI® without cells was used as the control group. Data are shown as mean ± standard deviation; * $p < 0.05$. DS = dry seeding; WI = wet seeding by injection.

cultures was higher as compared to the decrease in hMSCs monocultures. This, in combination with the results of de Windt *et al.* (2015), could indicate that, in co-culture, hMSCs might have a more stimulatory effect on the production of GAG and collagen from meniscus cells and contribute less to ECM production and replacement of avital native cells in damaged tissue; whereas, in monoculture, ECM production might be regulated by the hMSCs themselves.

The willingness to use allogeneic hMSCs for future *in vivo* experiments is reinforced by the possible pro-inflammatory effect triggered by the presence of allogeneic cells in the patient, which might cause a boost in the regenerative effect. Hare *et al.* (2017) have shown a superior effect of allogenic to autologous hMSCs in patients receiving transendocardial stem cell injections for non-ischemic dilated cardiomyopathy. Such patients have an improved endothelial function, a greater suppression of tumour necrosis factor alpha (TNF-α; suggesting a shift towards a less inflammatory phenotype of the immune cells) and clinical better outcomes (Hare *et al.*, 2017).

The present study showed an increase in GAG and collagen production in co-cultures as compared to monocultures of meniscus cells. Co-cultures with a higher percentage of hMSCs resulted in the highest ECM production. Similar results were previously described by Cui *et al.* (2012) and Matthies *et al.* (2013). Co-culture results were comparable to the results of co-cultures of hMSCs and chondrons, as shown by Bekkers *et al.* (2013), with the highest GAG/DNA production in 80 % and 90 % hMSCs, respectively. Nevertheless, monoculture of hMSCs resulted in the highest production of GAG and collagen per DNA, which is not described by others. A possible explanation could be that pellet culture might not be the optimal 3D culture method for ECM production by meniscus cells. In the native meniscus, cells are dispersed throughout the ECM and there is very limited contact between cells. In pellet culture, cells are aggregated together at high density without being surrounded by matrix, especially at the start of the culture. Consequently, results suggested that meniscus cells performed better in 3D hydrogels. This could partially explain the differences in ECM production between meniscus cells and hMSCs. However, Song *et al.* (2015) have shown less GAG and collagen production by MSCs as compared to co-cultures and monocultures of meniscus cells, similarly to Cui *et al.* (2012) and Matthies *et al.* (2013). Besides type of co-culture, the type of MSCs could significantly influence the difference in outcome after *in vitro* co-culture, as MSCs are a heterogeneous population of cells and their characteristics and regenerative potential is dependent on a variety of parameters, such as donor, location, harvest method, isolation method, expansion density and composition of expansion medium and culture medium (Fellows *et al.*, 2016). MSCs are often poorly characterised, making it challenging to compare the direct results of various studies. Synovium-derived mesenchymal stromal cells (SSC) were used by Song *et al.* (2015), differently from the marrow MSCs used in the present study. In addition, Song *et al.* (2015) co-cultured the pellets for a total of 2 weeks, half the time as compared to the current study. Therefore, it could be possible that MSCs started producing more ECM after the first 2 weeks of culture.

Due to the possible negative effect of co-culturing meniscus cells in pellets, the study included co-culturing in a type I collagen hydrogel to closer mimic the native environment of the meniscus cells. Results showed a significantly higher production of GAG/DNA for the 80 % and 90 % hMSC conditions and of total GAG/DNA for the 80 % hMSC condition, with a lower production of GAG in the hMSC monoculture. However, collagen production was hard to determine due to the collagen already present in the hydrogel. Collagen content corrected for DNA showed a significantly higher concentration in the conditions with 90 % and 100 % hMSCs, which could be either

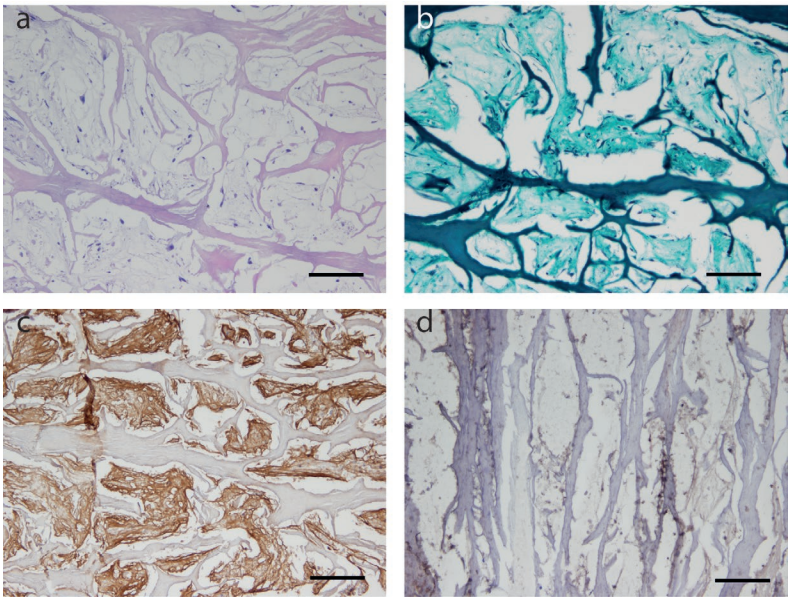

Fig. 6. Histological stainings. (**a**) H&E, (**b**) safranin O/fast green and (**c**) immunohistochemistry for type I and (**d**) type II collagen of dry-seeded CMI® with a 10 : 90 ratio of meniscus cells and hMSCs, cultured for 26 d. Scale bar: 100 μm.

the result of a higher collagen production by hMSCs and/or a higher break down of type I collagen hydrogel by the meniscus cells. These findings were different from those of McCorry *et al.* (2016), who have shown the highest GAG production in the 50 : 50 ratio. However, McCorry *et al.* (2016) have used bovine cells, passage 4 MSCs (cultured with fibroblast growth factor) and passage 0 meniscus cells as compared to hMSCs passage 3 and human meniscus cells passage 1 of the current study. In addition, in the present study, co-culture was harvested after 28 d, compared to the 15 d reported by McCorry *et al.* (2016). Perhaps, the most important difference is the fixed shape they have used for culturing type I collagen hydrogels, so that the collagen gel could not contract during the culturing period, which also has an influence on ECM production (Vickers *et al.*, 2006; Vickers *et al.*, 2010).

The most frequently-described seeding methods reported in the literature are static seeding, seeding by injection and centrifugal seeding (Godbey *et al.*, 2004; Thevenot *et al.*, 2008; Weinand *et al.*, 2009; Zhang *et al.*, 2015). Most studies are directed towards cell viability and distribution without considering the clinical applicability for a one-stage procedure where seeding of the scaffold has to be performed according to GMP-regulations. Zhang *et al.* (2015) have reported the best cell distribution of MSCs and meniscus fibrochondrocytes using centrifugal seeding, although these results were not significantly better than static seeding. When static seeding was used, Thevenot *et al.* (2008) have shown a high cell density in the top layer of the scaffold as compared to the centre and bottom. This result does not compare with the present study results where a homogeneous distribution of cells throughout the whole scaffold in vertical direction was shown. Besides the seeding method, scaffold material could also influence cell numbers and cell distribution after seeding. Demineralised cancellous bone and poly(lactide-co-glycolide) (PLGA) scaffolds were used by Zhang *et al.* (2015) and Thevenot *et al.* (2008), respectively, having different material characteristics as compared to the CMI®, including pore size. The CMI® has a wide range of pore sizes (50-400 μm), whereas Thevonot *et al.* (2008) (mean 212 μm, range 150-250 μm) and Zhang *et al.* (2015) (268 μm) used a smaller pore size. The smaller pore sizes could possibly negatively influence cell distribution after seeding. Moreover, the CMI® has a sponge-like structure, absorbing fluids rapidly when seeded onto the scaffold, providing a good distribution of the cells when static seeding is used. Multiple injections (in a wet environment) into the CMI® creates 'pockets' of cells instead of a homogenous distribution. This result is not illustrated in the literature, since previous authors performed the injected seeding with only one injection (Thevenot *et al.*, 2008; Weinand *et al.*, 2009; Zhang *et al.*, 2015).

Conclusion

The present study demonstrated the *in vitro* feasibility of a new one-stage cell-based procedure for meniscus regeneration in young and active patients with non-repairable meniscus tears. In co-culture hMSC stimulated meniscus cells to produce ECM by communication through gap junctions before going into apoptosis. The most optimal ratio for GAG and collagen production was 20 % meniscus cells and 80 % hMSCs. Static seeding resulted in a higher cell density and better cell distribution than wet seeding. The results of these *in vitro* experiments lay the foundation for clinical application of one-stage cell-based meniscus regeneration procedures.

Acknowledgements

The authors wish to thank Smith&Nephew and Ivy Sports Medicine for research funding and iMove Medical for providing research material.

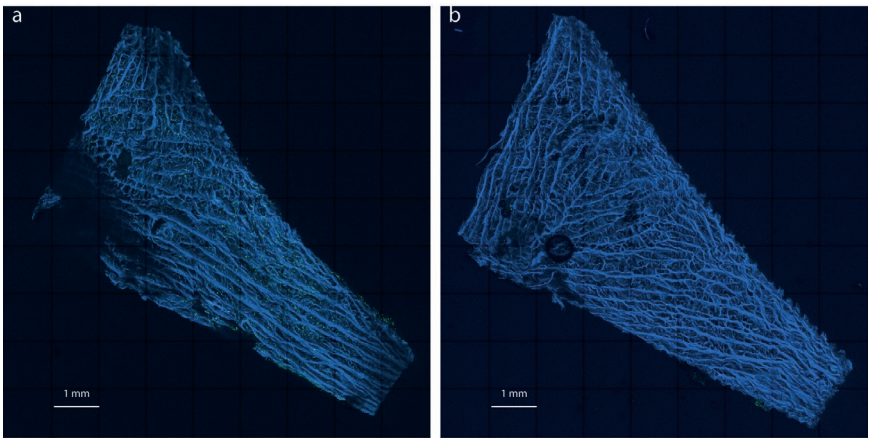

Fig. 7. Cell distribution throughout the CMI® for different seeding methods. Cells were stained with calcein AM (green) and the CMI® with DAPI (blue). 3D images were taken using a confocal microscope (Leica) showing cell distribution throughout the CMI® using (**a**) the dry static seeding method and (**b**) the wet-injected CMI®.

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Discussion with Reviewers

Norimasa Nakamura: What is the potential difficulty in transferring this model into an animal model?
Authors: Only one animal model is described in the literature (Martinek *et al.*, 2006, additional reference). The authors harvested fibrochondrocytes from sheep (n = 25), cultured them *in vitro*, seeded on CMI and implanted the seeded scaffolds where the meniscus used to be. An improvement of macroscopic and histological meniscus tissue as compared to implantation of non-seeded CMIs was shown. The study demonstrated that the principle of an animal study for seeded meniscus implants is an option. However, we do not think it will provide us with extra information. In a cadaveric study, Hagmeijer *et al.* (2018) showed that seeding before implantation of the scaffold results in a larger number of cells in the scaffold, a better distribution and no effect on cell survival as compared to seeding after implantation. de Windt *et al.* (2016) showed the safety of using allogeneic mesenchymal stromal cells for cartilage regeneration. Therefore, we think an animal model will not add extra evidence for proceeding with this new method of meniscus regeneration.

Reviewer 1: Did the authors isolate meniscus cells from the whole tissue or were the cells obtained from the inner and outer part, respectively? Did the authors perform some experiments to compare the inner and outer cells? In their opinion, could the difference in terms of ECM production be important to achieve the best outcome for the treatment?
Authors: Meniscus cells were isolated from whole meniscus and no difference was made between inner and outer part of the meniscus. No experiment was performed to distinguish between cells from inner and outer part of the meniscus in ECM production. Although, it is likely that there is a difference in ECM production from cells of the inner and outer part of the meniscus as their composition and loading patterns differ and also only the outer meniscus is vascularised. However, the present *in vitro* study was performed to look at the feasibility of a one-stage cell-based procedure for meniscus regeneration. In a clinical setting for such a procedure, it would be impossible to distinguish between the inner and outer meniscus. Moreover, cells from the torn meniscus would be used, which is automatically the part that needs to be regenerated. Therefore, in our opinion, these types of experiments would not contribute to a better clinical outcome.

Additional Reference

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Editor's note: The Scientific Editor responsible for this paper was Martin Stoddart.