

THE RESPONSE OF TENOCYTES TO COMMERCIAL SCAFFOLDS USED FOR ROTATOR CUFF REPAIR

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Abstract

Surgical repairs of rotator cuff tears have high re-tear rates and many scaffolds have been developed to augment the repair. Understanding the interaction between patients' cells and scaffolds is important for improving scaffold performance and tendon healing.

In this *in vitro* study, we investigated the response of patient-derived tenocytes to eight commercial scaffolds. Tested scaffolds included X-Repair, Poly-Tape, LARS Ligament, BioFiber (synthetic scaffolds), BioFiber-CM (biosynthetic scaffold), GraftJacket, Permacol, and Conexa (biological scaffolds). Cell attachment, proliferation, gene expression, and morphology were assessed.

After one day, more cells attached to synthetic scaffolds with dense, fine and aligned fibres (X-Repair and Poly-Tape). Despite low initial cell attachment, the human dermal scaffold (GraftJacket) promoted the greatest proliferation of cells over 13 days. Expression of collagen types I and III were upregulated in cells grown on non-cross-linked porcine dermis (Conexa). Interestingly, the ratio of collagen I to collagen III mRNA was lower on all dermal scaffolds compared to synthetic and biosynthetic scaffolds.

These findings demonstrate significant differences in the response of patient-derived tendon cells to scaffolds that are routinely used for rotator cuff surgery. Synthetic scaffolds promoted increased cell adhesion and a tendon-like cellular phenotype, while biological scaffolds promoted cell proliferation and expression of collagen genes. However, no single scaffold was superior. Our results may help understand the way that patients' cells interact with scaffolds and guide the development of new scaffolds in the future.

Keywords: Rotator cuff tears, scaffolds, materials, tenocytes, polymers, extracellular matrix, *in vitro*.

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Introduction

Shoulder pain is the third commonest cause of musculoskeletal pain in society, and 1 % of all adults see their doctor for new shoulder pain every year (Urwin *et al.*, 1998). In the UK, 14 % of the population report shoulder problems and rotator cuff problems are the most common cause of pain (Urwin *et al.*, 1998; Chaudhury *et al.*, 2010). Between 5 % (Neer, 1983) and 39 % (DePalma *et al.*, 1949) of the general population have rotator cuff tears, and prevalence is expected to increase as the population ages (Yamamoto *et al.*, 2010). Large and massive rotator cuff tears (tear size ≥ 3 cm) (Post *et al.*, 1983) do not heal spontaneously and often require surgical intervention (Cornwell *et al.*, 2009). Approximately 10,000 rotator cuff surgeries are performed in the UK every year (Judge *et al.*, 2014), in addition to the 75,000-300,000 surgeries performed annually in the United States (Aurora *et al.*, 2007; Vitale *et al.*, 2007; Marx *et al.*, 2009). However, many surgical repairs fail, with re-tear rates reported between 11 % (Lafosse *et al.*, 2007) and 94 % (Galatz *et al.*, 2004). Patients who re-tear have reduced functional outcome (Sugaya *et al.*, 2007; Gerber *et al.*, 2000) and fewer treatment options available. In light of this, scaffolds have become a popular method of stabilising surgical repairs.

A variety of scaffolds are now commercially available for the augmentation of rotator cuff reattachments (Table 1). These can be biological, synthetic or a combination thereof. Biological scaffolds are allografts or xenografts derived from decellularised mammalian tissue. Biological scaffolds are expected to retain biological (Bissell and Aggeler, 1987; Voytik-Harbin *et al.*, 1997) and structural (Bissell and Barcellos-Hoff, 1987; Badylak, 2007) cues that instruct cell migration (Reing *et al.*, 2009), proliferation (Ling *et al.*, 1990) and phenotype (Gong *et al.*, 2008) to promote repair (Derwin *et al.*, 2010; Zantop *et al.*, 2006). Popular biological scaffolds include GraftJacket (Wright Medical, Arlington, TN, USA), Permacol (Tissue Science Laboratories, Covington, GA, USA) – also marketed as Zimmer Collagen Repair Patch (Zimmer, Warsaw, IN, USA), TissueMend (Stryker Orthopedics, Mahwah, NJ, USA) and Conexa (Tornier, Inc., Edina, MN, USA).

Synthetic scaffolds are mostly made of polyester polymers, and examples include X-Repair (Synthasome, San Diego, CA, USA), Poly-Tape (Neoligaments, Leeds, UK), BioFiber (Tornier, Inc., Edina, MN, USA) and LARS Ligament (LARS, Arc-sur-Tille, Burgundy, France).

Table 1. Commercial scaffold information. Scaffold name, manufacturer, industrial supplier, source material, scaffold type, cross-linked, and terminally sterilised.

Scaffold Name	Manufacturer	Industrial Supplier	Source	Scaffold Type	Cross-Linked	Terminally Sterilised
X-Repair	Synthesome (San Diego, CA, USA)	Synthesome (San Diego, CA, USA)	Poly- L-lactic-acid	Synthetic	N	Y
LARS Ligament	LARS (Arc-sur-Tille, Burgundy, France)	LARS (Arc-sur-Tille, Burgundy, France)	Polyethylene Terephthalate	Synthetic	N	Y
Poly-Tape	Xiros plc, Neoligaments (Leeds, UK)	Neoligaments (Leeds, UK)	Polyethylene Terephthalate	Synthetic	N	Y
BioFiber	Tornier (Edina, MN, USA)	Tornier (Edina, MN, USA)	Poly(4-hydroxybutyrate)	Synthetic	N	Y
BioFiber-CM	Tornier (Edina, MN, USA)	Tornier (Edina, MN, USA)	Poly(4-hydroxybutyrate) + Bovine Collagen	Biosynthetic	N	Y
GraftJacket	LifeCell (Branchburg, NJ, USA)	Wright Medical Technology (Arlington, TN, USA)	Human Dermis	Biological	N	N
Permacol	Tissue Science Laboratories (Aldershot, Hampshire, UK)	Zimmer (Warsaw, IN, USA)	Porcine Dermis	Biological	Y	Y
Conexa	Tornier (Edina, MN)	Tornier (Edina, MN)	Porcine Dermis	Biological	N	Y

Synthetic scaffolds permit the customisation of specific mechanical (Zhang *et al.*, 2012) and chemical properties (Chen *et al.*, 2009) that can stabilise the repair (Derwin *et al.*, 2009). Recently, biosynthetic scaffolds have emerged, an example of which is BioFiber-CM (Tornier, Inc., Edina, MN, USA) which is a woven synthetic scaffold coated with bovine collagen.

Pre-clinical studies have reported favourable results using both biological (Cook *et al.*, 2008; Shea *et al.*, 2010) and synthetic scaffolds (Derwin *et al.*, 2009; McCarron *et al.*, 2010). However, results have not always been replicated in humans and several clinical studies have reported adverse reactions (Malcarney *et al.*, 2005; Phipatanakul and Petersen, 2009) and increased failure rates (Iannotti *et al.*, 2006; Sclamberg *et al.*, 2004) following scaffold use. This discrepancy illustrates the inadequacy of existing pre-clinical models to replicate human pathologies. Many studies artificially lacerate tendons in healthy animals (Dejardin *et al.*, 2001; Zalavras *et al.*, 2006) or use healthy tendon cells *in vitro* (Cook *et al.*, 2008; Shea *et al.*, 2010). Previous studies have demonstrated differences in tenocytes derived from healthy tissue compared to those derived from diseased tissue: specifically, differences in the expression of collagen (Maffulli *et al.*, 2000), inflammatory mediators (Dakin *et al.*, 2015) and apoptotic markers (Millar *et al.*, 2012) have been reported. Many previous studies that have investigated the response of tenocytes to biomaterials have overlooked this distinction.

Thus, the precise manner by which patients' cells interact with these scaffolds remains unclear. In addition, the large number of scaffolds and variety of experimental paradigms used to test them makes it difficult to compare different scaffolds.

Therefore, the purpose of this *in vitro* study was to compare the response of tenocytes from rotator cuff tear patients to 8 commercially available scaffolds in terms of initial cell attachment, cell proliferation, final cell number, gene expression, and cell morphology. We hypothesised that there would be significant differences in the parameters assessed between the different scaffolds tested.

Materials and Methods

Scaffold selection

Scaffolds were selected based on presence in the literature and commercial availability (Table 1). Biological, biosynthetic, and synthetic scaffolds were selected to represent a cross-section of the spectrum of available scaffolds.

Human tendon tissue acquisition

Biopsies of torn supraspinatus tendons were taken from three male patients aged 40–65 undergoing surgical repair for large or massive rotator cuff tears. Tendon biopsies were collected with informed donor consent under ethics from the Oxford Musculoskeletal Biobank (09/H0606/11) in compliance with the National and Institutional ethical requirements. During surgery, biopsies were immediately transferred into sterile tubes containing DMEM F12 media (Lonza, Slough, UK) and were then used for cell isolation.

Tenocyte cell isolation and culture

Tenocytes were extracted by cutting the tissue biopsies into small pieces, approximately 2 × 2 mm square and transferred to 6-well plates (Corning Inc., Corning, NY, USA). Cells were cultured in supplemented media: DMEM F12 media (Lonza, Slough, UK), 10 % foetal bovine serum (Labtech, Uckfield, UK), and 1 % Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Supplemented media was replaced every 3 d. Once cells had reached confluence, they were scraped and transferred to 10 cm Petri dishes (Greiner, Frickenhausen, Germany). Cells from each patient were used separately for each experiment and were used in passage 3 to avoid phenotypic drift experienced at higher passages (Yao *et al.*, 2006).

Scaffold preparation and tissue culture

Scaffolds were prepared according to manufacturer's instructions, then conditioned in supplemented media for 24 h prior to cell seeding in static culture. Cells were

seeded at a density of 100,000 cells/cm² for all experiments, except for cell morphology, where cells were seeded a density of 10,000 cells/cm². After cell seeding, all scaffolds were transferred to new well plates. Scaffolds were then transferred to new well plates with fresh media every 3 d such that only cells attached to the scaffolds were accounted for. Unless stated otherwise, tissue culture plastic (TCP) was used as a positive control and unseeded scaffolds were used as negative controls.

Environmental scanning electron microscopy (ESEM)

A 0.25 cm² piece of each scaffold was rinsed in phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) for 10 min then fixed in 2.5 % glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Scaffolds were rinsed in deionised water before undergoing a graded ethanol series (Sigma-Aldrich) (40, 70, 90, 95 and 100 % each for 10 min). Next, 100 µL of hexamethyldisilazane (Alfa Aesar, Ward Hill, MA, USA) was added to each scaffold for 24 h. Scaffolds were gold coated using a SC7620 Mini Sputter Coater System (Quorum Technologies Ltd, Laughton, UK) and images were taken at 50 × magnification using an Evo LS15 Variable Pressure Scanning Electron Microscope (Carl Zeiss AG, Oberkochen, Germany). The diameter of individual fibres was determined using ImageJ (National Institute of Health, Bethesda, MD, USA).

Initial cell attachment and final cell number

Six 1 cm² samples of each scaffold were seeded with 100,000 cells in a 24-well plate (Corning Inc., Corning, NY, USA). Three scaffolds were processed on day 1 to measure initial cell attachment. On day 13, the remaining scaffolds were processed to assess the final cell number.

For processing, scaffolds were rinsed twice in PBS (Sigma-Aldrich) then suspended in 350 µL of 0.2 % Nonidet P40 (Fluka Biochemika, Buchs, Switzerland). Scaffolds were freeze-thawed three times and sonicated twice for 10 s using an ultrasonic cell disruptor (Misonix, Farmingdale, NY, USA). The double stranded DNA (dsDNA) content of each well was quantified using the Quant-iT PicoGreen assay (Invitrogen, Paisley, UK) according to manufacturer's instructions.

Cell proliferation

Six 1 cm² samples of each scaffold were seeded with 100,000 cells and cultured in 24-well plates (Corning). A calibration curve was generated by seeding cells at the following densities in a 48-well plate (Corning): $5 \times 10^3/\text{cm}^2$, $1 \times 10^4/\text{cm}^2$, $5 \times 10^4/\text{cm}^2$ and $1 \times 10^5/\text{cm}^2$. This was used to approximate cell number throughout the experiment. Cell proliferation on scaffolds was quantified using AlamarBlue Assay, which has been validated previously (Ahmed *et al.*, 1994; Voytik-Harbin *et al.*, 1998). Every three days, scaffolds were incubated in 5 % AlamarBlue (Invitrogen) in supplemented media for 2 h. Fluorescence was measured at 485 nm excitation and 520 nm emission using a FLUOstar optima microplate reader (BMG Labtech, Ortenberg, Germany).

Gene expression

Three 1 cm² samples of each scaffold were seeded with 100,000 cells in a 24-well plate (Corning). After 13 d, scaffolds were rinsed in PBS (Sigma-Aldrich) then fixed in 1 mL TRIzol (Invitrogen). The mRNA was extracted using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) and mRNA quality was checked using a Nanodrop ND1000 (Nanodrop Technologies, Willington, DE, USA) and all samples had 260/280 value greater than 1.6. The mRNA was then converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Final cDNA was diluted to 5 ng / µL and 1 µL was used in a 10 µL reaction with Fast SYBR Master Mix (Applied Biosystems) according to manufacturer's protocol. The RT-qPCR reaction was performed using a ViiA7 real-time PCR machine (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and TATA-binding protein (*TBP*) were used as endogenous reference genes and gene expression was calculated using the comparative CT method (Schmittgen and Livak, 2008). QuantiTect primers for *COL1A1*, *COL3A1*, *GAPDH*, *COL2A1*, *COL6A1*, *TNC*, *DCN*, *SCX-B*, *TNMD*, *VCAN*, *ACAN* and *TBP* were used in the RT-qPCR reaction (Qiagen, Hilden, Germany).

Cell morphology

Six 0.5 × 0.5 cm samples of each scaffold were seeded with 2.5×10^4 cells in a 48-well plate (Corning). Three samples of each scaffold were fixed in 10 % formalin (Fischer Scientific, Loughborough, UK) on days 1 and 13. Samples were incubated in 1 mL 0.5 % Triton-X (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min, then rinsed in PBS (Sigma-Aldrich). A 100 nM solution of acti-stain 488 phalloidin (Cytoskeleton Inc., Denver, CO, USA) and 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Waltham, MA, USA) was generated in PBS (Sigma-Aldrich) and 500 µL of the solution was added to each scaffold and incubated for 2 h in the dark at 4°C. Scaffolds were rinsed in PBS (Sigma-Aldrich) and images were captured at 40 × magnification using a Zeiss LSM710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis

A Kolmogorov-Smirnov test was performed for each assay to determine normality. Technical replicates were included for assays where the number of biological replicates was not sufficient to determine normality. Unless otherwise stated, a one-way ANOVA was performed and a Tukey *post-hoc* test was completed to compare all scaffolds tested. To compare the ratios of collagen I to collagen III expression between dermal and synthetic/biosynthetic scaffolds, a two-tailed unpaired *t*-test was performed. Statistical analysis was performed using GraphPad PRISM (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was considered at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. Statistical significance represents the minimum statistical differences between the designated scaffold and every other scaffold. If more than one scaffold is significantly different from the other

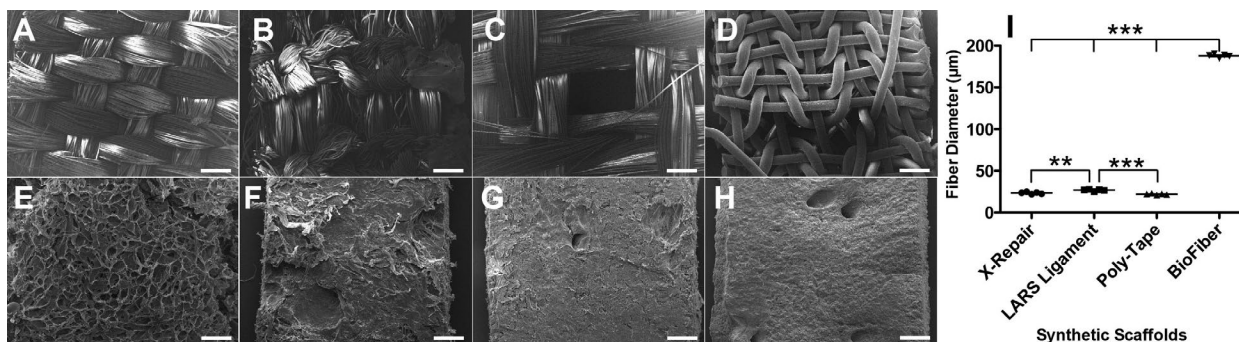


Fig 1. Scaffold morphology. ESEM micrographs showing the appearance of the scaffolds. X-Repair (A), LARS Ligament (B), Poly-Tape (C), BioFiber (D), BioFiber-CM (E), GraftJacket (F), Permacol (G), Conexa (H). 50 × magnification. Scale bar = 600 μm. The diameter of individual fibres of the synthetic scaffolds was measured from the ESEM images (I). Bar represents mean.

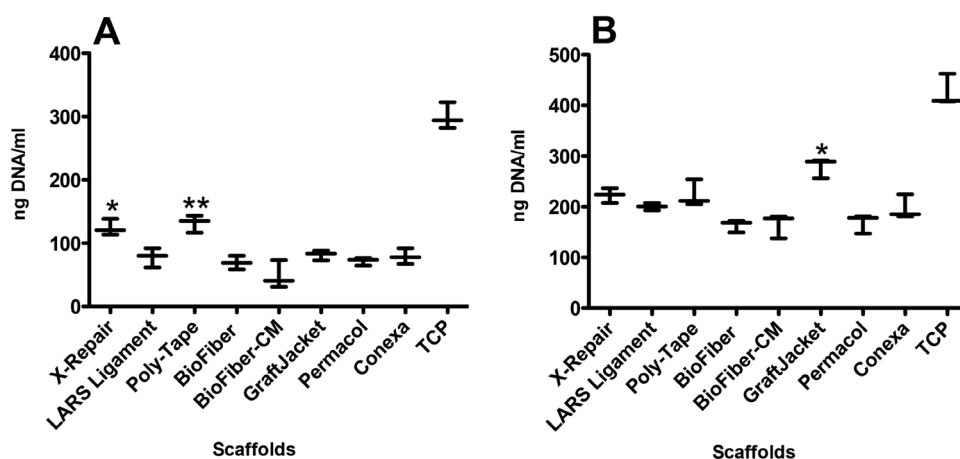


Fig 2. Amount of DNA extracted from cells on scaffolds on day 1 and day 13. DNA was quantified using PicoGreen and this was used to approximate the number of cells that attached to each scaffold on day 1 (A) and day 13 (B). On day 1, X-Repair had significantly more DNA than every other scaffold ($p \leq 0.05$) except Poly-Tape (A). This is shown in (A) as one asterisk above X-Repair. On day 1, Poly-Tape had significantly more DNA than every other scaffold ($p \leq 0.01$) except X-Repair (A). This is shown in (A) as two asterisks above Poly-Tape. On day 1, there was no statistical difference in DNA quantity between X-Repair and Poly-Tape (A). On day 13, GraftJacket had more DNA than every other scaffold ($p \leq 0.05$) (B). This is shown in (B) as one asterisk above GraftJacket. Graphs represent median with minimum and maximum ($n = 3$).

scaffolds, then there is no significant difference between the scaffolds that are different. For example, in Fig. 2A, X-Repair and Poly-Tape are significantly different from every other scaffold. However, there is no significant difference between X-Repair and Poly-Tape.

Results

Scaffold morphology

Synthetic scaffolds demonstrated different weave patterns (Fig. 1A–D) and fibre diameters (Fig. 1I). The average fibre diameter of BioFiber was larger than the three other synthetic scaffolds ($p \leq 0.001$) (Fig. 1I). The average fibre diameter of LARS Ligament was larger than X-Repair and Poly-Tape ($p \leq 0.01$ and $p \leq 0.001$ respectively) while the average fibre diameters of X-Repair and Poly-Tape were equivalent (Fig. 1I). X-Repair (Fig. 1A) and Poly-Tape (Fig. 1C) had plain weave patterns, with straight and perpendicular bundles of fibres. LARS ligament (Fig. 1B) had an array of straight fibres with the addition of twisted fibre bundles. BioFiber (Fig. 1D) had a lattice of parallel

fibres, interwoven with curved fibres. BioFiber (Fig. 1D) displayed thick fibres with high porosity, whereas BioFiber-CM (its bovine-collagen coated equivalent – Fig. 1E) appeared flat and dense. GraftJacket (Fig. 1F), the human dermal scaffold, appeared rougher than the two porcine dermal scaffolds of Permacol (Fig. 1G) and Conexa (Fig. 1H).

Cell attachment and proliferation

After 1 d, more cells attached to X-Repair ($p \leq 0.05$) and Poly-Tape ($p \leq 0.01$) than any other scaffold as measured by dsDNA content (Fig. 2A). All other scaffolds demonstrated similar amounts of dsDNA.

AlamarBlue was used to study the change in cell number (cell proliferation) on the scaffolds over 13 d. After 1 d, cell number/cm² was higher on X-Repair ($p \leq 0.05$) and Poly-Tape ($p \leq 0.05$) than any other scaffold (Fig. 3A). After 13 d, cell number/cm² was higher on GraftJacket ($p \leq 0.05$) than any other scaffold (Fig. 3A), which was confirmed measuring the total dsDNA (Fig. 2B). No statistical differences in cell number/cm² were observed between any other scaffolds (Fig. 3A).

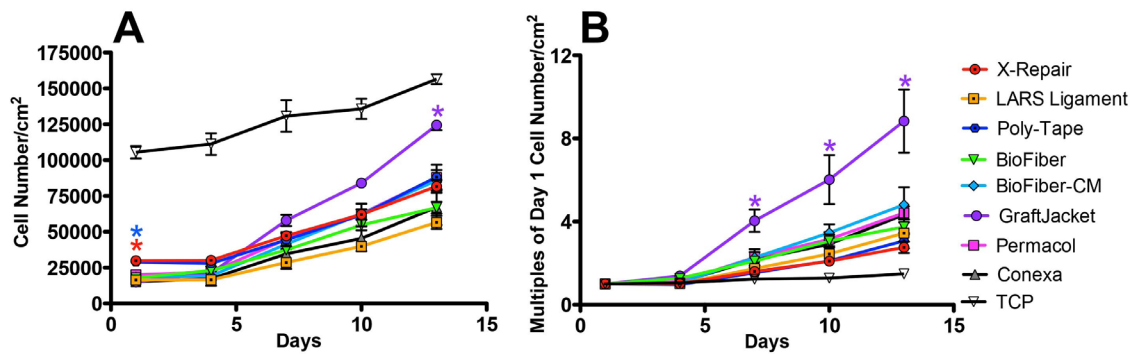


Fig 3. Proliferation of cells on scaffolds over 13 d. The absolute proliferation of cells on scaffolds was measured every 3 d for 13 d using AlamarBlue (A). On day 1, more cells were attached to X-Repair than every other scaffold ($p \leq 0.05$) except Poly-Tape (A). This is shown in (A) as one red asterisk above day 1. On day 1, more cells attached to Poly-Tape than every other scaffold ($p \leq 0.05$) except X-Repair (A). This is shown in (A) as one blue asterisk above day 1. On day 1, there was no statistical difference in cell number between X-Repair and Poly-Tape (A). On day 13, more cells attached to GraftJacket than every other scaffold ($p \leq 0.05$) (A). This is shown in (A) as one purple asterisk above day 13. Measurements were also standardised to day 1 to determine the relative proliferation of the cells that did attach (B). Of the cells that did attach, cells were significantly more proliferative on GraftJacket on day 7 ($p \leq 0.05$), day 10 ($p \leq 0.05$), and day 13 ($p \leq 0.05$) when compared to every other scaffold (B). These are shown in (B) as one purple asterisk above days 7, 10 and 13, respectively. Graphs represent median with minimum and maximum ($n = 3$).

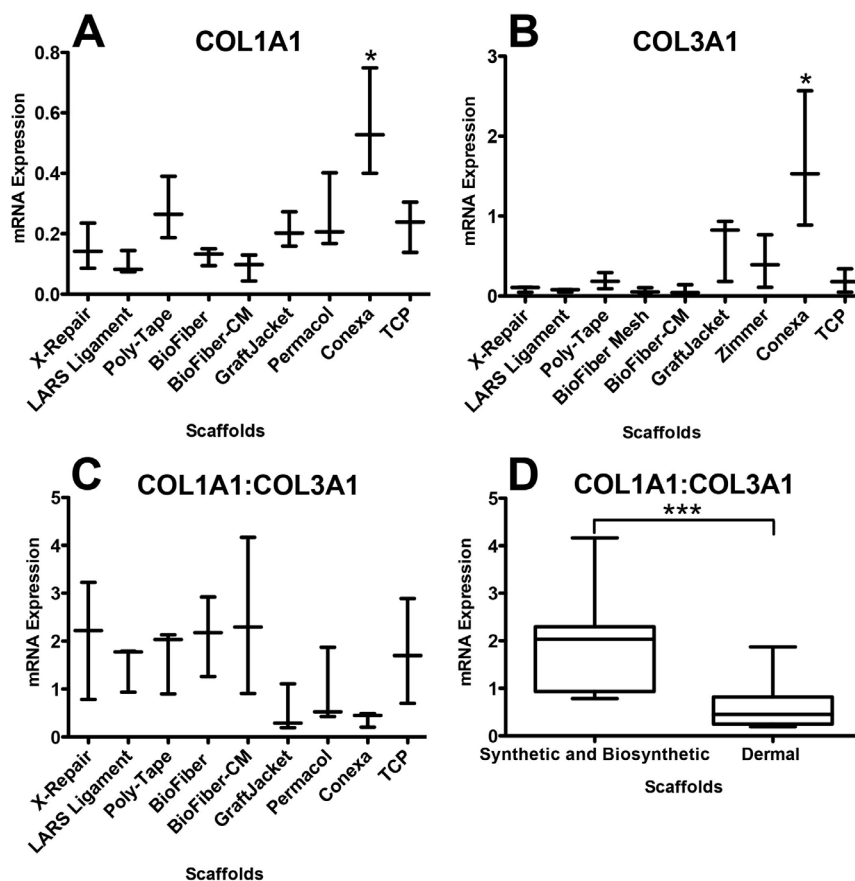


Fig 4. Day 13 mRNA expression. After 13 d, mRNA expression of *COL1A1* (A) and *COL3A1* (B) was calculated relative to *GAPDH*. After 13 d, *COL1A1* expression was greater on Conexa than every other scaffold ($p \leq 0.05$) (A). This is shown in (A) as one asterisk above Conexa. After 13 d, *COL3A1* was greater on Conexa than every other scaffold ($p \leq 0.05$) (B). This is shown in (B) as one asterisk above Conexa. The ratio of *COL1A1*:*COL3A1* mRNA after 13 d was also calculated (C). An unpaired *t*-test was performed to compare the ratio of *COL1A1*:*COL3A1* on synthetic and biosynthetic scaffolds, against dermal scaffolds (D). The ratio of *COL1A1*:*COL3A1* was higher on synthetic and biosynthetic scaffolds, compared to dermal scaffolds ($p = 0.0009$) (D). This is shown in (D) as three asterisks above line comparing the two groups. Graphs represent median with minimum and maximum ($n = 3$).

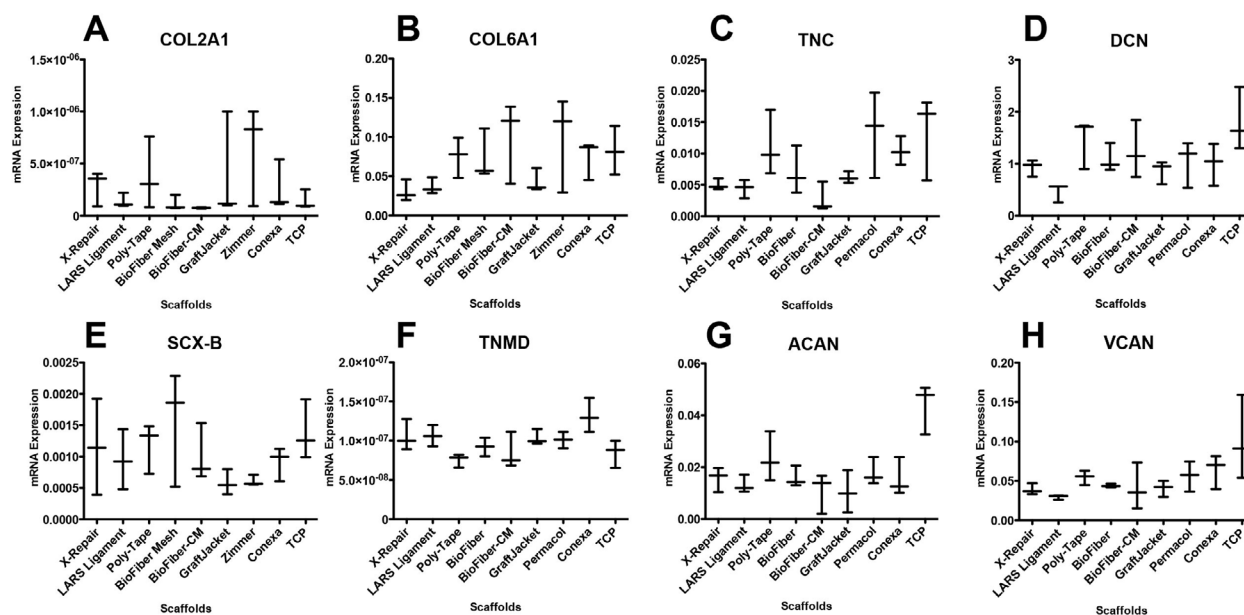


Fig 5. Day 13 mRNA expression. After 13 d, mRNA expression was calculated relative to *GAPDH*. *COL2A1* (A), *COL6A1* (B), *TNC* (C), *DCN* (D), *SCX-B* (E), *TNMD* (F), *ACAN* (G), *VCAN* (H). None of the scaffolds demonstrated significant differences for gene expression. Graphs represent median with minimum and maximum ($n = 3$).

Cell number *per cm*² for each scaffold was standardised to day 1 values to determine the relative proliferation of cells that attached to each scaffold (Fig. 3B). Cells that initially attached proliferated more on GraftJacket than any other scaffold after 7 d ($p \leq 0.05$), 10 d ($p \leq 0.05$) and 13 d ($p \leq 0.05$) (Fig. 3B). There were no statistically significant differences at any other time points.

Gene expression

After 13 d, mRNA expression of collagen types I and III were determined by RT-qPCR of cells on scaffolds. Expression of collagen types I (*COL1A1*) and III (*COL3A1*) were higher in cells on Conexa than all other scaffolds ($p \leq 0.05$) (Fig. 4A and 4B). The ratio of *COL1A1*:*COL3A1* mRNA expression was higher on synthetic and biosynthetic scaffolds (X-Repair, LARS Ligament, Poly-Tape, BioFiber, BioFiber-CM) than dermal scaffolds (GraftJacket, Permacol, Conexa) ($p = 0.0009$) (Fig. 4D). After 13 d, expression of *COL2A1*, *COL6A1*, *TNC*, *DCN*, *SCX-B*, *TNMD*, *VCAN* and *ACAN* were also assessed. No significant differences were seen between the scaffolds tested (Fig. 5)

Cell morphology

On day 13, cells on X-Repair, LARS ligament, and Poly-Tape looked like healthy native tenocytes, with extended lamellipodia aligning along the lengths of the scaffold fibres (Fig. 6B, D, F respectively). On day 13, cells on BioFiber aligned along fibres, but also exhibited filopodia that were transverse to fibre direction (Fig. 6H). In contrast, on day 13 cells on BioFiber-CM, GraftJacket, Permacol, and Conexa exhibited filopodia that extended in different directions (Fig. 6J, L, N, P respectively). After 13 d, cells on GraftJacket were smooth (Fig. 6L), while cells on Permacol (Fig. 6N) and Conexa (Fig. 6P) appeared dendritic and punctate.

Discussion

Rotator cuff tears are a prevalent pathology, and surgical repair is not always effective. Numerous scaffolds have been developed to augment the surgical repair and reduce re-tearing. However, little is known about how the degenerative tissue, and in particular the native cells, interact with these scaffolds. In this study, we compared the response of patient-derived rotator cuff tenocytes to 8 commercially available scaffolds. We show that X-Repair and Poly-Tape promoted the greatest cell attachment after 1 d and that GraftJacket promoted the greatest level of cell proliferation over 13 d. Expression of *COL1A1* and *COL3A1* was significantly upregulated in cells grown on Conexa after 13 d. In addition, the ratio of *COL1A1* to *COL3A1* mRNA was lower on all dermal scaffolds tested compared to synthetic and biosynthetic scaffolds.

For the implanted scaffold to be incorporated into the body, native tissue cells must attach to its surface and then migrate into the material. For adherent cells such as tenocytes, attachment is necessary for cell proliferation (Singhvi *et al.*, 1994) and survival (Folkman and Moscona, 1978; Ben-Ze'ev *et al.*, 1980). After 1 d, cell attachment was greatest on X-Repair and Poly-Tape, both synthetic scaffolds with dense arrays of aligned fibres with diameters of ~ 22 – $23 \mu\text{m}$. This result was unexpected as cells usually adhere more to hydrophilic surfaces and less to hydrophobic surfaces (Wei *et al.*, 2007; Webb *et al.*, 1998; Schakenraad *et al.*, 1986; Altankov *et al.*, 1996). X-Repair and Poly-Tape are composed of poly-L-lactic acid and polyethylene terephthalate respectively, both of which are hydrophobic (Van Wachem *et al.*, 1985; Hsieh and Cram, 1998). In contrast, GraftJacket, Permacol, and Conexa are derived from mammalian dermis which is hydrophilic (Gniadecka *et al.*, 1998). One plausible explanation is that synthetic micro-fibre scaffolds provide a large effective

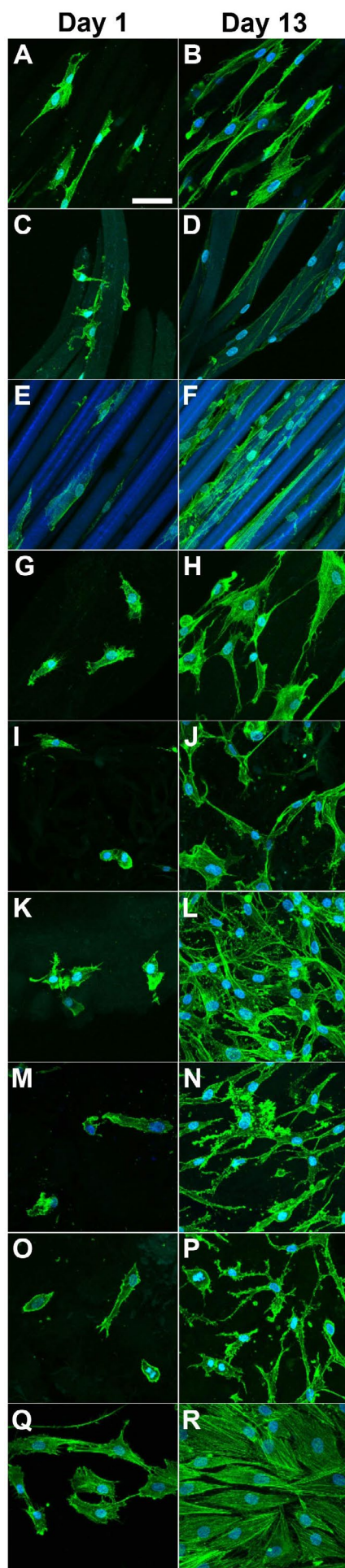


Fig 6. Fluorescent images of cell morphology on scaffolds. Cell morphology was assessed on days 1 and 13 using confocal microscopy. Cell cytoskeleton was stained with phalloidin (green) and nucleus was counterstained using DAPI (blue). Magnification 40 ×. Scale bar = 50 μm. X-Repair (A, B), LARS Ligament (C, D), Poly-Tape (E, F), BioFiber (G, H), BioFiber-CM (I, J), GraftJacket (K, L), Permacol (M, N), Conexa (O, P), TCP (Q, R).

surface area, allowing cells to attach above and within the scaffold. Previous studies have demonstrated increased cell attachment to biomaterials with increasing surface area (O'Brien *et al.*, 2005; Mo *et al.*, 2004; Murphy *et al.*, 2010). However, other material properties, such as elastic modulus, wettability, and roughness may also influence cell attachment.

Over 13 d, cell proliferation was greatest on GraftJacket, a minimally processed human dermis composed of collagen types I, III, IV and VII, elastin, proteoglycans and native growth factors, such as Fibroblast Growth Factor (FGF) (Coons and Barber, 2006). Many forms of FGF exist and their influence on cell proliferation has been well documented (Boilly *et al.*, 2000; Piotrowicz *et al.*, 1999). However, the mechanism behind the increase in cell proliferation on GraftJacket reported here remains to be investigated. While our study suggests that human cells proliferate more on human dermis than porcine dermis, it is unclear whether this response is due to difference in tissue source (human vs. porcine) or processing techniques used during manufacturing. GraftJacket is derived from human dermis that is not sterilised or cross-linked (Shea *et al.*, 2010) and is classified as human tissue for transplantation (Longo *et al.*, 2010) under the FDA Code of Federal regulations Title 21 Part 1270 (Ricchetti *et al.*, 2012). In contrast, Permacol is derived from porcine dermis and undergoes extensive mechanical and enzymatic processing (Longo *et al.*, 2010; Kimuli *et al.*, 2004) and is chemically cross-linked (Derwin *et al.*, 2006). Conexa is also derived from porcine dermis and is processed to minimise immunological reactions, but is not cross-linked (Ricchetti *et al.*, 2012). The proprietary nature of these processing techniques makes it difficult to determine their precise impact on the ECM and the subsequent cellular response. While several studies have investigated the effect of these processing techniques (Reing *et al.*, 2010; Brown *et al.*, 2011) on cellular response, only a small number of techniques were studied and therefore future investigations are required. It is noteworthy that the topography and structure of the scaffolds may have influenced the rate of cell proliferation. Previous studies suggest that cell proliferation varies between two-dimensional and three-dimensional scaffolds (Bott *et al.*, 2010; Cukierman *et al.*, 2001). Our presented data support this, with clear differences in cell proliferation on the three-dimensional scaffolds compared to the cells on two-dimensional TCP.

Despite increased cell attachment on X-Repair and Poly-Tape, there was no corresponding significant increase in cell proliferation over 13 d. This result is supported by previous studies, which have shown that surface area is correlated with increased cell attachment (O'Brien *et al.*, 2005; Mo *et al.*, 2004; Murphy *et al.*, 2010) but not

necessarily with increased cellular proliferation (Murphy *et al.*, 2010).

After 13 d, expression of *COL1A1* and *COL3A1* were significantly upregulated in cells on Conexa. Cellular production of new extracellular matrix proteins is critical for healing and neo-tissue formation. Collagen type I is the fundamental component of tendon (Amiel *et al.*, 1984; von der Mark, 1981) and affords the tendon with high strength (Jozsa *et al.*, 1984). In contrast, collagen III is associated with tendon healing and fibrosis (Leask and Abraham, 2004; Gurtner *et al.*, 2008) and is mechanically inferior to collagen I (Maffulli *et al.*, 2000). While no individual scaffold demonstrated a statistically different ratio of *COL1A1:COL3A1*, there was a clear difference between dermal scaffolds (GraftJacket, Permacol, Conexa) in comparison to biosynthetic (BioFiber-CM) and synthetic scaffolds (X-Repair, LARS Ligament, Poly-Tape, BioFiber). When stratified into separate groups, dermal scaffolds demonstrated a lower *COL1A1:COL3A1* ratio than biosynthetic and synthetic scaffolds. Lower ratios of collagen I to collagen III results in thinner collagen fibres (Dale *et al.*, 1996) and reduced tissue stability (Birk and Mayne, 1997; Klinge *et al.*, 2000). Decreased ratios of collagen I to collagen III expression is seen during skin scarring (Klinge *et al.*, 2000) and it is interesting to consider the possibility that residual physical and chemical cues in the dermal scaffolds promote this behaviour.

Our results contrast with a previous *in vitro* study by Shea *et al.* (2010) that found significantly increased collagen I mRNA expression on GraftJacket and Conexa, while demonstrating increased collagen III mRNA expression on GraftJacket, Conexa and Permacol. These differences may be due to different sampling time points or the use of different cell types; we utilised cells from rotator cuff tear patients, while Shea *et al.* used tenocytes derived from healthy tendons that were discarded during orthopaedic procedures (Shea *et al.*, 2010). Indeed, underlying changes in the tissue precede rotator cuff tears (Kannus and Jozsa, 1991). Therefore, the phenotype of cells within diseased tissues is likely different. The differential response between healthy and patient-derived cells may have important implications when evaluating the cellular response to biomaterials *in vitro*.

We observed dramatic differences in cell morphology on different scaffolds. After 1 d, cells on all scaffolds appeared rounded in comparison to tissue culture plastic (TCP), where cells were flattened. In contrast, after 13 d cell morphology differed markedly between the scaffold types tested. Cells on synthetic scaffolds aligned by following the orientation of the fibres, while cells on biosynthetic or dermal scaffolds were more isotropic and extended in numerous different directions, similar to TCP. We observed distinct differences in cell morphology between the dermal scaffolds; cells on human dermal scaffolds appeared smooth and interconnected, whereas cells on porcine dermal scaffolds appeared dendritic and punctate. Previous work suggests that the morphology of fibroblast-like cells transition from dendritic to smooth when the substrate they are on is sufficiently stiff (Tamariz and Grinnell, 2002).

The results from this study suggest that the addition of collagen to BioFiber to make BioFiber-CM did not significantly affect cell adhesion, proliferation or gene expression *in vitro*. Conflicting results exist in the literature, with some studies reporting improved cellular response with collagen-coated biomaterials (Nagai *et al.*, 2002; Röhlecke *et al.*, 2001) and some studies reporting no differences (Becker *et al.*, 2002; van den Dolder *et al.*, 2003). In tendon, tenocytes are organised into rows of connected cells within a pericellular matrix (Thakkar *et al.*, 2014) and their behaviour is greatly influenced by their connectivity with other cells (Schulze-Tanzil *et al.*, 2004). Examination of cell morphology after 13 d revealed a sparse distribution of cells which did not form the connected networks like those seen on BioFiber (Fig. 5H). This is rather surprising, and merits further investigation, and especially a comparison to an *in vivo* system. It may simply be that the deposited collagen did not form a pattern conducive to the formation of aligned cell arrays, or there may be another variable in the process of coating which may have affected the response of the cells to the surface.

There is increasing evidence that inflammation, and the cells that mediate it, are important in the pathogenesis and healing of tendon tears (Sharma and Maffulli, 2005; Millar *et al.*, 2010; Dakin *et al.*, 2015). Following surgical implantation, scaffolds will come into contact with a range of inflammatory cells, including monocytes, neutrophils, and macrophages (Anderson *et al.*, 2008; Anderson, 2001). Several studies have shown significant differences in the response of these inflammatory cells to materials used for hernia repair (Orenstein *et al.*, 2010; Grotenhuis *et al.*, 2013) and dental implants (Refai *et al.*, 2004; Stanford, 2010). However, the response of inflammatory cells to scaffold materials used for rotator cuff repair has not been thoroughly studied and warrants further investigation.

The main strengths of our work include comparing a large number of scaffolds and utilising patient-derived cells from diseased tissue. There are several limitations of our work, which must be considered. Firstly, we used cells from a small number of patients ($n = 3$) and the response of cells from other patients may differ. Secondly, we only studied one cell type, and it is likely that the interaction between native tenocytes and other cell types, such as macrophages, determine the patient's response to a scaffold. Finally, we performed the study in static tissue culture and were therefore unable to replicate the complex mechanical loading profile that the scaffolds would experience *in vivo*. These factors must be considered and as such, the relevance of our *in vitro* findings to clinical performance is limited.

Conclusion

Overall, this study suggests that synthetic scaffolds with large effective surface areas promoted increased cell adhesion, minimally processed human dermis promote cellular proliferation, and non-cross-linked porcine dermis promotes the expression of collagen types I and III in patient-derived tenocytes. By revealing potential advantages and disadvantages of each scaffold type, our

data may help determine which scaffold is most appropriate for augmenting surgical rotator cuff repairs.

Future work in the field should use cells from unhealthy tissue to better understand the native biological mechanisms involved in rotator cuff repair and how scaffolds can be used to enhance these processes. Also, well-designed randomised control clinical trials comparing different scaffolds will help surgeons determine the most appropriate type of scaffold for clinical use. The influence that industrial processing techniques and collagen coating have on the cellular response to biomaterials warrants further investigation.

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Discussion with Reviewers

Matthew Fisher: The severity of rotator cuff injury can vary wildly. Do the authors think the best scaffold is dependent on the severity of injury?

Authors: We agree that there is a broad spectrum of rotator cuff pathology. Previous work in our group has revealed distinct differences in patient groups when stratified by attributes such as tear size, levels of inflammation, and pain. Therefore, it is likely that the type of scaffold that is most appropriate for each patient will vary depending upon a range of different factors. Currently, scaffolds are reserved for patients with large or massive rotator cuff tears. This is because these patients are at much greater risk of re-tearing following a standard surgical repair and would therefore benefit from a scaffold-augmented repair. This is why we only used cells from patients with large or massive rotator cuff tears *i.e.* tear size ≥ 3 cm.

Matthew Fisher: Upon *in vivo* implantation, will the injured rotator cuff cells be the ones to repopulate the graft? What about other cell types?

Authors: Immediately following implantation, many inflammatory cells will invade the repair site. We believe that the way that these inflammatory cells interact with the implanted scaffold, and subsequently influence the healing response, are important aspects to understand. As such, this is the topic of an on-going research project. In terms of cell types repopulating the graft, most of the inflammatory cells are transient. This is because the healing tendon transitions from the acute inflammatory stage to the proliferative stage of repair after 1-7 d. We know that many other cell types have been detected around a healing tendon such as synovocytes, chondrocytes, and osteocytes, but the vast majority of cells in tendon tissue are resident tenocytes. Tenocytes are the main cell population responsible for maintaining and repairing tendon tissue. As such, we believe that tenocytes will be the main cell type that will repopulate the graft. While the identity of the healing cells in tendon is subject of much debate, we believe that tendon-derived proliferative cells play an important role in tendon healing. These cells are derived directly from human supraspinatus tendon tissue biopsies and are the cells that will directly interact with the scaffold following surgical implantation *in vivo*.

Gundula Gesine Schlulze-Tanzil: Only cells attached to the scaffold were accounted for. Were the detached cells also calculated separately to get an impression of the whole cell number?

Authors: AlamarBlue assays were performed on the old tissue culture plates after scaffolds were transferred out of them, to explore whether cells were detaching at a different rate from different scaffolds. However, after day 1, very few cells detached from any of the scaffolds and no differences between scaffolds were detected.

Gundula Gesine Schlulze-Tanzil: The AlamarBlue assay usually assesses only metabolic activity, please explain how the cell proliferation was calculated from the extinction shift produced by metabolised AlamarBlue.

Authors: AlamarBlue is an assay that measures the metabolic activity of cells. In order to calculate cell number from this, we had to assume that metabolism of the cells from each patient were approximately the same on the different scaffolds. We created a calibration curve for each patient using different cell concentrations. From this, we were able to convert each AlamarBlue fluorescence reading to an approximate cell number. We used the PicoGreen assay to validate this and confirm that changes were in fact due to more cells and not due to a change in cell metabolic activity on a particular material. Our PicoGreen results support our AlamarBlue results.

Gundula Gesine Schlulze-Tanzil: Cell adherence was measured in the study, but how about the cell penetration into the scaffold? It is important for the formation of new functional tissue.

Authors: We tried to analyse this, but with the porous synthetic scaffolds, cells were present on every side of the scaffold following cell-seeding. Therefore, it was impossible to determine whether the cells migrated there or whether they just adhered there following cell seeding. Because we were unable to make a fair comparison across all 8 scaffolds, we decided not to include these data.

Gundula Gesine Schlulze-Tanzil: Was a degradation of the biomaterials by cells observed?

Authors: In the preliminary study, SEM analysis of cells on scaffolds was performed and after 28 d in culture, no significant degradation of any of the scaffolds was observed. However, this was a crude evaluation, and it can be assumed that some degradation has occurred either through hydrolysis or enzymatic degradation from cell-secreted enzymes. We previously analysed collagen protein expression using an ELISA for collagen type 1, but decided to omit the results because we were unable to account for the enzymatic degradation of the collagen-based biological scaffolds.

Editor's Note: Scientific Editor in charge of the paper: Juerg Gasser.