Single-Stage Cell-Based Cartilage Regeneration Using a Combination of Chondrons and Mesenchymal Stromal Cells

Comparison With Microfracture

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Background: Autologous chondrocyte implantation (ACI) is traditionally a 2-step procedure used to repair focal articular cartilage lesions. With use of a combination of chondrons (chondrocytes in their own territorial matrix) and mesenchymal stromal cells (MSCs), ACI could be innovated and performed in a single step, as sufficient cells would be available to fill the defect within a 1-step surgical procedure. Chondrons have been shown to have higher regenerative capacities than chondrocytes without such a pericellular matrix.

Purpose: To evaluate cartilage formation by a combination of chondrons and MSCs in vitro and in both small and large animal models.

Study Design: Controlled laboratory study.

Methods: Chondrons and MSCs were cultured at different ratios in vitro containing 0%, 5%, 10%, 20%, 50%, or 100% chondrons (n = 3); embedded in injectable fibrin glue (Beriplast); and implanted subcutaneously in nude mice (n = 10; ratios of 0%, 5%, 10%, and 20% chondrons). Also, in a 1-step procedure, a combination of chondrons and MSCs was implanted in a freshly created focal articular cartilage lesion (10% chondrons) in goats (n = 8) and compared with microfracture. The effect of both treatments, after 6-month follow-up, was evaluated using biochemical glycosaminoglycan (GAG) and GAG/DNA analysis and scored using validated scoring systems for macroscopic and microscopic defect repairs.

Results: The addition of MSCs to chondron cultures enhanced cartilage-specific matrix production as reflected by a higher GAG production ($P < .03$), both in absolute levels and normalized to DNA content, compared with chondrocyte and 100% chondron cultures. Similar results were observed after 4 weeks of subcutaneous implantation in nude mice. Treatment of freshly created cartilage defects in goats using a combination of chondrons and MSCs in Beriplast resulted in better microscopic, macroscopic, and biochemical cartilage regeneration ($P \leq .02$) compared with microfracture treatment.

Conclusion: The combination of chondrons and MSCs increased cartilage matrix formation, and this combination of cells was safely applied in a goat model for focal cartilage lesions, outperforming microfracture.

Clinical Relevance: This study describes the bench-to-preclinical development of a new cell-based regenerative treatment for focal articular cartilage defects that outperforms microfracture in goats. In addition, it is a single-step procedure, thereby making the expensive cell expansion and reimplantation of dedifferentiated cells, as in ACI, redundant.

Keywords: chondron; ACI; MSC; 1 step; regeneration

Autologous chondrocyte implantation (ACI) is indicated for isolated lesions of the articular cartilage larger than 2 cm^2 in patients who wish to regain a high level of activity.[1] During a first arthroscopic procedure, a small biopsy sample is taken from the nonweightbearing trochlear margin in the knee. Articular chondrocytes are isolated from this specimen, expanded in vitro, and reimplanted in a second (mini) open surgical procedure.[3] Overall, the clinical improvement is good, and patients can reach a satisfying activity level. However, because of the low cellularity of the cartilage biopsy tissue, cellular expansion is necessary, leading to a procedure that requires 2 surgical interventions and entails cell dedifferentiation.[14,19]

The addition of another cell type to the freshly isolated chondrocytes from the cartilage biopsy tissue has been suggested to circumvent the expansion step and allow direct single-stage reimplantation of sufficient cells into the

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defect. Recently, stimulation of cartilage matrix production and upregulation of cartilage-specific matrix genes were observed when chondrocytes were combined with mesenchymal stromal cells (MSCs),[5,20,25] most likely by trophic factors derived from MSCs.[25] This finding indicates that MSCs could be an interesting cell population to combine with freshly isolated articular chondrocytes in a 1-step procedure.

In addition to adding MSCs to enhance cartilage regeneration, it has been suggested that the phenotype of chondrocytes currently used in ACI may be suboptimal. Within healthy articular cartilage, chondrocytes are surrounded by a pericellular matrix, together called the chondron. This pericellular matrix mainly consists of type VI collagen and has an important role in the metabolic activity of the chondrocyte and the mechanical signaling from and to the extracellular matrix.[7,13,24] The presence of a pericellular matrix has been shown to enhance matrix production by chondrocytes, suggesting that an intact pericellular matrix improves cellular cartilage regeneration.[13,22] However, current ACI treatment is based on isolation procedures that remove the territorial matrix from the chondrocytes. Our study aimed to determine the efficiency of cartilage formation using a combination of chondrons and MSCs in vitro and in vivo after subcutaneous implantation in nude mice. Finally, a combination of chondrons and MSCs was applied in a large animal model, aiming at the safe and scientific preclinical development of a single-step cell-based regenerative treatment for focal articular cartilage lesions.

MATERIALS AND METHODS

Study Outline

To test the hypothesis whether a combination of chondrons and MSCs stimulated cartilage formation, we started with a pellet culture (n = 5) consisting of a combination of chondrons and MSCs (chondron/MSC) at different ratios compared with a combined culture of chondrocytes and MSCs (chondrocyte/MSC). Cultures of only MSCs, chondrons, and chondrocytes were also included as controls. In vivo reproducibility of the positive effect of a chondron/MSC combination was tested subcutaneously in nude mice (n = 10) compared with chondron-only cultures. Finally, a new single-stage cell-based treatment, based on the combination of MSCs and chondrons in fibrin glue, was tested in a focal defect model in goats (n = 8) compared with microfracture.

Donors

The anonymous use of redundant material from patients with focal cartilage lesions undergoing surgery is part of the standard treatment agreement with patients in our institute. For the in vitro and in vivo studies on subcutaneous implantation, the debrided waste from focal articular cartilage lesions of 6 patients was obtained during ACI surgery. All debrided cartilage used in this study was derived from patients with either a grade III cartilage lesion or osteochondritis dissecans. Recent results from our group show that the chondrocytes obtained from debrided articular cartilage obtained from grade III and grade IV focal lesions have similar chondrogenic potentials and lead to, at least, a similar cartilage matrix production compared with those from the current nonweightbearing locations (unpublished results).

Cell Isolation

Chondrons were isolated using a rapid digestion protocol. For this, debrided cartilage was cut into small pieces and immersed in phosphate-buffered saline (PBS) with 1% penicillin/streptomycin, followed by 45 minutes of digestion at 37°C in 2% type II collagenase solution (Worthington Biochemical, Lakewood, New Jersey) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Bleiswijk, the Netherlands) with penicillin/streptomycin (100 U/mL or 100 μg/mL, respectively) (Invitrogen, Breda, the Netherlands). To show the presence of a pericellular matrix surrounding the cells after rapid digestion, isolated cells were cytospun and stained for safranin O and type VI collagen (see protocol below) and compared with isolated chondrocytes and isolated chondrons overnight for 3 donors using recently described isolation methods.[22]

The MSCs were obtained from and manufactured in our institutional Good Manufacturing Practices-approved cell therapy facility. Goat MSCs were derived from aspiration of iliac crest bone marrow in 2 Dutch milk goats, which were assigned to another experiment under the same study protocol. The bone marrow aspirate was cultured in αMEM supplemented with 15% fetal bovine serum (HyClone, Thermo Fisher Scientific, Leiden, the Netherlands), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 2 mM L-glutamine (GlutaMAX, Gibco). Adhering cells were cultured for 2 passages and then stored in liquid nitrogen for later use.

Pellet Culture

The debrided cartilage from a total of 5 patients was used for coculture of chondrons with MSCs, and cartilage from 3 of these patients was used for both chondrocyte and chondron isolation for direct comparison of the chondrogenic capacity of chondron/MSC pellets with chondrocyte/MSC cocultures. After isolation, chondrons and chondrocytes

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were combined with MSCs at different ratios: 0%, 5%, 10%, 20%, 50%, or 100% with a total cell number of 250,000 cells. Cells were pelleted by centrifugation and cultured for a period of 4 weeks in DMEM (Gibco) with 0.2 mM L-ascorbic acid 2-phosphate (AsAp, Sigma-Aldrich, Zwijndrecht, the Netherlands), 2% human serum albumin (Sanquin, Amsterdam, the Netherlands), penicillin/streptomycin (100 U/100 μg) (Invitrogen), 2% ITS-X (Invitrogen), and 5 ng/mL TGF-β2 (R&D Systems, Minneapolis, Minnesota). Medium was changed twice a week.

For all in vitro experiments (n = 5), a total of 3 pellets were created per coculture ratio. Pellets were analyzed for cartilage matrix production by glycosaminoglycan (GAG) and DNA quantification.

In Vivo Ectopic Cartilage Formation

Animal experiments were conducted in agreement with the committee for animal experiments and following institutional guidelines on the use of laboratory animals. To determine the optimal ratio of MSCs and chondrons in vivo, cells from 1 cartilage and MSC donor were embedded in 100 μL of fibrin glue (Beriplast, Nycomed, Zurich, Switzerland) at several ratios of chondron/MSC. The fibrin glue scaffolds with the chondron/MSC population contained 250,000 cells per scaffold with 0%, 5%, 10%, or 20% chondrons. In addition, Beriplast scaffolds were prepared that contained chondrons with the same absolute number as present in the chondron/MSC Beriplast scaffolds, but without the addition of MSCs (chondron-only scaffolds). Per ratio, for both the chondron/MSC and chondron-only scaffolds, a total of 5 scaffolds were created. After preparation, the scaffolds were kept overnight at 37°C and 5% CO_2 in DMEM (Invitrogen) containing 0.2 mM L-ascorbic acid 2-phosphate (AsAp, Sigma-Aldrich), 2% human serum albumin (Sanquin), and penicillin/streptomycin (100 U/100 μg) (Invitrogen). The next morning, the scaffolds were implanted subcutaneously in nude mice.

For implantation, the chondron/MSC and chondron-only scaffolds were randomly assigned to 4 separate subcutaneous dorsal implantation pockets in 10 female nude mice (Hsd-cpb:NMRI-nu, Harlan Laboratories, Horst, the Netherlands). Before surgery, 8 hours after surgery, and 1 day after surgery, the mice received pain medication by subcutaneous injections of buprenorphine (0.05 mg/kg) (Merck, Darmstadt, Germany). After 1 day of solitary housing, the mice were housed together (5 mice per cage) for 4 weeks at the Central Laboratory Animal Institute. The mice were sacrificed by cervical dislocation, and all scaffolds were harvested and analyzed for GAG and DNA content (see protocol below), while 1 scaffold per ratio was fixed in 10% buffered formalin overnight and further processed for safranin O histology and type I and II collagen immunohistochemistry (see protocol below).

In Vivo Large Animal Model

The goat experiments were performed following the guidelines of the local committee for animal experiments and by the institutional guidelines on the use of laboratory animals. Freshly created 5-mm cylindrical full-thickness cartilage defects were treated in a total of 8 female Dutch milk goats (age range, 2-4 years; mean weight, 69 ± 6 kg) with either a combination of chondrons and MSCs in Beriplast (chondron/MSC treatment) or microfracture treatment.

A standardized full-thickness chondral defect was created in the medial femoral condyles of both knees. Per goat, 1 defect was treated with the chondron/MSC treatment, while the other was treated with microfracture. The total study follow-up was 6 months, during which food and water were given ad libitum and health and general care conditions were monitored by the laboratory animal welfare officer.

After acclimatization for at least 1 week, and 1 day before surgery, a fentanyl patch was placed on the skin as pain medication, and the goats were weighed. All animals received intravenous premedication consisting of detomidine hydrochloride (0.04 mg/kg) (Pfizer, Capelle a/d IJssel, the Netherlands) and a single dose of amoxicillin-clavulanate (GlaxoSmithKline, Zeist, the Netherlands). Subsequently, induction of anesthesia was achieved with thiopental (6 mg/kg) (Rhône Mérieux, Eindhoven, the Netherlands). Throughout the surgical procedures, anesthesia was maintained with 0.8 mg/kg midazolam, 0.007 mg/kg Sufenta forte (Abbott Laboratories, Weesp, the Netherlands), and when necessary, isoflurane or propofol (Abbott Laboratories).

All surgical procedures were carried out under aseptic conditions. A medial parapatellar approach, without dislocation of the patella, was chosen to open the knee and obtain exposure of the medial femoral condyle. After macroscopic inspection, a standardized full-thickness chondral cylindrical cartilage defect (including the calcified cartilage layer) was created, using a hand-operated drill (5-mm diameter), in the full weightbearing center of the medial femoral condyle. A sharp surgical spoon was used to create a stable rim surrounding the defect. The debrided cartilage was collected in a sterile 50-mL tube with DMEM, containing 1% 100 U/100 μg penicillin/streptomycin (Invitrogen). Subsequently, the cartilage defect was treated by microfracturing. For this, the exposed subchondral bone at the bottom of the defect was perforated at the 12-, 8-, and 4-o'clock positions using a 1.5-mm K-wire until blood appeared in the created defect. Subsequently, the knee was closed in layers. In the meantime, preparations for the chondron/MSC treatment were performed. To this end, chondrons were isolated, using the rapid digestion protocol, from the debrided cartilage of the defect and combined with goat MSCs in a 10% chondron/90% MSC combination at a concentration of 1×10^6 cells/mL in the fibrinogen component of the fibrin glue. Next, the other knee was opened, and a chondral lesion was created as described above. The fibrinogen (with cells) and thrombin components of the fibrin glue were prepared for surgical application following the manufacturer's description. After this, the fibrin glue was injected into the defect and allowed to gelate for 5 minutes. The knee was flexed 10 times, to control for stability of the fibrin glue construct within the defect, and closed in layers.

After surgery, the animals were housed individually for a maximum of 24 hours, and direct full weightbearing was

allowed. In case of signs of wound infections or pain, adequate antibiotics or pain medication were provided, respectively, based on the judgment of the veterinarian. After 6 months, the animals were euthanized by an overdose of pentobarbital (Euthesate, Ceva Sante Animale, Maassluis, the Netherlands), and treated legs were removed and necessary cartilage explanted for further analysis.

Macroscopic and Microscopic Scoring

The macroscopic appearance of the articular cartilage of the medial and lateral femurs and tibias and the articular cartilage that directly articulated with the created defect were scored by 2 blinded observers using the Mastbergen score.[15] The Mastbergen score uses a 0- to 4-point scale to describe the surface damage of the articular cartilage, where an increasing score is related to increasing cartilage damage. Also, the macroscopic appearance of the treated articular defect was scored using the International Cartilage Repair Society (ICRS) macroscopic evaluation of cartilage repair score, which has recently been validated for cartilage regeneration.[21] This scoring system subdivides the quality of cartilage repair into a degree of defect repair, integration into the border zone, and macroscopic appearance using a maximum score of 12 points. The higher the score, the worse is the quality of defect repair. For both scoring systems, the scores of the 2 blinded observers were averaged. In the case the scores of the 2 observers differed more than 2 points on subitems of the scoring systems, a consensus was reached.

Full-thickness cartilage tissue samples were harvested from the anterior half of the defect and processed for GAG and DNA analysis. The posterior osteochondral half of the defect (including the cartilage surrounding this) as well as the articulating osteochondral medial tibia and osteochondral lateral femur and tibia samples were harvested and fixed in 10% buffered formalin for 48 hours. Subsequently, all samples were decalcified using Luthra solution (3.2% 11 M HCl, 10.0% formic acid in distilled water) and further processed for safranin O histology. Stained sections were scored by 2 blinded observers using the cartilage repair score (as described by O'Driscoll et al[17,18]) for the osteochondral samples derived from the posterior defect and using the Mankin score for the osteochondral samples from the lateral tibia and femur and from those having cartilage that articulated with and surrounded the defect. For the O'Driscoll system, a lower score is related to lower histological quality of the articular cartilage, while for the Mankin score, a higher score indicates a worse outcome.

Biochemical Analysis

Regenerated and native tissue cartilage samples were digested overnight in papain (250 μg/mL [Sigma-Aldrich] in 50 mM EDTA and 5 mM L-cysteine) at 56°C. The GAG content was determined using a dimethylmethylene blue (DMMB) assay[6] in which the complexation of GAG with DMMB was measured spectrophotometrically at 540 nm, using 595 nm as a reference. Chondroitin sulfate (shark) (Sigma-Aldrich) was used as a standard. Per sample, the DNA content was also determined from the papain digest using a PicoGreen DNA assay (Invitrogen) in accordance with the manufacturer's instructions.

Histological and Immunohistochemical Analyses

All fixed samples assigned for histological and immunohistochemical stainings were dehydrated by graded alcohol series, immersed in xylene, and embedded in paraffin. For histology, 5-μm sections were stained with safranin O (Merck) for GAG, and nuclei and cytoplasm were counterstained with, respectively, Weigert hematoxylin (Klinipath, Duiven, the Netherlands) and 0.4% Fast green (Merck).

Immunohistochemical staining was performed on 5-μm sections. Antigen retrieval steps, 30 minutes of Pronase (1 mg/mL in PBS) (Roche, Basel, Switzerland) followed by 30 minutes of hyaluronidase (10 mg/mL in PBS) (Sigma-Aldrich) at 37°C, were conducted for type I, II, and VI collagen staining, followed by blocking of the sections using a 5% PBS–bovine serum albumin (BSA) solution. The primary antibodies for type I collagen (20 ug/mL in 5% PBS-BSA) (CP17, Merck), type II collagen (1/100 in 5% PBS-BSA) (II-II6B3-s, Developmental Studies Hybridoma Bank, Iowa City, Iowa), and type VI collagen (1:10) (5C6, Developmental Studies Hybridoma Bank) were incubated overnight at 4°C. Next, the sections were washed in PBS-Tween and incubated with the secondary antibody for type I collagen (1/200 in 5% PBS-BSA) (biotinylated secondary anti-mouse antibody, RPN1001V, GE Healthcare, Little Chalfont, United Kingdom), type II collagen (5 μg/mL) (anti-mouse antibody conjugated with peroxidase, P0447, Dako, Glostrup, Denmark), and type VI collagen (1:100) (anti-mouse horseradish peroxidase [HRP], P0447, Dako) for 1 hour at room temperature. An extra enhancement step was performed for type I collagen, using streptavidin/HRP (1/500 in 5% PBS-BSA) (P0397, Dako) to allow visualization of the secondary antibody. Binding of the secondary antibody was visualized using 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich). Mayer hematoxylin was used as a counterstain.

Statistical Analysis

All statistical analyses were performed using SPSS version 15.0 (Chicago, Illinois). For both the in vitro and in vivo mice experiments comparing, respectively, chondron/MSC to chondrocyte/MSC and chondron/MSC to chondron only, differences in GAG and GAG/DNA were, per ratio, determined using a paired t test. Differences in GAG and GAG/DNA between the chondron/MSC ratios in the in vitro pellet cocultures were determined using a 1-way ANOVA with a post hoc Bonferroni test.

Regarding the in vivo goat experiments, the differences between the microfracture and chondron/MSC treatments for the macroscopic, microscopic, and biochemical analyses were tested using a paired t test. For all analyses, a difference of $P < .05$ was considered statistically significant, and outcomes are defined as mean ± standard deviation.

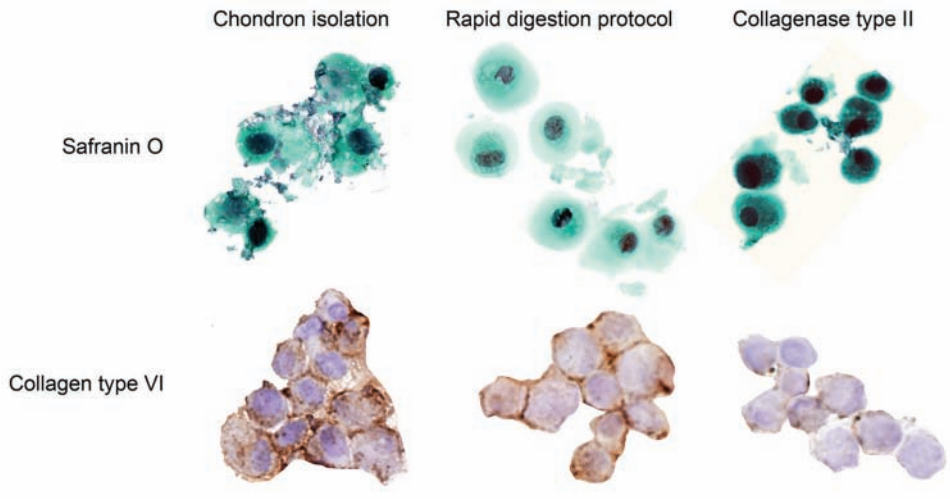

Figure 1. Type VI collagen and safranin O staining comparing chondron isolation, rapid digestion, and chondrocyte isolation. Type VI collagen and safranin O staining show a pericellular matrix comparing chondron isolation using standard versus rapid digestion protocols. The pericellular matrix is not present after standard overnight chondrocyte isolation.

RESULTS

Donor Material and Cell Isolation

On average, 0.72 ± 0.20 g of debrided tissue was obtained from the focal lesions, which yielded 1.06 ± 0.56 × 10^6 chondrons after rapid digestion. The mean efficiency of the rapid digestion protocol was thus calculated at 1.37 ± 0.50 × 10^6 chondrons per gram of debrided tissue. The type VI collagen and safranin O staining showed that the rapid digestion protocol indeed resulted in isolated cells with a preservation of their pericellular matrix in contrast to the overnight chondrocyte isolation (Figure 1).

Pellet Culture: Coculture of MSCs With Chondrocytes or Chondrons

Partial replacement of chondrocytes by MSCs (10%-50% chondrocyte/MSC cultures) resulted in a similar or higher amount of produced GAG per DNA compared with 100% chondrocyte cultures. Also, the 10% to 50% chondron/MSC pellets produced more total GAG ($P \leq .03$) and GAG per cell ($P \leq .001$) compared with the 100% chondron pellets. The total GAG content and GAG/DNA of the coculture pellets containing 10% to 50% chondrons were higher ($P \leq .03$) compared with the pellets with 10% to 50% chondrocytes (Figure 2). No differences ($P \geq .173$) in DNA content were observed within and between the chondron/MSC and chondrocyte/MSC cultures (data not shown).

In Vivo Subcutaneous Cartilage Formation

To determine the optimal chondron/MSC ratio, scaffolds containing chondron/MSC or chondron-only combinations were implanted subcutaneously in nude mice. A higher GAG content was found ($P \leq .04$) in the 5% to 20% chondron/MSC scaffolds compared with similar amounts of chondrons alone, indicating a stimulating effect on cartilage-specific matrix production upon the addition of MSCs to chondrons (Figure 3). The DNA content did not differ statistically ($P \geq .849$) between the chondron/MSC ratios, whereas the DNA content of the different ratios in the chondron-only scaffolds showed a decrease compared with the seeding density (data not shown). After correcting for the amount of DNA present after 4 weeks, the 10% to 20% chondron/MSC scaffolds still showed a higher ($P \leq .015$) GAG production per DNA compared with the related chondron-only scaffolds (Figure 3).

Both the chondron/MSC and chondron-only scaffolds stained positive for type II collagen, whereas safranin O staining was more intense in the chondron/MSC compared with the chondron-only scaffolds. Positive type I collagen staining was found surrounding the cells in the chondron/MSC scaffolds, while this was also present in the extracellular matrix for the chondron-only scaffolds (Figure 4).

Single-Stage Chondron/MSC Treatment Versus Microfracturing in Goats

Surgery was performed without complications. All articular surfaces were free from signs of degeneration at first inspection during surgery. After surgery, all goats were able to function with full weightbearing within 4 hours, and their mean maximum weight loss was 4.3% ± 1.4% from their preoperative weight. During follow-up, no signs of wounds or intra-articular infections were observed.

At 6 months after surgery, the macroscopic appearance of the defects treated with microfracture showed an incomplete defect fill (6 defects had 25%-50% and 2 defects had 50%-75% defect fill), while those treated with the chondron/MSC treatment were almost completely filled with cartilage-like tissue (7 defects had 75%-100% and 1

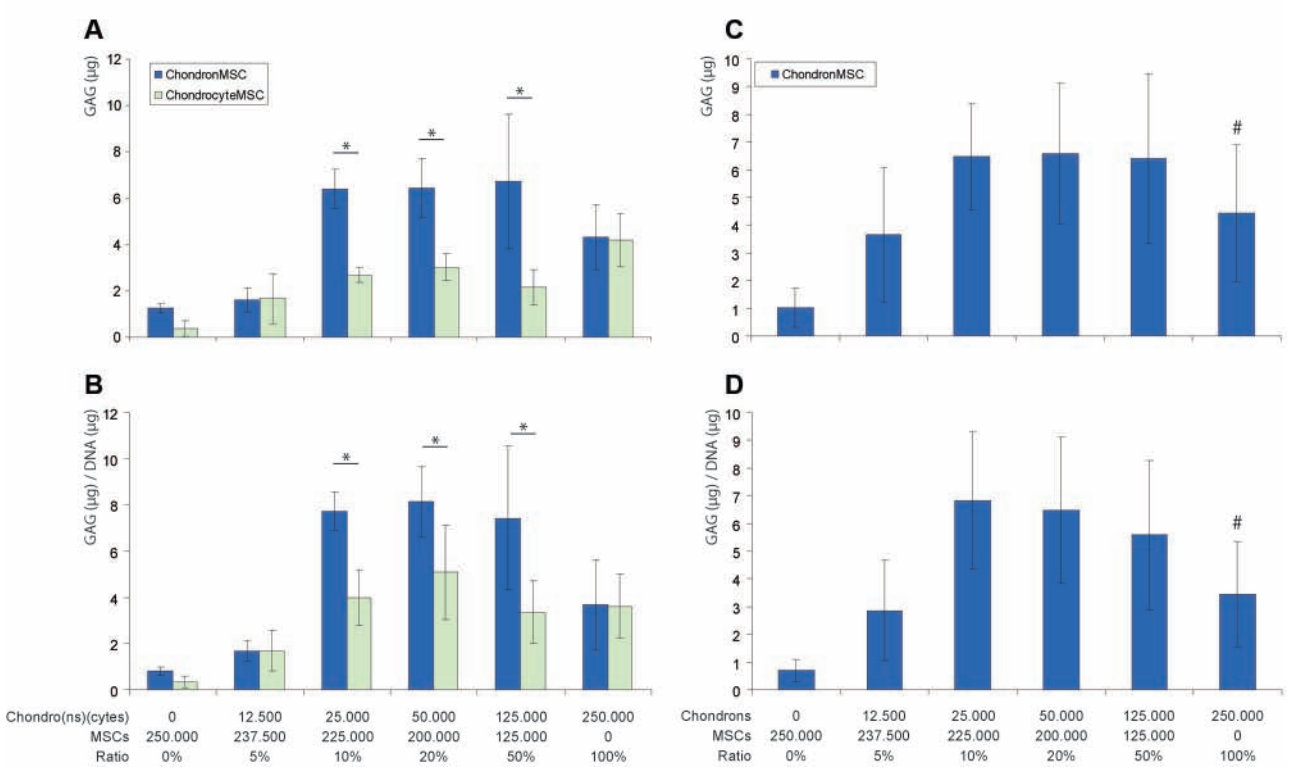

Figure 2. Pellet cocultures. Direct comparison between chondrocyte/mesenchymal stromal cell (MSC) and chondron/MSC cultures from the same cartilage donors (n = 3) (A, B) and separate chondron/MSC cultures (n = 5) (C, D). Absolute glycosaminoglycan (GAG) and GAG/DNA content resulting from 4 weeks of pellet culture. For the ratios of 10% to 50%, the chondron/MSC cultures show higher GAG and GAG/DNA compared with similar ratios between chondrocyte/MSC cultures. Also, a replacement of 50% to 90% chondrons in culture by MSCs improves GAG production and GAG/DNA content. Error bars represent standard deviations. $^{*}P \leq .003$; $^{\#}P \leq .03$.

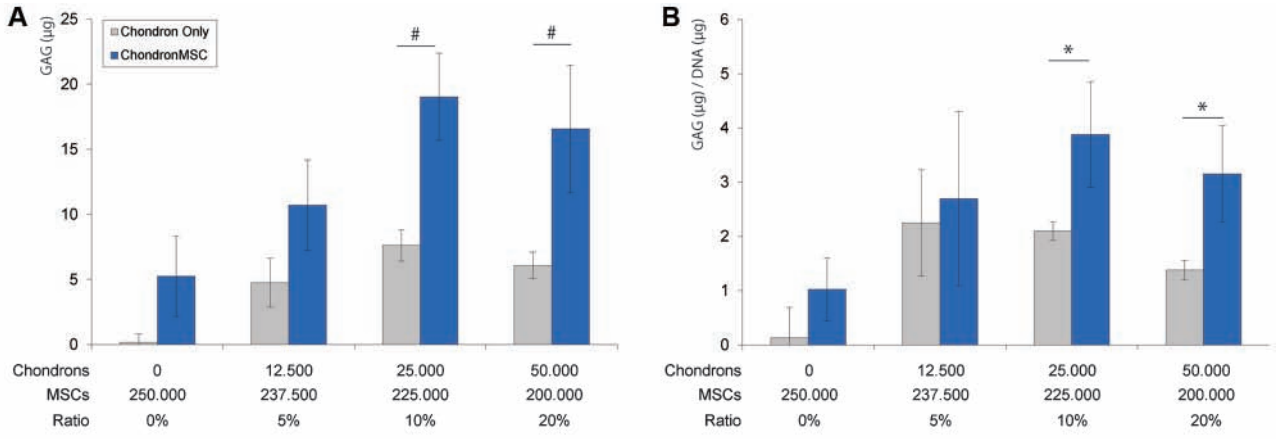

Figure 3. Chondron/mesenchymal stromal cell (MSC) versus chondron only after subcutaneous implantation. (A) Glycosaminoglycan (GAG) and (B) GAG/DNA content after 4 weeks of subcutaneous implantation of chondron/MSC scaffolds and chondron-only scaffolds. For ratios of 10% to 20%, the chondron/MSC scaffolds produced more GAG and GAG/DNA compared with similar absolute amounts of chondron-only scaffolds, indicating an added value of adding MSCs. Error bars represent standard deviations. $^{*}P \leq .04$; $^{\#}P \leq .015$.

defect had complete defect fill) (Figure 5). This was also confirmed by the mean ICRS macroscopic score for cartilage repair, which was significantly higher ($P = .002$) in the chondron/MSC–treated knees (6.63 ± 0.83) compared with the microfracture knees (4.13 ± 0.92) (Figure 6A). The macroscopic (Mastbergen) score of the other joint

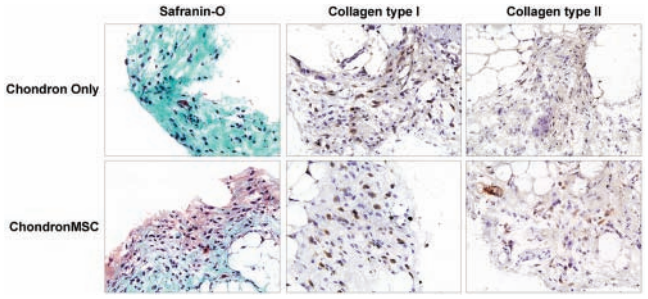

Figure 4. Cartilage-specific matrix staining after subcutaneous implantation. Chondron/mesenchymal stromal cell constructs show more safranin O staining for glycosaminoglycan, while type I collagen matrix staining is absent.

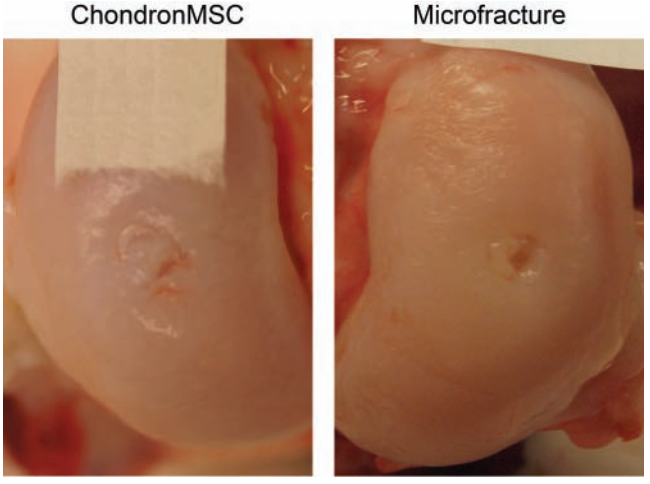

Figure 5. Macroscopic defect repair. Best macroscopic defect repair for both the chondron/mesenchymal stromal cell (MSC) and microfracture treatment. Chondron/MSC shows good defect fill with a relatively intact surface layer, while after microfracture, incomplete defect fill is present, and the rim of the created defect is still visible.

surfaces did not show a significant difference ($P \geq .17$) between the 2 interventions (Figure 6B).

The histological quality of the regenerated tissue was significantly ($P = .001$) higher (as determined using the O'Driscoll score) in the knees treated with chondron/MSC compared with the microfracture-treated defects (Figure 6C). Also, defect fill was more consistent in chondron/MSC–treated knees compared with microfracture-treated knees (Figure 7). Histological evaluation of the other joint surfaces showed no significant differences ($P \geq .48$) between the 2 treatments (Figure 6D). However, for both treatments, the Mankin scores were higher for the cartilage surrounding and articulating with the cartilage containing the treated defect compared with the cartilage in the lateral knee compartment (Figure 6D).

The mean absolute GAG production per gram of regenerated tissue was highest ($P = .022$) in the chondron/MSC–treated (0.083 ± 0.037 mg GAG/g tissue) compared with

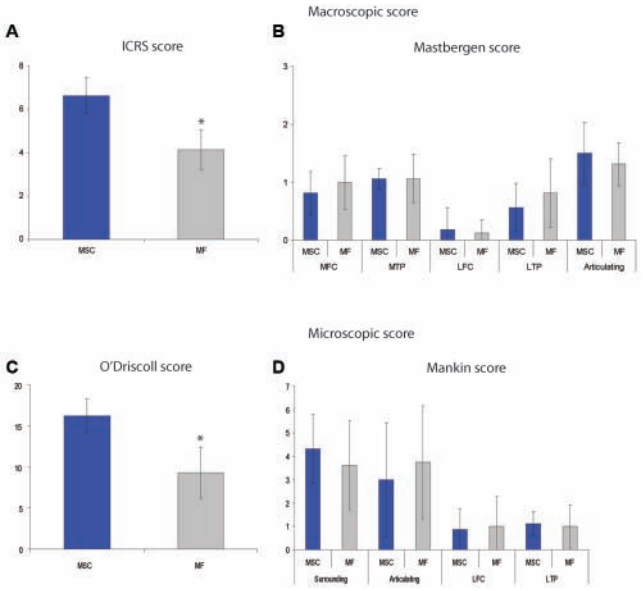

Figure 6. Macroscopic and microscopic scores. Macroscopic and microscopic scores of different anatomic locations of the goat knee at 6 months after surgery. Articulating, cartilage that directly articulates with the created defect; LFC, lateral femoral condyle; LTP, lateral tibial plateau; MF, microfracturimg; MFC, medial femoral condyle; MSC, chondron/mesenchymal stromal cell (MSC) therapy; MTP, medial tibial plateau; surrounding, cartilage that surrounds the created cartilage defect. For both macroscopic and microscopic cartilage scores, the MF-treated defects perform less compared with chondron/MSC–treated defects. Error bars represent standard deviations. *$P \leq .002$.

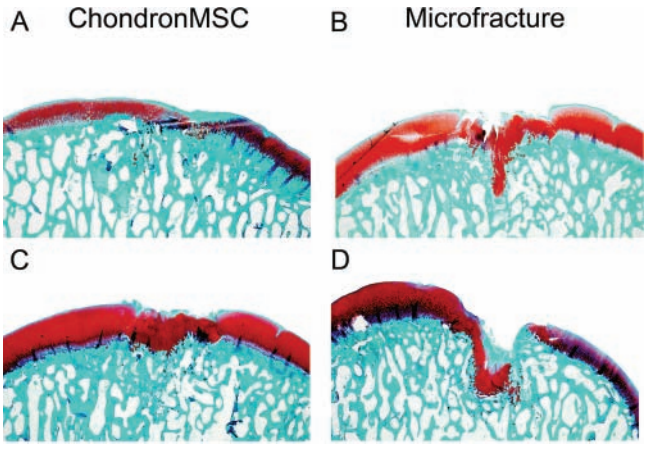

Figure 7. Microscopic defect repair. Slices were cut perpendicular through the middle of the created defect. For both the chondron/mesenchymal stromal cell (MSC) and microfracture treatments, the best (A, B) and worst results (C, D) are shown. For both chondron/MSC images (A, C), complete to almost complete defect fill is achieved, while for the microfracture images (B, D), the defect fill varied a lot between the different treated defects.

the microfracture-treated (0.041 ± 0.013 mg GAG/g tissue) defects. Also, when corrected for DNA, the neocartilage in the chondron/MSC group (502 ± 129 μg GAG/μg DNA) contained significantly more GAG (P = .016) compared with the microfracture group (301 ± 94 μg GAG/μg DNA). Both GAG per milligram of tissue and GAG per DNA did not show statistically significant differences between chondron/MSC and microfracture for other joint locations.

DISCUSSION

This study evaluated whether combining MSCs with chondrons is suitable for a single-stage cell-based regenerative treatment for focal articular cartilage lesions. We showed that in vitro combinations of 10% to 20% chondrons with MSCs produced more cartilage matrixes compared with chondrocyte/MSC cocultures. Moreover, in vivo, these combinations also showed more matrix production compared with scaffolds with only chondrons. In addition, a substitution of 80% to 90% chondrons by MSCs increased matrix production in vitro compared with the pellets in which chondrons were not substituted with MSCs. In vivo treatment of a freshly created articular cartilage defect in goats with a combination of 10% chondrons and 90% MSCs in a fibrin glue scaffold resulted in more matrix formation at the macroscopic, histological, and biochemical levels compared with microfracture.

Stimulation of cartilage-specific matrix production by a combination of chondrocytes and MSCs has already been shown in several in vitro models.[5,20,25] A substitution of 80% chondrocytes by MSCs was also shown to produce similar or more cartilage-specific matrix proteins, analogous to the current experiments using chondrons.[25] The mechanism underlying this effect is still under debate. However, in pellet cultures using MSCs and chondrocytes from different species, the MSCs disappeared over time as determined from polymerase chain reaction analysis.[25] This suggests that the MSCs stimulate chondrocyte proliferation and matrix production by trophic signaling instead of undergoing terminal chondrogenic differentiation themselves. Chondrocyte cultures under MSC-conditioned medium enhanced cartilage-specific matrix production but not to the same extent as when MSCs were actually present during culture.[25] This suggests that, next to trophic factors, direct cell-cell contact also plays a role in the stimulatory environment of the chondrocyte/MSC combination.

The added value of using chondrons rather than chondrocytes has been suggested previously. Preservation of the pericellular chondrocyte matrix in chondrons, in a 3-dimensional in vitro redifferentiation model, showed higher Col2a1 and lower MMP13 and Col1a1 gene expression when compared with chondrocytes without a pericellular matrix.[22] Also, other studies showed a higher GAG production in chondron cultures compared with chondrocyte cultures.[13] In our study, cocultures of chondrons produced more cartilage matrixes than chondrocyte/MSC cocultures. The pericellular matrix that surrounds chondrocytes plays a vital role in the communication between the cell and its environment.[7] Binding of growth factors to the surface-associated extracellular matrix molecules facilitates signaling events, which are crucial in processes such as cell proliferation, survival, and differentiation.[4,12] Therefore, trophic factors excreted by the MSC may have a larger effect when a surrounding matrix is present.

Approximately 1×10^6 chondrocytes per square centimeter of debrided defect are currently used to treat cartilage defects using ACI. In this study, we were able to isolate a mean $1.06 \pm 0.56 \times 10^6$ viable chondrons from debrided defect cartilage within 45 minutes. When mixed with allogenic MSCs, a combination containing 10% to 20% chondrons showed optimal cartilage formation in terms of quantitative matrix production and immunohistochemical collagen stainings. This indicates that defects up to 10 cm^2 could be treated in a single-stage cell-based approach using the fast digestion protocol and a combination of allogenic MSCs.

Despite the use of allogenic MSCs, intra-articular complications were not observed during follow-up, indicating that no clinically relevant graft rejection occurs. This is in accordance with the observation that allogenic MSCs have safely been applied in a number of clinical trials in humans.[10,11,23] In previous studies, when MSCs were encapsulated in hydrogels, only a gradual increase in the expression of major histocompatibility complex class I and II molecules was observed.[26]

Despite the improvement in cartilage regeneration compared with microfracturing, both treatments were shown to induce degeneration of the opposing cartilage, as was previously shown for microfracturing in the same in vivo model of cartilage trauma. Also, articular cartilage surrounding a focal lesion has been shown to be prone to accelerated matrix damage because of the higher tendency to deform under direct axial strain.[2,8,9] In this study, for both treatments, the cartilage surrounding and directly articulating with the treated defects by macroscopic evaluation appeared to be damaged. This suggests that trauma treatment, even if successful, still may negatively affect healing. However, direct weightbearing of the goats after surgery could have negatively contributed to the quality of the tissue that surrounded and opposed the cartilage defect.

Another disadvantage of the model used is the bilateral approach. While on the one hand, it provides the possibility to directly compare 2 treatments without intra-animal variation and reduces animal use, it is possible that one treatment affected the other because of possible asymmetrical weightbearing due to impaired healing. However, the goats in our study were directly able to fully bear weight in both their knees without loss of mobility during follow-up.

We have recently received ethical approval from the national ethical board on stem cell therapies to start a phase I trial in humans using the described technology (ie, allogenic stem cells and collagenase). This trial has briefly been described in a recently published review.[16] In conclusion, the current study showed that the combination of chondrons and MSCs increases cartilage matrix formation and that this combination of cells can successfully and safely be applied in a single-stage cell-based treatment of focal cartilage lesions, which outperforms microfracture in a goat model.

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