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TITLE:

A Novel Tenorrhaphy Suture Technique with Tissue Engineered Collagen Graft to Repair Large Tendon Defects

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SUMMARY

In this paper, we present an *in vitro* and *in situ* protocol to repair a tendon gap of up to 1.5 cm by filling it with engineered collagen graft. This was performed by developing a modified suture technique to take the mechanical load until the graft matures into the host tissue.

ABSTRACT

Surgical management of large tendon defects with tendon grafts is challenging, as there are a finite number of sites where donors can be readily identified and used. Currently, this gap is filled with tendon auto-, allo-, xeno-, or artificial grafts, but clinical methods to secure them are not necessarily translatable to animals because of the scale. In order to evaluate new biomaterials or study a tendon graft made up of collagen type 1, we have developed a modified suture technique to help maintain the engineered tendon in alignment with the tendon ends. Mechanical properties of these grafts are inferior to the native tendon. To incorporate engineered tendon into clinically relevant models of loaded repair, a strategy was adopted to offload the tissue engineered tendon graft and allow for the maturation and integration of the engineered tendon *in vivo* until a mechanically sound neo-tendon was formed. We describe this technique using incorporation of the collagen type 1 tissue engineered tendon construct.

INTRODUCTION

Tendon rupture may occur due to extrinsic factors such as traumatic lacerations or excessive loading of the tendon. Due to the external tensile forces placed on a tendon repair, a gap

inevitably forms with most tendon repair techniques. Currently, tendon defects/gaps are filled with auto-, allo-, xeno- or artificial grafts, but their availability is finite, and the donor site is a source of morbidity.

The tissue-engineered approach to fabricate tendon graft from a natural polymer such as collagen has the distinctive advantage of being biocompatible and can provide vital extracellular matrix (ECM) components that facilitate cell integration. However, due to a lack of fibrillar alignment, the mechanical properties of the engineered tendon (ET) are inferior to the native tendon. To increase mechanical properties of the weaker collagen, many methods have been used, such as physical cross-linking under vacuum, UV radiation, and dehydrothermal treatments[1]. Also, through chemical cross-linking with riboflavin, enzymatic and non-enzymatic methods increased collagen density and the Young's modulus of the collagen *in vitro*[2,3]. However, by adding cross-linking agents, biocompatibility of the collagen is compromised, as studies have shown a 33% alteration in mechanical properties and 40% loss of cell viability[3-5]. Gradual accruement of alignment and mechanical strength can be obtained through cyclic loading[6]; however, this can be efficiently acquired i*n vivo*[7].

For ET to integrate *in vivo* and acquire strength without the need for chemical alteration, one approach would be to use a stabilizing suture technique to hold the weaker construct in place. Most tendon repairs rely on the suture design to hold tendon ends together; hence modification of these existing techniques could provide a logical solution[8,9].

Until the 1980s, 2-strand repairs were widely used, but recent surgical literature describes the use of 4 strands, 6 strands or even 8 strands in repair[10,11]. In 1985, Savage described 6-strand suture techniques with 6 anchor points, and it was significantly stronger than the Bunnell suture technique that uses 4 strands [12]. Also, 8-strand repairs are 43% stronger than other strands in cadaver and *in situ* models, but these repairs are not widely practiced as it becomes technically difficult to reproduce the repairs accurately[13-16]. Therefore, a greater number of core suture strands relates to a proportional increase in biomechanical properties of the repaired tendon. However, there is a loss of cell viability around the suture points, and trauma from excessive suturing can be to the detriment of the tendon, which can compromise tendon healing[17]. Suture techniques should provide a strong geometric repair that is balanced and relatively inelastic to minimize tendon gapping after repair. In addition, the location of the suture and its knots have to be strategically placed in order for them not to interfere with gliding, blood supply and healing until accruement of adequate strength has been obtained[10,18].

To establish feasibility to secure weaker ET graft or other graft material in between ruptured tendon, we have developed a novel suture technique that can offload the graft so that it can mature and gradually integrate into the host tissue *in vivo.*

PROTOCOL:

Note: Experiment design and ethical approval were obtained from UCL Institutional Review Board (IRB). All experiments were carried out as per regulation of Home Office and guidelines of Animals

(scientific procedure) Act 1986 with revised legislation of European Directive 2010/63/EU (2013). Rabbits were inspected by a named veterinary surgeon (NVS) periodically and twice in a day by a named animal care and welfare officer (NACWO) (As per guidelines and regulations of Home office). They did not show any sign of pain until they were euthanized.

1. Preparation of Tissue Engineered Tendon (ET) Graft

1.1. To fabricate the collagen hydrogel, add 4 mL of rat tail collagen type 1 monomeric collagen solution (2.15 mg/mL in 0.6% acetic acid with 0.2% w/v of total protein) and 500 µL of 10x Minimal Essential Medium. Neutralize this by titrating against 5 M and 1 M sodium hydroxide and add 500 µL of Dulbecco's Modified Eagle Medium (DMEM).

1.2. Pour 5 mL of this solution into a custom built rectangular metal mold (33 mm × 22 mm × 10 mm, 120 g weight) (**Figure 1**). Keep the mold in a CO_2 incubator at 37 °C and 5% CO_2 for 15 minutes to allow matrix assembly[19].

2. Fabrication of the Graft

2.1. After polymerization, remove the collagen hydrogel from the mold and place in a standard plastic compression assembly (**Figure 2A**)[19].

2.2. Place the collagen hydrogel in between two 50 µm nylon mesh sheets and apply a static load of 120 g (total surface area 7.4 cm^2, which is a pressure equivalent to 1.6 kPa) for 5 minutes to remove interstitial fluid from the hydrogel (**Figure 2A**). Use four layers of filter paper to absorb the discharged fluid from hydrogels.

2.3. Use four layers of compressed gels rolled on top of each other (**Figure 2B**) and cut into 15 mm segments (**Figure 2C**) to fabricate the ET.

Note: New Zeland white male rabbits of age 16 - 25 weeks were used in the experiments.

2.4. Sedate animals with an intramuscular (i.m.) dose of Hypnorm (0.3 mg/mL) and euthanize by administering an overdose of pentobarbitone.

2.5. Immediately after euthanasia, trim the hair on both hind legs. Then with a size 20 surgical blade, make a 9 cm incision around the inferior tibiofibular area to expose the tibialis posterior (TP) tendon.

2.6. With the same sized surgical blade, excise lapine TP tendons with an average length of 70 mm and keep moist in PBS during the experimental process to avoid drying.

3. Developed Novel Tenorrhaphy Technique

Note: The sutures (see **Table of Materials**) are non-absorbable and made from an isotactic crystalline stereoisomer of polypropylene, which is a synthetic linear polyolefin. The core interlocking sutures were mainly consisting of 3-0 and the peripheral sutures were 6-0. These were the two main sutures used in all experiments.

3.1. With a surgical blade, cut the TP tendon at the midpoint. Excise a 15 mm segment of the tendon from the middle of the tendon and replace it with the ET collagen graft (**Figure 2D**). Interlock the 3-0 suture proximally away from native tendon ends (**Figure 3A**).

3.2. Pass the 3-0 core sutures above the entire length of the graft and interlock distally away from the cut end.

3.3. Secure both ends of the ET to the native tendon with 6-0 and continuous running sutures around the periphery by coupling two tendon ends (**Figure 3B**). This is done so that the graft can be moved easily on the suture by placing tension on the native tendon[20].

3.4. After securing the suture as described above, manually ensure that the tension on the sutures is appropriate and that there is no flaccidity in the entirety of the suture.

REPRESENTATIVE RESULTS

We have used collagen grafts fabricated from type I collagen, as this is the predominant protein found in the tendon. It constitutes almost 95% of total collagen in the tendon; hence, collagen has exhibited all ideal properties for mimicking tendon *in vivo* [21,22].

In this study, the type I collagen used was extracted from rat tail tendon and dissolved in the acetic acid (2.15 mg/mL). To polymerize this collagen, it was neutralized with sodium hydroxide *in vitro,* which formed non-cross-linked anisotropic collagen fibrils. This hydrogel contains 98% fluid and could mimic living tissue *in vivo* within 20 minutes during fabrication[23]. However, this hydrogel is mechanically weak; therefore, to increase mechanical properties, we have developed a method for rapid compression of collagen hydrogel by a technique known as 'plastic compression', where the degree of compression is directly proportional to the applied weight on the top and released fluid from the fluid leaving surface (FLS) [19] .

Spiral rolling of this graft increases its mechanical properties[19], but the graft remains significantly weaker than the native tendon. To address this issue, we have developed a novel modified suture technique by placing suture points, not at the edge of ruptured tendons but proximally and distally away. Thus, the strength of the repair is on the sutures and suture points and not on the mechanically weaker tendon graft.

To demonstrate the functionality of the developed novel suture technique, a lapine TP tendon was excised. The gap was filled with a 15 mm long tendon graft secured with 6-0 sutures, and 3-0 interlocked sutures were placed at 70 mm to act as load barriers (**Figure 3A**). The mean break strength of repair was 50.62 ± 8.17 N, which was significantly higher ($p < 0.05$) than that of the control Kessler repair of 12.49 ± 1.62 N (**Figure 4A**). Hence, core suture length and their

interlocking away from the tendon ends significantly influence resistance of the tendon and the repairs from failing at higher magnitude forces[24,25].

This resistance was inadequate in the control repairs which caused early repair failure and strain failure of more than 20% on the tendon. However, this is a physiological anomaly, as tendons *in vivo* are never subject to 20% strain due to there not being enough space for a tendon to extend that much; therefore to test feasibility of the suture technique *in vivo* models, we have performed repair *in situ* and calculated a mean break strength of 24.60 ± 3.92 N, which is significantly higher than the control mean break strength of 13.98 ± 2.26 N (**Figure 4B**).

FIGURES AND TABLE LEGENDS

Figure 1: Neutralized collagen hydrogel (pH 7.4) (pink color) cast in the stainless steel mold. Gel was allowed to remain in a CO_2 incubator at 37 °C for 20 minutes for fibrillogenesis to occur. The scale bar is shown at the bottom.

Figure 2: Plastic compression process. (A) The collagen hydrogel placed in between nylon meshes with a constant static load of 120 grams applied. Drained fluid was absorbed by four layers of filter paper. The arrow shows the fluid leaving surface (FLS) for the gel. **(B)** Four layers of compressed collagen sheets were rolled along the axis to form 'engineered tendon' (ET). **(C)** The section of ET was cut into 15 mm segments to mimic tendon. **(D)** The tendon defect was created in the native tendon (NT) by excising a 15 mm segment of the posterior tibial tendon, and the defect was filled with ET. This panel was modified from previous work[26].

Figure 3: (A) Tendon defect was filled with ET and secured with 6-0 sutures, and the 3-0 interlocking four strand suture technique was performed passing above graft in the 30 mm region. Block arrow shows the starting point for the suture and the blank arrow shows the end point of the suture. This panel was modified from previous work[26]. **(B)** Feasibility of performing developed suture technique in a space inside lapine model (*in situ*).

Figure 4: Mechanical strength. (A) A mechanical test output of the repair and **(B)** *in situ* mechanical test output (Error bars = SD; $*p < 0.05$, one-way ANOVA with Bonferroni correction). This panel was modified from previous work[26].

DISCUSSION

In this study, tissue engineered type I collagen grafts was chosen as a tendon graft because collagen is a natural polymer and used as a biomaterial for various tissue engineering applications[27,28]. Also, tendon collagen constitutes 60% of the dry mass of tendon, out of which 95% is type 1 collagen [21,29-32]. For successful engraftment to occur, mechanical properties of the graft should ideally match the native tendon[33]; however, with current engineering techniques, the mechanical properties of ET (4.41 N) are significantly inferior to the native tendon (NT) (261.08 N)[33]. It is proposed that this is due to the highly organized hierarchical arrangement of collagen fibril in the native tendon, which remains a challenge to engineer and match its mechanical properties[34]. We have tried to increase the density of the ET matrix by applying a

static weight of compression to the collagen hydrogel[33]; however, the architectural complexity from which the tendon acquires its strength is more intricate. Methods to accrue mechanical strength arguably are best attained *in vivo*, where the host biological processes can act on the remodelling of the extracellular matrix. Therefore, in this study, another strategy was adopted to modify the current suture technique as post tendon repair; the mechanical strength of the repaired tendon graft is entirely dependent on the suture technique[8,9]. Hence, by modifying existing suture techniques, we can offload the engineered tendon graft until cell and ECM induced remodelling occurs as a new approach.

To date, there are various suture techniques available to repair the tendon, none of which is a gold standard; however, the modified Kessler suture technique is widely used to repair tendons because it is less obstructive and damaging to tendons[35,36]. The flexor digitorum profundus muscle tendon of lambs, when sutured with the 6-strand Savage technique, was reported to have a break strength of 51.3 N, but when a modified Kessler suture technique was used, the break strength was 69.0 N[7]. However, in this study, when the tendon gap of 15 mm was filled with ET and repaired with Modified Kessler suture technique, the repair failed at an early stage with a break strength of 12.49 N (**Figure 4**). This low value makes the technique clinically irrelevant. Similar findings have been reported by De Wit *et al.* in a porcine flexor repair tendon model, suggesting that Kessler repair failed at suture rupture by reducing gapping by 15% as compared to cruciate repair, where gapping is reduced by 87% and repair failed at suture pull-out[38]. Thus, there is a need for another strong suture technique, which could hold mechanically weaker ET in place.

A novel modified suture technique was developed by using four core sutures over the entire length of the ET and above the opposite tendon. These sutures were interlocked onto the suture material itself at some distance away from each tendon end. This is mainly because it has been reported that putting suture knots at equal distance and equal load sharing tension on all suture strands increases their mechanical properties[39]. A balanced repair can also be achieved by keeping a continuous suture, and staggering the repair to allow for compression at the repair site[40].

In this study, 3-0 sutures were used for outer interlocked sutures considering that rabbit TP tendon has a length, width and thickness of 62.4 mm, 5 mm and 1.5 mm, respectively. 6-0 sutures were used to hold the ET in place. Although we have tried other absorbable suture materials, it would not be appropriate as they become weaker over a period *in vivo*[41]. A primary reason polypropylene sutures was selected is because they are a monofilament as well as non-absorbable and they do not cause structural or tensional modifications under load[42]. We tested all sutures from 2-0 to 7-0, but 3-0 and 6-0 were found to be ideal candidates for our experiments [26].

The primary reason for using 4 strand repair was to avoid excessive damage to ruptured tendon ends with a greater number of suture strands as it has been reported that a normal surgical suture in a tendon results in the formation of an acellular region[43]. It has been hypothesized that this is due to the cells migrating out from the compressive load that is put on the tendon, and

normally these cells are subject to tensile loading[17]. This migration of cells away from the suture could then cause weakening of the matrix as there is a paucity of cells to maintain and turnover the matrix, which could cause early tendon failure[17]. We can use more strands of sutures that are biomechanically twice as strong (*ex vivo)* than 4-strand sutures[11,12,44,45]; however, these repairs are not widely practiced and their clinical limitations are currently being evaluated[13-16].

The placement of the suture knot is important but there are arguments for and against externalizing the suture. Having the suture on the outer surface can potentially snag against structures like tendon pulleys and reduce glide. In a study, the areas where suture knots are placed inside illustrated a decrease in gliding resistance compared with the Kessler repair, which has suture knots outside[46]. Studies conducted in the canine model concluded that at a higher magnitude of the force, fewer suture knots located outside the repair and away from the tendon ends had survived compared with those located inside the repair[47,48]. However, internalizing the knot potentially reduces the contact surface of the healing tendon. There is also the consideration that tissue damage arises from the suture needle piercing the tendon and the greater number of passes relates to the increased tendon trauma[49].

To secure ET in between the tendon gap, a standard of running sutures[50] along the edge of the tendon and ET was performed. This was done because there was a need for peripheral sutures that are strong enough to hold the ET in place in the initial phase of healing until cell and ECM induced remodelling could occur[50]. The major problem was the variation in the mechanical properties of the NT and ET, which could result in early gap formation although the ET was stress shielded. On the other hand, applying a more secure technique such as horizontal mattress intrafiber sutures[51], Halsted continuous horizontal mattress sutures[52,53], cross stitch epitendinous repair techniques[54-57] or running lock sutures[58,59] would have ruptured ET as it is fragile. Thus, we chose running sutures as a peripheral suture technique which is simple and holds the ET intact in all directions.

From a tissue engineering perspective, we need to study whether this method can be used to fill a tendon gap greater than 1.5 cm. To use this graft in human clinical trials, we need to further investigate the immunological response to the xenogeneic source of collagen although this can be achieved by developing clinical grade collagen. The protocol described herein establishes the feasibility of the developed suture technique within available anatomical spaces in a porcine lapine model. This developed suture technique has suture points proximally and distally equidistance away from ruptured tendon ends so that engineered tendon graft could be off loaded. Hence, it could mature and integrate *in vivo*.

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DISCLOSURES:
The authors declare that they have no conflicts of interest.

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Figure

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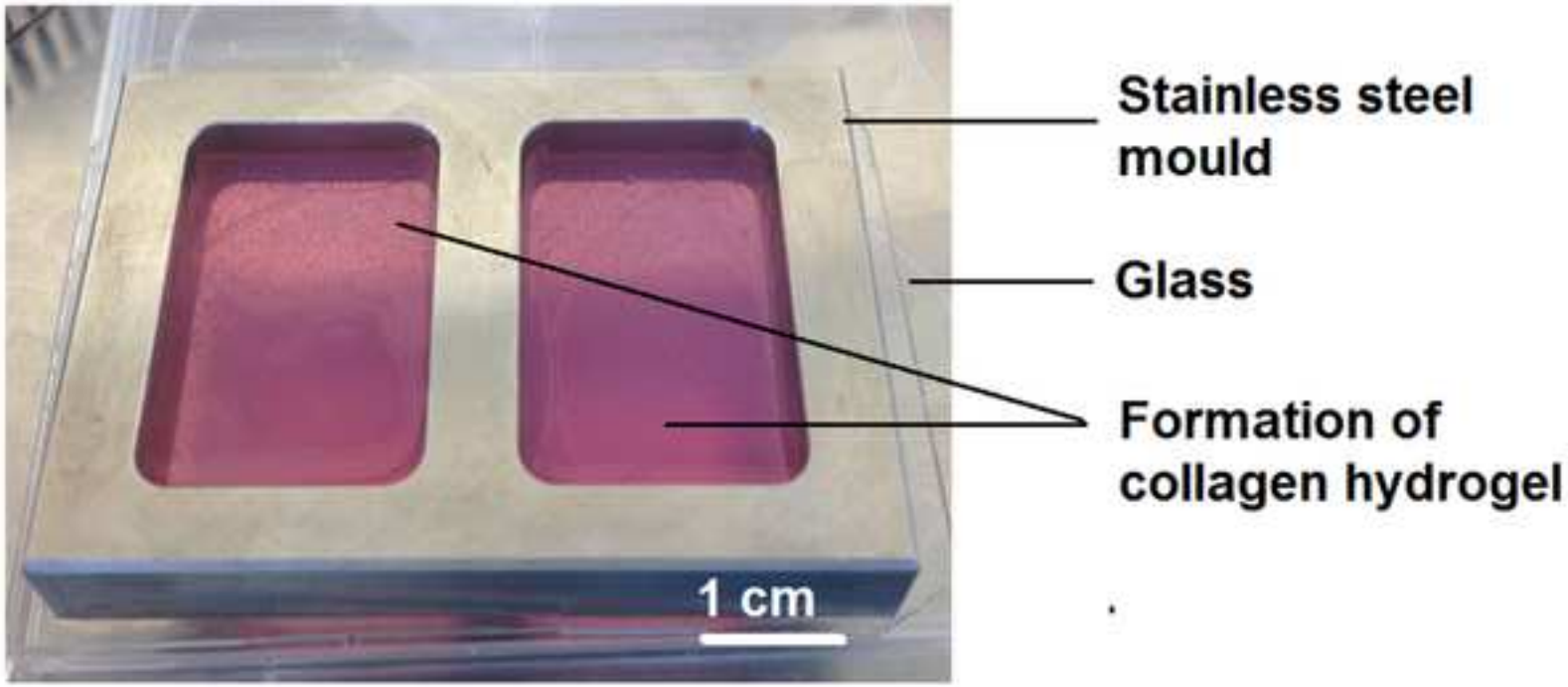

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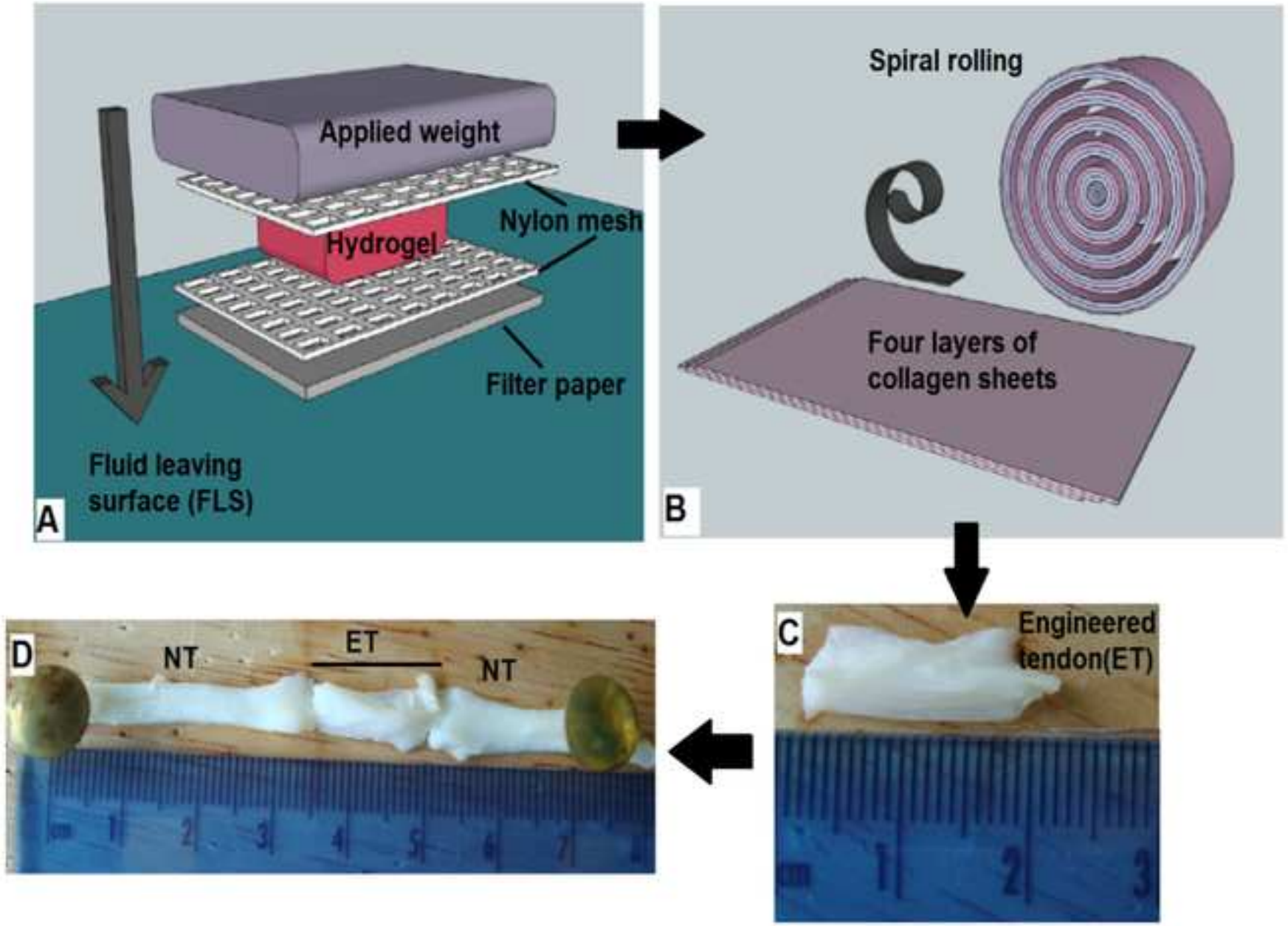

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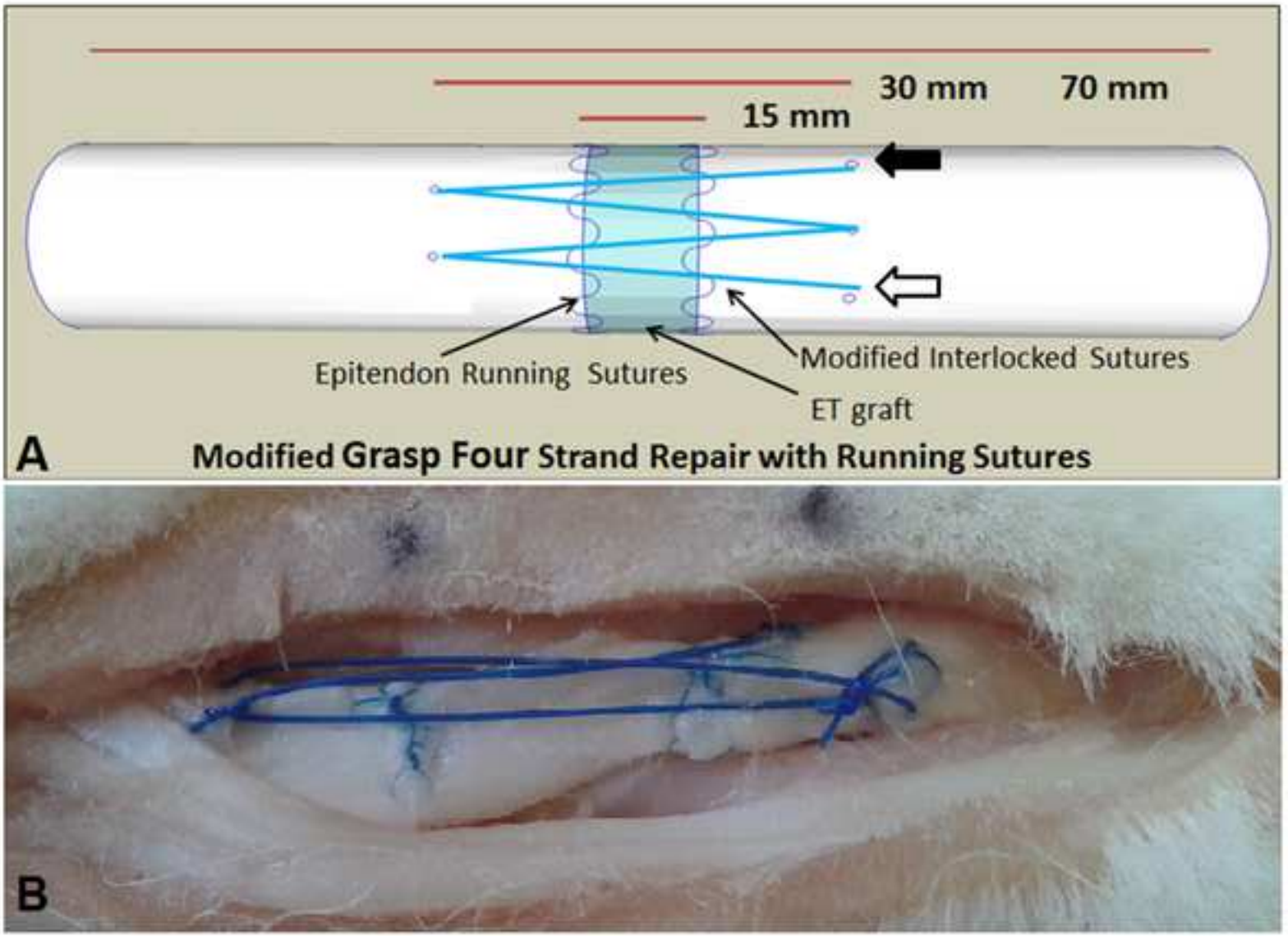

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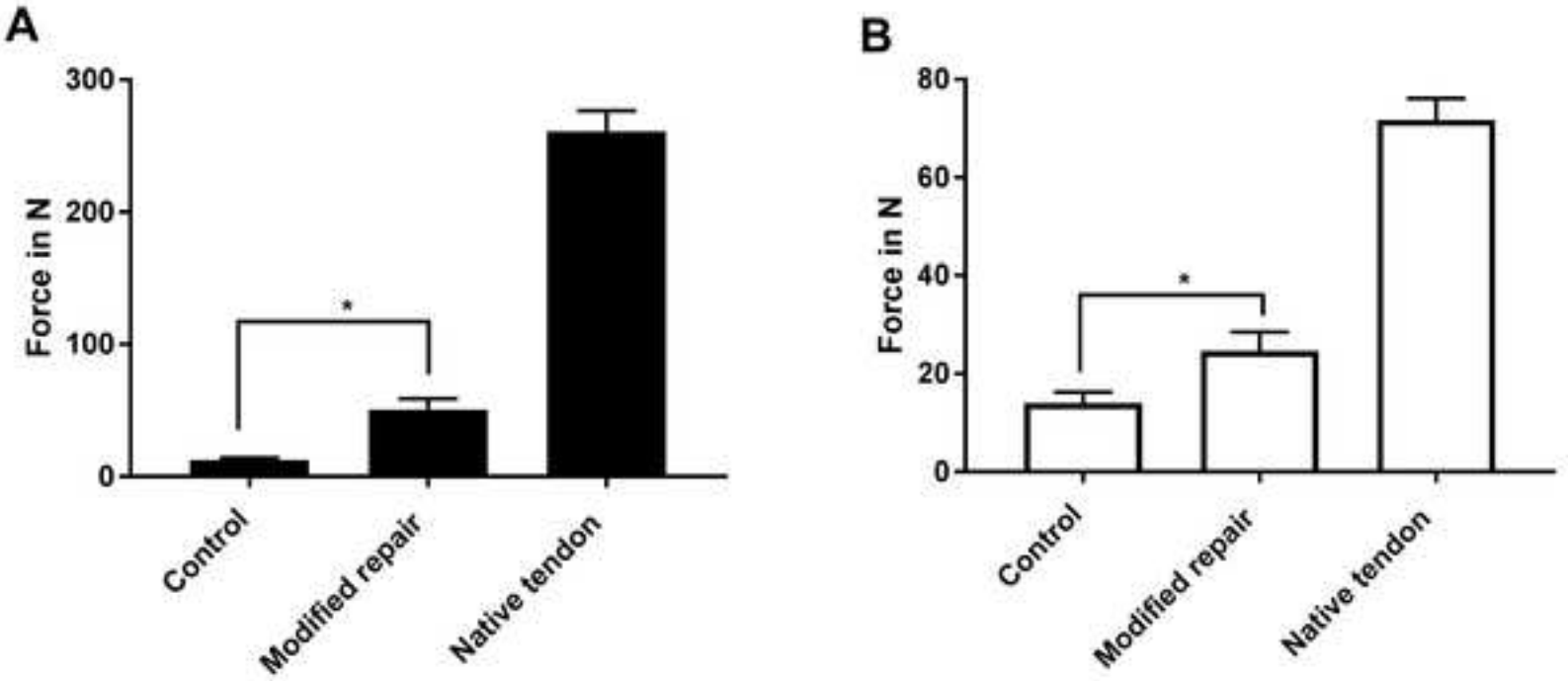

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Rat tail type 1 Collagen	First Link, Birmingham, UK	60-30-810	
prolene sutures 6-0	Ethicon Ltd, Edinburgh, U.K.	EP8726H	
prolene sutures 3-0	Ethicon Ltd, Edinburgh, U.K.	D8911	
Whatman filter paper	SIGMA-ALDRICH	WHA10010155	
Gibco™ DMEM, high glucose	Thermo Fisher Scientific	11574486	
Nylon mesh	Plastok® (Meshes and Filtration) Ltd.	NA	

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1. There are still grammar and usage errors; please proofread.

    - Manuscript has been proofread by all authors

2. 2.3: Please provide more details on this step. Which site will you be demonstrating on the video? How is excision done?

    - Site location and excision method has been mentioned in the manuscript.

3.1-3.2: While there is a figure demonstrating this, more written description of the sutures would be appropriate.

    - Suture description has been written in the section

4. 3.3: How exactly will you check tension?

    - It has been mentioned in the manuscript.

5. There still is not an appropriate Figure or Table for the Results section of the video, and I'm not sure what you mean by "real-time results can be provided during video recording". Please provide something demonstrating representative results, e.g., Figure 5A (modified as necessary) from your 2013 BioResearch paper.

    - An additional figure has been introduced in the manuscript (Figure 4) with mechanical data.

6. Figures 2D and 3A look to be from your 2013 paper; while permission does not seem to be necessary, as it was published under a CC license, please still cite the paper in the legends where appropriate.

    - It has been sited appropriate
    -

7. Figure 3: What are the white and black arrows here?

    - It has been described in the figure legends.

8. Discussion, second paragraph: Figure 1 is cited, but this does not seem to be correct.

    - Corrected

9. Please include more materials in the Table of Materials (e.g., media, nylon mesh).

    - Method section has been updated.