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EXPERIMENTAL STUDY

Osteochondral regeneration using a novel aragonite-hyaluronate bi-phasic scaffold in a goat model

E. Kon · G. Filardo · D. Robinson · J. A. Eisman · A. Levy · K. Zaslav · J. Shani · N. Altschuler

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Abstract
Purpose The objective of this study was to examine whether different mechanical modifications and/or impregnation of hyaluronic acid (HA) might enhance aragonite-based scaffold properties for the regeneration of cartilage and bone in an animal model.
Methods Bi-phasic osteochondral scaffolds were prepared using coralline aragonite with different modifications, including 1- to 2-mm-deep drilled channels in the cartilage phase (Group 1, $n = 7$) or in the bone phase (Group 2, $n = 8$), and compared with unmodified coral cylinders (Group 3, $n = 8$) as well as empty control defects (Group 4, $n = 4$). In each group, four of the implants were impregnated with HA to the cartilage phase. Osteochondral defects (6 mm diameter, 8 mm depth) were made in medial and lateral femoral condyles of 14 goats, and the scaffolds were implanted according to a randomization chart. After 6 months, cartilage and bone regeneration were evaluated macroscopically and histologically by an external laboratory.
Results Group 1 implants were replaced by newly formed hyaline cartilage and subchondral bone (*combined histological evaluation according to the ICRS II-2010 and O'Driscoll* et al. *34 ± 4 n = 7*). In this group, the cartilaginous repair tissue showed a smooth contour and was well integrated into the adjacent native cartilage, with morphological evidence of hyaline cartilage as confirmed by the marked presence of proteoglycans, a marked grade of collagen type II and the absence of collagen type I. The average scores in other groups were significantly lower (Group 2 ($n = 8$) 28.8 ± 11, Group 3 ($n = 8$) 23 ± *9* and Group 4 (empty control, $n = 4$) 19.7 ± *15*).
Conclusions The implants with the mechanical modification and HA impregnation in the cartilage phase outperformed all other types of implant. Although native coral is an excellent material for bone repair, as a stand-alone material implant, it does not regenerate hyaline cartilage. Mechanical modification with drilled channels and impregnation of HA within the coral pores enhanced the scaffold's cartilage regenerative potential. The modified implant shows young hyaline cartilage regeneration. This implant might be useful for the treatment of both chondral and osteochondral defects in humans.

Keywords Coral scaffold · Osteochondral defect · Cartilage regeneration · Bi-phasic implant

The study was conducted at the Research Unit of AssaF Haroffe Hospital, Israel.

E. Kon (✉) · G. Filardo
III Clinic—Biomechanics Laboratory, Rizzoli Orthopaedic Institute, Via Di Barbiano, 1/10, 40136 Bologna, Italy
e-mail: e.kon@biomec.ior.it

D. Robinson
Orthopedic Research & Foot and Ankle Unit, Rabin Medical Center, Petah Tikwa, Israel

J. A. Eisman
Garvan Institute of Medical Research, University of New South Wales, Sydney, Australia

A. Levy
Center for Advanced Sports Medicine Knee and Shoulder, Morristown, NJ, USA

K. Zaslav
Orthopedic Surgery Virginia Commonwealth University, Richmond, VA, USA

J. Shani
ChavatDaat Veterinary Specialist Center, Beit Berl, Israel

N. Altschuler
CartiHeal (2009) Ltd, Or Yehuda, Israel

Introduction

Chondral articular defects are a relevant problem in orthopaedic practice [27, 30]. The osteocartilaginous lesion is even more problematic, because tissue damage extends to the subchondral bone and often leads to joint degeneration. Tissue engineering aims to create 3D grafts by combining the patient's own cells and porous biomaterials as a template for tissue development. Such an engineered graft has several potential advantages, as the properties of the graft can be specifically tailored to introduce structural, biological and biomechanical cues into the affected joints that are necessary for a reproducible and durable repair [12, 29]. The optimal scaffold should allow bone repair in the subchondral area and migration of mesenchymal stem cells into the superficial cartilaginous layers, with cartilage repair at the articular surface and integration of the neocartilage with the surrounding cartilage.

Coral exoskeletons (aragonite) are a remarkably similar biological material to human bone including its 3D structure and pore interconnections and crystalline form of calcium carbonate ($CaCO_3$). These characteristics are thought to confer its osteoconductive ability and make it a suitable material for bone repair [10].

Corals are marine invertebrates from the Anthozoa class that include over 7,000 species [15, 25] with a wide variety of skeletal topologies, different morphologies and crystalline structures [51]. Corals used for medical applications are limited to a select number of species: Porites, Acropora, Lobophyllia, Goniopora, Polyphyllia and Pocillopora [10]. These species differ in size and interconnectivity of coral pores, which has been shown to be a critical factor in the rate of coral resorption and bone regeneration [4, 9, 17]. The minimal pore diameter for adequate colonization by connective and osteoid tissue has been found to be 100 μm [23, 46, 53]. In addition, pore connections of 100 to 200 μm are optimal for the development of Haversian systems and the essential entry of blood vessels [10].

Coralline biomaterial is reabsorbed gradually and leaves a functional bone tissue in its place [7, 17, 19, 20]. Moreover, due to its osteoconductive and osteogenesis properties [18, 31], it is used nowadays as a bone graft substitute and bone void filler in orthopaedic surgery, cranial and maxillo-facial surgery, as well as periodontal and plastic surgery [20, 33, 40, 43, 44, 48].

While coral is a good material for bone repair, as a stand-alone material, it cannot regenerate native hyaline cartilage. Shahgaldi et al. [45] performed implantations of coral plugs in the rabbit patellar groove, and although quality of surface repairs, indicated by 3D collagen structure and Safranin-O staining, was markedly better than that obtained for ungrafted empty defects, it did not regenerate normal articular hyaline cartilage.

Hyaluronic acid (HA) is a high molecular weight unsulfated glycosaminoglycan (GAG) present in all mammals with repeating disaccharide units composed of (β-1,4)-linked D-glucuronic acid and (β-1,3)-linked *N*-acetyl-D-glucosamine. HA is a critical molecule for the maintenance of the physicochemical characteristics of extracellular cartilage matrix (ECM). It has an important role in chondroprotection and chondrogenesis as it protects chondrocytes against apoptosis via CD44 [34] and I-CAM 1 [32]. It counteracts oxidative injuries in cultured human chondrocytes [5] and inhibits interleukin-1-evoked reactive oxygen species [14]. Hyaluronate also inhibits the Il-1b-stimulated production of matrix metalloproteinases [24]. HA bonded to a substrate exhibits a size-dependent stimulation of chondrogenic differentiation [32]. It influences cell motility, cell differentiation and cell development [50]. Finally, HA is a biodegradable and a biocompatible natural polymer allowing enhanced cell attachment and proliferation [2, 3, 11, 35, 37, 42, 47].

The purpose of this study was to evaluate the safety and regenerative potential of a newly developed aragonite-based scaffold (CartiHeal (2009) Ltd., Israel) for osteochondral defects in a goat model. The study hypothesis was that mechanical modification of drilled channels and impregnation of HA in the chondral phase would enhance the scaffold regenerative potential in the formation of bone and hyaline cartilage.

Materials and methods

Fourteen skeletally mature goats (Saanen, 55 ± 11 kg) were acquired from authorized farms and quarantined for at least 45 days before use. A total of 28 osteochondral lesions were performed (one of the implants was placed in an inappropriately deep position and removed from the analysis). Two locations were chosen for implantations, the medial femoral condyle (MFC) and the lateral femoral condyle (LFC). In each location, a single device was inserted, in the middle part of the condyle in a weight-bearing area. Each goat was treated with one type of implant only. Four defects in two animals were left untreated as controls.

Scaffold preparation

The basic scaffold consisted of the coralline aragonite. Following a machining process, a square grid pattern of 1- to 2-mm-deep drilled channels was made, in the chondral phase or in the bone phase, using Bungard CCD, a CNC drilling and routing machine and an appropriate drill-bit as described in US patent application 20120177702 and 20120189669. After extensive purification processes,

needed to treat and remove trapped particles, debris and organic remnants, the implants were sterilized by 25 kGy gamma radiation (Sor-Van Ltd., Israel).

Further modifications led to different prototypes, where half of the implants used in this study were impregnated with HA (Arthrease, Bio-Technology General Ltd., Israel) homogenously in the chondral phase of the implant.

Previous studies showed that a 6-mm defect size is a critical defect size, which does not heal spontaneously after 6 months [21]. Therefore, in the current study, the implants were cylindrically shaped with dimensions of 6 mm diameter and 8 mm length. The length of the material was chosen so that the distal part of the implant was bottomed and firmly embedded in cancellous bone beyond the subchondral bone plate.

Study design

Two different tissue repair strategies were compared: mechanically modified coral scaffold (scaffold vs. scaffold with drilled channels) and biologically modified coral scaffold (scaffold alone versus scaffold impregnated with HA).

In particular, two types of modified aragonite scaffolds distinguished mainly by the location of the mechanical modifications—drilled channels in the cartilage or bone phase were compared to unmodified coral and the empty defect. Within each group, half of the implants were impregnated with HA in the cartilage phase (Fig. 1).

- Group 1: implants with drilled channels in the cartilage phase, with impregnation of HA in the cartilage phase (Sub-group 1THA, $n = 3$) or without HA impregnation (Sub-group 1T, $n = 4$)
- Group 2: implants with drilled channels in the bone phase, with impregnation of HA in the cartilage phase (Sub-group 2BHA, $n = 4$) or without HA impregnation (Sub-group 2B, $n = 4$)
- Group 3: implants without any mechanical modifications, with impregnation of HA in the cartilage phase (Sub-group 3PCHA, $n = 4$) or without HA impregnation (Sub-group 3PC, $n = 4$)
- Group 4: control ($n = 4$)—empty defects of the same dimensions without any implant.

Surgical procedure

Surgery was conducted under general anaesthesia via a mini-arthrotomy approach. Standardized osteochondral defects 6 mm in diameter and 8 mm deep were made in two load-bearing areas: the medial and the LFC of the same knee joint. A minimal exposure of the implantation site was made using retractors with the limb placed at maximal flexion. The patella was kept intact and not moved from its natural position.

All implants were then inserted in a press fit manner into the implantation defect. Implant type was chosen according to a pre-prepared randomization chart (Fig. 2).

After the implantation process in the MFC was completed, a similar procedure in the LFC was performed, and again the patella was not dislocated. This is critical in a jumping animal such as a goat, as otherwise, due to retinaculum damage during the surgical approach, patellar dislocation after the operation is likely.

Antibiotics and analgesics were administrated postoperatively, and a veterinarian checked the animals' welfare routinely. All animals were killed 6 months after surgery, by pharmacological premedication and injection of 20 cc Pentothal 20 %.

Sample evaluation

Synovial fluid assessment

Immediately post-mortem, synovial fluid samples were taken for cell counts [white blood cells (WBC), red blood cells (RBC) and platelets] from every joint. The samples were used to detect signs of inflammation in the joints.

Macroscopic preparation and evaluation

Macroscopic evaluations were conducted after animal killing and joint harvesting. A cut was performed in the centre of each implantation site using a fine saw to allow

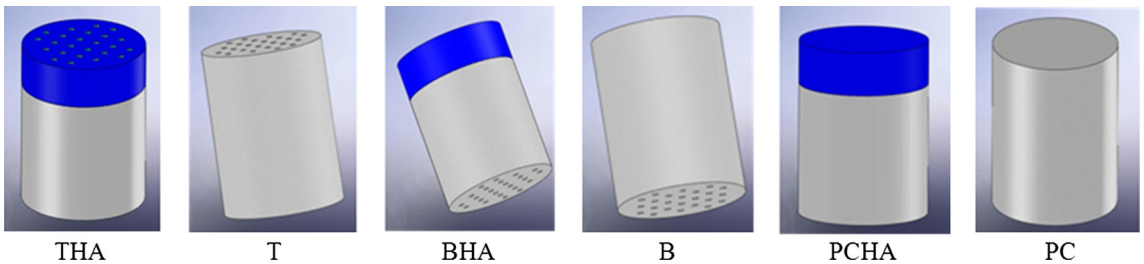

Fig. 1 Illustration of the implants evaluated in this study

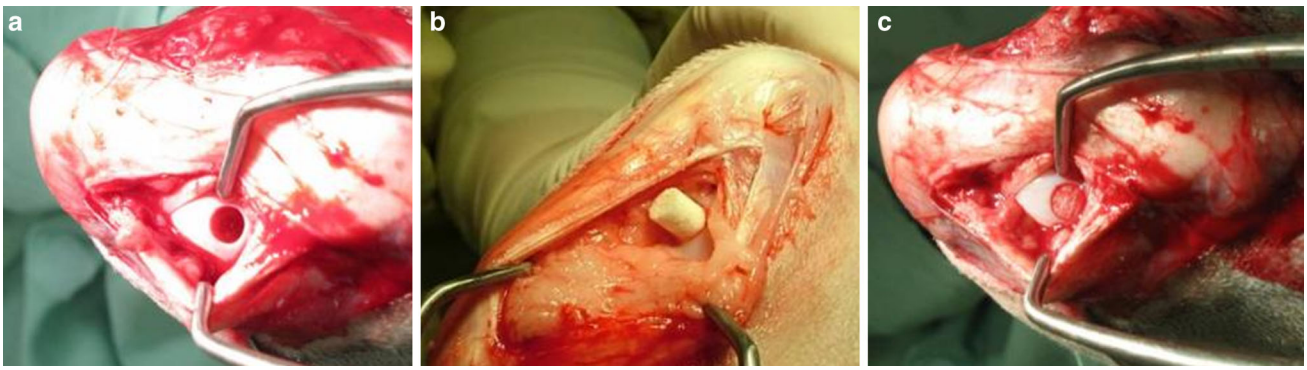

Fig. 2 Implant insertion: (**a**) site before implantation, (**b**) implant positioning, (**c**) implantation

Table 1 Results of gross appearance scaling system (modified from Fortier et al.)

		Surface texture of repair tissue	Percent area of defect filling	Graft recipient tissue integration	Bone cartilage interface appearance	Bone defect filling	Total (max = 15)
Group 1 ($N = 7$)	Mean	2.0	2.9	2.9	2.6	2.6	12.9
	SD	0.0	0.4	0.4	0.5	0.8	1.2
Group 2 ($N = 8$)	Mean	1.6	2.0	2.5	1.9	2.4	10.4
	SD	0.9	1.2	1.1	1.0	0.7	4.3
Group 3 ($N = 8$)	Mean	1.4	1.4	2.6	1.6	2.4	9.4
	SD	1.1	0.9	1.1	0.9	0.9	4.0
Group 4 ($N = 4$)	Mean	1.7	1.7	3.0	2.0	1.7	10.2
	SD	0.4	0.4	0.0	0.7	0.4	1.5

The Group 1 has the greatest mean gross appearance evaluation value of 12.9, whereas the other three groups have lower means. Group 3 has the lowest gross appearance evaluation value of 9.4. The Group 1 also has the lowest standard deviation. The *percent area of defect filling* was found to have greater mean values in the Group 1, and the difference is statistically significant ($F(3,2) = 3.8$; $p = 0.0249$). Analysis of the pairs shows that a statistically significant difference exists between Group 1 and Group 3 regarding percent area of defect filling ($t_{22} = 3.31$; $p = 0.0032$)

N number of implants analysed

evaluation of the cartilage and the subchondral bone. Semi-quantitative evaluations were performed in a blinded manner with magnification up to 50× (MOTIC Ltd., SMZ-168, D-35578 Wetzlar, Germany). A high-resolution scan was studied of the implant halves (sawed longitudinally, 600 dpi, 15 cm by 20 cm size photograph) as well as a photograph of the surface of the implant as photographed using a USB microscope at 40× magnification. The photographs were marked by animal number and implant code; this allowed completely blinded evaluation of the results. The photographs were coded and presented blindly to the orthopaedic surgeons who performed assessment independent of each other.

Macroscopic appearance of the implants and quality of healing were assessed using three scaling systems:

1. A modified scoring system by Fortier [31] that analyses surface texture of repair tissue, percent area of the defect filled and graft recipient tissue integration; the score was modified by adding the bone cartilage interface appearance and bone defect filling evaluation (Table 1).
2. Gross morphological evaluation using a scoring system by Niederauer [18], focusing on edge integration, smoothness of cartilage surface, cartilage degree of filling and colour of cartilage (Table 2).
3. The International Cartilage Repair Society (ICRS) macroscopic evaluation scoring system estimates three parameters (degree of defect repair, integration of new tissue with the border line, and macroscopic appearance) and enables division into four categories: "normal", "nearly normal", "abnormal" and "severely abnormal" (Table 3) [21].

Histological preparations and evaluations

Histological preparations and evaluations were conducted by NAMSA laboratory (NAMSA-BIOMATECH, Lyon, France), in a GLP manner and according to ISO 10993.

Table 2 Results of gross morphological evaluation (Niederaurer et al.)

		Edge integration	Smoothness of cartilage surface	Cartilage surface, degree of filling	Colour of cartilage	Total (max = 8)
Group 1 ($N = 7$)	Mean	2.0	1.3	1.9	1.1	6.3
	SD	0.0	0.5	0.4	0.4	0.8
Group 2 ($N = 8$)	Mean	1.7	1.0	1.1	1.0	4.9
	SD	0.7	0.9	1.0	0.5	2.6
Group 3 ($N = 8$)	Mean	1.7	0.6	0.6	1.1	4.1
	SD	0.7	0.7	0.7	0.6	2.4
Group 4 ($N = 4$)	Mean	2.0	0.7	0.7	1.2	4.7
	SD	0.0	0.5	0.5	0.5	1.3

Cartilage surface, degree of filling: The differences between the groups are statistically significant ($p = 0.0207$). Group 1 is significantly better than Groups 3 and 4 ($p = 0.0034$ and $p = 0.0239$, respectively)

Table 3 Results of ICRS macroscopic evaluation of cartilage repair

		Degree of repairing the defect	Integration in border zone	Macroscopic appearance	Total (max = 12)
Group 1 ($N = 7$)	Mean	3.9	3.9	2.9	10.6
	SD	0.4	0.4	0.4	0.8
Group 2 ($N = 8$)	Mean	2.5	3.5	2.1	8.1
	SD	1.6	1.4	1.4	4.0
Group 3 ($N = 8$)	Mean	2.2	3.5	1.9	7.6
	SD	1.2	1.4	1.4	3.4
Group 4 ($N = 4$)	Mean	3.0	3.5	2.0	8.5
	SD	0.4	0.0	0.8	1.2

The component of ICRS degree of repairing the defect was also found to have greater mean values in the Group 1; this difference was found to be borderline statistically significant ($F(3,22) = 2.84$; $p = 0.0615$)

The specimens were pre-fixed in 10 % neutral buffered formalin for 24 h after animal killing. The specimens were completely decalcified in EDTA solution, dehydrated in alcohol solutions of increasing concentration and then cleared in xylene before embedding in paraffin. The embedded sites were then longitudinally cut in the middle of the implantation site (5 µm thickness ± 0.5 µm) using a microtome (MICROM®, France). For each site, five central serial sections were performed. Three sections were stained, respectively, with safranin–haematoxylin–eosin (SHE) for analysis of the inflammatory reaction, Masson trichrome for general morphology assessment, and Safranin-o/Fast green to assess the proteoglycan content. The other two sections were used for immunohistochemistry determination of collagen type I (bone and fibrous tissue marker) and collagen type II (hyaline cartilage marker). Analysis was performed in a blinded manner with respect to the treatment groups, involving evaluation of the inflammatory reaction (polymorphonuclear cells, lymphocytes, plasma cells, macrophages and giant cells/osteoclasts), fibrin, necrosis, osteolysis and new vessels. Efficacy parameters were graded according to a combination of the ICRS II grading scale [36] and the O'Driscoll et al. [39] grading scale: nature of predominant tissue (tissue morphology and Safranin-o staining of the matrix), structural characteristics (surface regularity, structural integrity, thickness, bonding to the adjacent cartilage, basal integration, tidemark, subchondral bone abnormalities/marrow fibrosis and abnormal calcification/ossification, cancellous bone regeneration), freedom from cellular changes of degeneration (hypocellularity and chondrocytes clustering), freedom from degenerative changes in adjacent cartilage and overall assessment (Table 4). In addition, all slides were evaluated under polarized light for the determination of the collagen organization to detect fibrous organization. The immunolabelled slides were evaluated for the presence of collagen type I and type II (Table 5).

Animal care and surgery were approved by the Ethical Committee of the Experimental Center and performed under the Animal Welfare Law and ISO 10993 Standards.

Statistical analysis

Power analysis was performed using Russ Lenth Java Applets for Power and Sample Size(http://homepage.stat.uiowa.edu/~rlenth/Power/). Assuming 4 groups with 8

Table 4 Histological results: cartilage and bone regeneration (combined ICRS II-2010 and O'Driscoll et al.)

		Overall assessment (max = 4)	Total score (max = 40)
Group 1 ($N = 7$)	Mean	3.7	34.4
	SD	0.8	5.8
Group 2 ($N = 8$)	Mean	3.0	28.9
	SD	1.8	11.4
Group 3 ($N = 8$)	Mean	2.2	23.0
	SD	2.0	9.9
Group 4 ($N = 4$)	Mean	2.0	19.7
	SD	2.3	16.0

Overall assessment, ANOVA $F = 4.14$, $p < 0.02$, total score ANOVA $F = 4.7$, $p < 0.02$

Table 5 Immunohistochemistry results. Score scale: (0)—Absence (1)—Slight presence (2)—Moderate presence (3)—Marked presence

		Collagen type II	Collagen type I
Group 1 ($N = 7$)	Mean	3.0	0.3
	SD	0.0	0.5
Group 2 ($N = 8$)	Mean	2.7	0.9
	SD	0.5	0.8
Group 3 ($N = 8$)	Mean	2.2	1.4
	SD	0.9	0.7
Group 4 ($N = 4$)	Mean	1.5	0.5
	SD	1.7	1.0

Collagen type II: The differences between the group means are statistically significant ($p = 0.0461$). Group 1 is significantly different from the control ($p = 0.0100$). Collagen type I: The differences between the group means are marginally significant ($p = 0.0698$). Group 1 is significantly different from Group 3 ($p = 0.0129$)

implants in each group and standard deviation of 0.5 and assuming an effect size of 0.8, then the power of the study is 80 % for detecting an intergroup difference at the 0.05 level.

A general linear mixed model (SAS PROC MIXED procedure) was used to compare the four study groups. Model estimated means (LSMeans) and 95 % confidence intervals were calculated, as well as the standard deviations and respective 95 % CIs. If the main effect was found to be statistically significant or borderline significant (defined as a p value of <0.08), then pairwise tests of least squared means were performed.

Statistical analyses were performed using SAS® V9.2 (SAS Institute, Cary NC USA). A p value of 0.05 or less was considered statistically significant.

Results

All goats recovered from anaesthesia and were able to stand and walk with weight bearing (e.g. immediate load bearing and free movement were allowed) on the day of surgery. During the first 10 days, all goats were placed in protected stalls with limited space for movement (2 m × 2 m) and with an individual supply of food and water, which allowed traceability of nutritional consumption. After 10 days, the stitches were removed and the goats were transferred to a common shelter for the entire cohort group. During the first 2 weeks after surgery, all goats returned to normal behaviour, normal weight, normal gait, and normal food and water consumption.

All goats showed fast recovery with no evidence of any negative influence on their behaviour. There was no evidence of any restriction of movement or use of joint, including joint locking, in any of the goats during the 6-month follow-up period.

One animal presented a stitch abscess, which was considered a procedure-related adverse event, and not implant related. The infection was treated by drainage and antibiotics, and no signs of infection were detected after several days.

In all methods of evaluation, an advantage was seen in favour of the implants impregnated with HA in the chondral phase of the implant. The details of macroscopic and histologic/immunohistochemical evaluations are reported, respectively, in Tables 1, 2, 3, 4, and 5.

Synovial fluid specimens were analysed after killing for the presence of cells, and were found to uniformly contain less than 100 cells per ml, without evidence of crystals in the fluid.

Group 1: implants with drilled channels in the cartilage phase ($N = 7$)

Sub-group 1THA outperformed Sub-group 1T and all other tested groups. In sub-group 1THA (impregnated with HA in the cartilage phase), the examined sites were healed and showed a cartilaginous repair tissue with morphological evidence of hyaline cartilage, confirmed by the marked presence of proteoglycans and appearance of collagen type II. The repair tissue did not show collagen type I. The weight-bearing surface mostly showed a smooth contour. The repaired cartilage was well integrated into the adjacent

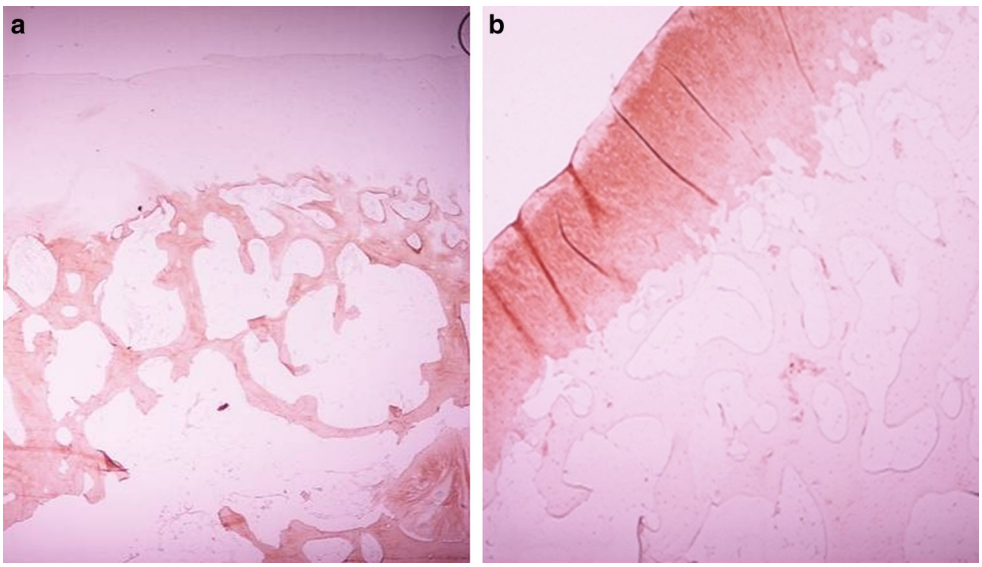

Fig. 3 Immunohistochemistry stains of Sub-group 1THA, (**a**) collagen type I—not present in cartilage phase, (**b**) collagen type II—present in newly formed hyaline cartilage

native cartilage and basal tissues. The newly formed tidemark was well defined between cartilage and bone, and the subchondral plate was fully reconstructed. The underlying cancellous bone was almost completely reconstructed with the replacement of the implant. Some chondrocyte clustering combined with a noticeable density of individual chondrocytes infiltrating the cartilaginous repair tissue was seen. Very limited signs of inflammation with infiltration of macrophages were observed in the underlying bone tissue area. Neovascularization was of moderate amount (Figs. 3, 4).

In sub-group 1T, three out of four sites showed a cartilaginous repair tissue with morphological evidence of hyaline cartilage, which was confirmed by the moderate-to-marked presence of proteoglycans combined with appearance of collagen type II. Two of the healed sites showed a very slight presence of collagen type I and two were completely devoid of collagen type I within this cartilaginous repair tissue. The cartilaginous repair tissue was well integrated into the adjacent native cartilage and basal tissues, and the underlying cancellous bone was moderately to completely reconstructed with the replacement of the implant. Some chondrocyte clustering combined with a noticeable density of individual chondrocytes infiltrated the cartilaginous repair tissue. The subchondral plate was partially reconstructed in all the sites. Slight signs of inflammation with infiltration of macrophages were observed in the underlying bone tissue area. Neovascularization was of moderate grade.

Group 2: implants with drilled channels in the bone phase ($N = 8$)

Sub-group 2BHA showed less healing performance than Group 1 (both T and THA), but better healing performance compared to sub-group 2B, Group 3 and Group 4. In sub-group 2BHA, three sites showed cartilaginous repair tissue with morphological evidence of hyaline cartilage, confirmed by the moderate-to-high levels of proteoglycans combined with a marked amount of collagen type II. Two of the healed sites were devoid of collagen type I, whereas the other two sites showed a slight presence of collagen type I. A newly formed tidemark was only observed in the two sites devoid of collagen type I, which showed better signs of healing compared with the other sites. Some chondrocyte clustering was combined with a noticeable density of individual chondrocytes infiltrating the cartilaginous repair tissue. The weight-bearing surface mostly showed a smooth contour. The cartilaginous repair tissue was well integrated into the adjacent native cartilage and basal tissues. The subchondral plate was not fully reconstructed, except for one of the healed sites. The underlying cancellous bone was almost completely reconstructed with the replacement of the implant. One site was not healed, and the defects were laterally filled with a fibrous and fibrocartilaginous tissue. Slight signs of inflammation with infiltration of macrophages and a few giant cells/osteoclasts were observed in the underlying bone tissue area. Neovascularization was of moderate amount.

In this sub-group 2BHA, two out of four sites yielded advanced signs of healing. No local adverse effects were observed in the healed sites.

In sub-group 2B, three sites showed cartilaginous repair tissue with morphological evidence of hyaline cartilage. The hyaline nature was confirmed by the marked presence of proteoglycans and collagen type II. One of the healed sites was devoid of collagen type I, whereas in the other two healed sites, a slight presence of collagen type I was

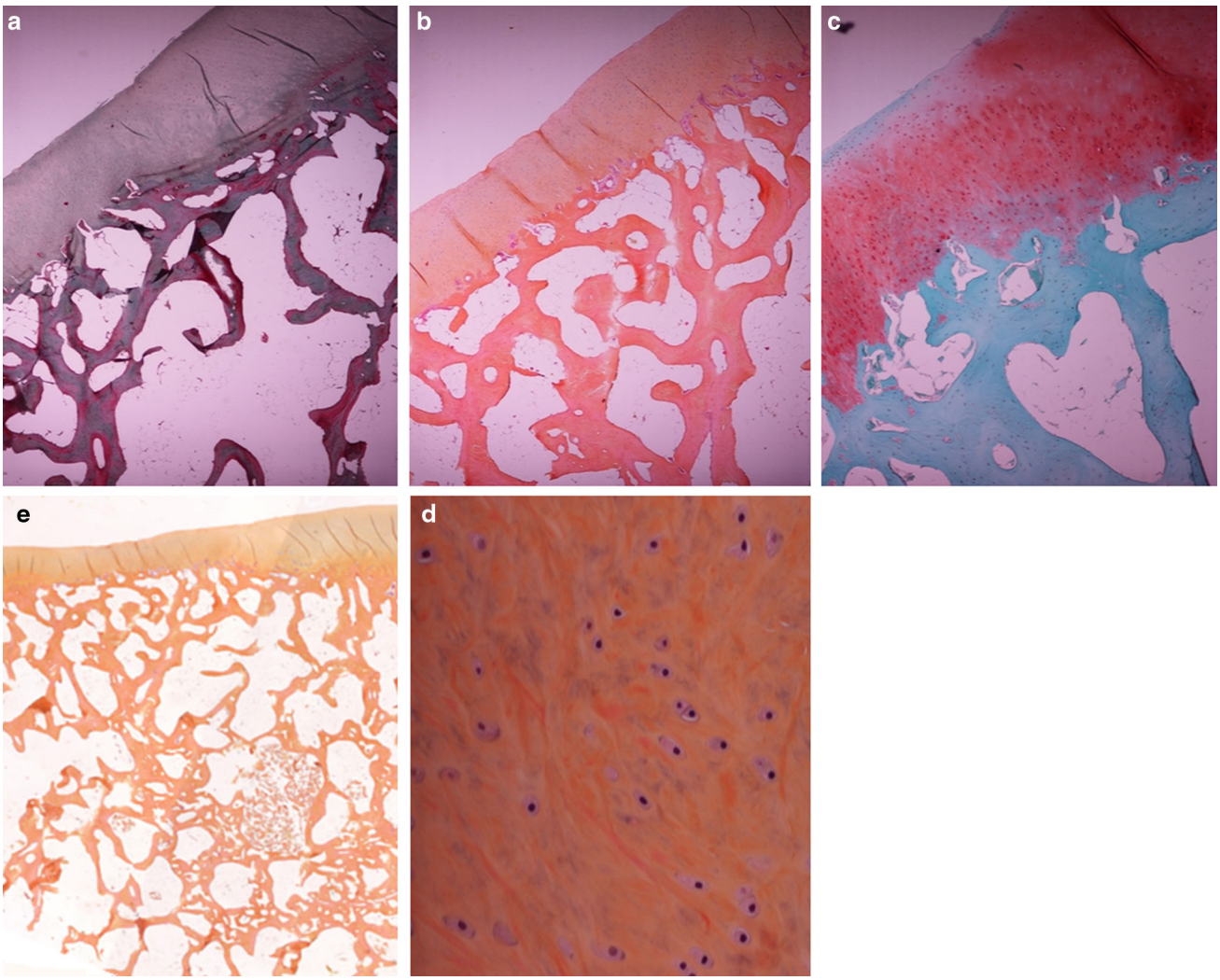

Fig. 4 Histology stains of Sub-group 1THA, (**a**) Masson trichrome, (**b**) safranin–haematoxylin–eosin, (**c**) Safranin-O/Fast green, (**d**) implantation site: regenerated and native tissues (SHE), (**e**) chondrocytes within newly formed articular hyaline cartilage

observed. A newly formed tidemark was only observed in the site devoid of collagen type I, which showed better signs of healing compared with the other two sites. Some chondrocyte clustering was observed, combined with a noticeably increased density of individual chondrocytes infiltrating the cartilaginous repair tissue. The weight-bearing surface showed some irregularities. The cartilaginous repair tissue was well integrated into the adjacent native cartilage and basal tissues. The subchondral plate was not fully reconstructed. The underlying cancellous bone was almost completely reconstructed with replacement of the implant. One site was not healed, and the defects were laterally filled with a fibrous and fibrocartilaginous tissue. Slight signs of inflammation with infiltration of macrophages, giant cells/osteoclasts and a few lymphocytes were observed in the underlying bone tissue area. Neovascularization was of moderate amount.

Group 2 achieved the second highest score. However, although those implants had some healing potential, the risk of inadequate defect filling appeared high and unpredictable. This does not support the use of this group of implants.

Group 3: implants without mechanical modification ($N = 8$)

In sub-group 3PC, three sites were not healed with the defect laterally filled with fibrous tissue. The remaining site showed some cartilaginous repair tissue with moderate presence of proteoglycans, a marked amount of collagen type II and a slight presence of collagen type I. Some chondrocyte clustering was observed, combined with a noticeable density of individual chondrocytes infiltrating the cartilaginous repair tissue. The weight-bearing surface showed some irregularities. A slight-to-moderate amount of a newly formed tidemark was observed between the

cartilage and the bone tissues. The subchondral plate was not fully reconstructed. Moderate signs of inflammation with infiltration of macrophages, giant cells/osteoclastic cells and a few lymphocytes were observed in the underlying bone tissue area. Neovascularization was of moderate amount.

In sub-group 3PCHA, three sites showed cartilaginous repair tissue, which was confirmed by the moderate-to-marked presence of proteoglycans combined with a marked amount of collagen type II. One of the healed sites was devoid of collagen type I, whereas in the other two healed sites, collagen type I was observed within this cartilaginous repair tissue. A newly formed tidemark was only observed in the site devoid of collagen type I, which showed better signs of healing compared to the other three sites. Some chondrocyte clustering was combined with a noticeable density of individual chondrocytes infiltrating the cartilaginous repair tissue. The weight-bearing surface showed some irregularities. The cartilaginous repair tissue was well integrated into the adjacent native cartilage and basal tissues. However, the subchondral plate was not fully reconstructed. The underlying cancellous bone was moderately to markedly reconstructed, replacing the implant. Slight signs of inflammation with infiltration of macrophages, giant cells/osteoclastic cells and a few lymphocytes were observed in the underlying bone tissue area. Neovascularization was of moderate amount.

Together with the empty defect (Group 4), Group 3 presented the lowest healing performance. The scaffolds impregnated with HA in the cartilage phase yielded better healing outcomes, but still less than the other groups.

Group 4: empty defects ($N = 4$)

There was no healing in two sites, and the defects were laterally filled with a fibrous tissue. The other two sites showed some cartilaginous repair tissue with a moderate presence of proteoglycans and marked degree of collagen type II. Some chondrocyte clustering was combined with a noticeable density of individual chondrocytes infiltrating the cartilaginous repair tissue. The weight-bearing surface showed irregularities in all sites. A moderate-to-marked amount of newly formed tidemark was observed between the cartilage and the bone tissues. However, the subchondral plate was not fully reconstructed neither was the underlying cancellous bone even when defect closure had occurred. Slight signs of inflammation with infiltration of macrophages and a few lymphocytes were observed in the underlying bone tissue area. Neovascularization was of moderate amount.

Together with Group 3, this group presented the lowest healing performance (Fig. 5).

Discussion

The aim of this study in a goat model was to evaluate the local effects and healing performance of aragonite-based implants used for cartilage and bone regeneration after 6 months of implantation and to test whether mechanical modification with or without the addition of HA enhanced the scaffold's regenerative potential.

The goat model was chosen for these experiments because it is widely accepted and recommended as a model for cartilage and bone repair. Goats provide a relatively large joint size and good cartilage thickness. The anatomy and biomechanical properties of the femorotibial joint of goats are similar to those of humans as are the proportions of cartilage and subchondral bone [1]. Moreover, this animal presents relevant loading conditions and allows a similar surgical technique to that used in humans [1].

Results of this study appear to indicate that the coral-based scaffolds did not induce any significant adverse local tissue effects or joint-related adverse effects. Regarding implant biodegradability, tissue voids that might correspond to residual particulate scaffold remnants were rarely observed in any group. This indicated that the scaffold was nearly completely biodegraded and replaced by newly formed tissue by 6 months after implantation. At this time, an overall regeneration of both chondral and bone tissues was observed. However, marked differences were found among the tested implant groups and sub-groups.

The 6-month follow-up results indicated the expected poor spontaneous healing of the critical-sized osteochondral defect. Interestingly, not only the empty defect group but also group 3, coral without mechanical modifications, showed poor healing, resulting mostly in unclosed defects or fibrocartilaginous repair.

Two strategies were evaluated for improving the scaffold's regenerative potential: mechanical modification with drilled channels and impregnation with HA. The combination of coral scaffold with the biological polymer HA in the chondral phase of the implant was designed to mimic the organic–inorganic composite of developing cartilage and bone, while the aragonite scaffold aimed at triggering initial cell adhesion and interactions and stimulate mesenchymal stem cell (MSCs) differentiation [13, 18, 31, 41, 52]. Hyaluronan was added to enhance chondrogenic differentiation [2, 35, 47]. The combination of the two strategies showed the best results; in fact, the sub-group that showed the most advanced cartilage repair was the sub-group with drilled channels in the cartilage phase and HA impregnation (1THA). The examined sites of these implants were healed and showed a cartilaginous repair tissue with morphological evidence of hyaline cartilage that was confirmed by the marked presence of proteoglycans, a marked amount of collagen type II and absence of

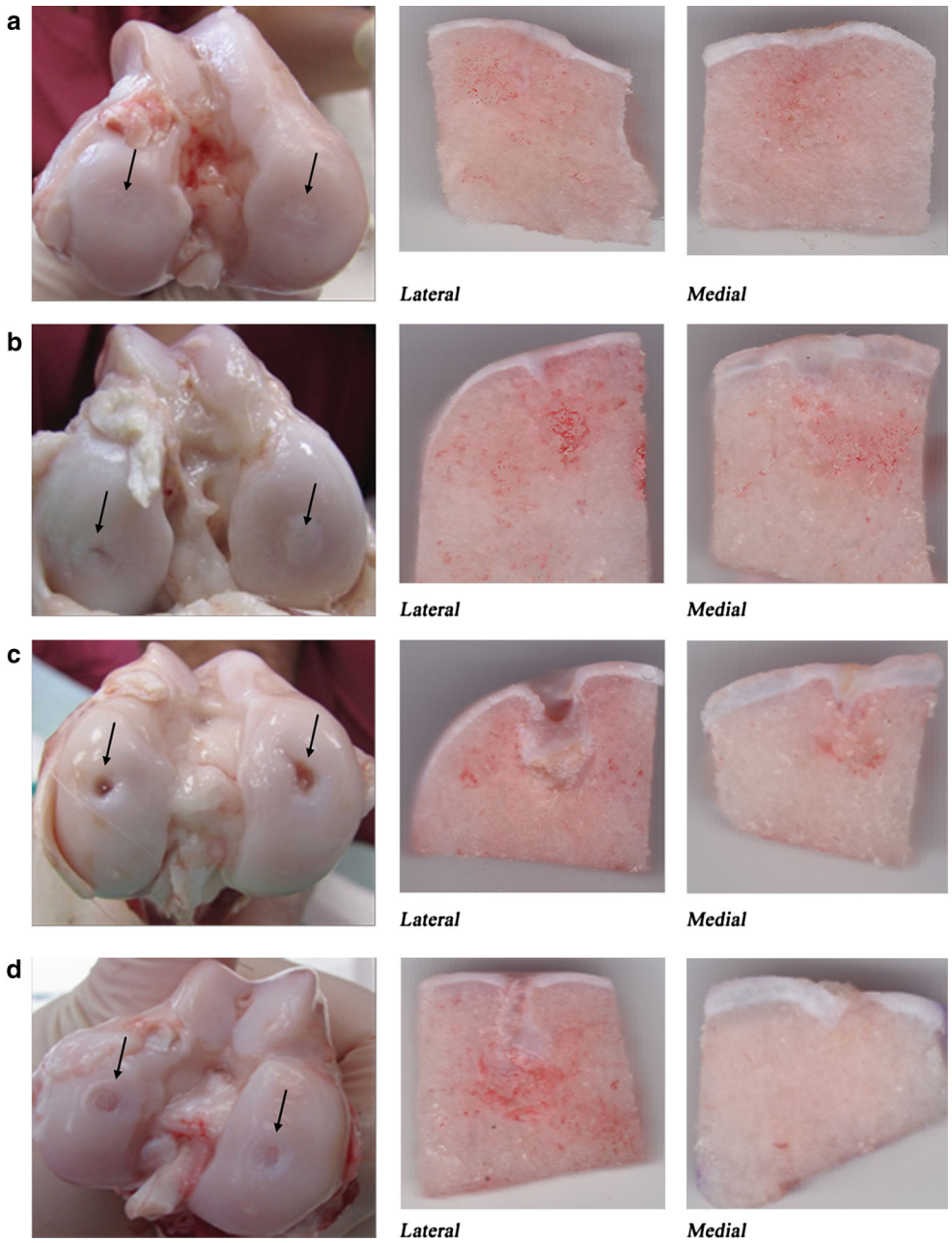

Fig. 5 Representative appearance of the implantation sites 6 months after surgery: (**a**) Sub-group 1THA, (**b**) Sub-group 2BHA, (**c**) Sub-group 3PC, (**d**) Group 4

collagen type I. The weight-bearing surface mostly showed a smooth contour. The cartilaginous repair tissue was well integrated into the adjacent native cartilage and basal tissues. Moreover, a newly formed tidemark was observed between the cartilage and the bone tissues, the subchondral plate was fully reconstructed, and the underlying cancellous bone was almost completely reconstructed, replacing the implanted scaffold. As stated above, there was a trend for better results when the hyaluronate and channels were combined relative to pre-drilling alone or when using hyaluronate alone without pre-drilling. This might be related to the better adherence of the hyaluronate to the perforated surface as compared with undrilled coral.

There are several potential reasons why these implants lead to better healing performance:

1. The drilled channels increase the porosity of the implant in the cartilage phase which might better

simulate the normal biomechanical environment of the surrounding cartilage tissue.
2. The voids improve nutrient supply.
3. The drilled channels reduce the amount of aragonite material in the cartilage phase and so facilitate repair.

Besides its chondrogenic properties, HA probably lubricates the articular side of the implant.Corals without any modification did not support hyaline cartilage tissue regeneration. Mechanical modification by drilling in the chondral phase of the implant improved the regeneration of hyaline cartilage, and the healing performance was even further improved with HA impregnation in the chondral phase of the implant.

The combined mechanical and biological modifications in the cartilage phase in implants of Sub-group 1THA appear to be an optimal prototype design. These implants elicited good osteochondral regeneration potential, meeting many of the requirements of an ideal scaffold.

These results indicate that a properly designed cell-free scaffold can support cartilage and bone regeneration. This implant acts as a homing device and enhances the adhesion, proliferation and differentiation of MSCs. The material appears to support recruitment of MSCs from the surrounding subchondral bone, as well as invasion of chondrocytes from the surrounding native cartilage. This is consistent with a concept which is gaining growing popularity among the treatment strategies for cartilage and osteochondral regeneration, advocating the relevant contribution of MSCs attracted from the subchondral bone marrow towards autologous matrix-induced tissue regeneration [6, 8, 16, 22, 26].

A major obstacle for the wide spread use of cartilage repair strategies such as autologous cell implantation has been the need to make a customized implant for each patient. This leads to high cost and the need for scheduled repeated surgery in most cases. Moreover, even though augmentation strategies could play a role in the regeneration process [38, 49], it has to be underlined that the literature also suggests a cautious approach due to controversial findings: in fact, cells and growth factors introduce a degree of biological variability and even potentially negative effects on tissue regeneration have been documented [28]. On the other hand, osteochondral autologous grafting requires donor site killing of healthy tissue.

An off-the-shelf, cell-free and cost-effective implant that can regenerate cartilage and osteochondral defects would be an easier and less expensive one-step surgical procedure with respect to other techniques. The implants evaluated in the present study, placed in an implantation site where they are fully surrounded by healthy tissue to enable integration of the surrounding cartilage area with the newly formed repaired tissue, proved to be a valid treatment option. The very good results documented for the mechanically and biologically optimized coral scaffold highlight the potential in promoting bone and cartilage tissue restoration by itself and can be considered as a promising cell-free treatment strategy to be evaluated for osteochondral tissue regeneration in humans. Possible limitations of the current study are the size of the plugs used. Most plugs used in humans are expected to range in size between 8 and 10 mm in diameter. Such plugs cannot be tested in a goat model due to size limitations imposed by the caprine anatomy. While the caprine bone metabolism is considered similar to that of humans, the different sizes needed to reconstruct the clinically significant defects might influence the results in humans. Other limitation of the study might be related to the relatively another number of implants in each subgroup that prevented detailed subgroup analysis. However, the results do indicate significant advantage of the implant with pre-drilled cartilage channels and hyaluronate as compared with other groups. The advantage appears to be not only in the average score but also in the consistency of the repair reaction.

Conclusions

Although native coral is a good material for bone repair, as a stand-alone material, its implantation does not lead to hyaline cartilage regeneration. Mechanical modification with drilled channels and biological augmentation with HA enhances the scaffold's regenerative potential. In particular, 6 months after implantation in goats, the combined mechanical and biological modifications in the cartilage phase suggested that this is an optimal prototype design, eliciting a high level of osteochondral defects healing with both articular hyaline cartilage and subchondral bone regeneration.

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Conflict of interest Some authors declare to have conflicts of interest: E. Kon, MD is a consultant for Cartiheal; D. Robinson, MD, PhD is a consultant for Cartiheal; JA. Eisman, MB BS, PhD, FRACP is a consultant for Cartiheal; A. Levy, MD is a consultant for Cartiheal; K. Zaslav, MD is a consultant for Cartiheal; J. Shani, DVM Dipl. ECVS, Bc.S is a consultant for Cartiheal; N. Altschuler, Bc.S, MBA is a founder and CEO of Cartiheal.

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