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One-stage focal cartilage defect treatment with bone marrow mononuclear cells and chondrocytes leads to better macroscopic cartilage regeneration compared to microfracture in goats

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SUMMARY

Objective: The combination of chondrocytes and mononuclear fraction (MNF) cells might solve the expansion induced dedifferentiation problem of reimplanted cells in autologous chondrocytes implantation as sufficient cells would be available for direct, one-stage, implantation. Earlier *in vitro* work already showed a positive stimulation of cartilage specific matrix production when chondrocytes and MNF cells were combined. Therefore, this study aimed to evaluate cartilage regeneration using a one-stage procedure combining MNF cells and primary chondrocytes for the treatment of focal cartilage lesions in goats compared to microfracture treatment.

Design: Freshly created focal cartilage defects were treated with either a combination of chondrocytes and MNF cells embedded in fibrin glue or microfracture treatment. After 6 months follow-up local regeneration as well as the general joint cartilage health were evaluated using validated scores and biochemical assays.

Results: Macroscopic ($P = 0.015$) scores for the cartilage surface at the treated defect were, after 6 months, significantly higher for the chondrocyteMNF treatment compared to microfracture-treated defects, but microscopic scores were not ($P = 0.067$). The articulating cartilage showed more ($P = 0.005$) degeneration following microfracture treatment compared to chondrocyteMNF treatment. Biochemical glycosaminoglycans (GAG) evaluation did not reveal differences between the treatments. Both treatments had resulted in a slight to moderate cartilage degeneration at other locations in the joint.

Conclusion: In conclusion, treatment of focal articular cartilage lesions in goats using a combination of MNF cells from bone marrow and unexpanded chondrocytes leads to better macroscopic regeneration compared to microfracture, however needs further fine-tuning to decrease the negative influence on other joint compartments.

Introduction

Autologous chondrocyte implantation (ACI) is frequently used to treat focal cartilage lesions in the knee. Due to the limited number of cells that can be harvested from a biopsy, *in vitro* cell expansion followed by reimplantation of this precultured population is inevitable and considered to be the main drawback of this relatively successful procedure. A combination of cells could be an alternative for the expansion of harvested chondrocytes in ACI. When isolated chondrocytes are directly combined with another cell type, which also would improve the chondrogenic potential of reimplanted cells, the whole procedure could be performed within one surgery and the quality of regenerated cartilage improved.

The combination of cells derived from native tissue with other cell types has recently gained attention to address currently defined challenges in regenerative medicine[1]. For example, vascular endothelial cells and urothelial cells were both able to differentiate adipose-derived stem cells into respectively the

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osteogenic and urothelial lineage in order to enhance bone and urothelial tissue engineering[2,3]. Articular cartilage matrix production of expanded articular chondrocytes has been shown to be positively influenced by primary chondrocytes and human adipose-derived and embryonic stem cells *in vitro*[4–6]. In addition, the chondrogenic phenotype of dedifferentiated chondrocytes was improved when cocultured with mesenchymal stromal cells (MSC)[7–9]. As these effects were also achieved when conditioned medium and non-contact culture systems were used, the underlying mechanism could be explained by the communication between the cells through trophic factors[5,8,10]. The mononuclear fraction (MNF) in bone marrow is a major source of trophic factors and can easily be obtained in the timeframe of one surgery[11]. Delivery of this bone marrow fraction to full-thickness cartilage defects in horses showed good cartilage healing[12]. For this reason, combination of primary isolated chondrocytes and MNF cells could be the basis for a one-stage cell-based regenerative treatment for focal articular cartilage defects. Therefore, this study aimed to evaluate cartilage regeneration using a one-stage procedure combining MNF cells and primary chondrocytes for the treatment of focal cartilage lesions in goats compared to microfracture treatment.

Materials and methods

Experimental design

This experiment was approved by and conducted following the guidelines of the animal care committee of the University of Utrecht under number DEC 2011.III.03.026. A full-thickness chondral lesion was created in the medial condyle of both knees in female Dutch milk goats. Per goat one defect was treated with a combination of chondrocytes and MNF cells in fibrin glue (Beriplast®, Nycomed, CSL Behring, The Netherlands) and the other by microfracture. The animals were euthanized at 6 months after surgery. Defect healing and the quality of articular cartilage in all other compartments of the joint were determined using macroscopic, microscopic and biochemical analysis.

Animals

A total of nine adult Dutch milk goats (age 3–5 years old, weight 75 ± 10 kg) were used in this study. The necessary number of animal ($n = 9$) was determined by a power analysis (power of 0.90 and an α of 0.05) based on the estimated amount of glycosaminoglycans (GAG) (32.2 ± 2.3) at 6 months after cartilage surgery[13].Once arrived at the animal facility the goats were allowed to acclimatize for at least 1 week before surgery. During follow-up food was provided *ad libitum* and general health was assessed by the veterinarian of the institutional animal care facility. Up to 2 weeks after surgery an additional health scoring system, focusing on wound infection, limping and general activity, was scored.

Surgery

One day prior to surgery, the goats were weighed and prophylactic pain medication was provided by a fentanyl skin patch. Intravenous premedication (0.4 mg/kg detomidine hydrochloride (Pfizer, The Netherlands) and a single dose of Augmentin (GlaxoSmithKline, The Netherlands)) was followed by induction anesthesia using thiopental (6 mg/kg, Rhône Mérieux, France). During surgery, anesthesia was maintained by a combination of midazolam 0.8 mg/kg (Abbott Laboratories, The Netherlands) and sufenta forte 0.007 mg/kg (ASTPharma, The Netherlands) and, if necessary, isoflurane or propofol (Abbott Laboratories).

Surgery was performed under aseptic conditions. Bone marrow was obtained by needle aspiration from the iliac crest. For this a Jamshidi® needle was tapped into the iliac crest. Bone marrow was aspirated using 20 ml sterile syringes and stored in heparin-coated tubes. After this the whole bone marrow was spun down (300 g, 10 min) and the cell pellet diluted 50 times in red blood cell lysis buffer (Sigma, The Netherlands) during 45 min. Following this the remaining cells were spun down by centrifugation and washed twice in phosphate buffered saline (PBS), thus producing the MNF fraction.

A medial parapatellar approach was performed to expose the medial femur condyle. After macroscopic scoring of the medial cartilage surface, using the Mastbergen score[14], a 5 mm cylindrical chondral lesion was created in the central weight bearing region using a hand-operated drill. A bone curette was used to debride the remnants of articular cartilage and to create a stable defect rim. The debrided defect cartilage was digested using a rapid-digestion which resulted in chondrocytes with similar chondrogenic potential, in terms of cartilage matrix formation, compared to chondrocytes obtained after standard overnight isolation (data not shown). For this, cartilage was cut into small pieces and digested during 45 min in 2% collagenase type II solution (Worthington, Lakewood, USA) in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) with penicillin/streptomycin (100 U/ml/100 μg/ml, Invitrogen, The Netherlands) under continuous shaking at 37°C. Following digestion the collagen was washed out by three washing steps. This digestion protocol resulted in a digestion efficiency of $1.37 \pm 0.50 \times 10^6$ viable chondrocytes per gram debrided tissue. Meanwhile, the debrided defect was treated using the microfracture technique by creating four holes through the subchondral bone using a 1.5 mm K-wire. Microfracture treatment was confirmed by the presence of blood entering the defect from the drilled holes. Following this the knee was closed in layers.

The other knee was opened, the medial cartilage surface scored and a chondral defect created using similar procedures as described above. A mixture of 10% rapidly isolated chondrocytes and 90% MNF cells were suspended in the fibrinogen component of Beriplast® (Nycomed) at a final concentration of 1×10^6 cells per milliliter. The fibrinogen component and thrombin component of the Beriplast® were prepared for application following the manufacturers' protocol and injected into the cartilage defect. After 5 min of gelation time the knee was flexed 10 times to check the stability of the graft into the defect and the knee was closed in layers.

Direct full-weight bearing was allowed following surgery. The animals were housed individually for a period of 24 h. Additional pain medication and antibiotics were provided, based on the judgment of the veterinarian, with approval of JEJB. The animals were euthanized after 6 months using an overdose of pentobarbital (Euthesate®) and both hind legs explanted for further analysis.

Macroscopic cartilage evaluation

Soft tissues were removed from the explanted hind legs and high-resolution pictures obtained from the medial femoral condyle (MFC) and lateral femoral condyle (LFC) and tibial cartilage surfaces (medial MTP, lateral LTP) and from the cartilage defect. Pictures were coded for blinded scoring by two observers. The articular cartilage of the medial and lateral tibia and femur as well as the medial tibia cartilage that directly articulated with the treated defect were scored using the macroscopic Mastbergen score[14]. The Mastbergen score is a four-point scale ranging from a macroscopically healthy and smooth cartilage surface (0 points) to cartilage degeneration characterized by deep grooves and surrounding

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