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The use of a cartilage decellularized matrix scaffold for the repair of osteochondral defects: the importance of long-term studies in a large animal model

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SUMMARY

Objective: To investigate the effect of decellularized cartilage-derived matrix (CDM) scaffolds, by itself and as a composite scaffold with a calcium phosphate (CaP) base, for the repair of osteochondral defects. It was hypothesized that the chondral defects would heal with fibrocartilaginous tissue and that the composite scaffold would result in better bone formation.

Methods: After an 8-week pilot experiment in a single horse, scaffolds were implanted in eight healthy horses in osteochondral defects on the medial trochlear ridge of the femur. In one joint a composite CDM–CaP scaffold was implanted (+P), in the contralateral joint a CDM only (−P) scaffold. After euthanasia at 6 months, tissues were analysed by histology, immunohistochemistry, micro-CT, biochemistry and biomechanical evaluation.

Results: The 8-week pilot showed encouraging formation of bone and cartilage, but incomplete defect filling. At 6 months, micro-CT and histology showed much more limited filling of the defect, but the CaP component of the +P scaffolds was well integrated with the surrounding bone. The repair tissue was fibrotic with high collagen type I and low type II content and with no differences between the groups. There were also no biochemical differences between the groups and repair tissue was much less stiff than normal tissue ($P < 0.0001$).

Conclusions: The implants failed to produce reasonable repair tissue in this osteochondral defect model, although the CaP base in the −P group integrated well with the recipient bone. The study stresses the importance of long-term *in vivo* studies to assess the efficacy of cartilage repair techniques.

Introduction

In the quest for techniques to produce a cartilage repair tissue that will remain functional life-long, a wide range of approaches have been explored[1]. Although some techniques, such as various variants of autologous chondrocyte implantation (ACI)[2] and joint distraction[3], yield promising clinical results, none of these approaches has yet been proven to fully restore proper hyaline cartilage. An alternative technique that has yielded very promising results in other areas of regenerative medicine, such as restoration of bladder, trachea and gut, is the use of scaffolds based on decellularized extracellular matrix (ECM)[4]. The advantage of this technique is that an environment of natural ECM elements is created that may still contain a variety of appropriate bioactive cues without the presence of cellular components, hence avoiding immunological issues[5,6]. Moreover, it does allow for a xenogenic approach as ECM proteins are highly conserved across species[4]. *In vitro* studies have already demonstrated the potential of cartilage-derived matrix (CDM) scaffolds, as abundant new glycosaminoglycan (GAG)- and collagen type II-containing cartilaginous matrix was formed by cultured mesenchymal

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stromal cells[7]. The potency was further underscored by *in vivo* studies in small animal models both at ectopic[6,8] and orthotopic locations[9,10].

The CDM scaffolds are potentially interesting for the repair of osteochondral defects, as they can be custom-shaped into osteochondral plugs that can be inserted in a press-fit fashion, avoiding insecure or complicating fixation techniques that may induce damage to the surrounding and opposing tissue[11]. They also have the potential to become an off-the-shelf product, as there is no need for pre-implantation culturing. This approach, however, requires the simultaneous regeneration of both bony and cartilaginous tissues. This can in theory be realized by using either a composite scaffold, or a single scaffold that will allow formation of both tissues, depending on the surrounding original tissue.

In this study, a CDM-based scaffold is developed and used. The CDM consists of predominantly collagen type II, in the absence of GAGs and cells[12] and is used either on its own, or combined with a three-dimensionally (3D) printed calcium phosphate (CaP) cement-based proven osteogenic scaffold[13,14] to fill osteochondral defects in the femoropatellar joints of horses. The equine model is seen as one of the best and most challenging models for orthopaedic ailments[15,16]. It was hypothesized that the chondral portion of the osteochondral defects would heal with neo-tissue coming close to hyaline cartilage and that the composite scaffold would show better bone formation and lead to better overall anatomical reconstitution.

Methods

Animals

The protocols and studies described were approved by the ethical and animal welfare committees of Utrecht University (pilot study) and National University of Costa Rica (long-term study). For the pilot study one Dutch Warmblood horse (age: 6 years, 490 kg) was used, while for the long-term study eight healthy Criollo breed horses (age 4–9 years; weight 275–350 kg) were used. The horses were free of lameness and without any clinical or radiographic evidence of acute or chronic injuries. They were housed in individual boxes, and fed a standard maintenance ration of concentrate with hay *ad libitum* and free access to water.

Scaffold preparation

Full-thickness cartilage was harvested from cadaveric femoropatellar joints of horses ($n = 5$; age 3–10 years), euthanized for reasons other than joint disease, with owner permission. The cartilage particles were pooled and decellularized according to a protocol previously described[11]. Finally, particles were milled in liquid nitrogen (A11 basic analytical mill, IKA, Staufen, Germany) and sieved through pores of 300 μm.

The CaP scaffold [Fig. 1(A), (B)] was manufactured by 3D printing of tricalcium phosphate (TCP) powder with diluted phosphoric acid, as described previously[14]. Briefly, TCP was synthesized by heating a mixture of $CaHPO_4$ and $CaCO_3$ (both Merck, Germany) in a 2:1 molar ratio to 1400°C for 5 h, followed by quenching to room temperature. The sintered cake was manually crushed and sieved <125 μm and finally ground in a ball mill (PM400, Retsch, Germany) for 10 min. Printing was performed on a Z-Corp 310 powder printer (Z-Corporation, Burlington, USA) by using the TCP powder and 20 wt% phosphoric acid as printing liquid.

The suspended decellularized particles were placed in a 11 mm diameter cylindrical mould, either directly (−P) or placed on top of the CaP scaffolds (+P) while ensuring that they penetrated all macropores of the CaP scaffold. The scaffolds were then lyophilized for 24 h and subjected to ultraviolet light overnight to allow for crosslinking, before sterilization using ethylene oxide gas.

Experimental design

Scaffolds were implanted in 11 mm diameter and 10 mm deep cylindrical defects that were surgically created at the axial side of the medial femoral trochlear ridge. The 8-week pilot study was performed to evaluate the short-term response to the CDM scaffold. To reduce the use of experimental animals, a horse was used that was destined to be sacrificed for educational purposes. No CaP scaffold was used in this case. In the main 6-month study, each horse received both the treatment with CDM scaffold alone (−P) and with a composite scaffold consisting of CDM and a 3D printed CaP scaffold (+P). Horses 1–4 received treatment −P and +P for the left and right femoropatellar joints, respectively. For horses 5–8 this was inverted.

Surgical procedure

After premedication with xylazine (1.1 mg/kg, Pisa) intravenously (IV), horses were induced with ketamine (2.2 mg/kg, Holliday) and midazolam (0.05 mg/kg, Holliday) IV and positioned in dorsal recumbence. General anaesthesia was maintained with isoflurane in oxygen.

A cranial femoropatellar mini-arthrotomy was performed through a 5 cm incision made between the middle and medial patellar ligaments[17]. Osteochondral defects at the middle aspect of each medial femoral trochlear ridge were created using a power-driven drill. Defect site and joints were flushed with saline solution (Careflex) before implantation. Scaffolds were press-fit implanted into each defect. Wounds were sutured in four layers (joint capsule, deep fascia, superficial fascia, and skin) and a stent bandage was applied over the incision.

Post-operative care and rehabilitation

Post-operatively, horses received antibiotics for 8 days (procaine penicillin 15,000 IU/kg intramuscularly SID, Alfasan, and IV gentamicin 6.6 mg/kg BID, Alfasan), and non-steroidal anti-inflammatory drugs (phenylbutazone (2.2 mg/kg, Lisan, orally BID)) during the first 14 days.

The pilot horse was subjected to the following rehabilitation protocol during the next 8 weeks: from week 2, the horse was hand-walked daily starting with 5 min/day with increments of 5 min per week until 20 min/day was reached. In the main study, from week 3, horses were hand-walked starting with 5 min/day with increments of 5 min/week, until 30 min/day was reached, which was then kept stable until week 16. In months 5 and 6, horses were walked, trotted and cantered for 2 min at each gait.

Monitoring during experimental period

The animals were subjected to daily monitoring of clinical parameters (temperature, heart rate and respiratory rate). Blood parameters were checked and lateromedial radiographs of the femoropatellar joints were taken before surgery and at months 1, 2, 4 and 6 after the intervention to check for general health.

Euthanasia and sample harvesting

The pilot horse was euthanized at 8 weeks by a combination of detomidine (0.01 mg/kg, Vetoquinol), ketamine (2 mg/kg, Vetoquinol), midazolam (0.1 mg/kg, Actavis) and pentobarbital (50 mg/kg, AST Farma). The horses of the main study were euthanized at 6