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EXPERIMENTAL STUDY

Collagen fibre implant for tendon and ligament biological augmentation. In vivo study in an ovine model

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Abstract
Purpose Although most in vitro studies indicate that collagen is a suitable biomaterial for tendon and ligament tissue engineering, in vivo studies of implanted collagen for regeneration of these tissues are still lacking. The objectives of this study were the following: (1) to investigate the regeneration of the central third of the ovine patellar tendon using implants made of an open array of collagen fibres (reconstituted, extruded bovine collagen); and (2) to compare two collagen crosslinking chemistries: carbodiimide and carbodiimide associated with ethyleneglycoldiglycidylether.
Methods Forty-eight Welsh Mountain sheep were operated on their right hind leg. The central third of patellar tendon was removed and substituted with carbodiimide ($n = 16$) and carbodiimide–ethyleneglycoldiglycidylether-crosslinked implants ($n = 16$). In the control group the defect was left empty ($n = 16$). The central third of contralateral unoperated tendons was used as positive controls. Half of the sheep in each group were killed at 3- and 6-month time points. After proper dissection, tendon sub-units (medial, central and lateral) were tested to failure ($n = 6$ for each group), whilst 2 non-dissected samples were used for histology.
Results Both the implants had significantly lower stress to failure and modulus with respect to native tendon at both 3- and at 6-month time points. The implants did not statistically differ in stress to failure, whilst carbodiimide-crosslinked implants had significantly higher modulus than carbodiimide–ethyleneglycoldiglycidylether-crosslinked implants both at 3 and at 6 months. Histology showed carbodiimide-crosslinked implants to have a better integration with the native tendon than carbodiimide–ethyleneglycoldiglycidylether-crosslinked implants. Carbodiimide-crosslinked implants appeared partially resorbed and showed increased tissue ingrowth with respect to carbodiimide–ethyleneglycoldiglycidylether-crosslinked implants.
Conclusions To deliver collagen implants as an open array of fibres allows optimal tendon–implant integration and good ingrowth of regenerated tissue. In the present study the resorption rate of both the examined implants was too low due to the high level of crosslinking. This led to only minor substitution of the implant with regenerated tissue, which in turn produced a low-strength implanted region. Further studies are needed to find the right balance between strength and resorption rate of collagen fibres.

Keywords Tissue engineering · Collagen fibre · Augmentation · Collagen crosslinking · Tendon repair

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Introduction

Tendon and ligament injuries often result in large tissue gaps requiring appropriate grafting to augment or substitute

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the injured tissue and restore natural function [2, 18, 21, 24, 27]. However, autografting, allografting and synthetic grafting all suffer specific complications such as donor site morbidity [29], immunoreaction [28] and fatigue tearing [31]. Consequently, an off-the-shelf, resorbable scaffold capable of withstanding load and able to progressively regenerate native tissue would be a useful treatment option in selected cases.

Collagen scaffolds have been widely studied both in vitro and in vivo in an attempt to regenerate tendon and ligament tissue [5, 7, 12]. Collagen fibres included in a collagen matrix have been adopted to regenerate the anterior cruciate ligament (ACL) in a dog model and the Achilles tendon in a rat model [9, 15]. However, collagen fibres arranged in an open array have never been implanted in a large animal model.

Reconstituted collagen fibres are weak and degrade very quickly even in in vitro culture conditions [4]. Therefore, crosslinking chemistry is the main factor capable of influencing collagen fibre strength and resorption rate. Many physical and chemical crosslinking methods have been explored [16]; however, to date the optimal compromise between strength, biocompatibility and resorption rate has not been found. Recently, the association of carbodiimide (EDC) with an epoxy compound (EGDE) has gained interest as a novel collagen crosslinking chemistry capable of yielding strong collagen with good biocompatibility [33].

It has been found that a single EDC/EGDE fibre could resist stresses of more than 10 MPa [10], which is thought to be the amount of load a human ACL withstands during normal daily activities [25].

This study has been conceived to develop a resorbable collagen implant for tendon and ligament biological augmentation. This study aimed to (1) evaluate the tissue regeneration potential of an EDC-crosslinked open array of collagen fibres implanted in the central third of sheep patellar tendon; and (2) compare the EDC crosslinking chemistry with the novel EDC/EGDE crosslinking chemistry in terms of mechanical properties and tissue integration.

Materials and methods

Implant manufacturing

Acid-swollen type-I collagen from bovine corium was dispersed at 6 mg mL^{-1} in 2 mM HCl for at least 15 h, blended and then degassed at 20 mTorr and stored in 30-mL syringes at 4 °C for up to 2 weeks.

The collagen gel was extruded from an 0.51-mm microbore tubing at 0.3 mL min^{-1} using a syringe pump (Chemxy fusion 200, KR Analytical, Cheshire, UK) into a stainless steel bath containing "fibre formation buffer" (FFB), which consisted of phosphate-buffered saline (PBS) and 20 % w/v poly(ethylene glycol) (PEG) $M_w \sim 8000$ (Sigma-Aldrich, Dorset, UK) at 37 °C. The fibre was incubated for at least 5 min in FFB to allow coagulation and partial fibrillogenesis to occur.

The fibre was then wound onto a spool using a custom-built automated system that utilized a rotating and translating spool. The individual fibres were visible in the final dried assembly (Fig. 1a).

The dried collagen/PEG composite was simultaneously crosslinked and washed on the spool in an 80 % acetone/water solution. The assembly was then crosslinked in either: (1) a solution of 25 mM EDC and 12.5 mM *N*-hydroxysuccinimide NHS in 80 % acetone/PBS for 2 h or (2) a solution of 25 mM EDC and 12.5 mM NHS in 80 % acetone/PBS for 2 h followed by incubation in a solution of 1 % (v/v) ethyleneglycoldiglycidylether (EGDE) in PBS for 5 days.

After crosslinking, the assembly was washed on the spool with PBS for 2×30 min and ultra-high-purity water ($R > 18$ MΩ) for 2×30 min. The hydrated and crosslinked assembly was then air dried overnight. Removal of PEG was confirmed via both differential scanning calorimetry and Fourier transform infrared spectroscopy in comparison with unwashed controls (data not shown). The dried assembly was then sterilized using γ-irradiation at 25 kGy. The end product is shown in Fig. 1.

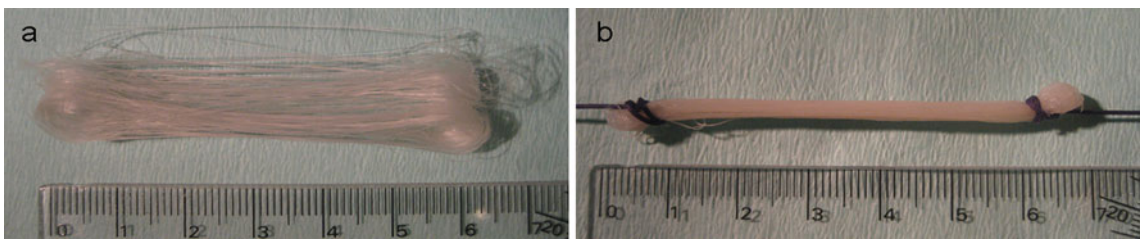


Fig. 1 **a** Dehydrated collagen fibre implant; **b** hydrated implant under tension

Surgical technique

Operations were carried out on forty-eight Welsh Mountain sheep aged between 3 and 5 years and weighing 30–50 kg. There were 3 operative groups (EDC implants, EDC/EGDE implants and empty controls) and 2 time points (3 and 6 months). Under general anaesthesia, the sheep right leg was clipped and hair was removed with a wet cloth. Scaffold implantation was performed under strictly aseptic conditions (Fig. 2). A knee midline skin incision was performed, and the paratenon was incised and preserved. A double-bladed knife (blades 4 mm apart) was used to remove the central third of the patellar tendon. Two drill holes of 4 mm ø and 5 mm depth were made in the tibial tuberosity and in the patella at the bone–tendon junction. Through *K*-wire holes, 3–0 nylon sutures were passed within the 4-mm ø drill holes and used to fix the hydrated implant in place under tension. Three 4–0 silk sutures were placed on each side of the implant on the normal tendon tissue to identify the boundary between the implant and the remnants. All animals were allowed to move freely without immobilization after surgery. Post-operatively, sheep were kept inside in small groups for 2 days and then moved to larger pens in community. For the majority of the experimental period, they were allowed free access to open-field grazing. Sheep were killed at 3 and 6 months after surgery. The hind knee joints were immediately dissected and kept at −80 °C.

Mechanical testing

Six specimens from each group were used for mechanical testing. Immediately after killing, the patella–patellar tendon–tibial tuberosity complex was isolated from each knee (right and left). In the right knee (treated), all the scar tissue surrounding the tendon was removed, the implant (or the regenerated tissue in the empty defect) was identified and separated from the two remnants, and the patella was sawed so as to obtain two patella–tendon–tibia units (medial and lateral) and a central patella–implant/regenerated tibia unit (Fig. 3c, e). In the left knee (control), the patella–tendon–tibial tuberosity complex was isolated, the double-blade knife was used to separate the central third of the patellar tendon, and the patella was sawed to obtain three patella–tendon–tibia units. After dissection, samples were stored at −80 °C.

Before mechanical testing, samples were allowed to defrost overnight at room temperature in glass jars containing deionized water. The cross-sectional areas of the tendons were measured using vernier callipers at 4 locations, at equal spacing from the top to the bottom of the tendons. Bone blocks were gripped using grips with serrated faces. The bottom bone block was held at an angle of 20° using custom-made grips to ensure that all tendon fibres were loaded simultaneously. The mechanical test setting is shown in Fig. 3g and h. Mechanical testing was carried out using an ESH mechanical testing machine fitted with a 10 kN load cell (accuracy 0.1 N). A program was written to control and run the tests automatically.

For each sample, the first third to be tested was always the central third, followed by the lateral third and finally the medial third. Whilst one section of tendon was being tested, the remaining parts were kept wrapped in wet surgical cloth, and a mister was used during testing to ensure that samples remained fully hydrated.

The mechanical testing procedure used was as follows: (1) a small preload of 1 N was applied; (2) a preconditioning step was carried out, in which 10 cycles from 0 to 1 % strain were performed at a rate of 0.5 Hz; (3) the load was removed and the sample was allowed to rest for 2 min; (4) the sample was then extended to 5 % strain at 1 %/s and held for 5 min, in order to investigate the stress relaxation; and (5) finally, the sample was tested to failure at 0.5 %/s.

The load-to-failure test was performed by increasing the tensile load continuously with a speed of 10 mm/min. The tensile load and elongation were recorded, and stiffness was determined by the slope of the load–deformation curve.

Histological evaluation

Two knee joints from each group were dissected into patella–patellar tendon–tibial tuberosity complexes. The complexes were fixed in 4 % paraformaldehyde and embedded in paraffin. Transverse sections of 5 μm were cut, collected on slides and stained with haematoxylin and eosin (HE) for histological evaluation.

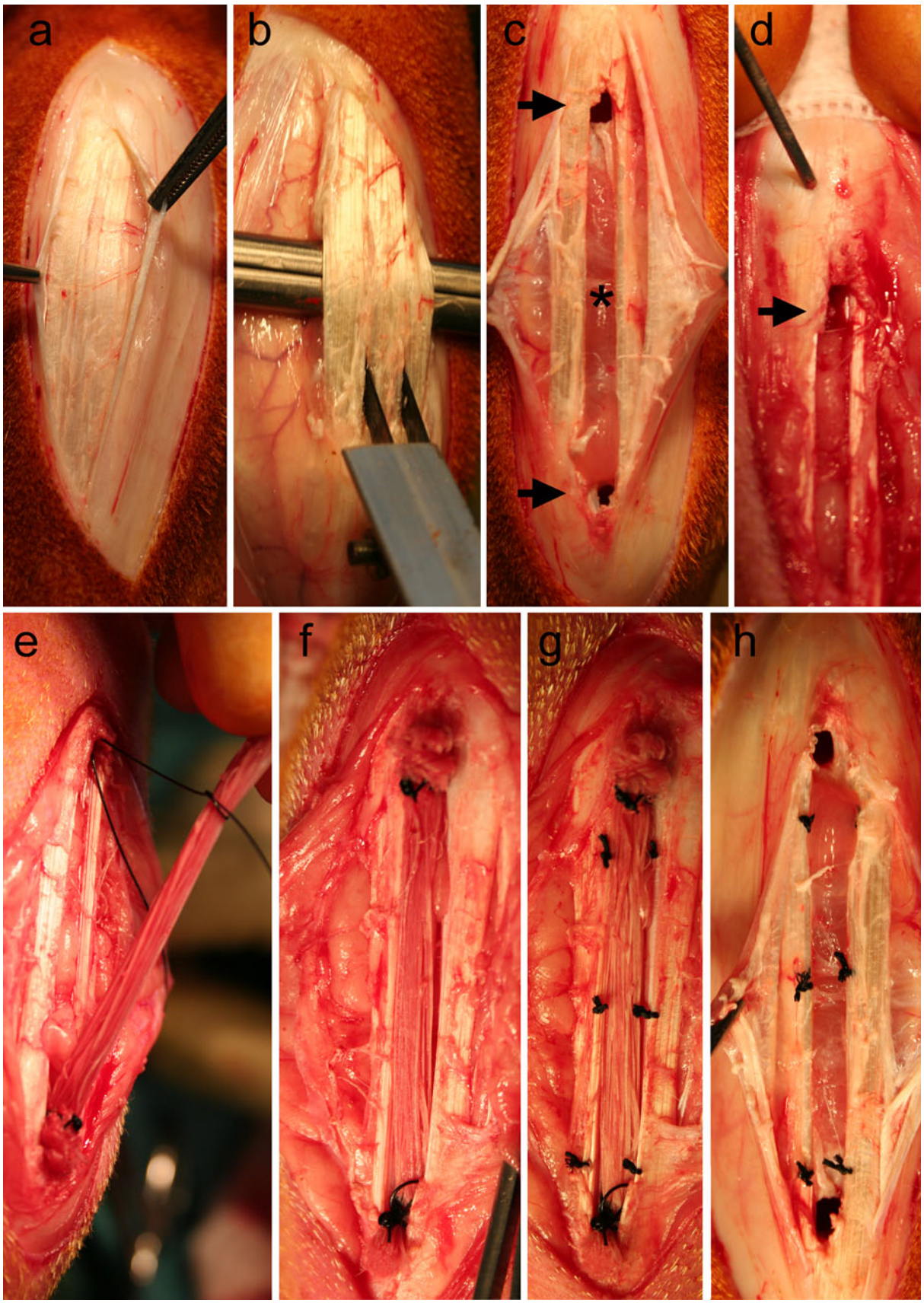
Statistical analysis

All data were analysed using SPSS 13.0 software, and statistically significant differences were defined as $p < 0.05$. The mechanical properties were expressed as mean ± standard deviation (SD). The difference was detected using one-way analysis of variance (ANOVA) test, and differences between individual test groups were analysed by a post hoc Tukey's pairwise multiple comparison procedure.

Results

Post-operative course

Sheep recovered well following surgery, the external wound healed rapidly, and there were no infections. Sheep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◀ **Fig. 2** Surgical procedure. **a** Paratenon was excised and preserved; **b** patellar tendon central third was sectioned with a double-blade knife; **c** two holes were drilled (*arrows*) at the insertion of the dissected central third. The fat pad is clearly visible (*asterisk*) between the outer thirds of the tendon; **d** two *K*-wires were driven from the patella to the drilled hole (*arrow*) to allow implant suture fixation. The same procedure was performed on the tibial side (not shown); **e** implant fixation with sutures emerging from the drilled bone holes; **f** implant tensioned in place; **g** three pairs of sutures were placed at the boundary between implant and native tendon to facilitate future dissection; **h** suture marks were placed in the empty group (fat pad is visible within the two tendon remnants)

were able to rise and stand normally and regained full load bearing on the operated leg at between 2 and 7 days.

Gross observation

The knee joint was exposed and carefully examined. No signs of patellar osteoarthritis or synovial membrane reactions were detected.

Scar tissue was present around patellar tendons of the three operative groups, which had to be carefully removed to expose the tendon/implant fibres. The presence of scar tissue was slightly more abundant around implanted tendons with respect to empty tendons. In the empty group at both 3 and 6 months, the outer thirds of the tendon appeared to be very close or in touch with one another with almost no regenerated tissue in between (Fig. 3a, c, d). In the EDC and EDC/EGDE groups at both 3 and 6 months, the implant was always well recognizable in the centre of the tendon and no gross resorption of the implant was observed (Fig. 3b, e, f). Fat pad adhesion to the operative site was always observed for both empty and implanted defects.

The process of further dissecting the tendons in thirds for mechanical testing was straightforward for the implanted tendons, but was difficult for the empty group, in that it was almost impossible to exclude native tendon fibres from the dissection of the central third (Fig. 3d, arrows).

Mechanical testing

When samples were tested to failure, the rupture always occurred in the tendon mid-substance and bony avulsion was never observed. The most part of the central third samples failed neatly at a point within the tendon, and a few implants were observed to fail near the bottom bone block. Of the unoperated thirds, a large number failed by some fibres pulling away from the bottom bone block and some pulling away from the top bone block, and the others failed neatly within the tendon.

The mechanical properties of the studied groups are shown in Table 1. At the time of dissection, the two remnant thirds of the empty group were often found to have collapsed together (Fig. 3a). For this reason, the separation procedure was difficult, and often native tendon fibres were comprised in the sectioned central third (Fig. 3d). Therefore, the results showed in Table 1 and in Fig. 4 (dotted

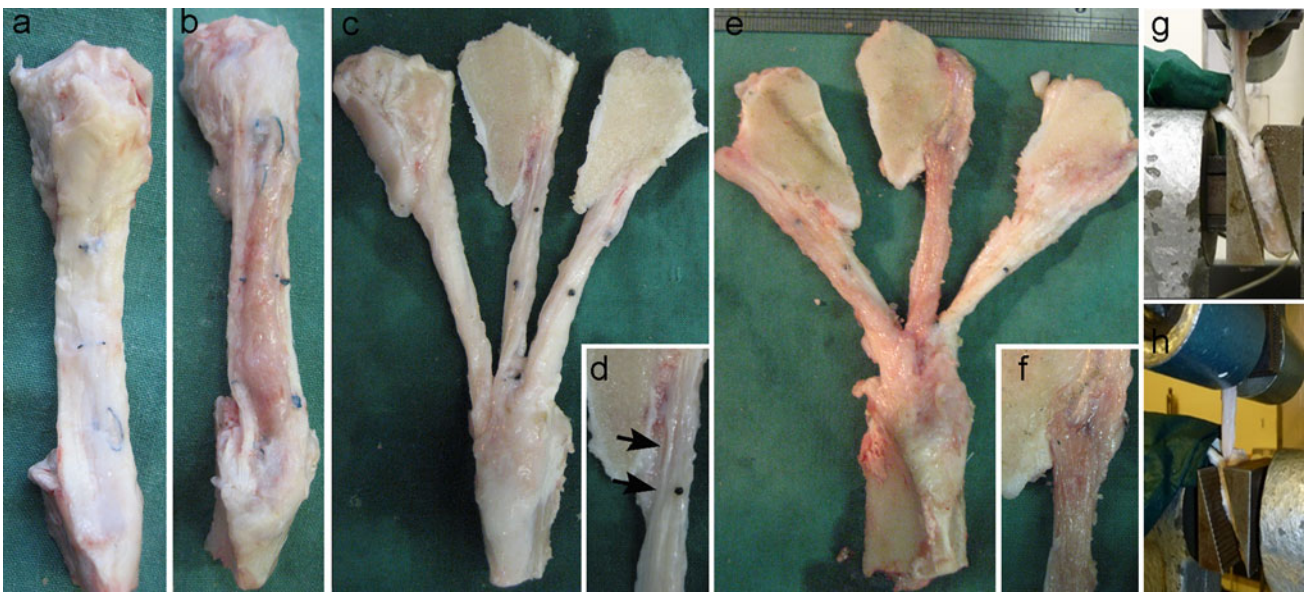


Fig. 3 **a** Empty sample (6 months) of whole patellar tendon from the patella (*top*) to tibial tuberosity (*bottom*). **b** Implanted sample (EDC, 6 months). **c** Empty sample (3 months) whose patella and tendon have been sectioned in thirds to perform mechanical testing. **d** Detail of central third tendon to bone (patella) insertion. *Arrows* show native tendon fibres in the central third, where only regenerated tissue should be observed. **e** Implanted sample (EDC/EGDE, 3 months) sectioned in thirds. **f** Detail of implant to bone (patella) insertion showing good macroscopic integration. **g, h** Mechanical test setting: note the tibial tuberosity inclination of 20° to allow even fibre tensioning

Table 1 Mechanical properties of bone–tendon–bone units

	Mean stress (MPa) ± SD		Mean strain (%) ± SD		Mean modulus (MPa) ± SD		Mean area (mm^2)	
	3 months	6 months	3 months	6 months	3 months	6 months	3 months	6 months
Lateral thirds								
EDC	15 ± 7	21 ± 15	18 ± 5	17 ± 4	125 ± 53	259 ± 62	23 ± 5*[b]	20 ± 4
EDC/EGDE	14 ± 6*[b]	14 ± 3*[b]	19 ± 2	18 ± 4	131 ± 58	118 ± 41	29 ± 9*[b]	17 ± 2
Empty	18 ± 6	26 ± 12	21 ± 4	20 ± 3	150 ± 38	253 ± 116	22 ± 6*[b]	18 ± 4
Control	30 ± 11*[a]	19 ± 3	222 ± 70	13 ± 2*[a]				
Central thirds								
EDC	3 ± 2*[b,‡a]	4 ± 2*[b,§a]	16 ± 3	15 ± 4	26 ± 15*[b,‡a,%a]	51 ± 22*[b,§a,$a]	23 ± 5*[b,‡b]	18 ± 4
EDC/EGDE	1 ± 1*[b,‡a,]	1 ± 1*[b,§a]	16 ± 5	14 ± 2	10 ± 4*[b,‡b,%a]	15 ± 10*[b,§b,$a]	25 ± 5*[b,‡b]	25 ± 5*[b,‡b]
Empty	8 ± 5[‡b,%a]	13 ± 6[§b,%a]	18 ± 5	15 ± 4	65 ± 28 [%b,£a]	141 ± 32[$b,£a]	19 ± 4[‡b]	11 ± 2[‡a]
Control	39 ± 12*[a,%b]	21 ± 2	293 ± 72*[a,£b]	14 ± 3*[a]				
Medial thirds								
EDC	7 ± 6*[b]	19 ± 6	17 ± 3	17 ± 3	73 ± 56	198 ± 63	30 ± 12	22 ± 7
EDC/EGDE	11 ± 5	13 ± 7	25 ± 6	18 ± 6	68 ± 41*[b]	115 ± 28	31 ± 5*[b,‡b]	23 ± 8
Empty	10 ± 6	25 ± 11	19 ± 4	19 ± 3	104 ± 52	241 ± 66	29 ± 5*[b]	21 ± 3[‡a]
Control	21 ± 8*[a]	20 ± 4	146 ± 45*[a]	16 ± 3*[a]				

*[a] Significantly different from *[b,‡a] significantly different from [‡b,§a] significantly different from [§b,%a] significantly different from [%b,$a] significantly different from [$b,£a] significantly different from [£b] ($p < 0.05$). $N = 6$ for each group. *SD* standard deviation

line columns) have to be interpreted carefully. Stress to failure and modulus of the empty group were significantly lower than those of unoperated controls, but significantly higher than those of both implants at each of the two time points (Fig. 4b, h ampersand, dollar sign, pound sign symbol). The strain to failure of the empty central thirds was not statistically different from that of the implants and of unoperated thirds at both time points.

The stress to failure of the implants was always statistically lower than that of unoperated controls (Fig. 4b). The EDC group had a stress to failure almost twice than that of the EDC/EGDE group at both time points, but it was not statistically significant (Fig. 4b). In the outer thirds (Fig. 4a, c), all the operative groups at 3 months showed a decrease in stress to failure with respect to unoperated controls. However, all the operative groups except for EDC/EGDE showed an increase ($p > 0.05$) in stress to failure from the 3- to the 6-month time point.

The strain to failure of the implants (Fig. 4e) was slightly lower but never statistically different from that of unoperated controls, with a slight, non-significant decrease from 3 to 6 months (Fig. 4d, f). In the outer thirds, strain to failure was never significantly different from that of unoperated controls.

The modulus of the implants was always significantly lower than that of the unoperated controls (Fig. 4h asterisk). Modulus of EDC implants was significantly greater than that of EDC/EGDE implant at both 3- and 6-month time point (Fig. 4h double dagger, section sign). In the outer thirds (Fig. 4g, i), all the operative groups at 3 months showed a decrease in modulus with respect to unoperated controls. However, all the operative groups but EDC/EGDE showed a non-significant increase in modulus ($p > 0.05$) from 3- to 6-month time point.

When the in vivo force to failure of the implants at 3 and 6 months was compared with the force to failure of the hydrated pre-implanted scaffold (Fig. 4j), a statistically significant increase was detected between pre-implanted EDC and implants at both 3 and 6 months. On the contrary, there was a significant decrease in force to failure between pre-implanted EDC/EGDE and implants at both 3 and 6 months.

Histological evaluation

At both 3 and at 6 months, the outer thirds of the patellar tendon of the empty group were joined by a thin layer of loose connective tissue rich in large vessels (Fig. 5c, i, l). In one of the 6-month specimens (Fig. 5f), there is an interposition of tissue which has a tendon-like appearance and yet has a different morphology to the outer thirds, being more cellular and with bundles that did not separate during sectioning. It is possible that this is newly regenerating tendon tissue. All the implants (EDC and EDC/EGDE) were clearly visible in the central third of the patellar tendon. No gross resorption occurred at the examined time points even though there appeared to be a decrease in the number of fibres for the EDC implants with respect to EDC/EGDE implants at both time points (Fig. 5a, b, d, e). All the implants at both time points were

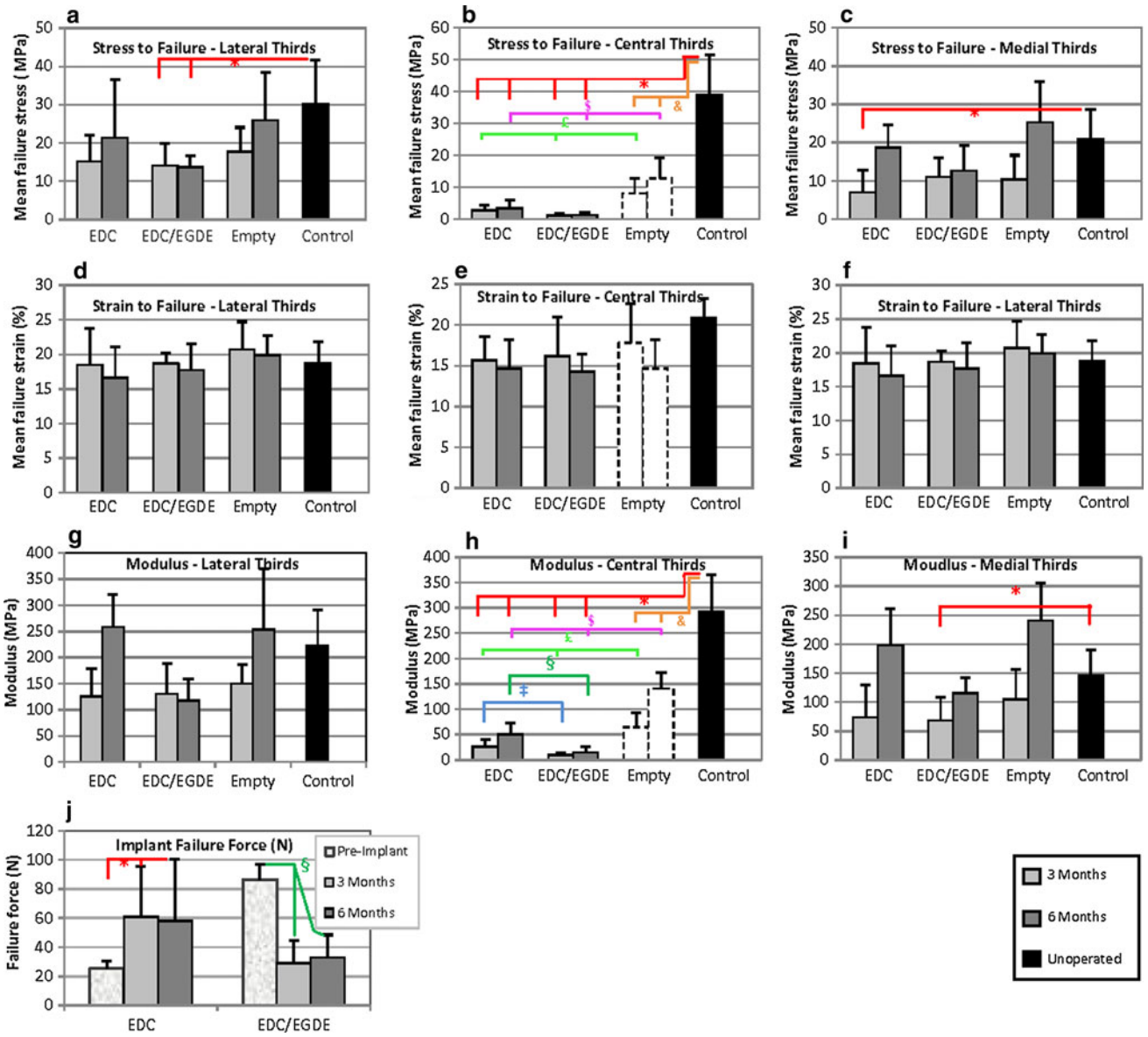
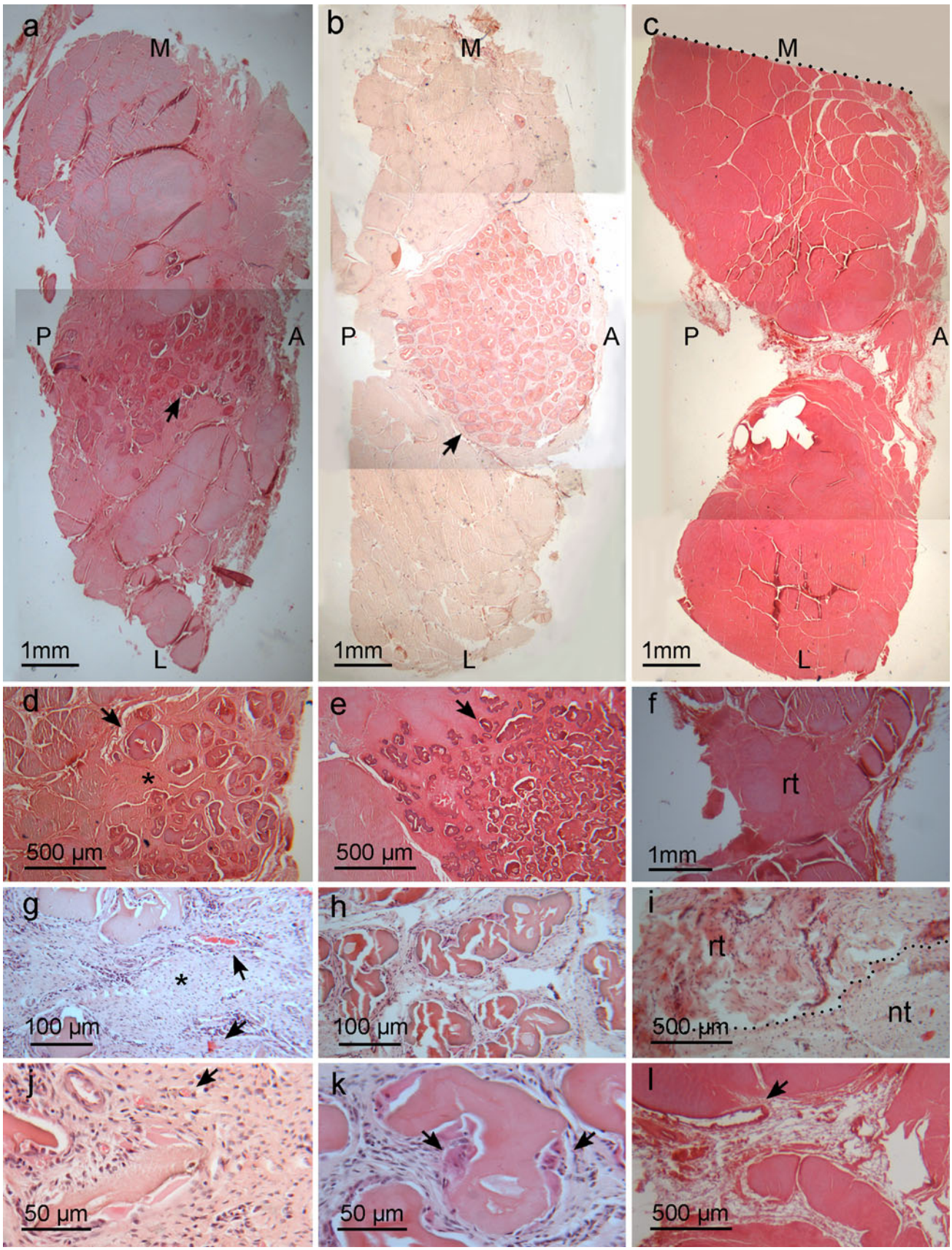


Fig. 4 *Bar* graphs illustrating tendon thirds/implants stress to failure (**a**, **b**, **c**), strain to failure (**d**, **e**, **f**), modulus (**g**, **h**, **i**) and failure force (**j**). Statistically significant difference ($p < 0.05$) is highlighted by *red* (*asterisk symbol*), *blue* (*double dagger symbol*), *green* (*section sign symbol*), *orange* (*ampersand symbol*), *light green* (*pound sign symbol*) and *purple* (*dollar sign symbol*) lines

in close touch with the surrounding tendon; however, the EDC samples appeared more integrated and with a higher level of regenerated tissue between the fibres when compared to the EDC/EGDE samples (Fig. 5d, e, g, h). The regenerated tissue between the fibres appeared more cellular and more vascular (Fig. 5g, j) than the normal tendon tissue. It was mainly composed of tendon fibroblasts, with a few polymorphonuclear cells and multinuclear cells adhering on the fibres (Fig. 5k). In both groups, no encapsulation of the implant occurred in contrast to the encapsulation described by other authors [9, 15].

In the EDC group, a certain degree of fibre fragmentation and resorption was observed at both 3 and 6 months (Fig. 5j); in fact, in some cases the boundary between the collagen fibre and the surrounding tissue was not clearly visible (Fig. 5j). On the other hand in the EDC/EGDE group, a neat distinction between the fibre and the tissue was always observed (Fig. 5h, k). Interestingly, in the EDC group at both time points, tendon fibroblasts often entered the interstices resulting from fibre fragmentation (Fig. 5g) and just a few multinuclear cells could be observed adhering on the fibres. On the contrary, tendon fibroblasts rarely entered EDC/EGDE fibres, and multinuclear cells could be observed adhering on the periphery of the fibre itself (Fig. 5k). It is likely that many of the empty spaces within the EDC/EGDE fibres are tissue processing artefacts (Fig. 5h).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◀ **Fig. 5** Histological samples showing cross-sections of operated tendons. Orientation is described by upper-case letters: *A* anterior, *P* posterior, *M* medial, *L* lateral. **a** EDC implant at 6-month time point (6 m). Non-resorbed EDC fibres are evident in dark pink (*arrow*); **b** EDG/EGDE implant (3 m). Non-resorbed EDC/EGDE fibres are evident in dark pink (*arrow*); **c** empty group (6 m) showing loose connective tissue between tendon remnants; **d** EDC implant (6 m) showing EDC fibres (*arrow*) and good integration and regenerated tissue deposition between fibres (*asterisk*); **e** EDC/EGDE implant (6 m) showing EDC/EGDE fibres (*arrow*) and good integration but scarce deposition of regenerated tissue between fibres; **f** empty group (6 m) with tissue having the appearance of regenerated tissue between the outer thirds (rt); **g** EDC implant (3 m) showing dense regenerated tissue deposition between fibres (*asterisk*) and small vessels (*arrows*); **h** EDC/EGDE implant (3 m) showing packed fibres with scarce tissue deposition. The *white* spaces between fibres are artefacts from cutting and possibly from shrinkage of the tissue; **i** empty group (3 m). *Dotted line* highlights the boundary between native tendon (nt) and regenerated tissue (rt) with fibroblasts randomly organized; **j** EDC implant (3 m) showing fibre resorption and small vessels (*arrows*); **k** EDC/EGDE implant (3 m) showing multinuclear cells adhering onto the collagen fibre (*arrows*); **l** empty group (6 m) showing loose connective tissue and a big vessel (*arrow*)

Discussion

The main findings of this study were the following: (1) the open-array fibre scaffold designed for tendon regeneration allowed tissue ingrowth and integration and avoided implant encapsulation; (2) the EDC crosslinking method adopted in this study did not yield a strength of the collagen fibre comparable with native tendon and did not allow a consistent fibre resorption at 6-month follow-up; and (3) the EDC/EGDE crosslinking produced an inferior mechanical performance of the implant compared to EDC crosslinking, probably due to an even slower resorption rate. These results are consistent with the view that, whilst one of the functions of the open fibre array is to provide space in the defect to allow new tissue ingrowth, it is also going to slow down the healing process if it is not resorbed as the new tissue is formed.

Even though substantial biomechanical differences between EDC and EDC/EGDE implants were not found, some interesting comparisons can be made. In particular, the trend to the increase in modulus from 3- to 6-month time point for the EDC implant suggests that some degree of tissue ingrowth and maturation occurred. This is likely to be associated with the resorption phenomena observed in the histological sections. Similarly, the significant increase in load to failure for the EDC group from the pre-implanted condition to the latest time point (Fig. 4j) suggests that load-bearing ECM deposition occurred between and in place of the implant fibres (Fig. 5d).

In comparison, the EDC/EGDE implant, in which no fibre resorption and tissue substitution were observed, did not show increased mechanical properties from 3 to 6 months, but conversely had a statistically significant decrease in load to failure from the pre-implanted condition to the 6-month time point (Fig. 4j).This phenomenon is likely to be linked to the high level of implant crosslinking which can decrease implant–cell interactions and possibly interfere with the implant resorption and substitution [10]. It is conceivable that if substitution does not take place, the implant could undergo fatigue and fibre tearing which would lead to the progressive weakening of the structure.

There was a decrease in stress to failure and modulus of the outer thirds of the operated patellar tendon with respect to unoperated controls at the 3-month time point (Fig. 4a, c, g, i). This decrease in mechanical properties is a common finding in both small and large animal models and depends on remodelling by resident tendon fibroblasts due to increased load [14, 30]. This decrease resolved by 6 months for the EDC and empty groups, but not for the EDC/EGDE group (Fig. 4a, c, g, i), which seemed to consistently interfere with the strength recovery of the outer thirds of the tendon. Since the mechanical properties of the implant are too low to implicate stress shielding over the remnants, a certain degree of local toxicity of the EDC/EGDE fibres may be postulated.

The empty central thirds had stress to failure and modulus significantly higher than those of EDC and EDC/EGDE implants at both time points. Empty stress to failure values were 21 and 33 % compared to unoperated controls at 3 and 6 months, respectively. These values are in line with other studies in the literature [17, 22]. However, since the presence of native tendon fibres has been documented (Fig. 3d), these results are difficult to interpret. Moreover, histological sections of the empty group showed the presence of collapsed remnants and loose connective tissue between them (Fig. 5 c, i, l), suggesting that the strength detected may be an overestimation of the real condition. However, there was histological evidence for tendon regeneration in one of the specimens (Fig. 5f) and if present in the tested samples may also have contributed to the mechanical strength.

Kato et al. [15] substituted the rat Achilles tendon with glutaraldehyde or EDC/dehydrothermal treatment (DHT) crosslinked collagen fibres cast in a non-crosslinked collagen matrix. At 12 months, the EDC/DHT implant was completely resorbed and produced a yield strength of 22 MPa and a modulus of 41 MPa which were 60 and 22 % with respect to normal tendon. At the same time point, the implant crosslinked with glutaraldehyde resulted in fibrous encapsulation and only partially resorbed produced a yield strength of 22 MPa and a modulus of 192 MPa, which were 60 and 104 % with respect to normal tendon. The same group of authors demonstrated that the EDC/DHT implant was completely resorbed already by 10 weeks after implantation [13]. The fact that resorption occurred as early as 10 weeks for the EDC/DHT group

may be explained by the adoption of the DHT treatment, which has been shown to weaken the collagen structure [32].

Similar results were obtained when the same implants were adopted to substitute canine ACL [9]. By 20 weeks, the entire EDC/DHT crosslinked implant had been resorbed and replaced by a crimped repair tissue which yielded to a stress to failure (17 MPa) of about 35 % of that of normal ACL (49 MPa). At the same time point, the glutaraldehyde crosslinked implant was only partially resorbed and encapsulated by fibrous tissue and inflammatory cells and had a stress to failure (10 MPa) of about 20 % of that of normal ACL. Interestingly in both these studies [9, 15], the mechanical properties of the EDC/DHT implant consistently improved from the pre-implant condition to the latest time point, indicating that load-bearing matrix was being deposited into the scaffold, whist the contrary happened for the glutaraldehyde implant.

In the present study by 6 months, the EDC implant had a stress to failure of 4 MPa (9 % of unoperated control) and the EDC/EGDE implant had a stress to failure of 1 MPa (3.6 % of unoperated control). Several factors may help to account for the differences observed between these results and those of the two models described above [9, 15]. Firstly, the Achilles and ACL studies were substitution models, whilst we used an augmentation model in which the stress shielding operated by the remnants may have contributed to the lower implant strength and new tissue formation. Secondly, the difference in crosslinking chemistry, implant positioning (anatomical place, intra- or extra-articular) and species used may all have contributed to the differences in outcome.

It is intuitive that substitution devices are of more interest to clinicians when compared with augmentation devices. In fact, whilst reparable rotator cuff tears may be a potential application for augmentation scaffolds [20, 23], irreparable cuff tears, ACL tears and Achilles tendon tears with a large gap all require substitution devices. The scaffold used in the present study may bear loads that the human tendon withstands in everyday life [10]; however, it would rupture at a higher level of activity. Therefore, since we could not control the animal activity levels or immobilize the hind limb in a cast for a long time, we opted for a tendon augmentation model. Moreover, the central third of the patellar tendon offered the possibility to study tendon healing in a more favourable condition of vascularity and load protection with respect to other intra-articular sites (ACL).

The present study had a number of limitations. Firstly, the mechanical results of the empty group may have been affected by the difficulties in dissecting the repair tissue and therefore do not represent a reliable negative control. However, this does not prevent the valid comparison of the two implant materials. Secondly, in order to limit the number of animals used, duplicates only were used in each group for histological analysis. Thirdly, the tendon–bone junction was deliberately not explored because this study was designed to look at the tendon regeneration, and a direct implant to bone insertion was not created.

In recent years, many different materials for tendon and ligament tissue engineering have been proposed. Extracellular matrix (ECM)-derived scaffolds have already been introduced in clinical practice and are FDA approved as augmentation devices [6, 19], but they have shown a number of important limitations [1]. Knitted silk has shown promising results in a large animal model for ligament substitution [11]. Poly-lactide-co-glycolide (PLGA) and polyglycolic (PGA) scaffolds were investigated in small animal models for tendon substitution with good biomechanical results [3, 26]. However, despite extensive research, the material with the right combination of strength and biocompatibility has not been found.

Whilst collagen fibres can be crosslinked to produce materials with different mechanical and biological properties [16], they share the same basic structure and carry inherent limitations such as intrinsic weakness and the difficulty of manufacturing a reproducible product [34]. If a stronger fibre is required, a high level of crosslinking has to be used, and the biological interactions between cells and fibres will deteriorate accordingly [8]. The results of the present study and of other studies in the literature [9, 15] suggest that the right trade-off between strength and resorbability may not exist for collagen-derived fibres for both augmentative and substitutional approaches to tendon and ligament repair. However, novel crosslinking chemistries are being developed [16], and the association between scaffolds, cell therapy and growth factors will possibly be the key to overcome the shortcomings of collagen scaffolds alone [19].

This study is clinically relevant because it demonstrated that a scaffold made of an open array of fibres is easy to handle and to deliver on surgical site and allows optimal tissue regeneration and ingrowth.

Conclusion

The present study aimed to develop a fibrous collagen implant for tendon and ligament biological augmentation. The free fibres allowed good tendon–implant integration; however, more efforts have to be made to produce implants that combine good initial fibre strength with an optimal resorption rate in order to favour prompt substitution of the implant by regenerated tissue.

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