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Engineering of a functional tendon using collagen as a natural polymer

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Abstract

Reconstruction of a tendon rupture is surgically challenging as each end of the tendon retracts leaving a substantial gap and direct repair is often not feasible. Hence, to restore function a tendon graft, it is required to bridge this defect. Presently, these gaps are filled with auto-, allo- or, synthetic grafts but they all have clinical limitations. To address this issue, tissue engineered grafts were developed by a rapid process using compressed type I collagen, which is the most dominant protein in the tendon. However, bio-mechanical properties were found to be unsuitable to withstand complete load bearing in vivo. Hence, a modified suture technique was previously developed to reduce the load on the engineered collagen graft to aid integration *in vivo*. By using this technique, engineered collagen grafts were tested *in vivo* on a lapine model in three groups up to 12 weeks without immobilisation. Gross observation at 3 and 12 weeks showed the bridged integration without adhesions with a significant increase in the mechanical, structural and histological properties as compared to 1 week. Insertion of tissue engineered collagen graft, using a novel load bearing suture technique which partially loads in vivo showed integration, greater mechanical strength and no adhesion formation in the time period tested. This collagen graft has inherent advantages as compared to the present-day tendon grafts.

1. Introduction

Tendon is a fibrous connective tissue which is anatomically arbitrated between muscle and bone to withstand physiological loading during muscle contraction and provide stability to the body. [1] Tendons have the property to alter their physical and mechanical function by tissue mechanical adaption mechanism in response to applied physiological and mechanical stimuli. [2-4]

In the event of tendon rupture, it is not always feasible to perform direct repair because tendons are usually under physiological tension and the following rupture, tendon ends can retract leaving a substantial gap to bridge. Current strategies for tendon repair are auto, allo and xenografts but these strategies have inherent weaknesses and clinical complications such as: autografts lead to donor site morbidity and have limited availability, allografts may elicit an immune response and are expensive, xenografts match mechanically to the human tendon but they carry a risk of an immune rejection. [5]

Tissue engineering is based on the nature-inspired design of tissue constructs that can perform native tissue's function. In the last 30 years, advances in material science have shown that using artificial scaffolds made up of the natural and synthetic polymer can mimic the architecture of native tissue and over time, can replace damaged tissues/organs. [6-8] In this study, we have fabricated tendon graft using collagen, which is a naturally occurring polymer and is highly conserved across species. It is the predominant protein in tendon and therefore, using collagen as a biomaterial for tendon grafts is a rational advance.[9] [10] In this study, collagen grafts were fabricated using plastic compression techniques. Although they were mechanically stronger to the other tissue types, as a tendon graft, they were weak compared to the native tendon. To address this issue, we have successfully developed and tested a modified suture technique which can reduce loading to the tendon graft by placing suture points, not at the edge but

proximally and distally away from the ruptured tendon so the strength of the repair is on suture points rather than on the tendon graft. [11]

There are many opinions in literature around cell sources for tendon tissue engineering. Allogenic tendon cells can be obtained from the patient and cultured in vitro before transplantation, ensuring immunocompatibility. However, total cell yield is marginal in appropriately sized biopsies and tendon fibroblasts are not available as "off the shelf" product.[12] The use of xenogeneic cell sources is controversial because it requires additional engineering to facilitate immune acceptance and there are major concerns regarding zoonosis and germ line mutation. Alternatively, allogenic cell sources can be available off the shelf but are also subject to immune acceptance issues. For the lapine implantation model, allogeneic cell sources can be immunocompatible [13], therefore in this study, we have used cells from the allogenic source. This *in vivo* study shows successful implantation and function of tissue engineered collagen graft incorporating allogenic tendon fibroblast cells fixed with a novel suturing technique. This tendon graft has inherent advantages over currently used tendon graft treatments.

2.   Materials and Methods

2.1   *Cell isolation and culture*

A tenotomy was performed on rabbits of age 16-25 weeks in order to excise PT tendons. Extracted tendons were washed with PBS and the peritendinous tissue layer was removed. Tendons were minced into small fragments and digested with 5% collagenase type 1 (Sigma, UK) at 37ºC for 3 hours. Digested solution was filtered with 70 μm cell strainer and tendon

fibroblasts (TF) cultured with Dulbecco's Modified Eagle Medium (DMEM) (Sigma, UK), 10% fetal calf serum (First Link, UK), 1% Penicillin and Streptomycin (Invitrogen, UK) in T225cm^2 tissue culture flasks with standard culture conditions. Cells were passaged routinely at 80% confluency and all experiments were carried out at passage 2.

2.2   *Fabrication of Tissue engineered tendon grafts (ET)*

The ET was fabricated as previously described by Sawadkar et al;. [11] In short, collagen hydrogel was fabricated by using 4 ml (80%) rat tail collagen type I (First Link, Birmingham, UK) monomeric collagen solution (2.16 mg/ml in 0.6% acetic acid with total protein concentration 0.2% w/v) and 500µl (10%) of 10X Minimal Essential Medium (Invitrogen, Paisley, UK), (pH indicator) neutralised using 5M and 1M sodium hydroxide (Sigma-Aldrich, Dorset, UK) and 500µl (10%) of Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Irvine, UK) containing 2.5×10^5 cells a standard plastic compression was performed [14] . To mimic the posterior tibial tendon of rabbit, four layers of compressed collagen constructs were rolled together. The construct was cut into segments of 15mm which formed the tissue engineered tendon grafts (ET) for fixation into surgically created tendon defects in posterior tibial (PT) tendon.

2.3   **Cell viability**

To study the viability of the cells in the ET a florescence based live/dead test was performed by using double staining kit (Invitrogen, UK). A solution of 0.2% (V/V) calcein-AM and 0.1% (V/V) propidium iodide were prepared in PBS and added to the ET and it was incubated with

5% CO_2 for 30 minutes. Images were obtained by using an upright fluorescent microscope Olympus BX61 (Olympus, Tokyo, Japan).

2.4   *Cell proliferation*

To study a proliferation of the cells incorporated in the ET a resazurin based Alamar blue assay was performed. In total 10^6 cells were seeded in the ET and cultured for 1, 3 and 7 days. At each time point 1:10 (vol/vol) AlamarBlue® (BIO-RAD, UK) reagent was added to the media and incubated at 37°C with 5% CO_2 for 3 hours. Absorbance was measured at 570 nm (600 nm as a reference wavelength).

2.5   *In vivo study design*:

Experiment design and ethical approval were obtained from the UCL Institutional Review Board (IRB). All experiments were carried out as per the regulation of Home Office and guidelines of Animals (scientific procedure) Act 1986 with revised legislation of European Directive 2010/63/EU (2013). Thirty (30) New Zealand White male rabbits were purchased from B&K Universal (Grimston, UK) at weights between 3 and 3.5 kg. All animals were housed in an animal facility provided at Northwick Park Institute of Medical Research (NPIMR). For each time point (1, 3 and 12 weeks), 10 animals were randomly divided into 3 groups (Table 1) and before implantation, ETs were tested for microbial contamination.

2.6   *Surgical procedure*

Rabbits were sedated with intramuscular (i/m) Hypnorm (0.3mg/ml) (Janssen Pharmaceutica, Beerse, Belgium). The posterior aspect of the right hind leg was shaved with a clipper and disinfected using Iodine solution. Animals were anesthetised intravenously (i/v) with 70%

Hypnorm (0.3mg/ml) (Janssen Pharmaceutica, Beerse, Belgium) and 30% Diazepam (5 mg/ml, 0.1ml/kg). A constant infusion of 0.9% saline (Baxter, UK) (10ml/kg) through a 23gauge butterfly cannula was given to maintain fluid balance. Animals were maintained in a prone position throughout surgery and constant supply of oxygen was given at the flow rate of 1 L/min through a facial mask. To maintain anaesthesia during surgery, Hypnorm (0.1ml/kg) was administrated every 25- 30minutes and anaesthesia monitored with an ear pinch reflex method. A surgical area was scaled (Figure 1A) and an incision using a size 20 surgical blade was made 3 cm around the inferior tibiofibular area to expose posterior tibial (PT) tendon (Figure 1B). A tendon defect of 1.5 cm was created in the PT tendon (Figure 1C). This defect was infilled with ET [acellular grafts (AG) and cellular grafts (CG)] (Figure 1D) and for control, 1.5cm PT tendon was excised, rotated through 180° degrees and placed into the tendon defect (Figure 1E). Tenorrhaphy suture techniques were carried out as described previously in short for control modified Kessler repair with tendon graft and for AG and CG developed modified suture technique of four-strand interlocked proximally and distally away from the rupture ends were used. [11] 1) Control group:- Modified Kessler repair with Tendon graft (TG), 2) Acellular graft group (AG):- A novel modified suture technique with acellular ET 3) Cellular graft group , (CG):- novel modified suture technique with cellular ET. Wounds were closed with Vicryl 4-0 (Ethicon, Edinburgh, UK) by subcutaneous running suture technique. The second layer of reverse mattress sutures was performed with 3-0 prolene suture (Ethicon, Edinburgh, UK) (Figure 1F). A schematic of modified Kessler suture technique (Figure 1G) and modified suture technique was applied for AG and CG (Figure 1H). Post- surgery, subcutaneous 4mg/kg Carprofen (Pfizer Ltd, Sandwich, UK) analgesia was administered to the animals and veterinary antibiotic wound powder (Smith & Nephew, London, UK) was applied at the closure site to avoid infection.

2.7 *Tissue harvest*

At the end of each time point (1, 3 and 12 weeks), the animals were sedated by intramuscular i/m dose of Hypnorm (0.3mg/ml) (Janssen Pharmaceutica, Beerse, Belgium), and euthanized by overdose of intravenous pentobarbitone (Lethobard, 200mg/ml, 140 mg/kg) (Arnolds, Reading, UK). Immediately after euthanasia, the operative site was trimmed to remove the hair and an incision with surgical blade size 12 was made lateral to the implantation site. The entire PT tendon was excised and stored in 20 ml of 10X Phosphate buffer saline (PBS) until further use at room temperature.

2.8 *Mechanical testing*

The mechanical tests were conducted immediately after euthanasia to avoid any physical or chemical alteration in the PT tendon. Before mounting PT tendon on the loading machine, all load-bearing outer sutures (Prolene 3-0) were removed so that the mechanical properties of the repair could be assessed. All specimens were tested under tension to failure using Zwick/Roell (Z005 model Ulm, Germany) tensiometer with an extension speed of 50 mm/min for PT tendon repair and 30 mm/min for ET. A difference in the test speed was because ETs were weaker than PT tendon repairs.

2.9 *Histopathology*

Tissue was fixed in 10% formalin saline and processed to embedding in paraffin wax routine automated procedures. Sections at 3 μm were de-waxed, de- and re-hydrated as per standard protocol and stained with H & E. The slides were examined using a light microscope (Olympus BH-2) fitted with an Olympus Camedia 2020 and images were obtained at 10X and 40X to show ET and NT interface.

2.10 *Collagen maturation:*

A 3 μm tissue histopathological tissue section was dewaxed, rehydrated and stained with picro-sirius staining standard protocol. To quantify this birefringence, images of polarised light were processed by converting them from 24-bit RGB (Red, Green, and Blue) to 8-bit HSB (Hue, Saturation, Brightness) stack using ImageJ software (NIH, USA). By using threshold function, colour intensity was defined as 2-9 and 230-256 for red, 10-38 orange, 39-51 yellow and 52-128green. The hue intensity for 129-229 was defined as non-birefringent collagen and total hue range considered as collagen pixel percentage [16].

2.11 *Scanning electron microscopy (SEM):*

Samples for scanning electron microscopy were prepared according to a standard routine protocol, mounted on stubs and sputter-coated with gold –palladium. All images were obtained using a secondary electron detector in a Philips XL 30 Field Emission SEM, operated at 5 kV and average working distance was 10 mm.

2.12 *Atomic force microscopy (AFM):*

A dewaxed tissue section of 3 μm on a glass slide was imaged using Park systems XE-100 AFM in a contact mode, in conjunction with Bruker Antimony doped Silicon (001-0.025 Ohm.cm) contact tips. The spring constant of the cantilever used was 0.01 N/m and error signals and images were obtained. A topology difference was calculated by using Gwyddion software 2.38 (Czech Metrology institute, Czech) for each image topology difference and fibril diameter was calculated by using measure function.

2.13   *Statistical analysis*

 In this study, all results presented in mean ± standards deviation. The significance was calculated at 95% confidence interval with two tail alpha level 0.05, (p-value) and parametric tests were used for multiple groups analysis of variance (ANOVA) with post hoc Bonferroni correction.

3. Results

3.1   *Material characterisation*

The mechanical and structural properties of the ET and native tendon (NT) were studied. In both samples, collagen fibril could be observed (Figure 2 A and B). In the ET orientation of the fibrils was random with no constant D -periodic banding pattern seen but in the NT all collagen fibrils were unidirectional and collagen bundles were arranged in longitudinal directions with D -periodic banding pattern with a constant interval of 45 ±1.23nm. The collagen fibril alignment was studied in detail by analysing diameter and angle of alignment of fibrils. In NT, fibril diameter (269.23± 19 nm) was significantly and higher than ET 65.17± 29.49 nm with the angular alignment of 179.76± 0.19º for NT. Whereas in ET, collagen fibrils were random and no particular angular alignment was calculated (angle range was between 10~176º) in the direction of applied force. This random fibril arrangement in the ET affected its mechanical property as the mechanical strength of a single rolled sheet of collagen was 270.02 ±34.35 mN and two rolled sheets was 1678 ± 54.56 mN. To mimic the size of native tendon, four sheets of rolled collagen were used which had the mechanical strength of 4410 ± 80 mN. (Figure 2 C) which was significantly lower than the NT.[11] When this ET was seeded with tendon fibroblast then it resulted in continual proliferation and there was no significant change in the viability over a period of on day 1, 3 and 7 days (Figure 2D, E and F)

3.2   *Gross observation*

At the end of implantation period (i.e. 1, 3 and 12 weeks), grafts were visualised bridging the tendon defect with integration at both approximation ends and there were no gaps between ET and NT (Figure 3). These grafts appeared viable (visual screening) without any scar tissue formation, no adhesion to the surrounding tissue and no hyperemia was present. The absence of adhesions was confirmed using the method described by Oryan et; al. [17] However, there was a large amount of inflammation observed by the end of 1 week (visual screening) (Figure 3B). The inflammatory response was reduced by 3 weeks (Figure 3C) and by 12 weeks (Figure 3D), there was a marginal inflammation associated with the operative site. In the control studies of 1, 3 and 12 weeks, grafts were avulsed from the suture point (Figure 3A). This avulsion was due to the active mobilisation of the animals, where standard suture technique (Modified Kessler) was not able to cope with the force exerted by the native tendon during locomotion. Whereas, the modified suture technique coped with the force exerted by the tendon without constraining standard practise post-operative movement. [18] The average transverse diameter of the graft was 8 ± 0.23 mm at day 0. This diameter increased significantly within a week to 23 ± 8 mm for AG (Figure 4A) and to 28 ± 12 mm for CG ($P< 0.05$) (Figure 4 B) but ETs were intact between NTs. This diameter was reduced by the end of 3 weeks in AG to 12.4 ± 4.29 mm (Figure 4C) and in CG to 11.17 ± 3.19 mm ($P < 0.05$) (Figure 4D). By 12 weeks, there was a statistically significant reduction [AG 8.34 ± 0.89 mm (Figure 4E) and CG 6.18 ± 0.71 mm) (Figure 4F) ($P< 0.05$)] in the diameter compared to 1 week and 3 weeks. This reduction resulted in tendon graft diameters consistent with that of the tendon (5.3 ± 1.2 mm). However, in control groups 1, 3 and 12 weeks, in all cases, tendon grafts were avulsed from the proximal with no change in the diameter of tendon grafts (Average diameter was 5.4 ± 2.3 mm). A

decrease in the diameter of the ET over time was an indication that there was a reduction in the inflammatory response.

3.3   *Mechanical properties of the repair*

In the AG group, the break strength at hour 0 was equivalent to the CG (hour 0). In AG, at 1 week, this break strength was decreased compared to hour 0. However, by the end of 3 weeks, there was a significant increase in the break strength and it was highest in 12 weeks. In the CG group, there was a significant increase in the mechanical break strength for 3 and 12 weeks as compared to 1 week (Figure 5A). The results also showed at 12 weeks there was a significant increase in the break for AG and CG, therefore, encapsulating ET with tendon fibroblast (TF) was beneficial. The calculated modulus of the ET for the pre implantation (day 0) was 0.44 MPa for AG and 0.42 MPa for CG. After implantation, the modulus decreased in CG group within one week, followed by a marginal increase by 3 weeks, however, by 12 weeks, there was a significant increase in the modulus of the AG and CG (Figure 5B). These results indicated that within 1 and 3 weeks, ET modulus was less due to the progression of maturation of ET in vivo, therefore, it yielded lower modulus than 12 weeks for AG and CG. At the end of 12 weeks, modulus was highest. The stiffness of the ET is an important factor, as it is related to the resistance towards deformation under applied force by the NT over a period of time. At 1 week, strain grafts were increased for AG and CG compared to 0 hours. The increase in the strain resulted in less stiff and weaker grafts at 1 week, however, at the end of 3 weeks grafts increased in stiffness and resulted in less strain for AG and CG. The grafts continued to gain stiffness over a period of 12 weeks (Figure 5C).

3.4   *Mechanical properties of the graft*

Break strength of the graft at 0 hours (pre implantation) was 4.56 N for AG and 4.58 N for CG. This break strength was reduced when grafts were implanted in vivo at the end of 1 week for AG and for CG, but at the 3 weeks, the mechanical properties were significantly higher than 1 week. This trend continued up to 12 weeks where calculated break strength was maximum, and ET was able to remodel and mature over time (Figure 5D). A calculated modulus of the AG and CG at time 0 was higher than at 1 week, however, modulus of ETs was increased significantly by 3 weeks in both groups. Similarly, by 12 weeks there was a significant increase in the modulus to AG and CG (P<0.05) (Figure 5E). The stiffness of the graft at 1 week was increased for AG compared to time 0 but strain% was reduced at 3 weeks in both AG and CG. In the CG, the graft was stiffer by 12 weeks as calculated strain was higher than AG (Figure 5F). The mechanical properties of the ET and repaired tendon were increased significantly at each time point. Although, at 1 week the graft was weaker as compared to time 0, over a period of time these mechanical properties were increased significantly in all groups. Modulus and stiffness of ET and PT tendon (ET incorporated) was also increased over time 1, 3 and 12 weeks. All these characteristics are seen more in the CG group than AG, therefore, it can be concluded that it was beneficial to seed cells in the ET.

3.5   *Microscopical inflammatory response*

In the AG, within a week, there was an inflammatory reaction in the implanted ET grafts. This resulted in the rapid migration of the inflammatory cells inside ET at 1 week (Figure 6A). The total inflammatory cells were reduced by the end of 3 weeks and significantly reduced by 12 weeks (Figure 6C and E). In the NT, there was no evidence of inflammatory cells was seen (Figure 6G). In the CG there was higher inflammatory reaction was seen by 1 week , (Figure 6B) but, over the period by 3 weeks there was marginal reduction in the inflammation (Figure 6D) and , by 12 weeks there was a significant reduction in the in the inflammatory reaction

(Figure 6F); in comparison, in NT this count was marginal (Figure 6H). This signifies that at post-implantation of the graft, there was rapid cross-reaction to the ET tendon graft and allogeneic cells by the end of 1 week. This could be correlated to the gross observation where at 1week, the graft was seen swollen from its normal size, but this inflammatory reaction was reduced marginally end by 3 weeks (partial integration was seen of the graft in the gross) and significantly by 12 weeks (complete integration was seen in the gross).

3.6   *Interface between NT and ET at fascicle level*

At 1 week, both AG (Figure 7A) and CG (Figure 7B) showed disturbance of the internal architecture of the NT and ET. The fascicle orientation of the NT at the proximal and distal end was irregular, and in the ET, at the junction point, grafts were polymerised. This puckering effect was due to suturing of the mechanically weaker collagen grafts to the mechanically strong NT. At the end of 3 weeks, this puckering was not seen in either AG (Figure 7C) or CG (Figure 7D), and ET was showing integration into NT. By 12 weeks both AG (Figure 7E) and CG (Figure 7F) showed complete integration to the NT. In the case of AG, there was an integration margin visible, but in CG this was not seen. All SEM images at 250X show that by 12 weeks in AG and CG, ET was integrated into NT, but in the case of CG, integration was compact. This compact integration without any gapping indicates that ET was able to withstand the force exerted by the NT and was able to match material properties (strain and modulus) to the NT at 12 weeks as compared to 3 weeks and 1 week integration.

3.7   *Interface between ET and NT at fibril level*

Fibril integration between ET and NT was an important key factor to analyse in this study. The NT displayed the expected presence of highly aligned mature collagen fibrils, characterised by their banding periodicity and uniform diameter (Figure 8G and H). At 1 week in AG (Figure 8A) the ET fibrils were observed to be immature with small diameter interspaced by non fibrillar regions. At the junction point, there was a lack of integration between ET and NT with a distinct boundary. However, fibrils at the junction point were seen to have a more defined structure than ET fibrils, although they remained immature. In CG (Figure 8B), the ET did not show the presence of mature fibrils, showing an abundance of regions with non-fibrillar structure. In NT fibrils found close to the junction point with ET showed well defined morphology associated with mature fibrils and were highly oriented. The mean topological difference across the junction point was measured to be 496 ±182 nm for AG and 168±65 nm for CG (Figure 8I). After 3 weeks implantation AG (Figure 8C) showed maturation in the morphology of ET fibrils without non-fibrillar components. The fibrils were highly disordered and immature with no constant banding periodicity. In CG, the fibrils were mostly immature and interspaced by non-fibrillar regions. At the junction point, fibrils were more structured, and there were regions where small diameter mature fibrils were observed (Figure 8D), showing an intermediary stage of fibril maturity between ET and NT. The mean topology difference (variation in the surface topology of NT and ET) across the junction point was 468±109 nm for AG and 177±49 nm for CG. After 12 weeks implantation, AG (Figure 8E) showed extensive remodelling of the ET fibrils resulting in mature fibril formation with some remaining non- fibrillar regions. The bundles of NT fibrils were decreased in diameter and highly oriented, additionally, they were found to be well integrated into the ET regions at the junction point. In CG (Figure 8F) there was a significant change in the morphology of the ET

fibrils at the junction point. The ET fibrils were matured and similar in morphology to the NT fibrils. At the junction point NT and ET fibrils were integrated with ET fibrils aligning parallel to NT fibrils. The mean topological difference across the junction point was measured to be 251±56 nm for AG and 75±48 nm for CG.

3.8   *Quantitative assessment of collagen in the ET*

A difference in birefringence could be distinguished between NT and ET. At 1 week it was evident that birefringence of the ET was less than the NT in both AG and CG cases (Figure 9A and B). These gradients were seen to improve from 3 weeks post-implantation (Figure 9C and D) to 12 weeks (Figure 9E and F) in both AG and CG. It is well-established that in *in vivo* implantation models, collagen fibrils become thicker by tissue based remodelling over a period of time.[20-22] The birefringence colour intensity percentage calculated for each time point in AG and CG intensity decreased for green and increased for yellow and red for 1,3 and 12 weeks. This indicates that over a period of time, collagen was becoming more mature with an increase in birefringence from yellow to red. However, there was no evidence found for green percentages in an NT as there was no immature collagen seen. Conversely, significantly higher percentages were found in yellow orange and red mature and remodelling state (Figure 9G). Furthermore, the gradient was quantified by hues associated with the birefringence at each time point, [20] and percentages were calculated by dividing the total pixel area of the ET. The percentage collagen content in the ET at the time of 1 week was 26.2± 5.44% for AG and 35.4 ± 3.84 % for CG but this percentage was increased significantly by 3 weeks (58.8±4.32% for AG and 75.8± 5.11 for CG p< 0.05) and 12 weeks (83.2±4.56 % for AG and 92.8±3.03%) (Figure 9H). The increase in the collagen content was likely to be a manifestation of ET maturation and this was probably a chronic response to the mechanical loading of ET. An

increase in the gradient of hues associated with the birefringence at each time point (1, 3 and 12 weeks) indicated that collagen remodelling and graft maturation at 12 weeks was higher than at 3 and 1 week.

4. Discussion

The mechanical properties of the tendon tissue entirely depends upon its composition, nano-, micro- and macro- scale properties, therefore, in this study we have evaluated structural properties of ET and ET at all levels. Structural properties were investigated at fibril level to understand why ET was weaker to NT. In the NT, alignment (179.76°) and thicker fibrils (269.23± 19 nm) were key factors for its mechanics. This is due to a multistep process of collagen fibrillogenesis, remodelling and maturation along with constant D -periodic banding pattern of 45nm, which has been proposed to act as shock absorbers during locomotion in vivo. [23] However, ET was fabricated within 20 minutes and fibrillogenesis had occurred due to the static weight applied on the collagen hydrogel during the compression process. [11] This rapid fabrication of ET formed random (angle range was between 10~176º), immature (diameter 65.17± 29.49 nm) and mechanically weaker collagen fibrils as compared to NT in which self-assembly, parallel angular alignment and compaction of collagen fibrils with higher diameters were key factors for higher mechanical strength. [1]

To make a functional tendon graft, the mechanical properties of the ET should be adequate. To increase mechanical properties of ET, there was a choice to use either physical or chemical cross-linking, but it will never address mechanics of native tendon, therefore, we have adapted a strategy to modify suture technique and so, a novel load bearing suture technique was developed. The concept of suture technique and stress shielding mechanics has been reported

previously [11]. In this study, we have demonstrated a functional tendon graft in the lapine model. The posterior tibial (PT) tendon was chosen as an implantation site as PT tendon was easy to access anatomically. It is the longest (average size 10-12 cm), as well as the strongest tendon (254.02N) in rabbit hence it is an ideal tendon to test developing load bearing suture techniques and 90% stress shielded ET . [11] An additional advantage of PT tendon was that as animals were actively mobilised, this could enable the loading of tendon post-surgery hence encouraging rapid integration and remodelling of ET. [24-25]

In the control group, the grafts were avulsed from the proximal end due to the fact that all animals were actively mobilised. [26] In these animals, the modified MK technique could not withstand physiological loading and mechanical forces exerted on the sutures during locomotion. The mechanical property of ET was weaker post implantation (1 week) but the modified suture technique was strong enough to withstand the physiological load and ET was intact up to 12 weeks. An additional key finding in this study was that there were no adhesions of ET to the surrounding tissues and the tendon was able to glide without any obstruction. A potential reason for this was that these animals were neither immobilised nor passively mobilised post-surgery. To support these findings, similar studies conducted on the flexor tendon in a murine model showed that when mice were actively mobilised, the repaired flexor tendon cell biology initiated a standard wound healing response. Implanted ET should match the mechanical property of the native tendon at least up to biological break strength for it to be considered as a functional tendon graft. It has been reported that various biological and synthetic tendon grafts gained mechanical and physical properties *in vivo* over a period of time. [28-29] In this study, mechanical properties of repair and ET were decreased at 1 week as compared to time 0. This may have been due to an influx of the fluid from the surrounding tissue. [30] An initial inflammatory response and immune cell infiltration were key factors for weaker mechanical properties.[13] Over a period of 3 weeks, although AG was weaker than CG,

mechanical properties of the repair were increased probably because the added cells were able to remodel the matrix by increasing production of type I collagen and its deposition. [31-33] This indicates better integration due to seeding of cells. At the end of 12 weeks, ET was reshaped (diameter AG 8.34mm, CG 6.18 mm) to the size of the native tendon (average diameter was 5.4 mm), collagen fibrils were matured and aligned with the applied stress. At this time point, it is likely that higher levels of collagen were being synthesised and cellularity was being reduced whilst retaining mechanical properties. [33-35] All these characteristics are seen more in CG group than AG, therefore, it was beneficial to add cells in the ET.

We propose that an increase in the mechanical properties also correlated to the active mobilisation of the animals because when canine flexor digitorum profundus tendons were actively mobilised, they gained significantly higher mechanical properties than immobilised tendons in 42 day studies. [26] An interesting observation was made: in both AG and CG at Time 0 and 1 extension breakpoint was at midgraft whereas for 3 and 12 weeks both AG and CG break point was at the junction between ET and NT. An increase in stiffness over a period of time is an important characteristic which defines mechanical integrity and energy storing capacity of ET. [36] The increasing stiffness could be correlated with an increase in force transmission, energy storing and absorption capacity of the ET, [37] which showed better integration by SEM. The ET was able to obtain stiffness equivalent to the native tendon but it is unlikely that it would become stiffer than the native tendon as stiffness is directly proportional to the cross-sectional area. [36]

The ET was fabricated from allogeneic cells. An advantage of this cell source is the off the shelf availability without any additional harvesting procedure from the autologous source, which causes donor site morbidity. [38] In contrast, many researchers suggest the use of autologous cell sources as allogeneic cells elicit increased inflammatory reaction over autologous cells. [39] An ACL reconstruction using tissue engineered grafts using allogeneic cells

in an ovine model after 6 months reported similar histological and mechanical properties to autologous cells. [40]

A major concern in this study was immunogenicity of the implanted ET graft as they were fabricated from a xenogenic source of collagen. Mudera et al., implanted these collagen grafts in vivo in lapine models at intercostal spaces to show their biocompatibility. Furthermore, these implanted grafts showed the migration of host endothelial cells resulting in angiogenesis and revascularisation. [13] However, in this study, endothelial docking and angiogenesis formation may have resulted in an impaired tendon graft as the natural tendon is a poorly vascularised tissue, [41-42] but in ET, angiogenesis and revascularisation were absent. We conclude that ET as tendon graft has mimicked native tendon vascularity in a lapine model.

An investigation of this repair at fibril level was conducted to understand the remodelling mechanism and pattern of the collagen. [43-44] Collagen fibrils from the ET were remodelled *in vivo* at the junction point over time. The characteristics of fibril remodelling varied depending on whether the engineered tendon was seeded with cells or was acellular. An abundance of non-fibrillar collagen was found in ET for the AG at both 1 week and 3 weeks. This might be accounted for by the presence of cells depositing excess non fibrillar collagen. The presence of excess deposited collagen may explain why the topological difference between the AG and CG planes is smaller than for the acellular grafts. The mass of the cellular engineered scaffold consists of engineered tendon and deposited collagen. The mass of the acellular engineered scaffold consists of the just engineered tendon. This explains why the initial topological difference for the cellular scaffold is far lower than the AG. The topological difference across junction point for CG was far smaller than for AG for all time points (including week 1). Additionally, the topological difference was reduced over time for both cellular and acellular scaffolds reflecting the increased integration of ET and NT over time.

Fibril alignment is a crucial factor for gaining appropriate mechanical properties in the tendon. In the process of ET remodelling, collagen fibril density was increased, and some degree of alignment was seen. The fibril alignment is dependent on the shear stress and length of fibrils so it is likely that these forces were present within ET cases. This is supported by the finding of maturation of collagen fibrils in tendon under stress in animal models. [45-46] In this study, we have reported that addition of cells to ET has improved the mechanical property as compared to acellular constructs. Hence, we can conclude that cell seeded ET was able to lay down non fibrillar neo-collagen over time and decreased the topological difference between ET and NT. These findings lead us to understand the difference between mechanical characteristics elicited by cellular and acellular grafts as reported above.

Compared to the current tendon grafting treatments, the ET with a novel load bearing suture technique has distinct advantages: 1) ET was recellularised from an allogeneic cell source and fabricated with a xenogeneic collagen source (rat tail tendon) which has proved to be biocompatible as tissue engineered tendon graft; 2) A major advantage of ET is that it could be off the shelf, readily available, easy to handle and reproduce; 3) All tissue engineered grafts (described in the literature) have adequate biomechanical indices and histology but are never able to succeed in attaining mechanical strength equivalent to the natural tendon. [47] ET was able to improve mechanically over a period of time and achieved mechanical properties of least biological equivalence in terms of extension induced separation (2.38N at 1 week and 51.87N at 12 weeks); 4) Cellular attachment to the scaffold is a crucial factor for the tissue engineered tendon graft. The advantage of ET was that it is able to mimic natural tendon cellularity (due to the addition of the cells during graft fabrication). Seeding commercially available scaffolds is challenging because cells are not able to migrate in between collagen fibres and attach to the graft due to its diameter and lack of porosity in the early stage post implantation; 5) The ET was non-adhesiogenic (visual screening) whereas adhesion development in association with

currently available tendon grafts is a clinical problem; 6) Tearing is an issue in currently available tendon grafts, no tearing of the ET was seen in these studies due to load bearing sutures being placed to ameliorate some of the functional loads; 7) This procedure offers some fiscal economy compared to other currently available tendon reparative solutions.

5. Conclusions

In this in-vivo preclinical study we have successfully demonstrated that by the end of testing period (1, 3 and 12 weeks), ET showed the bridged integration of the graft without any adhesion formation with a significant increase in the mechanical for 12 weeks and 3 weeks as compared to 1 week. Histological analysis showed that tendon fibroblasts from the native tendon were able to migrate into the graft with higher collagen remodelling and graft maturation at 12 weeks. Insertion of tissue engineered collagen graft using a novel load bearing suture technique, which partially loads the graft *in vivo*, showed integration, greater mechanical strength and no adhesion formation in the time period tested and the potential for ET as a candidate for tendon grafting. Further work is needed to understand immunological responses to xenogeneic sources of collagen, although, some of this would be obviated by the development of clinical grade collagen. With the successful progression of this philosophy, ET offers clinically attractive alternatives to the present-day tendon graft.

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For Table of Contents Use Only

Applied weight

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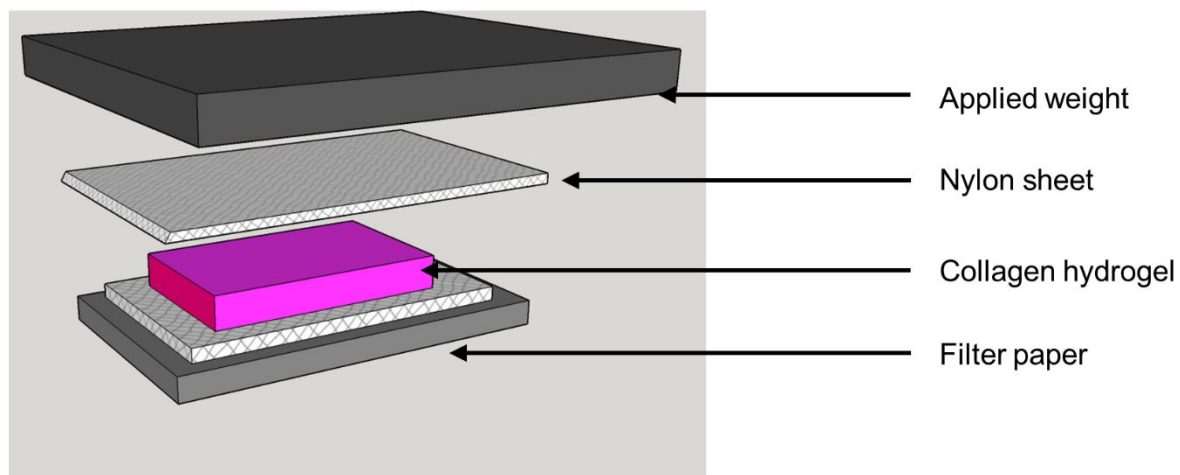
Plastic compression of collagen

Manuscript title:- "Engineering of a functional tendon using collagen as a natural polymer"

Authors:- Prasad Sawadkar , Paul Sibbons , Tarek Ahmed, Laurent Bozec and Vivek Mudera
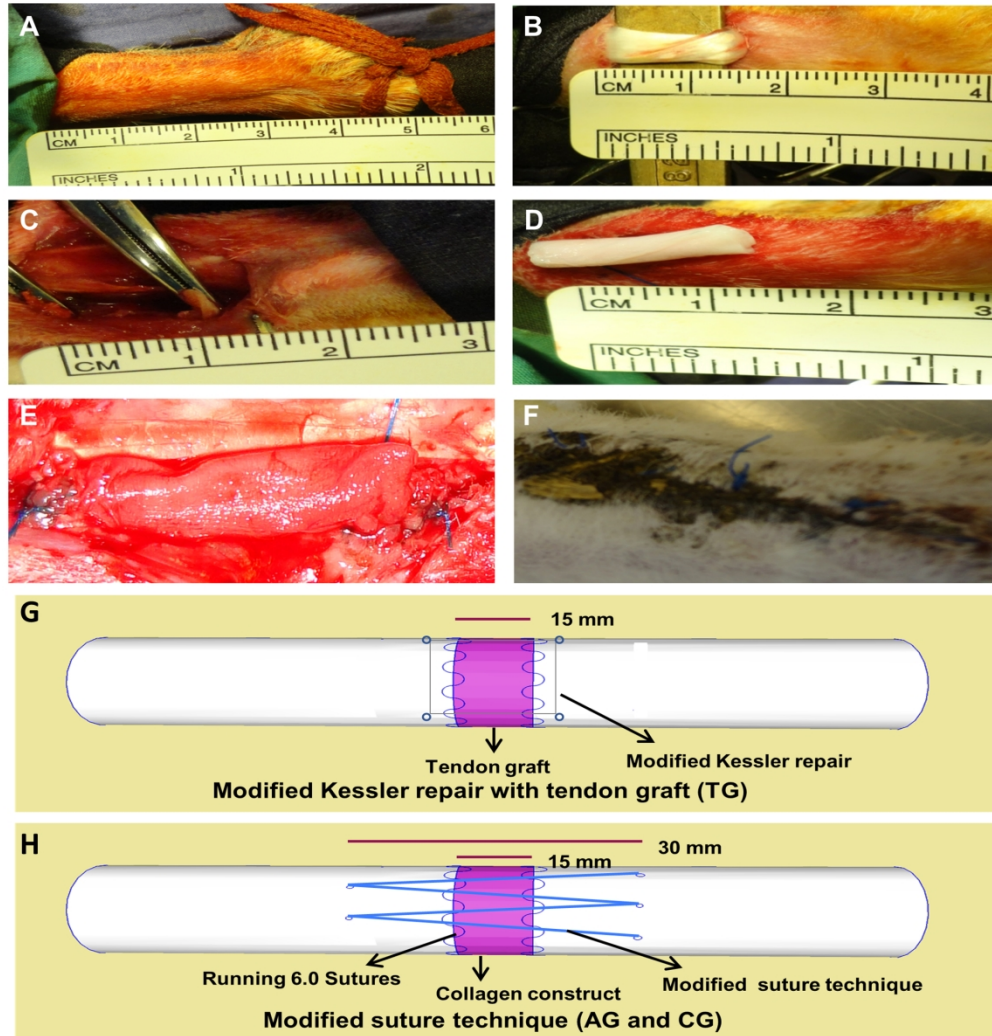
Surgical procedure, an Op site on the posterior area of the rabbit right hind leg (A). An insertion was made in the inferior tibiofibular area (B). A tendon defect of 1.5 cm was created in the PT tendon (C). This defect was filled with engineered tendon (ET) (D) ET was secured with 6-0 prolene sutures and sutures techniques were applied as per group (E). Wound was closed with Vicryl 4-0 sutures and 3-0 prolene suture (F). A schematic diagram of modified Kessler repair (G) and modified suture technique (H)
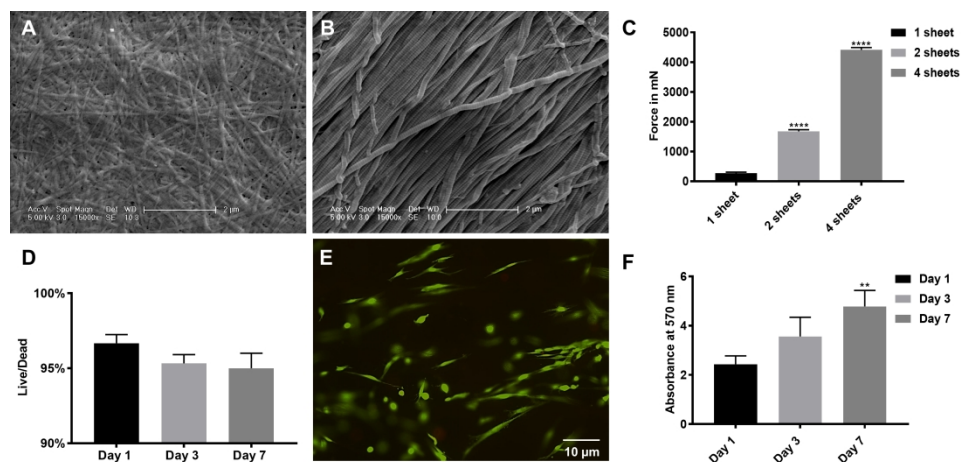
Figure 2 Material characterisation of ET and NT. Fibril alignment of ET and NT (A and B). Mechanical property of ET (C). Live/dead percentages for day 1,3 and 7 (D) with live cell morphology (E) and cell proliferation profile for day 1,3 and 7. * , ** and ****denotes statistical significance of P< 0.05, P< 0.01 and P < 0.0001 from day 1.

338x190mm (300 x 300 DPI)

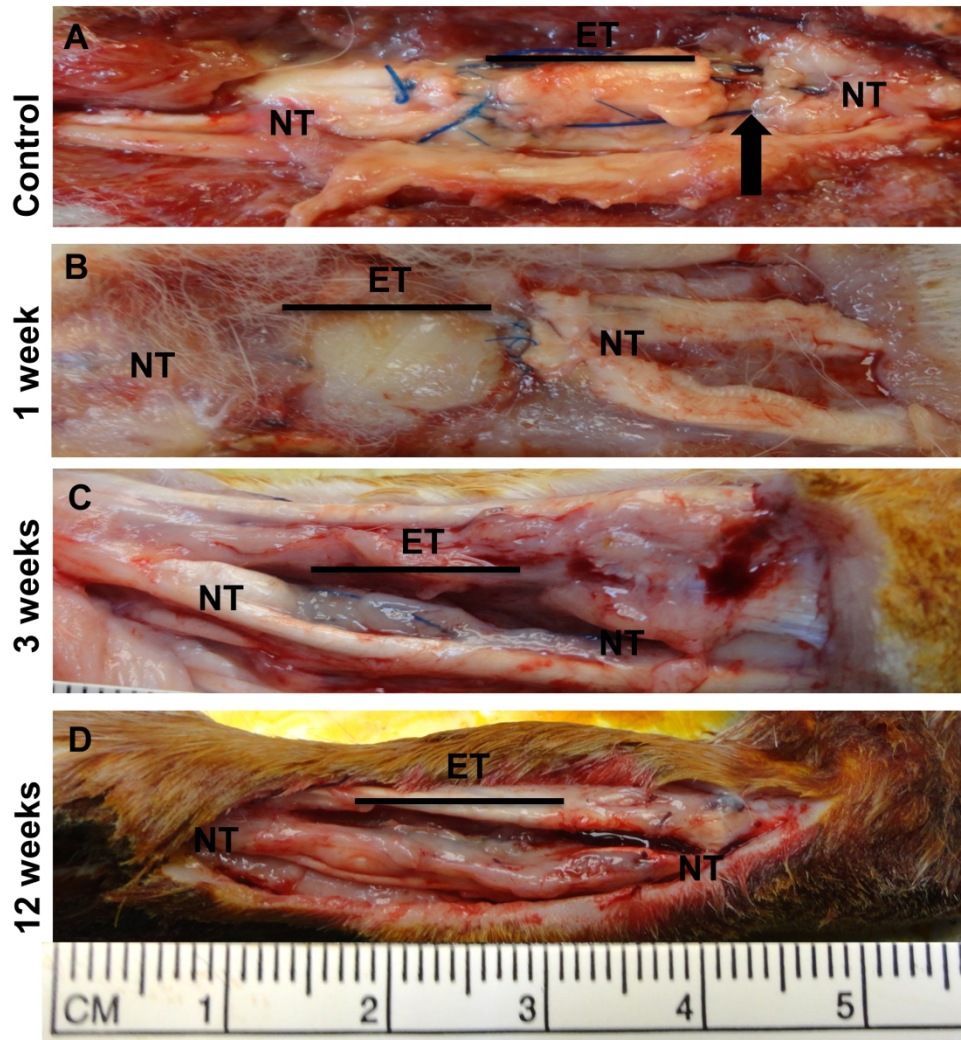Figure 3 Engraftment of the ET to the NT. In control group TG avulsed from the proximal end, an arrow showing gapping between tendons ends (A), In the 1week ET is in intact between NT with the increase in the size (B), by 3 and 12 weeks graft is still persisted in between NT. There no adhesion was seen (C) and (D).

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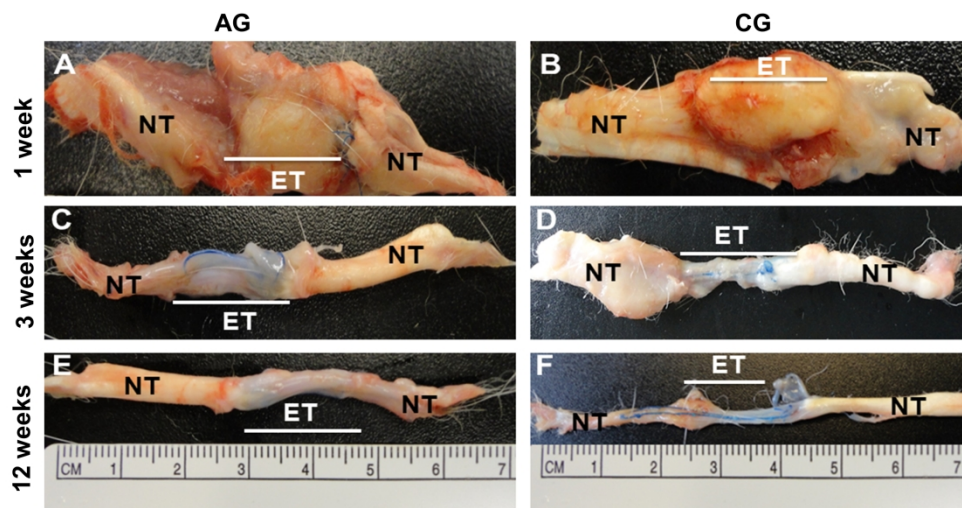
Figure 4 Gross observation of the tendon with ET incorporated at the end of 1 week for AG and CG where ET diameter was increased (A and B), but this diameter decreased by 3 weeks (C and D) and further decrease in the diameter was seen by 12 weeks (E and F) for AG and for CG.
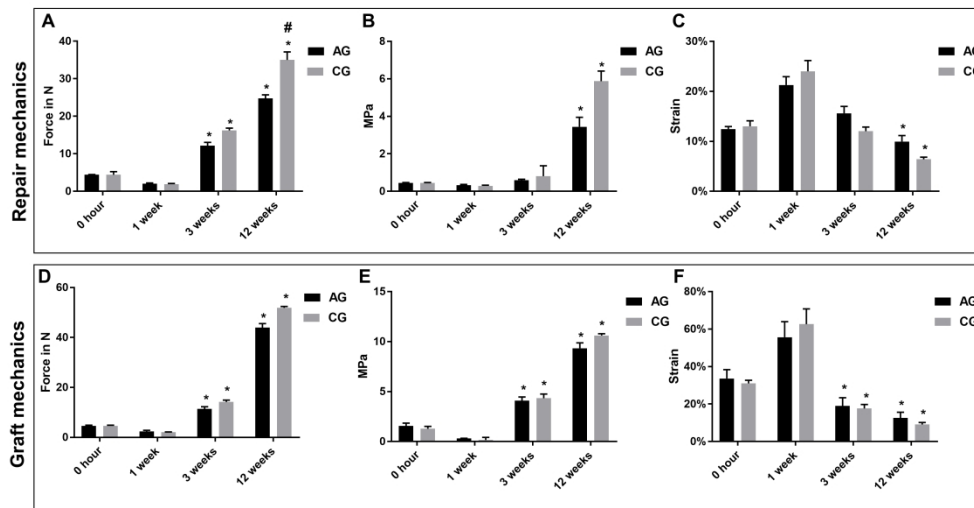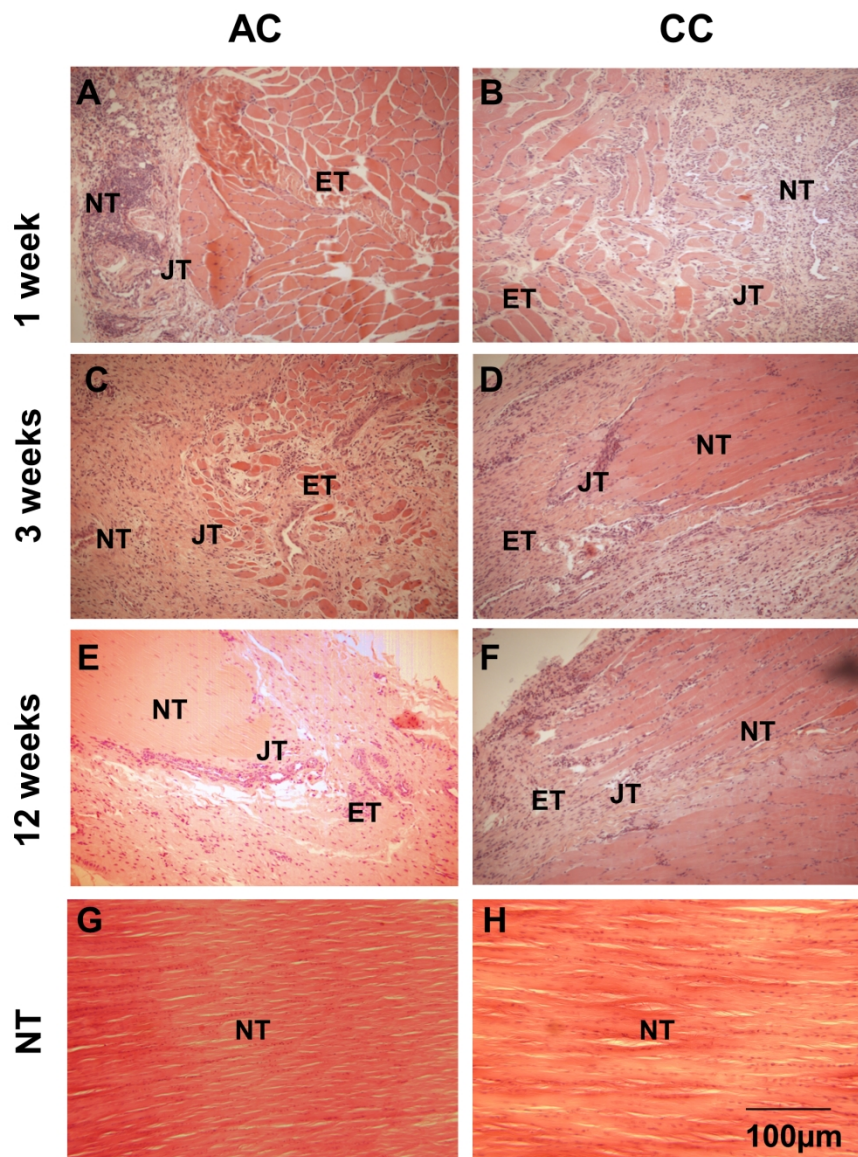
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Figure 5 Mechanical properties at 0 hour, 1, 3 and 12 weeks. Mechanics of repair, break strength(A), modulus(B), strain(C) and mechanics of graft break strength(D), modulus (E), strain (F). * denotes statistical significance of $P < 0.05$ from 1 week and # denotes significance between AG and CG at 12 weeks.

338x190mm (300 x 300 DPI)

Histological section around junction tendon (JT) between AC and CC stained with H and E stain at the time 1 week (A and B) , 3 weeks (C and D) and 12 weeks (E and F). Native tendon NT (G and H).

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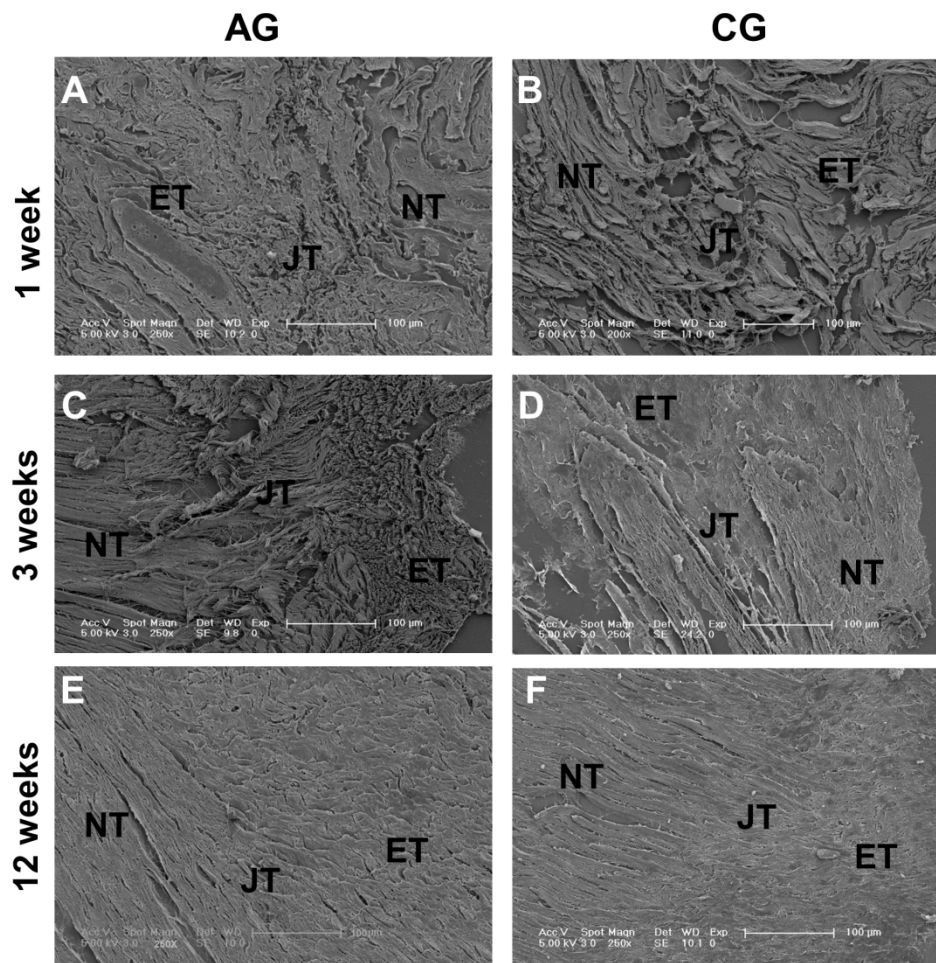
SEM images for AG and CG at 250X for damage tendon ends at junction tendon (JT) between engineered tendon (ET) and native tendon (NT) post-surgery at 1 week (A and B). At the end of 3 weeks this puckering was not seen at JT site (C and D) and by 12 weeks complete integrated ET to NT was seen (E and F).

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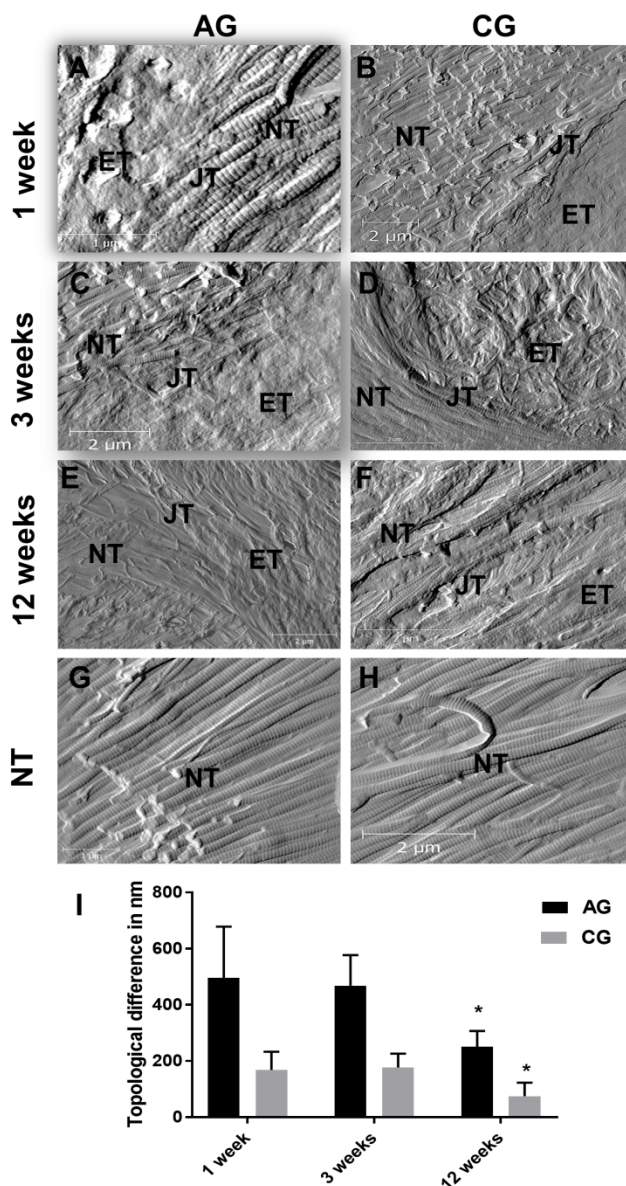
AFM images for AG and CG. 1 week (A and B), 3 weeks (C and D), 12 weeks (E and F). Native tendon (NT) displayed the expected presence of highly aligned mature collagen fibrils, characterised by their banding periodicity and uniform diameter (G and H). Topological differences between NT and ET for the AG and CG group at 1, 3 and 12 weeks at the JT (I). * denotes statistical significance of $P < 0.05$.

190x254mm (300 x 300 DPI)
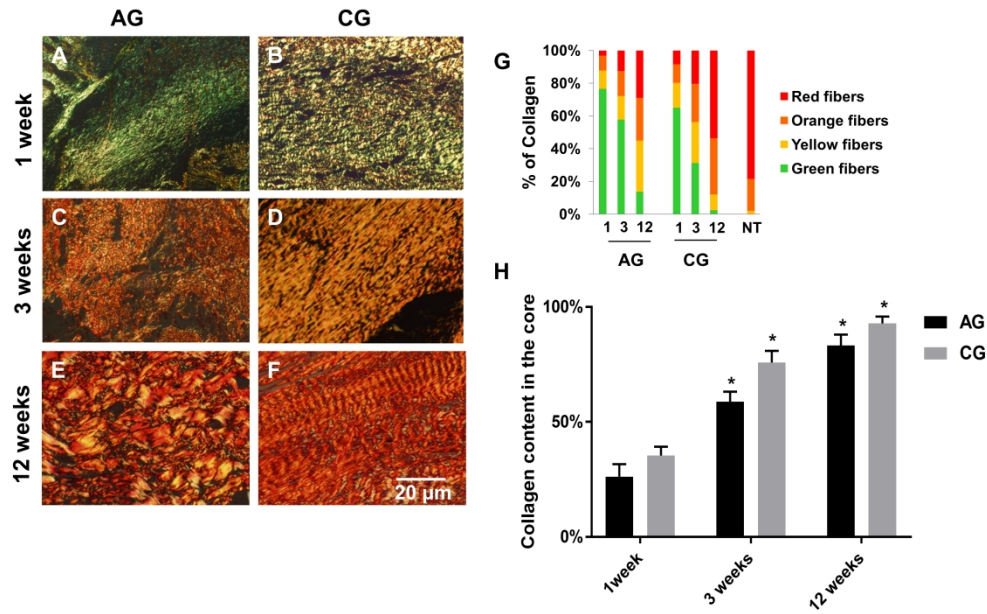
Birefringence colour intensity under polarised microscope indicates that over a period of time, collagen was getting mature with increase in birefringence from green to yellow to orange to red over time 1 (A and B) , 3 (C and D) and 12 (E and F) weeks. Collagen birefringence distribution for the ET at 1, 3 12 weeks (G). Collagen percentages at the core for ET (H). * denotes statistical significance of P< 0.05

883x551mm (96 x 96 DPI)