Articular cartilage restoration in load-bearing osteochondral defects by implantation of autologous chondrocyte-fibrin constructs

AN EXPERIMENTAL STUDY IN SHEEP

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Ovine articular chondrocytes were isolated from cartilage biopsy and culture expanded *in vitro*. Approximately 30 million cells per ml of cultured chondrocytes were incorporated with autologous plasma-derived fibrin to form a three-dimensional construct. Full-thickness punch hole defects were created in the lateral and medial femoral condyles. The defects were implanted with either an autologous 'chondrocyte-fibrin' construct (ACFC), autologous chondrocytes (ACI) or fibrin blanks (AF) as controls. Animals were killed after 12 weeks. The gross appearance of the treated defects was inspected and photographed. The repaired tissues were studied histologically and by scanning electron microscopy analysis.

All defects were assessed using the International Cartilage Repair Society (ICRS) classification. Those treated with ACFC, ACI and AF exhibited median scores which correspond to a nearly-normal appearance. On the basis of the modified O'Driscoll histological scoring scale, ACFC implantation significantly enhanced cartilage repair compared to ACI and AF. Using scanning electron microscopy, ACFC and ACI showed characteristic organisation of chondrocytes and matrices, which were relatively similar to the surrounding adjacent cartilage.

Implantation of ACFC resulted in superior hyaline-like cartilage regeneration when compared with ACI. If this result is applicable to humans, a better outcome would be obtained than by using conventional ACI.

Articular cartilage has limited healing potential.¹ Cartilage injuries, either partial thickness or full thickness with osteochondral pathology, can lead to premature arthritis² and produce deleterious effects in the knee.³ In the hope of treating these injuries, numerous studies have been carried out in laboratory animals focusing mainly on transplantation of the reconstructed hybrid 'scaffold-cells' construct. These experiments have required the incorporation of cultured cells into polymer scaffolds, such as medical-grade polycaprolactone (mPCL),⁴ bilayer type I/III collagen membrane,⁵ atelocollagen sponge/poly-L-lactic acid (PLLA) mesh composite,⁶ poly(D,L)-lactide-co-glycolide (PLGA)-based biomaterials,⁷ and fibrin glue.⁸ Alongside the current advancement of tissue engineering and regenerative technology, attempts to incorporate cultured cells into suitable biodegradable scaffolds are essential to improve the biological and structural properties of the engineered construct.

Several researchers have studied the potential of osteochondral restoration, in either the femoropatellar groove,^{6,9,10} the trochlear groove¹¹ or the femoral condyles⁵ of the rabbit.

Most have reported good results. Theoretically the femoropatellar and trochlear groove defect models are not representative of the situation faced in most patients. Anatomically, these two sites bear less weight and the lining of articular cartilage is thinner than in high weight-bearing areas.⁴ Therefore, we decided to study the restoration of osteochondral defects within high weight-bearing areas in a sheep model in an attempt to better simulate the clinical situation. We initiated these pre-clinical experiments by maintaining the animal model up to three months post-transplantation, based on a previous study¹² in which it was reported that there were no significant differences in cartilage healing at three months or six months in an animal model.

Autologous chondrocyte implantation (ACI) was first described by Brittberg et al¹³ and subsequently reviewed in 2001.¹⁴ It has been widely used to promote the healing of symptomatic articular defects in patients with success rates of about 90%.¹⁵ However, there have been reports of failure.^{16,17} The implantation of a tissue-engineered cartilage construct was one of the early treatments proposed to restore chondral defects.^{10,18}

We have carried out several *in vitro* studies to engineer 'chondrocyte-fibrin' constructs using different sources of chondrocytes,¹⁹ specifically human articular chondrocytes,²⁰⁻²² nasal septum chondrocytes²³⁻²⁵ and auricular chondrocytes.²⁶⁻²⁸ Although fibrin is unstable and quickly disintegrates, its use as a natural polymer scaffold for tissue engineering is increasing.²⁹ Fibrin is easily polymerised and moulded from its basic constituents.³⁰ Moreover, fibrin is non-cytotoxic, biocompatible and biodegradable.^{31,32} A study of *in vivo* auricular cartilage repair in rabbits using autologous fibrin and chondrocytes showed encouraging results.³³ A slow degradation rate was achieved by stabilising the autologous 'fibrin-chondrocyte' construct using anti-fibrinolytic agents such as aprotinin and tranexamic acid.³³ However, some studies suggest that fibrin glue is not an ideal carrier to restore large articular cartilage defects in weight-bearing areas as fibrin is rapidly degraded⁴ and actively disintegrated.⁸ The latter leads to the repaired tissues having insufficient potential to resist mechanical forces. To date, we have successfully engineered our own laboratory-grown chondrocyte-fibrin construct³⁴⁻³⁶ and have investigated an autologous chondrocyte-fibrin construct (ACFC) in a sheep model.^{37,38}

In this study, we aimed to evaluate the potential of the novel ACFC implantation technique to obtain early hyaline-like cartilage regeneration in an osteochondral defect. We evaluated the gross appearance of the treated defects, histological changes and integration of the implanted ACFC with the adjacent tissues. We assessed the performance of ACFC compared with conventional ACI and autologous fibrin (AF) blank. We hypothesised that ACFC would promote early chondrogenesis and induce hyaline-like cartilage regeneration at 12 weeks post-operation.

Materials and Methods

With the approval of the Animal Ethics Committee³⁹ and the Research Committee of the Medical Faculty, National University of Malaysia, 12 healthy six-month-old male sheep with a mean weight of 20 kg (15 to 25) were used in the study. Two arthrotomy steps were required, first to harvest cartilage biopsy for *in vitro* expansion of chondrocytes, and secondly to implant the ACFC or ACI.

All operations were performed under general anaesthesia. After premedication with an intramuscular injection of 0.1 mg/kg xylazine (Xylaxil-20, Troy Laboratories Pty Limited, Smithfield, Australia) and 2.0 mg/kg of tramadol (Domadol, Unichem Laboratories Ltd, Mumbai, India), intravenous (IV) access was established at the elbow. Sheep were pre-oxygenated and induced with 5.0 mg/kg to 10.0 mg/kg IV ketamine (Bioketan, Vetoquinolo Biowet, Gorzów, Poland). The animals were intubated and maintained on halothane with 100% oxygen. Ventilation was controlled to maintain end-tidal carbon dioxide of less than 45 mmHg. A bolus of normal saline was administered at 20 ml/kg IV, immediately after induction, followed by continuous infusion at 5 ml/kg/h to 10 ml/kg/h. Intra-operative

monitoring consisted of electrocardiography, pulse oximetry, non-invasive blood pressure and end-tidal carbon dioxide measurements. All animals received intramuscular antibiotics, 1.0 ml/10 kg amoxicillin (Betamox LA injection, Norbrook Laboratories Limited, Carlisle, United Kingdom) for peri-operative infection prophylaxis and every other day for one week afterwards. Intramuscular meloxicam (Metacam, Boehringer Ingelheim Pty Limited, Auckland, New Zealand) 0.2 mg/kg was used for three days for post-operative pain relief.

Isolation and cultivation of articular chondrocytes. The right stifle joint region was shaved and disinfected. A medial incision of about 5.0 cm long was made, followed by a medial parapatellar incision of the capsule. The patella was dislocated laterally and parts of Hoffa's fat pad were resected. A full-thickness punch biopsy was harvested at the trochlear groove using a cylindrical hollow punch with an inner diameter of 5.0 mm and a wall thickness of 0.5 mm. The cartilage layer was separated from the underlying bone and was used for chondrocyte isolation, *in vitro* cultivation, and preparation of the ACFC.

The cartilage was rinsed with phosphate-buffered saline (PBS; Gibco/Invitrogen, Grand Island, New York) and diced into approximately 1.0 mm³ segments. Chondrocytes were isolated enzymatically using 0.6% (w/v) collagenase type II (Gibco/Invitrogen) in an orbital incubator (Stuart Scientific, Redhill, United Kingdom) at 37°C for six hours. Chondrocytes were strained from undigested tissue debris and cell clumps using a 40.0 µm polyethersulfone mesh (Falcon, Franklin Lakes, New Jersey). The isolated chondrocytes were washed twice with PBS to remove residual collagenase. After final centrifugation at 6000 rpm for 5 min at 37°C (Jouan centrifuge, Duguay Trouin, St Herblain, France) the cell pellet was resuspended in PBS for total cell count using a haemocytometer (Weber Scientific International Ltd, Middlesex, United Kingdom). Cell viability was determined using 0.4% trypan blue dye exclusion test (Gibco/Invitrogen). Isolated chondrocytes were seeded in six-well plates (Falcon) with an initial seeding of 5000 cells/cm² in F12:DMEM (1:1) supplemented with 10% (v/v) Lamb Serum (Gibco/Invitrogen), antibiotic and antimycotic (Gibco/Invitrogen), 200 mM l-glutamine (Gibco/Invitrogen), 50 µg/ml of ascorbic acid (Sigma Aldrich, St Louis, Missouri) and 15 mM N-2-hydroxyethylenepiperazine-N'-2-ethanesulfonic acid (HEPES) buffer 1M (Gibco/Invitrogen). All cultures were maintained at 37°C in a humidified 5% CO₂ incubator (Jouan). The culture medium was changed every two to three days. Phase-contrast light microscopy (Olympus, Tokyo, Japan) was used to observe chondrocyte morphology on a daily basis.

Autologous chondrocyte-fibrin construct formation. Articular chondrocytes were cultured in 175 cm² cultured flasks (Falcon) at a density of 5000 cells/cm². Once confluent, the cells were detached using 0.05% trypsin-EDTA (Gibco/Invitrogen) and counted with a haemocytometer. Approxi-

Table I. Modification of the O'Driscoll histological scoring scale⁴¹ for evaluation of regenerated cartilage and bone according to Frenkel et al⁴² (reprinted with permission from Elsevier)

Category		Points	
I.	Percentage hyaline articular cartilage:	80 to 100	8
		60 to 79	6
		40 to 59	4
		20 to 39	2
		0 to 19	0
II.	a. Surface regularity:	Smooth & intact	2
		Fissures	1
		Severe disruption, fibrillation	0
	b. Structural integrity:	Normal	2
		Slight disruption, including cysts	1
		Severe lack of integration	0
	c. Thickness:	100% of normal adjacent cartilage	2
		50% to 99% of normal cartilage or thicker than normal	1
		0% to 49% of normal cartilage	0
	d. Bonding to adjacent cartilage:	Bonded at both ends of grafts	2
		Bonded at one end/partially at both ends	1
		Not bonded	0
III.	Freedom from cellular changes of degeneration:	Normal cellularity, no clusters	2
		Slight hypocellularity, < 25% chondrocyte clusters	1
		Moderate hypocellularity/hyper, > 25% clusters	0
IV.	Freedom from degenerative changes in adjacent cartilage:	Normal cellularity, no clusters, normal staining	3
		Normal cellularity, mild clusters, moderate staining	2
		Mild or moderate hypocellularity, slight staining	1
		Severe hypocellularity, poor or no staining	0
V.	Reconstitution of subchondral bone:	Complete reconstitution	2
		More than 50% reconstitution	1
		50% or less reconstitution	0
VI.	Bonding of repair cartilage to <i>de novo</i> subchondral bone	Complete and uninterrupted	2
		< 100% but > 50% complete	1
		< 50% complete	0
VII.	Safranin O staining	> 80% homogeneous positive stain	2
		40% to 79% homogeneous positive stain	1
		< 40% homogeneous positive stain	0
		Total score (maximum 27 points)	

mately 30 million cells/ml were incorporated into fresh autologous fibrin.³⁴⁻³⁶ This mixture was then polymerised with 250 mM of calcium chloride (CaCl₂). Fibrin was used as a temporary scaffold material to support the cells in a three-dimensional (3D) environment. This fresh fibrin was derived from the sheep's own plasma. The plasma was extracted from whole sheep blood collected in sodium citrate tubes by 4800 rpm centrifugation for 5 min, at 4°C (Jouan centrifuge). The resulting 3D gel-like ACFC was maintained for one week *in vitro* in a complete culture medium prior to the actual implantation. A small ACFC segment was sent for histological assessment.

Implantation of the autologous chondrocyte-fibrin construct. During surgery the left stifle joint was opened. Full-thickness punch hole defects penetrating into the subchondral bone (about 2.5 mm total depth) were created in the lateral and medial femoral condyles. One defect was implanted with ACFC (n = 12) and the other was implanted with either autologous chondrocytes (ACI) (n = 4) or autologous fibrin blank construct (AF) (n = 6). The untreated defects were left empty (n = 2). Precisely fitting periosteal flaps taken from the proximal medial tibia were applied to cover the defects, with the cambium layer facing the subchondral bone. The flaps were fixed securely by interrupted 6.0 Vicryl sutures

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1d

Macroscopic view of the repaired osteochondral defects three months after implantation. The colour of a) autologous chondrocyte-fibrin construct-treated defects and b) autologous chondrocyte-treated defects was relatively similar to that of normal adjacent cartilage, whereas the colour of c) the autologous fibrin-treated defects and d) the untreated defects varied from white to tan-purple or red. The texture ranged from irregular to smooth.

(Ethicon, Johnson & Johnson, Brussels, Belgium). Before the implantation step, autologous fibrin was applied to the surrounding rim of the defects. A watertight seal was confirmed by injecting saline solution under the flap. Cultured chondrocytes were then injected beneath the periosteal flap as described by Brittberg et al.¹³ Before closure, the joint was rinsed thoroughly and the capsule sutured with resorbable stitches, 3.0 Vicryl (Johnson & Johnson). The total number of transplanted grafts was 22 (12 ACFC, 4 ACI, 6 AF) and two blank defects. The sheep were extensively observed for one week after surgery. They were allowed to move freely without immobilisation and to undertake unrestricted outdoor activities.

Macroscopic assessment. After 12 weeks, the animals were killed by IV injection of 8.0 ml pentobarbital 200 mg/ml (Dorminal-20%, Alfasan, Woerden, Holland) together with 2.0 g of ketamine (Bioketan, Vetoquinol

Biowet) given intramuscularly. Both stifle joints were harvested, grossly evaluated and photographed. The gross appearance of repaired tissues was assessed in accordance with the International Cartilage Repair Society (ICRS) classification.⁴⁰ Biologically acceptable repairs were defined as having smooth, firm repair tissue that filled the defect and appeared to be attached to the adjacent cartilage. The ICRS classification score for overall repair assessment as: grade I normal, 12 points; grade II, nearly normal, 11 to 8 points; grade III abnormal, 7 to 4 points and grade IV, severely abnormal; 3 to 1 points was used. Samples were excised *en bloc* for histological analysis and divided into two sections, of which one was fixed in 10% buffered formalin for 24 hours for histological analysis, and another fixed in 4% glutaraldehyde for six hours for scanning electron microscopic analysis.

Histological evaluation. After 10% buffered formalin fixation, all *in vivo* specimens were decalcified in 8.0% hydrochloric acid (HCl) solution saturated with ethylenediamine tetraacetic acid (EDTA) for ten days. *In vitro* ACFC and *in vivo* samples were dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned at a thickness of 5.0 μ m using a Leica microtome (Leica Microsystems, Wetzlar, Germany). Haematoxylin and eosin (H&E) and safranin O staining was performed on all sections and visualised using a light microscope (Olympus). All sections were assessed according to the modified O'Driscoll, Keeley and Salter⁴¹ histological-histochemical grading scale.^{10,42} Perfect healing of the defect was characterised by 27 points (Table I). In this study, scores were determined using histological sections and included both the defect area and the adjacent native cartilage.

Scanning electron microscopy. After primary fixation in 4% glutaraldehyde, all samples were soaked with 0.1M sodium cacodylate for 10 minutes and washed three times. Samples were transferred into 1% osmium tetroxide for two hours as post fixation. All were then washed again with 0.1M sodium cacodylate. Samples were dehydrated in a graded series of acetone, placed into the critical point dryer (CPD Baltec-030) for 30 minutes, mounted on to a stub, sputtered with gold coating in Sputter Coater Polaron E-5100 scanning electron microscopy SEM Coating Unit and viewed under a JEOL-JSM 6400 scanning electron microscope unit.

Statistical analysis. Because of the nature of the semi-quantitative scale scoring system the Wilcoxon Mann-Whitney test was used to compare the two populations. This test is used to test the null hypothesis that two populations have identical distribution functions against the alternative hypothesis that the two distribution functions differ only with respect to location (median). Small significance values ($p < 0.05$) indicate that the two groups have different locations.

Table II. Scores according to the International Cartilage Repair Society⁴⁰ classification*

Treatment	Three months after implantation
Autologous chondrocyte-fibrin construct	10.0
Autologous chondrocyte implantation	9.5
Autologous fibrin blank	8.5
Empty (control)	8.0

* Values are given as the median score (in points) for each category. Biologically acceptable repairs were defined as a smooth, firm repair tissue that filled the defect and appeared to be attached to the adjacent cartilage. There was no significant difference ($p > 0.05$) between the total median score for ACFC *versus* ACI ($p = 0.07$), but the ACFC score was significantly better than AF blank ($p = 0.04$) and empty control ($p = 0.003$). However, the total median score for each treatment correspond to a nearly normal appearance. Overall repair assessment:

Grade I	12 points
Grade II	11 to 8 points
Grade III	7 to 4 points
Grade IV	3 to 1 points

Results

All sheep tolerated both unilateral arthrotomies well. They were able to stand on all four limbs immediately after surgery, and could walk without limping after five to seven days. No infections or complications occurred throughout post-transplantation monitoring.

Macroscopic observations of cartilage defects. After 12 weeks there was no evidence of infection, arthrofibrosis or fibrinous overgrowth of the joint surface. There was no osteophyte formation, altered synovium or degenerative arthritis. The surface of the repaired tissues showed a nearly normal contour and the texture ranged from irregular to smooth. The colour of ACFC-treated defects (Fig. 1a) and ACI-treated defects (Fig. 1b) was mainly white, which was similar to the adjacent cartilage. However, the colour of fibrin blank (AF)-treated defects (Fig. 1c) and the untreated defects (Fig. 1d) varied from white to tan-purple or red. Visually, the transplanted graft integrated well with the adjacent cartilage. All transplants could be identified easily, as they showed relatively clear demarcation between transplanted graft and adjacent cartilage except for the ACFC-treated defects, which had good integration with the surrounding cartilage. Using the ICRS classification all defects treated with ACFC, ACI and AF exhibited median scores of 10.0, 9.5 and 8.5, respectively, which corresponded to a nearly normal appearance (Table II). Statistically, there was no significant difference between the ACFC and ACI scores.

Standard histological evaluation. After one week, stabilised *in vitro* ACFC specimens had a homogenous distribution of immature chondrocytes. Underdeveloped proteoglycan-rich protein in the *in vitro* construct was evidenced by the negative safranin O staining, similar to previously reported results.¹⁹

After 12 weeks, significant cartilage formation with high cell density was noted extending into the ACFC (Fig. 2) and ACI-treated defects (Fig. 3). Cells were located within a typical lacunar space surrounded by metachromatic matrix. The cells resembled well-differentiated chondrocytes embedded within cartilaginous matrix sim-

ilar to native tissue (Fig. 4). Subchondral bone formation was evidenced in both ACFC and ACI specimens, although the repaired tissues did not shown columnar alignment like that of the native tissue. Histological sections were stained positive with safranin O (orange-red colour) to indicate the presence of accumulated proteoglycan-rich matrix. The transplanted grafts revealed both lateral and basal chondral and osseous integration with the adjacent cartilage in most cases: ACFC (Fig. 2) and ACI (Fig. 3). A hypocellular area could be clearly identified at the demarcation line. However, no sign of nonunion, surface fibrillation or fissure was seen. On the other hand, the defect treated with AF blank exhibited minimal cartilage ingrowth (Fig. 5). The untreated defect (empty control) was fully occupied with disorganised fibrous tissue (Fig. 6) which did not restore a continuous articular surface with the adjacent cartilage. Also, ACFC and ACI significantly enhanced cartilage repair with respective median histological scores of 21.5 and 16.0, compared with AF blank-treated defects (7.5) and untreated defects (0) (Table III).

Scanning electron microscopy examination. Scanning electron micrography of ACFC-treated defects (Fig. 7a) showed a spherical chondrocyte structure attached to the dense extra-cellular matrix and was similar to the native cartilage in Figure 7b. However, in the sagittal cross-section of ACI-treated defects (Fig. 7c) the cell was structurally more elongated and was attached to a thin loose collagen fibril network compared with native cartilage (Fig. 7b) and ACFC (Fig. 7a). Interestingly, the presence of a chondrocyte-secreting matrix was evident in both ACFC- and ACI-treated defects, indicated by the presence of cells with closely packed microvilli on the surface (Fig. 7d). In some fields the presence of healthy proliferative cells in the repaired tissue was indicated by round chondrocytes and flattened, branched pseudopodia-like structures in the lacunar space (Fig. 7e). However, the presence of sponge-like pattern cells in a few fields indicated damage to the chondrocyte structure (Fig. 7f). At

Fig. 2a

Fig. 2b

Fig. 2c

Fig. 2d

Histological examination of the ACFC (a-d) using haematoxylin and eosin and safranin O staining. After 12 weeks there was significant cartilage formation with high cell density (L) extending into the defect (a-d original magnification $\times 50$). Note the formation of new bone (black arrows). The histological sections stained positive with safranin O, indicating the presence of proteoglycan cartilage-rich matrix. The connection between the grafts and adjacent tissues revealed excellent chondral and osseous integration (white arrows). There was no evidence of nonunion.

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3d

Histological examination of the ACI (a-d) using haematoxylin and eosin and safranin O staining. After 12 weeks there was significant cartilage formation with high cell density (L) extending into the defect (a-d original magnification $\times 50$). Note the formation of new bone (black arrows). The histological sections stained positive with safranin O, indicating the presence of proteoglycan cartilage-rich matrix. The connection between the grafts and adjacent tissues revealed excellent chondral and osseous integration (white arrows). There was no evidence of nonunion.

low magnification, SEM micrographs of ACFC (Fig. 7g) and ACI (Fig. 7h) revealed excellent chondro-osseous and chondrochondral integration.

Discussion

Regenerative medicine using tissue-engineering techniques has been given much attention in orthopaedic fields, espe-

Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4d

Histological examination of the normal cartilage (a-d) using haematoxylin and eosin and safranin O staining (L, chondrocytes in lacunae).

Fig. 5a

Fig. 5b

Fig. 5c

Fig. 5d

Histological examination of the AF blank-treated defect (a-d) using haematoxylin and eosin and safranin O staining. In contrast to Figures 2 to 4, the defect treated with autologous fibrin blank only exhibited minimal chondral ingrowths (marked with arrows) (a-d original magnification $\times 50$).

cially in the management of cartilage repair.^{3,37} To date, there is very little information available for sheep in the NIH GenBank.⁴³ Unfortunately, there is no information quoted for articular cartilage in the nucleotide databases.

Therefore we evaluated the repaired tissues thoroughly using gross inspection and histological examination.

The three-month results demonstrated that chondrocytes trapped within the fibrin network were able to differentiate

Fig. 6a

Fig. 6b

Fig. 6c

Fig. 6d

Histological examination of the untreated defect (empty control, a-d) using haematoxylin and eosin and safranin O staining. Generalised fibrosis deposits were present throughout these empty controls (a-d original magnification $\times 50$).

Table III. Scores according to the modified histological grading scale⁴²

	Category*										Total median score
	I	II				III	IV	V	VI	VII	
Treatment		A	B	C	D						
ACFC	6.0	1.0	2.0	1.0	2.0	2.0	2.5	1.0	2.0	2.0	21.5
ACI	5.0	1.0	2.0	0	2.0	1.0	2.0	1.0	1.0	1.0	16.0
AF blank	2.0	1.0	1.5	0	2.0	0	1.0	0	0	0	7.5
Empty (control)	0	0	0	0	0	0	0	0	0	0	0

* Values for each category are given as the median of the score (in points). Perfect healing of the defect was characterised by 27 points. The Wilcoxon Mann-Whitney test was used to compare two populations. There was a significant difference between the total median score for ACFC *versus* ACI (Wilcoxon Mann-Whitney test, $p = 0.001$). ACFC and ACI significantly enhanced cartilage repair compared to AF blank and empty control (Wilcoxon Mann-Whitney test, $p < 0.05$)

and produce good-quality cartilage-like tissue. This supports the hypothesis that ACFC implantation would influence hyaline-like cartilage regeneration. Additionally, this study confirms the findings of Ruszymah and co-workers.^{19-28,36-38} In parallel, several researchers have successfully fabricated injectable tissue-engineered cartilage using fibrin glue matrix seeded with chondrocytes from calf³⁰ and swine.^{31,32} Another animal study by Shao et al,⁴ using allogeneic bone marrow mesenchymal stem cells (BMSCs) seeded on to fibrin glue matrix and mPCL, also demonstrated that the cell was able to differentiate and produce cartilage-like neo-tissue after three months in a rabbit model. However, some of the transplants had deteriorated by six months, which they considered to be a result of degradation of the fibrin glue matrix.

Macroscopically, ACFC- and ACI-treated groups had a similar colour to the adjacent cartilage. However, ACFC demonstrated a smoother surface than ACI and AF blank. Surface erosion and fissure formation were rarely observed. There was a variation in thickness of repaired tissues in all cases, as some of the area appeared slightly thicker than the adjacent cartilage. Ito et al,⁶ who studied cartilage repair using rabbit articular chondrocytes and an atelocollagen sponge/PLLA mesh composite, reported that the repaired tissue had a smoother surface and the thickness of the tissue was about twice that of normal cartilage after 12 weeks. No tissue hypertrophy was observed in this experiment, although there are several studies in humans reporting that approximately 10% to 15%⁴⁴ or up to 40%¹⁵ of

Fig. 7a

Fig. 7b

Fig. 7c

Fig. 7d

Fig. 7e

Fig. 7f

Fig. 7g

Fig. 7h

Scanning electron micrographs. a) A sagittal cross-section of autologous chondrocyte-fibrin construct-treated defect. The dense extracellular matrices were similar to the native cartilage (b), with spherical chondrocyte located within lacunar spaces ($\times 3000$), b) the native cartilage showed dense extracellular matrices with spherical chondrocyte attached to the surface (original magnification $\times 3000$), c) micrograph of the autologous chondrocytes implantation-treated defect. The cell is more elongated and attached to a network of thin/loose collagen fibrils ($\times 3000$); chondrocyte-secreting matrix is evidenced by the presence of cells with closely packed microvilli on their free membrane surface, d) $\times 5000$, e) $\times 700$ shows the chondrocytes (arrow) in the lacunar space. The presence of cells with a sponge-like pattern during SEM observation indicates that the ultrastructure of chondrocytes was destroyed, f) $\times 2500$; low-magnification views of sagittal cross-sections of the defect areas treated with autologous chondrocyte-fibrin construct, g) $\times 330$ and autologous chondrocyte implantation, and h) $\times 150$. The repaired tissues integrated well with the adjacent cartilage (integration line marked with arrow) (SCB, subchondral bone; NC, native cartilage; T, implant).

patients had hypertrophy after receiving periosteal flap and chondrocyte applications.

The histomorphological features and matrix composition of ACFC-treated defects seem superior to those of ACI and AF blank. Most of the repaired tissues demonstrated minimal fibrocartilage formation in the middle of the defect, where the healing process takes place most slowly. However, the fibrocartilage formation in this study should be regarded as newly organised repair tissue, because the invasion of BMSCs from the subchondral bone may produce fibrous tissue during osteochondral repair.⁷ In a few cases a mild inflammatory reaction was seen at the defect site, and in one case neovascularisation was observed in the middle of the ACI-treated defect. Healing of the underlying subchondral bone is essential, as it offers support for the overlying cartilage regeneration. The formation of subchondral bone was evident in the ACFC and ACI

groups compared with AF blank, although we have implanted single-phase transplants regardless of bone formation at the defect sites. Thus, the infiltration of host BMSCs into a fibrin matrix network should be considered to facilitate subchondral bone formation. The integration of the repaired tissues with the normal adjacent cartilage was excellent, but the margin of the original defect can be easily identified. However, because of excellent bonding between transplants and the native cartilage, Niederauer et al⁷ had difficulty in identifying the original margin in their study of osteochondral repair in a goat model using multiphase implants consisting of poly (d,l) lactide co-glycolide as a base scaffold material.

Scanning electron microscopic analysis on the morphology of repaired tissues showed the organisation of chondrocytes and matrices in both ACFC- and ACI-treated defects, which was relatively similar to that of

native articular cartilage. The ability of chondrocytes to retain a spherical configuration suggests a well-differentiated phenotype. Chondrocytes characteristically had no direct cell-cell contact and were separated by a sparse territorial matrix, as normally seen in native tissue. The chondrocytes that were retained in the collagenous lacunar space are dedicated to resynthesising and reorganising the associated matrix. In some cases, production of a pericellular matrix by the chondrocyte was clearly visible. Matrix composition was increased from a few collagenous fibrils to form fully established fibril networks that were morphologically indistinguishable from those of normal tissue.

In this study, expansion of articular chondrocyte culture and the formation of an autologous 3D chondrocyte-fibrin construct has allowed us to bypass some methodological concerns regarding the treatment currently in clinical use. This pragmatic concern includes the shortage of donor tissue, donor site morbidity for autograft transplantation, immune rejection, and pathogen transfer with allograft transplantation, delayed inflammatory response in most resorbable scaffolds, and the limited service life or toxicity of arthroplasties. However, in the search for ways to re-establish normal joint function using minimally-invasive surgery, we performed arthrotomy on the animal joints in this study. This technique was employed because of the size of the sheep stifle joints. However, in the future we anticipate the possibility of an arthroscopic procedure on a larger joint such as the human knee.

These findings support the hypothesis that ACFC promotes early chondrogenesis by inducing hyaline-like cartilage regeneration at 12 weeks post-surgery. From the macroscopic and histological findings we concluded that cartilage healing in ACFC was superior to that in controls. Moreover, chondro-osseous and chondrochondral integration between implanted ACFC with surrounding tissues was excellent. ACFC implantation could improve the outcome following conventional ACI. The next step is to evaluate the repaired tissue biomechanically and biochemically over a longer period.

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