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Extruded collagen fibres for tissue engineering applications: effect of crosslinking method on mechanical and biological properties

Davide Enea · Frances Henson · Simon Kew · John Wardale · Alan Getgood · Roger Brooks · Neil Rushton

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Abstract Reconstituted collagen fibres are promising candidates for tendon and ligament tissue regeneration. The crosslinking procedure determines the fibres' mechanical properties, degradation rate, and cell–fibre interactions. We aimed to compare mechanical and biological properties of collagen fibres resulting from two different types of crosslinking chemistry based on 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide (EDC). Fibres were crosslinked with either EDC or with EDC and ethylene-glycol-diglycidyl-ether (EDC/EGDE). Single fibres were mechanically tested to failure and bundles of fibres were seeded with tendon fibroblasts (TFs) and cell attachment and proliferation were determined over 14 days in culture. Collagen type I and tenascin-C production were assessed by immunohistochemistry and dot-blotting. EDC chemistry resulted in fibres with average mechanical properties but the highest cell proliferation rate and matrix protein production. EDC/EGDE chemistry resulted in fibres with improved mechanical properties but with a lower biocompatibility profile. Both chemistries may provide useful structures for scaffolding regeneration of tendon and ligament tissue and will be evaluated for in vivo tendon regeneration in future experiments.

D. Enea (✉) · J. Wardale · A. Getgood · R. Brooks · N. Rushton
Orthopaedic Research Unit, Department of Surgery, Cambridge University, Addenbrooke's Hospital, Hills Rd, CB2 2QQ Cambridge, UK
e-mail: davidenea@tiscali.it

D. Enea
Clinica Ortopedica, Polytechnic University of Marche, Ospedali Riuniti Ancona, Via Tronto 10, 60126 Ancona, Italy

F. Henson
Department of Veterinary Medicine, University of Cambridge, Madingley Road, CB3 OES Cambridge, UK

S. Kew
Orthomimetics Laboratories, Byron House, Milton Rd, CB4 0WZ Cambridge, UK

1 Introduction

Tendon and ligament injuries are common and often require surgical reconstruction with auto-, allo-, or synthetic grafting to restore natural function [1–3]. To avoid complications related to these grafting options, such as donor site morbidity [4], immunoreaction [5] and tearing of the synthetic implant [6] tissue engineering aims to develop a degradable and biocompatible "off-the-shelf" bioscaffold which is capable of undergoing remodelling and progressive substitution by the de novo synthesised tissue in order to regenerate a normal anatomical structure [7].

In the last few years a renewed interest in the use of type I collagen for tissue engineering of tendon and ligament as well as for bone and cartilage has developed. Crosslinking methods [8–10], fibre manufacturing [10, 11] and coupling with other materials [12–14] have been explored in order to enhance bio-mechanical properties of the scaffold.

Type I collagen is the main constituent of normal tendon and ligament structure. Bovine or porcine type I collagen is easy to purify, has low antigenicity and promotes cell adhesion and growth [15, 16]. These properties make it an attractive candidate to build a resorbable scaffold for tendon and ligament tissue regeneration. Moreover, collagen can be extruded in fibres of small diameter, which can be arranged in a parallel array to mimic the normal anatomy of tendon and ligament tissue [11, 17].

Reconstituted collagen fibres degrade quickly under in vitro culture conditions and are not suitable for in vivo

applications [8, 18]. Therefore, different methods of crosslinking have been developed in order to both increase the strength of collagen fibres and to control the biodegradation rate [8, 9, 18–21]. However, studies which investigated the in vivo implantation of bundles of type I collagen fibres, have shown that an appropriate crosslinking chemistry to form fibres with both favourable mechanical and biological properties has yet to be found [22, 23].

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) crosslinking of collagen fibres has been widely used to yield fibres with reasonable mechanical strength while preserving good biocompatibility [8, 24]. However, the *"zero-length"* carbodiimide-mediated crosslinking of collagen structures, whilst biocompatible, is known to provide materials with inferior mechanical properties compared with longer crosslinking reagents such as glutaraldehyde. It is possible that *zero-length* crosslinkers such as EDC embrittle and stiffen the structure as evidenced by the reduction in strain-to-failure for extensively crosslinked materials [25]. On the other hand, *non zero-length* crosslinking compounds are usually more toxic than EDC and have to be removed from the crosslinked material to an appropriate level prior to use in vivo. The epoxy compound ethylene-glycol-diglycidyl-ether (EGDE) is a *non zero-length* crosslinker which yielded high mechanical properties of collagen fibres [10] and exhibited acceptable cytotoxicity [26].

There is evidence that the combination of EDC with epoxy compounds may provide an advantageous balance in terms of biocompatibility, mechanical properties and resistance towards enzymatic degradation in comparison to epoxy compounds alone [27, 28].

Therefore, in order to improve the bio-mechanical properties of the collagen fibres a novel chemistry including both EDC and the epoxy compound EGDE was developed in this study.

The aim of this study was to compare mechanical and biological properties of EDC and EDC/EGDE crosslinked collagen fibres. The ultimate scope of the investigation will be the manufacture of an implant to be used for an in vivo tendon augmentation model.

2 Methods

2.1 Fibre production

Collagen fibres were produced according to a method adapted from Kato et al. [17]. Briefly, 3 g of bovine dermal collagen (Devro, Moodiesburn, UK) was hydrated in 100 ml 20 mM HCl overnight. The resulting acid gel was homogenised for eight minutes, loaded into a disposable 10 ml syringe and extruded through 0.51 mm inner diameter microbore tubing at 0.2 ml min^{-1}. The collagen gel was extruded into "fibre formation buffer" (FFB) comprising 135 mM NaCl, 30 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid and 30 mM Na_2HPO_4 (Sigma, Poole, UK). After the extrusion time plus 60 min in FFB at 35°C the fibres were transferred to phosphate buffered saline (PBS) at 22°C for 15 min and deionised water at 22°C for at least 15 min. The fibres were allowed to air dry under tension.

2.2 Fibre crosslinking

All chemicals were purchased from Sigma-Aldrich (Poole, Dorset UK) unless otherwise indicated. The collagen:EDC:NHS (N-hydroxysuccinimide) mass ratio was kept constant for all reactions at 1:2:0.5. After each reaction the fibres were washed via incubation in two 500 ml portions of deionised water and then air dried under tension. *Method 1 (EDC)*: fibres were hydrated in 50 ml 0.01 M MES buffer, pH 5.5, T = 22°C to which was added 50 ml MES buffer containing EDC and NHS. The fibres were crosslinked for 1 h. *Method 2 (EDC/EGDE)*: fibres were first hydrated in 40 ml PBS. To this was added 50 ml of PBS containing NHS and EDC and 10 ml of 50% v/v ethylene-glycol-diglycidyl-ether (EGDE) to give a final concentration of 5% v/v. The fibres were incubated at 37°C for 5 h.

2.3 Fibre mechanical testing

Stress–strain curves for the extruded collagen fibres were obtained using an Instron 3343 universal testing machine (Instron Ltd. Buckinghamshire, UK) after they had been hydrated in PBS for 24 h. The fibres were tested at an extension rate of 10 mm/min based on a nominal gauge length of 20 mm. The cross sectional area of the fibres was determined by measuring the diameter of the hydrated fibre at five points using a L2000 HTG microscope (GX Microscopes, UK) with calibrated eye-piece and polarising filters. The failure strain was calculated using vernier calipers to measure the initial length to an accuracy of approximately ±10 μm. The modulus was determined by taking a chord between 5 and 15% strain.

2.4 Cell isolation

Patellar tendon tissue was harvested in a sterile fashion from sheep and washed 3 times in fresh Dulbecco's modified essential medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml gentamicin. The tissue was finely minced and approximately 5 g

wet weight of tissue were put into a 175 cm^2 flask to which was added 50 ml of 0.2% collagenase 1A (Sigma) in supplemented DMEM. Tissue was then incubated at 37°C with shaking for 2–3 h. The resulting cell suspension was spun at 1500 rpm for 5 min and plated in 175 cm^2 flasks.

2.5 Cell culture

One-hundred collagen fibres 40 cm in length were placed in parallel, sutured with 1-0 nylon thread every 1 cm and then cut to size (final ø 3 mm). Scaffolds were placed in 24 well plates and sterilised in ethanol. Bundles were seeded with 10^5 cells in 20 µl DMEM taking care to pipette the 20 µl drop onto the bundle. The same amount of cells was seeded in micro-masses on 24 well plates in triplicate as controls. After seeding, the bundles were incubated for 4 h before 2 ml of phenol red free supplemented DMEM was added to each well. For each group of crosslinked fibres, bundles were seeded in triplicate for the cell proliferation assay and in duplicate for the immunohistochemistry and scanning electron microscope (SEM) imaging. Cells were cultivated in standard conditions: humidified, 37°C, 5%CO_2, 95% air. The media was changed every third day, and an aliquot stored for dot-blotting (day 1, 3, 6, 9, 12). Cells and culture plates were harvested by snap-freezing at 1, 7 and 14 days. Bundles were replated at every media change and before the cell counting.

2.6 Cell proliferation assay

On the day of the experiment, plates were thawed and analysed for DNA content as a measure of cell number using the CyQuant assay (Invitrogen) according to the manufacturer's instructions.

2.7 Dot-blotting

Medium from each well was diluted in PBS and filtered through the transfer membrane (Immobilon-P, Millipore Corp., Bedford, MA) using a dot-blotting apparatus (Bio–Dot Apparatus, Bio-Rad, UK). The membranes were blocked with PBS containing 0.1% Tween-20 and 5% non-fat dry milk PBS (PBSTM) for 1 h and incubated overnight with primary antibodies: anti-human tenascin-C mouse IgG1 (IBL International—4F10TT) 1:1000 dilution, and anti-collagen type I rabbit IgG (Rockland 600-4001-103-0.1) 1:1000 dilution. Membranes were then washed and incubated for 2 h with the appropriate species horseradish peroxidise-conjugated secondary antibody at 1:10000 dilution in PBSTM. Blots were visualised using the ECL Plus blotting system (GE Healthcare, UK) as per the manufacturer's instructions. Developed films were scanned using a GS-710 Calibrated Imaging Densitometer and analyzed using Quantity One software (Bio-Rad, UK).

2.8 Immunoistochemistry

Frozen sections of 7 µm thickness were obtained from each bundle at each culture time point. The sections were fixed in 4% paraformaldehyde for 20 min at room temperature and washed in PBS. Immunohistochemistry was performed on bundles using a standard fluorescent secondary antibody detection method. The primary antibodies used as described above. The anti-collagen type I antibody was used at a dilution of 1:100 and the anti-tenascin at 1:200. The secondary antibodies used were FITC-labeled anti-mouse and FITC-labeled anti-goat (Sigma, UK). Sections were counterstained with DAPI (Vector Laboratories, UK) to allow identification of the nucleus. All fluorescent-labeled sections were imaged with a Laborlux 12 fluorescence microscope (Leitz, UK) using digital image acquisition.

2.9 Scanning Electron Microscopy (SEM)

Tendon fibroblasts were seeded on collagen bundles as previously described. Furthermore, EDC and EDC/EGDE crosslinked collagen films (6.4 mm ø) were hydrated for 1 h and seeded with TFs at a seeding density of 10^4 cells in 200 µl of complete DMEM. Both bundles and films were cultivated for 24 h, 7 days and 14 days, fixed in 4% glutaraldehyde in 0.1 M Pipes buffer at pH 7.4, post-fixed in 1% osmium ferricyanide dehydrated in increasing ethanol concentrations, CPD-dried, mounted on stubs and gold sputtered. They were then viewed with an FEI-Philips XL30 FEG SEM microscope (Philips, UK).

2.10 Statistics

Data were analyzed by one-way ANOVA and differences between individual test groups were analyzed by a posthoc Tukey pairwise multiple comparison procedure. Statistical significance was set at $P < 0.05$ (two-tailed). Data were analyzed with SPSS 17.0 (SPSS Inc, Chicago, IL, USA). All data are presented as mean ± SEM.

3 Results

3.1 Mechanical properties

The shapes of the stress–strain curves, shown in Fig. 1, exhibited the non-linearity expected for hydrated collagen fibres with lower modulus toe region and a higher modulus linear region occurring before sample failure. The modulus of the EDC/EGDE crosslinked fibres was significantly higher than that of the fibres crosslinked with EDC ($P < 0.05$) (see Table 1). Similarly, the stress at failure

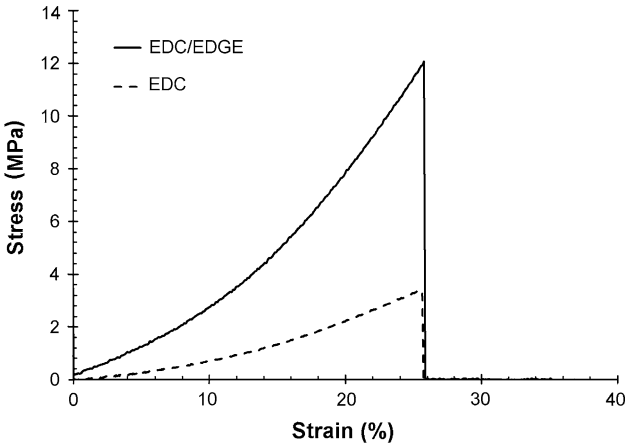

Fig. 1 Representative stress–strain curves for EDC and EDC/EGDE crosslinked fibres

was significantly greater for the EDC/EGDE fibres (10.5 ± 1.3 MPa) with respect to that for the EDC (4.6 ± 0.4 MPa) ($P < 0.05$). The strain-to-failure was not significantly different between the two crosslinking chemistries. The diameter of the EDC/EGDE fibres was on average 137 μm and resulted significantly smaller than that of the EDC fibres (215 μm) ($P < 0.05$).

3.2 Cell proliferation

At day 1 the mean number of TFs on the two bundle types was compared with the mean number of cells at the bottom of the respective wells, i.e. non-adherent (data not shown). The resulting seeding rate was 92%. The different groups showed significantly different levels of cell proliferation ($P < 0{,}001$) (Fig. 2). At day 1 no significant difference in cell number was found between any of the conditions. At day 7 and at day 14 the cell number on the EDC bundles and on controls was significantly greater than that on the EDC/EGDE bundles ($P < 0.05$), and the cell number on controls was significantly greater than that on EDC bundles ($P < 0.05$). Cell number on the EDC bundles significantly increased from day 1 to day 7 ($P < 0.05$). Cells on the controls increased from day 1 to day 14 ($P < 0.05$). Cell number on EDC/EGDE bundles significantly decreased from day 1 to day 7 ($P < 0.05$) and then remained stable.

3.3 Matrix protein production

Collagen type I production (Fig. 3) in the medium of control wells increased significantly from day 1 to day 3 and from day 3 to day 6, then remained stable ($P < 0.05$). Collagen type I production in the medium of the EDC bundles increased significantly from day 3 to day 6 and then significantly decreased from day 6 to day 9 ($P < 0.05$). Collagen type I production in the medium of the EDC/EGDE bundles peaked at day 6 being significantly greater than that at other time points ($P < 0.05$). Collagen type I levels in control wells were always significantly greater than those in bundles wells but at day 6, when they were not significantly higher than those in EDC bundles ($P > 0.05$). Collagen type I production in the medium of EDC bundles was always significantly greater than that of EDC/EGDE bundles ($P < 0.05$).

Levels of tenascin-C (Fig. 4) were not detectable for any of the samples at day 1 and for any of the samples but the control wells at day 3. Tenascin-C production in the control wells significantly increased from day 6 to day 9 and then remained stable ($P < 0.05$). Tenascin-C production in the medium of EDC and EDC/EGDE bundles increased significantly from day 9 to day 12 ($P < 0.05$). At day 9 production of tenascin-C was significantly higher for controls than for EDC bundles ($P < 0.05$). Production of tenascin-C in the medium of control wells and EDC bundles was always significantly higher than in that of EDC/EGDE bundles ($P < 0.05$).

3.4 Immunohistochemistry

DAPI nuclear staining (blue staining) demonstrated that TFs were predominately arranged singly on the surface of the fibre. At every time point collagen I and tenascin-C immunoreactivity (green staining) followed a similar pattern: it was detected associated with TFs on the EDC bundles (Fig. 5a, c, e, g), but were not readily identifiable associated with TFs on the EDC/EGDE bundles (Fig. 5b, c, f, h). A low cellularity was detected at day 7 (data not shown) and at day 14 on the EDC/EGDE bundles (Fig. 5d, h). In all cases the immunoreactivity was detected within the cell cytoplasm. In multi-cellular areas green staining appeared to form a layer surrounding the fibre (Fig. 5a, c, e, g, white arrows).

Table 1 Mechanical properties of crosslinked fibres. Values are expressed as mean ± SEM

Crosslinking method	Stress at failure (MPa)	Strain at failure (%)	Modulus (MPa)	Fibre diameter (μm)
EDC ($n = 9$)	4.6 (±0.4)	23.2 (±2.0)	19.3 (±1.7)	215 (±12.3)
EDC/EGDE ($n = 8$)	10.5 (±1.3)	23.1 (±2.3)	46.2 (±4.9)	137 (±16.6)

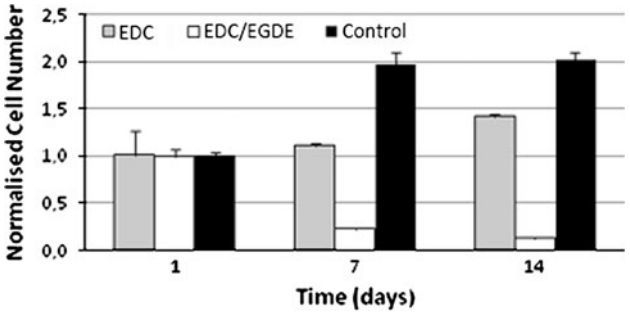

Fig. 2 Number of TFs (normalized with respect to control at day 1) that attached on EDC bundles, EDC/EGDE bundles and on plastic wells (control). Data are shown as mean ± SEM at different time points

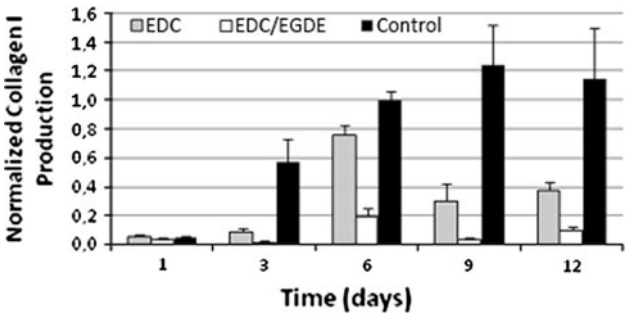

Fig. 3 Production of collagen type I (normalized with respect to control at day 6) detected in the culture media of EDC bundles, EDC/EGDE bundles and on plastic wells (control). Data are shown as mean ± SEM at different time points

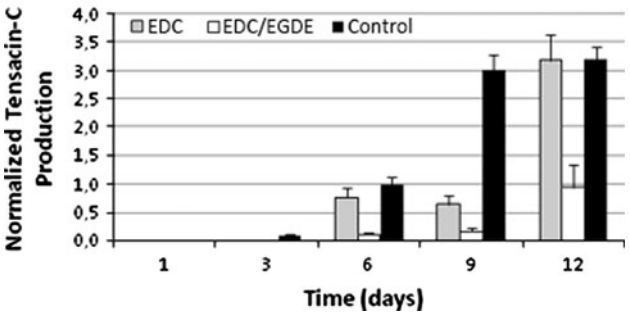

Fig. 4 Production of tenascin-C (normalized with respect to control at day 6) detected in the culture media of EDC bundles, EDC/EGDE bundles and on plastic wells (control). Data are shown as mean ± SEM at different time points

3.5 Scanning electron microscopy (SEM)

The pattern of TFs adhesion was noticeably diverse between the different chemistries in use. *EDC*: at day 1 TFs evenly distributed on collagen films (Fig. 6a, b) and fibres (Fig. 6e, f). They were mainly disposed singularly or in little groups and showed a flattened or elongated appearance. Both on films and on fibres the cell number was comparable with that on the EDC/EGDE crosslinked collagen at day 1. TFs increased in number at day 7 (data not shown) and reached confluence at day 14 on collagen films (Fig. 7a, b) and appeared flattened and elongated on fibres (Fig. 7e, f). *EDC/EGDE*: at day 1 both on films (Fig. 6c, d) and on fibres (Fig. 6g, h) TFs distributed singularly and maintained a round appearance. They decreased in number both at day 7 (data not shown) and at day 14, when they appeared slightly flattened on collagen films (Fig. 7c, d). At day 14 TFs maintained a round appearance on fibres, sometimes appearing more elongated than at day 1 (Fig. 7g, h).

4 Discussion

The mechanical properties of implanted collagen fibres have been found to be rapidly modulated by the tissue healing process in vivo and even with fibres that are mechanically weaker than the native tissue, biological enhancement can provide comparable properties to autogenous grafts [29]. High strength collagen fibres have been reported based on the use of extreme dehydration to cause condensation crosslinking, with break stresses as high as 92 MPa [25]. However, these high strength and presumably highly crosslinked collagen materials have been liable to mechanical failure in vivo despite their mechanical strength being comparable to native ligament [23]. A likely cause for this behaviour is the rapid enzymatic degradation of dehydrothermally treated collagen fibres compared with even uncrosslinked collagen [30], which may have resulted in a rapid reduction in mechanical properties. Therefore, the most critical parameter for the success of a collagen fibre scaffold that regenerates ligament or tendon may be the rate at which fibroblasts are able to attach, proliferate and remodel the scaffold matrix and not necessarily the initial mechanical properties. In order to identify collagen fibre structures that achieve more rapid regeneration of tendon and ligament tissue, it may actually be necessary to reduce the amount of crosslinking.

The fibres utilised in this study were crosslinked using EDC, which has been shown to provide a lower crosslinking density than DHT or UV treatment [24]. In the present study, it was found that the strongest fibres tested were crosslinked with the combination of EDC and EGDE. It is likely that the length of the EGDE crosslinker enabled a larger number of the available nucleophilic sites to be used for the crosslinking reaction compared with the *zero-length* EDC chemistry alone. Recent studies by Zeugolis et al. [10] support our findings. Furthermore, it was found that the strain-to-failure was ca. 20%, indicating that the additional strength achieved using EGDE did not cause embrittlement of the material, possibly due to the length of the crosslinker.

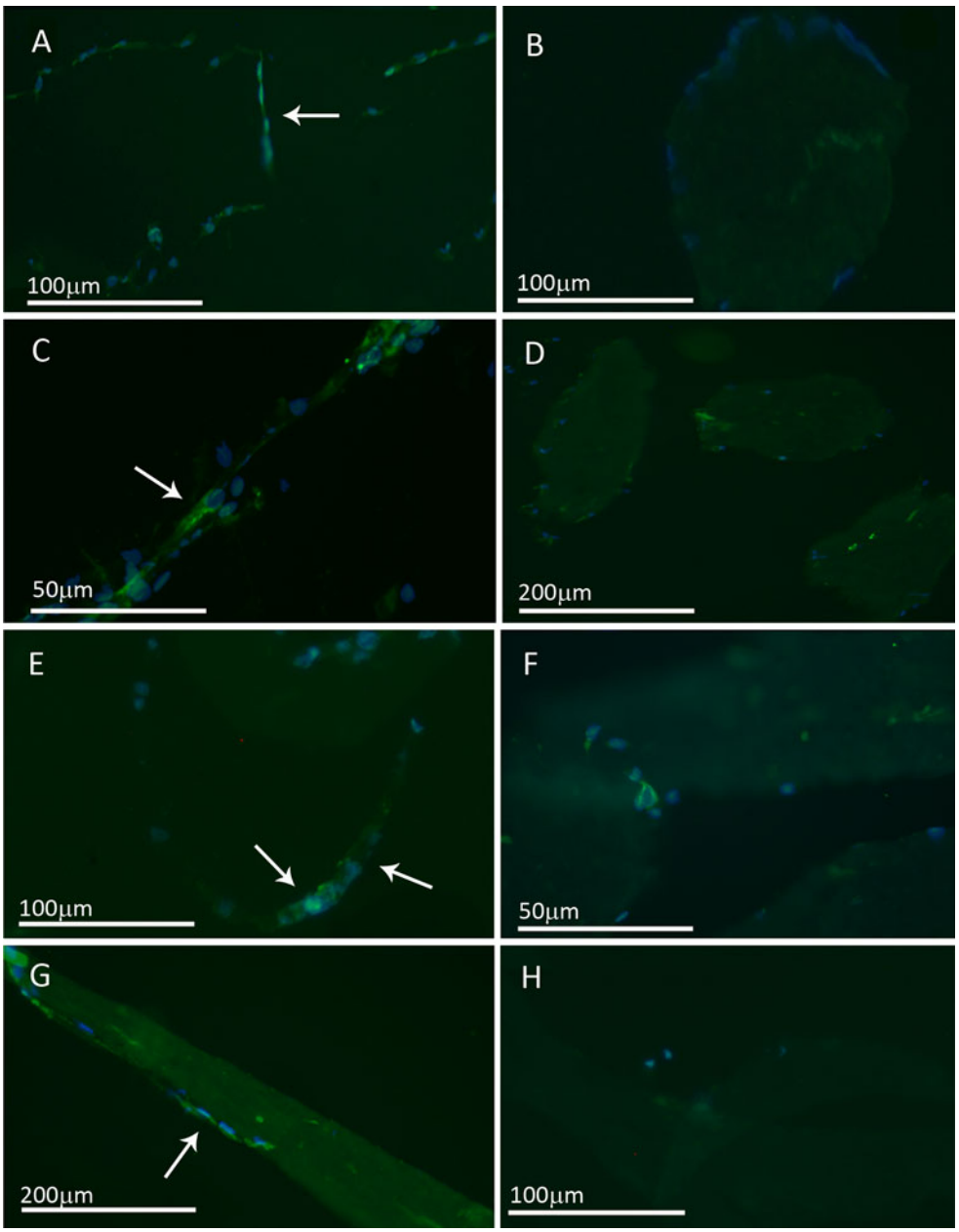

Fig. 5 Type I collagen (**a**, **b**, **c**, **d**) and tenascin-C (**e**, **f**, **g**, **h**) protein immunoreactivity detected at day 1 (**a**, **b**, **e**, **f**) and day 14 (**c**, **d**, **g**, **h**) time-points. TFs were cultivated on EDC (**a**, **c**, **e**, **g**) and EDC/EGDE (**b**, **d**, **f**, **h**) bundles. See text for description

It is well established that the crosslinking chemistry [24] and the 3D architecture [15] of a collagen scaffold determine the ability of the cells to proliferate and differentiate on the construct. Moreover the association of collagen with other biomaterials such as silk, polycaprolactone and chitosan has been shown to be beneficial to optimize cell adhesion, proliferation rate and differentiation [12–14].

However, to our knowledge, only a few studies assessed direct fibroblast attachment and proliferation on crosslinked collagen fibres not embedded in collagen gels, and none of them focused on the EDC/EGDE crosslinking chemistry [19, 31]. In this experiment the seeding rate at day 1 (i.e. the number of cells adhering to the collagen fibres after seeding) was higher than 90%. Our data are consistent with those of similar studies in which the cell attachment was evaluated on collagen fibres and on flat dishes coated with collagen fibrils [21, 32]. However, in two other studies, skin and ligament fibroblasts seeded on fibrous collagen scaffolds with a similar seeding technique had an attachment rate after 1 day, which ranged only from 4–8% [19, 31]. The discrepancies in the observed attachment rate can be explained by the different crosslinking

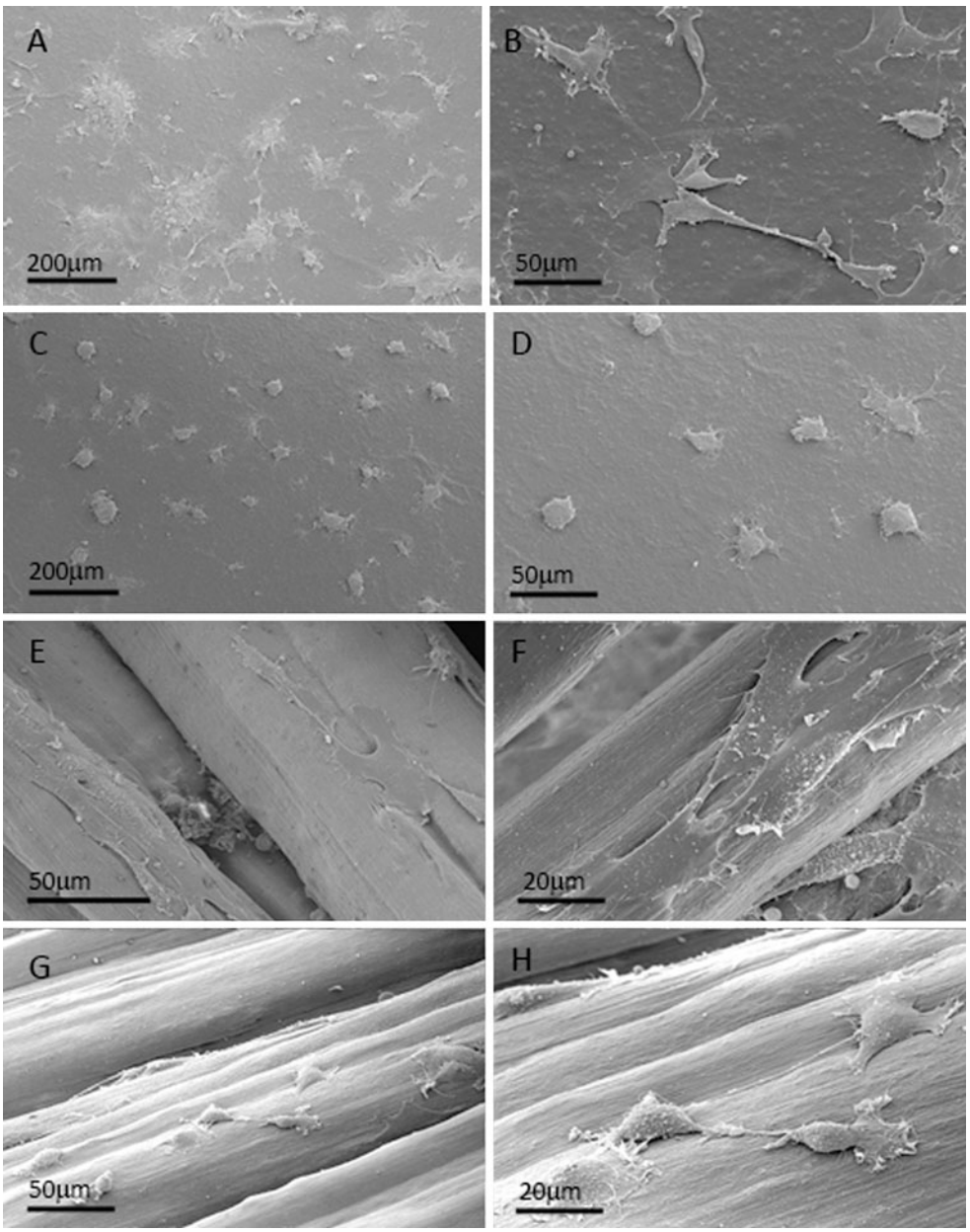

Fig. 6 The pictures show TFs cultivated for 24 h on EDC crosslinked films (**a**, **b**) and fibres (**e**, **f**) and on EDC/EGDE crosslinked films (**c**, **d**) and fibres (**g**, **h**). See text for description

method [19] or by the different crosslinking time [31]. In fact a longer crosslinking time can alter the surface biochemistry of the collagen removing chemotactic epitopes as more free primary amino and carboxyl groups react and thus impairing cell adhesion. The adhesion data at day 1 matched the results of SEM microscopy in that EDC and EDC/EGDE fibres showed the same level of cellularity. However, the SEM at day 1 revealed a noticeable difference in TFs adhesion pattern between EDC and EDC/EGDE crosslinked collagen. The roundness of TFs on EDC/EGDE may indicate contact toxicity and explain the decreased cell number on EDC/EGDE fibres at later time points. SEM confirmed this decreased cellularity, although TFs assumed a more elongated shape by day 14 (Fig. 7c,d, g, h) with respect to earlier time points (Fig 6c, d, g, h).

We assume that the cell number on the EDC/EGDE bundles may have decreased due to tardive cell detachment or death, as it may be hypothesised that the EGDE crosslinker will react with more nucleophilic groups and that any residual crosslinker will have a higher toxicity for the EDC/EGDE crosslinking method compared to the EDC method [31, 33, 34]. On the other side we exclude that the

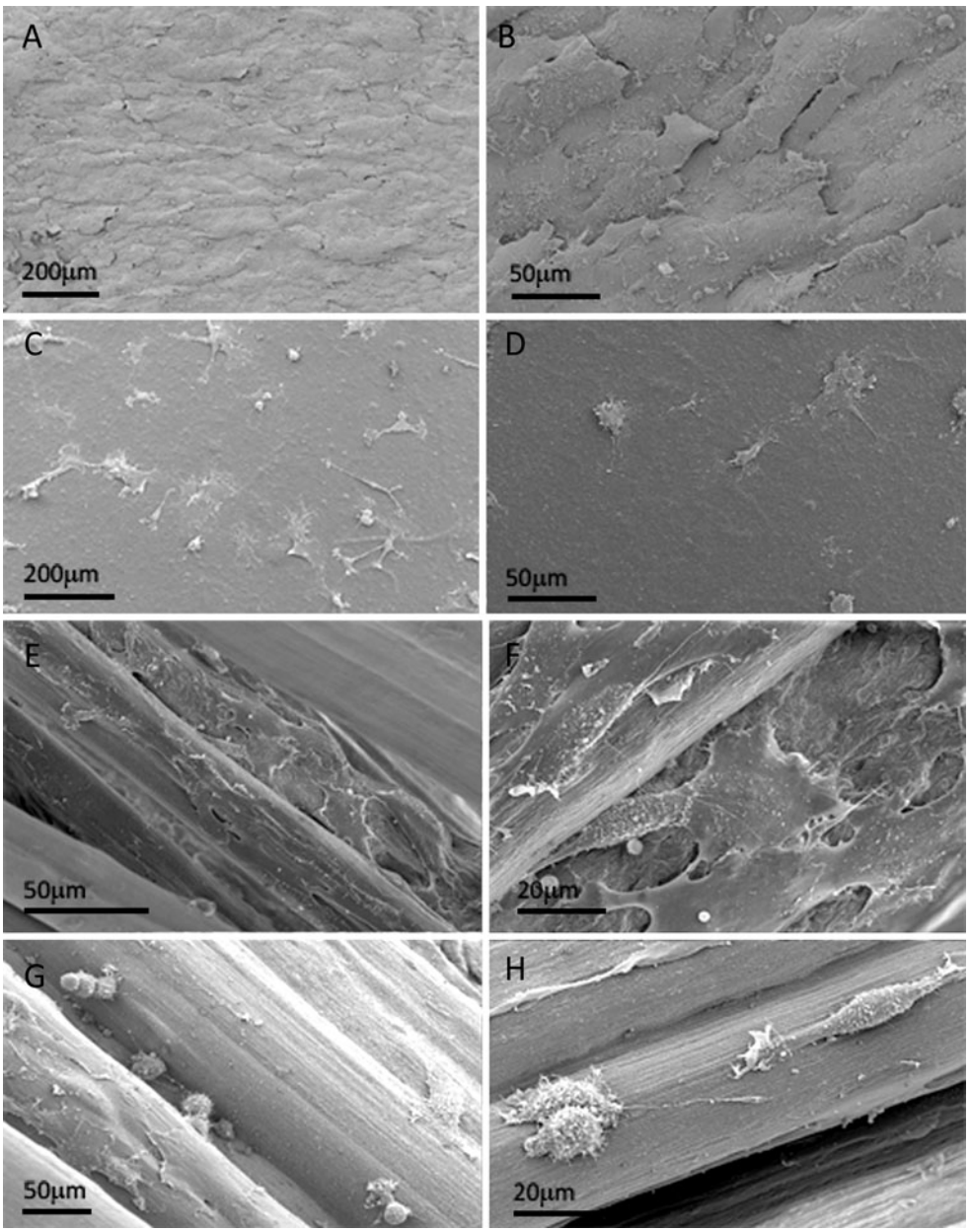

Fig. 7 The pictures show TFs cultivated for 14 days on EDC crosslinked films (**a**, **b**) and fibres (**e**, **f**) and on EDC/EGDE crosslinked films (**c**, **d**) and fibres (**g**, **h**). See text for description

EDC/EGDE chemistry was particularly toxic for TFs because living cells were imaged on the fibres at day 14 (Fig. 7c, d, g, h).

There is no specific marker to characterise the fully differentiated TF, therefore we used a panel of markers to increase the specificity of this cell type detection [35]. In addition to collagen type I, which is the main marker for TFs we looked at tenascin-C which is a glycoprotein involved in fibrillogenesis [36]. In the present study collagen type I and tenascin-C production in the medium were comparable between EDC bundles and controls, and were much inferior for the EDC/EGDE bundles. Overall, the production of collagen type I and tenascin-C in the medium was time-dependent and related to the actual number of cells on the respective bundles.

TFs seeded on EDC bundles were found to be positive for collagen type I and tenascin-C immuno-staining. On the other hand the low number of TFs attached on EDC/EGDE bundles demonstrated almost no positivity for the same staining procedures. These results matched the immuno-blotting of the culture media for the same proteins and indicate that TF matrix protein production is maintained on EDC bundles.

The different crosslinking chemistries adopted in this study critically affected the properties of extruded collagen

fibres. Even though EDC crosslinked fibres showed better biocompatibility than EDC/EGDE crosslinked fibres at 14 days follow up, the highly improved mechanical properties of the latter group make them a viable option for tendon and ligament regeneration purposes. It is conceivable that only the in vivo application of these differently crosslinked fibres will be able to elucidate which feature, mechanical strength or biocompatibility, will be the most effective for tissue repair.

An in vivo animal trial is currently under way, in which a fibrous implant (either crosslinked with EDC or EDC/EGDE chemistry) has been adopted to augment a defect created in the central third of sheep patellar tendon.

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