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NANOSTRUCTURED FIBRIN-BASED HYDROGEL MEMBRANES FOR USE AS AN AUGMENTATION STRATEGY IN ACHILLES TENDON SURGICAL REPAIR IN RATS

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Abstract

Hydrogels are polymeric biomaterials characterised by their promising biological and biomechanical properties, which make them potential alternatives for use in tendon repair. The aim of the present study was to generate *in vitro*, and determine the therapeutic efficacy *in vivo*, of novel nanostructured fibrin-based hydrogels to be used as an augmentation strategy for the surgical repair of rat Achilles tendon injuries. Fibrin, fibrin-agarose and fibrin-collagen nanostructured hydrogels (NFH, NFAH and NFCH, respectively) were generated and their biomechanical properties and cell-biomaterial interactions characterised *ex vivo*. Achilles tendon ruptures were created in 24 adult Wistar rats, which were next treated with direct repair (control group) or direct repair augmented with the generated biomaterials (6 rats/group). After 4 and 8 weeks, the animals were euthanised for macroscopical and histological analyses. Biomechanical characterisation showed optimal properties of the biomaterials for use in tendon repair. Moreover, biological analyses confirmed that tendon-derived fibroblasts were able to adhere to the surface of the generated biomaterials, with high levels of viability and functionality. *In vivo* studies demonstrated successful tendon repair in all groups. Lastly, histological analyses disclosed better tissue and extracellular matrix organisation and alignment with biomaterial-based augmentation strategies than direct repair, especially when NFAH and NFCH were used. The present study demonstrated that nanostructured fibrin-collagen hydrogels can be used to enhance the healing process in the surgical repair of tendon ruptures.

Keywords: Tissue engineering, tendon repair, fibrin-based hydrogel, natural biomaterials, *in vivo* regeneration, orthopaedics.

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List of Abbreviations

CD	cluster of differentiation
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
H&E	haematoxylin and eosin
NFAH	nanostructured fibrin-agarose hydrogel
NFCH	nanostructured fibrin-collagen hydrogel
NFH	nanostructured fibrin hydrogel
PBS	phosphate-buffered saline
SD	standard deviation
TDF	tendon-derived fibroblasts
TE	tissue engineering
WST-1	water-soluble tetrazolium salt-1

Introduction

Tendons are structures composed of a highly organised collagen-rich connective tissue that connects the muscles and bones of the musculoskeletal

system, thereby making body locomotion possible. The structure and function of tendons are generally damaged in tendinopathies and traumatic injuries. Of relevance, alterations within the musculoskeletal system are the second largest contributor to disability and more than 30 million tendinopathies are diagnosed annually worldwide (Costa-Almeida *et al.*, 2019). The surgical repair of tendons is indicated when conservative treatments fail. Different strategies are available, ranging from direct surgical repair to the use of autografts, allografts or commercial artificial grafts. However, the results of these conventional strategies have not been completely satisfactory (Holm *et al.*, 2015; Kadakia *et al.*, 2017). The variability in outcomes is probably related to poor biological tissue healing (Müller *et al.*, 2015).

The use of new biomaterial-based strategies, within the framework of a TE approach, holds potential to improve the biological processes involved in the regeneration of repaired tendons, thus favouring positive clinical outcomes (Domingues *et al.*, 2015). During recent decades, TE approaches have progressively achieved the generation of bioartificial substitutes that have shown promising results in many biomedical applications (Santisteban-Espejo *et al.*, 2018; 2019). Among the most widely used biomaterials to produce engineered tissues or organs by TE are natural hydrogels, which are characterised by their high hydration rate, polymeric nature and high biocompatibility – features that often mimic the texture and properties of the native ECM (Lee and Kim, 2018; Scionti *et al.*, 2014; Sudhakar *et al.*, 2015). Fibrin is a biodegradable protein widely used in TE because of its low cost, good biocompatibility, porous fibrillar structure and availability from the patient's own plasma (Ahmed *et al.*, 2008; Campos *et al.*, 2020). Although these properties are suitable for the generation of several artificial substitutes by TE, their biomechanical properties and their stability *in vivo* still constitute an important limitation (Reddy *et al.*, 2015). In this regard, the use of different crosslinking strategies (physical, chemical or a combination of both) was shown to modulate and improve natural hydrogel microstructure, dimensions and biochemical properties depending on the native tissue or organ to be repaired (Campos *et al.*, 2016; Campos *et al.*, 2018; Carriel *et al.*, 2017b). Different engineered tissues, such as the cornea and skin, have recently been developed using these techniques together with a fibrin hydrogel – an approach which has already been transferred to clinical practice as an advanced therapy medicinal product (Alaminos *et al.*, 2006; Carriel *et al.*, 2011b; González-Andrades *et al.*, 2017; Rico-Sánchez *et al.*, 2019).

Recently, González-Quevedo *et al.* (2020) have developed a fibrin-agarose biomedical model enhanced by crosslinking protocols, specifically through physical nanostructuring and the use of genipin, to repair damaged tendons. This strategy made it possible to improve biomechanical properties and biocompatibility of the replacement tissue as well as the healing process. Moreover, collagen-based biomaterials used to generate tendon substitutes have also been recently developed (Buschmann and Meier Bürgisser, 2017; Gabler *et al.*, 2018). Although these biomaterials have excellent properties, such as low immunogenicity, hydrophilicity and easy processing, they tend to shrink. In addition, substitutes made of collagen are not thermostable and have poor physical and chemical plasticity (Copes *et al.*, 2019; Gu *et al.*, 2019; Liu *et al.*, 2019).

Previous studies (González-Quevedo *et al.*, 2020) have hypothesised that the use of nanostructured fibrin-based hydrogels for tendon ruptures could enhance tendon healing, resulting in a better structural and functional organisation. Accordingly, the main aims of the present study were to develop and characterise nanostructured fibrin, fibrin-agarose and fibrin-collagen hydrogels *in vitro* and to investigate their histological effects *in vivo* in a rat model of Achilles tendon rupture.

Materials and Methods

The experimental methods are summarised in Fig. 1.

Ex vivo biomaterial generation and characterisation

42 nanostructured hydrogels were generated and used for *ex vivo* (8 animals per group) and *in vivo* (6 animals per group) studies. The biomaterials were generated as reported below.

Generation of hydrogels

To prepare 30 mL of fibrin hydrogel, 25.8 mL of human plasma from blood donors (Centro Regional de Transfusiones Sanguíneas, Granada, Spain) were used as the source of fibrinogen. To prevent spontaneous fibrinolysis of the resulting fibrin hydrogels, 450 μL of tranexamic acid (Amchafibrin, Fides-Ecofarma, Valencia, Spain), an anti-fibrinolytic agent, were added to the solution. Then, 3 mL of 2 % $CaCl_2$ were added to trigger formation of the fibrillar fibrin hydrogel. This solution was carefully mixed and placed in 55 mm diameter Petri dishes until complete gelation under standard culture conditions (37 °C and 5 % CO_2).

In accordance with previously described protocols (Campos *et al.*, 2020), 22.8 mL of human plasma, 2.25 mL PBS and 450 μL tranexamic acid were used to obtain 30 mL of fibrin-agarose hydrogel. To promote gelation, the solution was mixed with 3 mL of 2 % $CaCl_2$ and 1.5 mL of melted 2 % type VII agarose (Sigma-Aldrich). The solution was carefully mixed and kept in 55 mm diameter Petri dishes until complete gelation, which took 1 h under standard culture conditions.

To obtain 30 mL of fibrin-collagen hydrogel, 12 mL of type I collagen from bovine dermis (Sigma-Aldrich) was neutralised using 2 mL PBS containing 0.01 mol/L NaOH, followed by careful mixing and storage at ~ 4 °C under mild agitation until use.

Then, the fibrin solution was prepared (12.275 mL human plasma, 1 mL PBS, 1.5 mL $CaCl_2$ and 225 µL tranexamic acid) and carefully mixed with the collagen solution. This solution was placed in 55 mm diameter Petri dishes and kept for at least 2 h under standard culture conditions until complete gelation.

Hydrogel nanostructuring

To proceed with nanostructuring of the fibrin-based hydrogels, the hydrogels were first placed between two nylon filter membranes with a pore size of 0.22 µm (Merck-Millipore) and, then, compressed between two sheets of Whatman absorbent paper using a flat glass device, according to previously described procedures (Campos *et al.*, 2016). After uniform mechanical pressure (5100 Pa) was applied for 3 min, a NFH, a NFAH and a NFCH with a thickness of 40-60 µm were obtained (Campos *et al.*, 2020; Carriel *et al.*, 2019; Chato-Astrain *et al.*, 2018).

Biomechanical evaluation

5 small samples (30 × 10 × 0.05 mm) of each generated biomaterial (NFH, NFAH and NFCH) were harvested and subjected to tensile tests using a universal testing machine (Model 5943, Instrom, Needham, MA, USA) prepared for 50 N load-cell sampling. For these tests, the samples were oriented lengthwise along the direction of tension and clamped at each end, leaving a constant distance of 10 mm between the clamps, in accordance with previously described protocols (Carriel *et al.*, 2017a; Scionti *et al.*, 2014). Young's modulus, the stress at break (σ break) and the strain at break or deformation (ε break) were determined at a constant strain rate of 5 mm/min at room temperature. Young's modulus was calculated as the tangent modulus of the initial linear portion of the stress-strain curve in each experimental run, whereas stress and strain at break values were determined by identifying the point of the stress-strain curve where the fracture occurred.

Cell-biomaterial interaction

To determine the *ex vivo* cytotoxicity or biocompatibility of the generated biomaterials, TDF were used as previously described (González-Quevedo *et al.*, 2020). Cells were isolated from rat tail tendons of 3 healthy donor rats (see section Laboratory animals for further details). The tendons were carefully dissected, cleaned of the surrounded connective tissue, mechanically fragmented into small pieces and, then, digested for 7 h at 37 °C using 0.3 % type I collagenase solution (Gibco). Once the ECM was fully digested, TDF were harvested by centrifugation and, then, cultured in basal medium [450 mL DMEM, 5 mL antibiotic-antimycotic cocktail solution (100 U/mL penicillin G, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin B), 50 mL foetal bovine serum] at 37 °C and 5 % CO_2. The culture medium was replaced every 3 d and cells were cultured until they reached 80 % confluence. All reagents used to prepare the basic culture medium were purchased from Sigma-Aldrich.

Each biomaterial was cut into 8 mm diameter discs to be used in 24-well plates. TDF from passages 4-6 were harvested and cultured on top of each nanostructured hydrogel at 2 × 10^4 cells/scaffold. To assess cell function and viability, the following procedures were used as previously described (Campos *et al.*, 2016; 2018): live/dead assay (LIVE/DEAD™ Viability/Cytotoxicity Kit, Thermo Fisher Scientific), WST-1 assay (Roche Diagnostics) and quantification of released DNA. In addition, TDF cultured on chamber slides (2 × 10^4 cells/chamber) were used as a positive technical control (2D positive

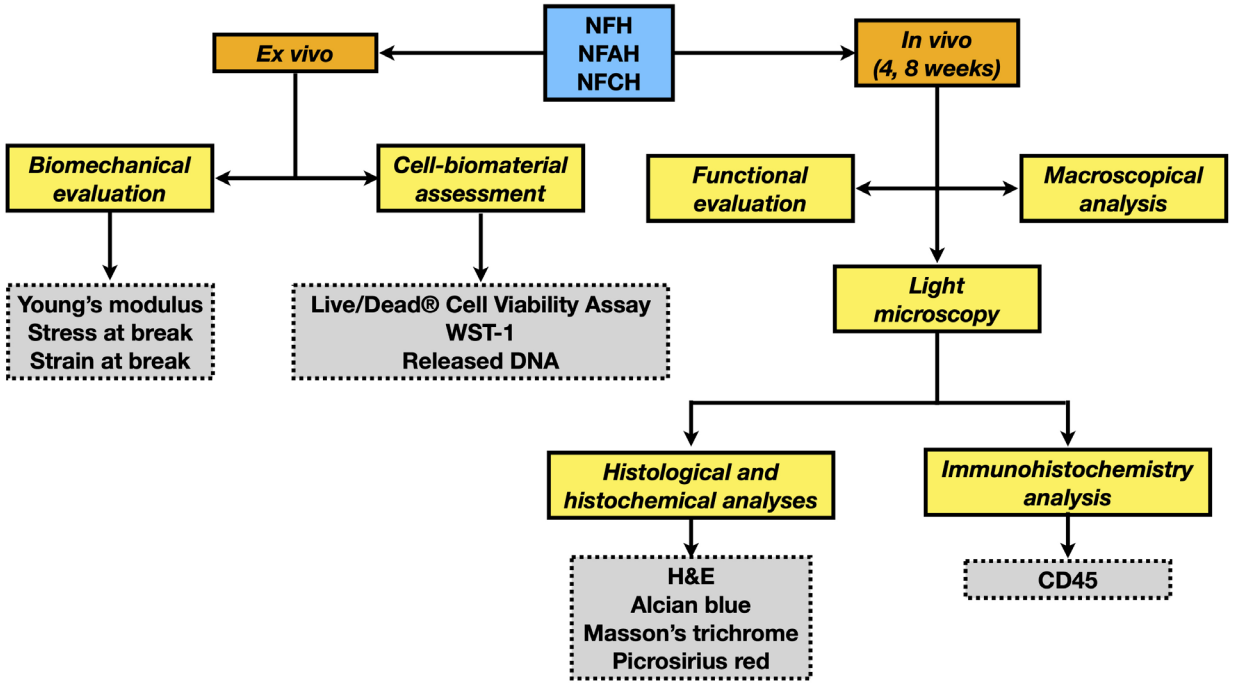


Fig. 1. Description of the experimental methods used.

control), while cells seeded and incubated with 2 % Triton X-100 were used as a technical negative control (2D negative control). These analyses were performed in quintuplicate.

In vivo evaluation

Laboratory animals

27 12-week-old male Wistar rats (body weight 250-300 g) were provided by and maintained at the Experimental Unit of of the Insituto de Investigación Biosanitaria Ibs.GRANADA (Granada, Spain). This work was conducted with the approval of the University of Granada Ethics Committee (approval number: 231-CEEA-OH-2018). The animals were housed in individual cages in a temperature-controlled room (21 ± 1 °C) on a 12 h light/dark cycle, with *ad libitum* access to tap water and standard rat chow.

Surgical procedure and experimental groups

The surgical procedures were performed under general anaesthesia [intraperitoneal injection of a mixture of acepromazine (0.001 mg/g body weight; Calmo-Neosan®, Boehringer Ingelheim,), ketamine (0.15 mg/g body weight; Imalgene 1000®, Boehringer Ingelheim) and atropine (0.05 μg/g body weight, B.Braun, Melsungen, Germany)]. The skin area where surgery was performed was shaved and disinfected with 10 % povidone iodine solution.

Under sterile conditions, a longitudinal incision was made above the gastrocnemius muscle to the calcaneus bone in the left legs of 24 animals, which were then randomly divided into 4 groups of 6 rats each. In the control group, the central portion of the Achilles tendon was sectioned and immediately suture-repaired following a modified Kessler method and using 4-0 nylon suture. In experimental groups, the tendons were repaired as described for the control group and, then, externally covered or augmented with NFH, NFAH or NFCH. The biomaterials were cut (30 × 10 × 0.05 mm) and wrapped around the repaired tendon, without suturing.

In all groups, the surgical procedure was performed by the same orthopaedic surgeon (DGQ) following the same surgical technique and the plantar tendon was used for internal splitting. The skin was closed with interrupted non-absorbable sutures. After the surgical procedure, the rats were kept in cages and allowed to move without restriction throughout the study period. Healthy animals ($n = 3$) and also the right Achilles tendon in each experimental animal were used as a native or healthy control for the different analyses described in the following sections.

Native and repaired tendons were harvested from each group at 4 and 8 weeks after surgery ($n = 3$ in each group). Animals were euthanised using anaesthesia followed by an intracardiac injection of Euthanex® (INVET, Bogotá, Colombia). These cut-off times were chosen according to postoperative findings reported in previous studies (Battaglia *et al.*, 2003; González-Quevedo *et al.*, 2020).

Functional evaluation

Walking track analysis was used to evaluate motor function in the operated leg in each animal. The hind paws were painted with water-based blue ink and the animal was allowed to walk through an enclosed walkway approximately 80 cm long, 10 cm wide lined with white paper. Tracks were evaluated in each animal at 4 or 8 postoperative weeks. Sensitivity was tested with the pinch test of sensory recovery, for which a mild pinching stimulus was applied with a forceps to the toes of the operated leg until a withdrawal reaction was observed (Chato-Astrain *et al.*, 2018). The responses in operated animals were compared to that in healthy animals (control).

Macroscopical and histological analyses

Normal and repaired Achilles tendons were harvested from each animal after euthanasia, fixed for 48 h in 10 % neutral buffered formalin, examined macroscopically and, then, processed for histological analyses.

The aim of macroscopical qualitative analysis was to observe whether the operated tendons maintained their anatomical characteristics, dimensions and the connection between the two tendon stumps. In addition, the appearance of any pathological process (inflammation, rupture, deformation, biomaterial rejection, granuloma formation or tissue degeneration) was also carefully explored.

For histological analyses, fixed samples were dehydrated, embedded in paraffin-wax and 5 μm-thick sections were obtained using a microtome Microm HM315 (Thermo Scientific). Histological sections were dewaxed, hydrated and stained with H&E for general histological evaluation. In addition, acid proteoglycans were identified by alcian blue histochemical staining (pH 2.5). The characteristic collagen network (only fibrillar collagen fibres) in tendons was evaluated using Masson's trichrome and picrosirius red histochemical methods. Alignment of the fibrillar collagen fibres was evaluated using picrosirius red staining (which enhances the natural birefringence of collagens) under polarised light microscopy, as previously described (Carriel *et al.*, 2011a; 2013). The presence or infiltration of inflammatory cells within regenerating tendons was determined using indirect immunohistochemistry to identify the leucocyte common antigen (CD45; 1:40, ab10558, Abcam). A ready to use horse anti-rabbit IgG conjugated with peroxidase (Polymer Kit, MP-7401, Vector Laboratories) and DAB substrate kit peroxidase (SK-4100, Vector Laboratories) were used to visualise antigen-antibody reaction. For histological analyses, tendons from healthy animals as well as those obtained from the right (unoperated) leg in experimental animals were used as native controls.

All micrographs of the sutured region were obtained using a Nikon Eclipse 90i microscope equipped with a Nikon DS-Ri2 digital camera and NissElements software.

Table 1. Histological scoring system.

Collagen fibres/ECM organisation	
Normal (wavy, compact, parallel)	3
Mild	2
Moderate	1
Abnormal	0
Cell alignment and distribution	
Normal (uniaxial and homogeneous)	3
Mild	2
Moderate	1
Abnormal	0
Cell nucleus morphology	
Normal (tenocytes)	3
Mild	2
Moderate	1
Abnormal (larger, polymorph)	0
Degenerative changes/tissue metaplasia	
Normal	3
Mild	2
Moderate	1
Severe	0
Vascularisation (angiogenesis)	
Normal	3
Mild	2
Moderate	1
Severe	0
Inflammation	
Normal	3
Mild	2
Moderate	1
Severe	0
Native tendon	18

Quantitative analyses

Cellularity was determined in control and experimental groups using Image J software (NIH) according to a previously described procedure (Carriel *et al.*, 2013; 2017a). Briefly, H&E nuclear staining was selected in each slide with the "threshold" function and the images were converted to binary format. Once the images were converted, the percentage of area occupied by cell nuclei within the entire area of analysis was automatically determined with the "measure" function of Image J software.

Histological scoring was performed using a grading system reported previously (Stoll *et al.*, 2011) and recently modified (Table 1) (González-Quevedo *et al.*, 2020). In this system, a native Achilles tendon (without lesions) is assigned a score of 18.

Statistical analysis

To identify statistically significant differences between conditions, the Shapiro-Wilk test was used to verify the normal distribution of values. Given that the values presented a non-normal distribution, statistically significant differences and power were determined with the Kruskal-Wallis test, followed by *post-hoc* analysis between groups with the non-parametric Mann-Whitney test. For each analysis, a Bonferroni correction for multiple comparisons was used, which yielded a statistically significant $p < 0.005$ for the biomechanical analysis, $p < 0.00454$ for WST-1 and DNA and $p < 0.00138$ for cellularity and histological score. All statistical tests were two-tailed and all analyses were carried out with SPSS 24.0 software (SPSS Inc.) and G*power 3.1.9.6 (Universität Kiel, Kiel, Germany).

Data availability

The data supporting the findings of this study are available upon reasonable request to the corresponding author.

Results

***Ex vivo* properties of biomaterials**

Biomechanical characterisation of biomaterials

The average value of Young's modulus was the highest in the NFAH group, followed by rats treated with NFH and NFCH (Table 2), but no significant differences among the three biomaterials were observed performing the Kruskal-Wallis test ($p = 0.01640$) or *post-hoc* comparisons (Table 2). The stress-at-fracture values were higher in the NFAH group compared to the other biomaterials, with no significant differences measured by Kruskal-Wallis test ($p = 0.02187$) or *post-hoc* comparisons (Table 2). However, the deformation capacity of NFH was higher than the strain at fracture of NFAH and NFCH, but with no significant differences among groups when performing the Kruskal-Wallis test ($p = 0.47237$) or *post-hoc* comparisons (Table 2).

Ex vivo cell-biomaterial interactions

Functional and morphological analyses by LIVE/DEAD™ assay, conducted after 48 h of cell culture, confirmed the presence of viable cells (green fluorescence) on the surface of all nanostructured fibrin-based hydrogels used (Fig. 2). This morpho-functional assay showed that viable TFD mostly exhibited a flat, fusiform morphology comparable to the morphological pattern observed in the positive control group (Fig. 2). Some dead cells (red fluorescence) appeared on the surface of NFH and NFAH, but not NFCH (Fig. 2).

Quantitative biochemical analysis of cell metabolic activity, as measured by WST-1 assay, showed statistically significant differences ($p < 0.0001$, 99.9 % power) with the Kruskal-Wallis test. Normalised WST-1 values obtained in cells cultured on the surface of each of the three biomaterials were significantly higher than in the negative technical control ($p < 0.00001$, 99.9 % power). There were no statistically significant differences between the technical positive control and NFAH cultures. Interestingly, the

Table 2. Tensile mechanical results and statistical analysis. Data are shown as mean ± SD. Exact *p*-values for the Mann-Whitney non-parametric test are shown for each comparison.

		NFH	NFAH	NFCH
Young's modulus (MPa)	Mean ± SD	0.39 ± 0.06	0.46 ± 0.10	0.03 ± 0.01
	vs. NFH		0.19127	0.02016
	vs. NFAH			0.02016
Stress at fracture (σ break, MPa)	Mean ± SD	0.44 ± 0.21	0.52 ± 0.12	0.02 ± 0.01
	vs. NFH		0.24821	0.38648
	vs. NFAH			0.77283
Strain at fracture (ε break, %)	Mean ± SD	246.33 ± 69.48	182.23 ± 45.14	197.40 ± 75.88
	vs. NFH		0.56370	0.01796
	vs. NFAH			0.01706

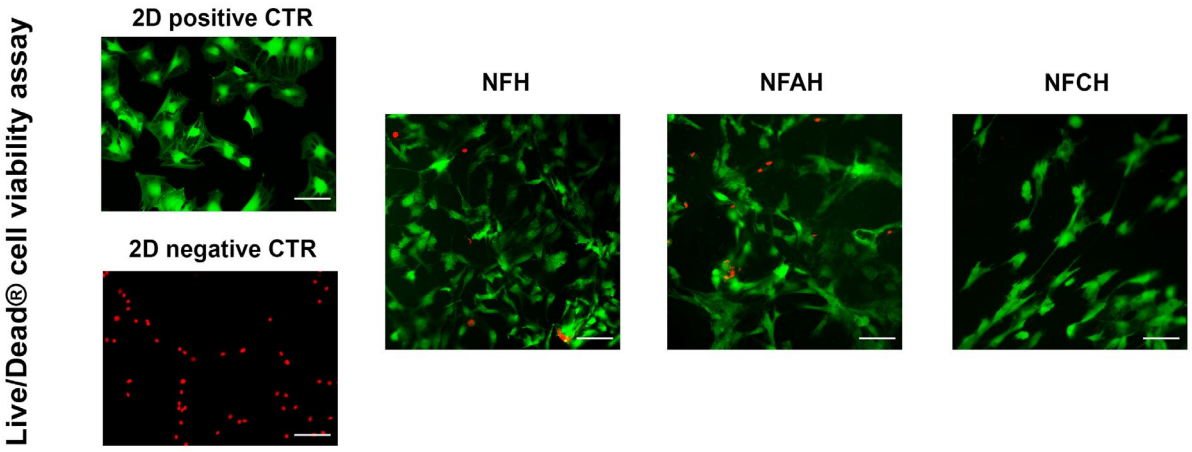


Fig. 2. Tendon-derived behaviour in experimental groups. Fluorescence microscopy with the LIVE/DEAD™ cell viability assay showing viable cells (green) and dead cells (red) in technical controls (2D positive CTR and 2D negative CTR) and in the biomaterials. Scale bar: 100 μm.

WST-1 values in cells cultured on NFH and NFCH biomaterials were lower than the values obtained in the positive technical control and NFAH (Fig. 3) but the differences were significant only when comparing NFH to both NFAH and the positive control (Fig. 3).

Spectrophotometric analysis of the DNA released by cultured cells revealed irreversible nuclear membrane damage. Only 0.13 % of the DNA was released into the culture medium in the 2D positive control, in contrast to 98.86 % in the 2D negative control (Fig. 3). The Kruskal-Wallis test yielded significant differences ($p < 0.00001$, 100.0 % power) among groups. Specifically, values in all experimental cultures were significantly lower than in the negative control, as determined by *post-hoc* comparisons. In addition, *post-hoc* comparisons showed that cells cultured with NFH and NFCH had significantly higher values than both the positive control and NFAH ($p < 0.00001$, 100.0 % power). Interestingly, the values for NFAH were significantly lower than in the 2D positive control (Fig. 3).

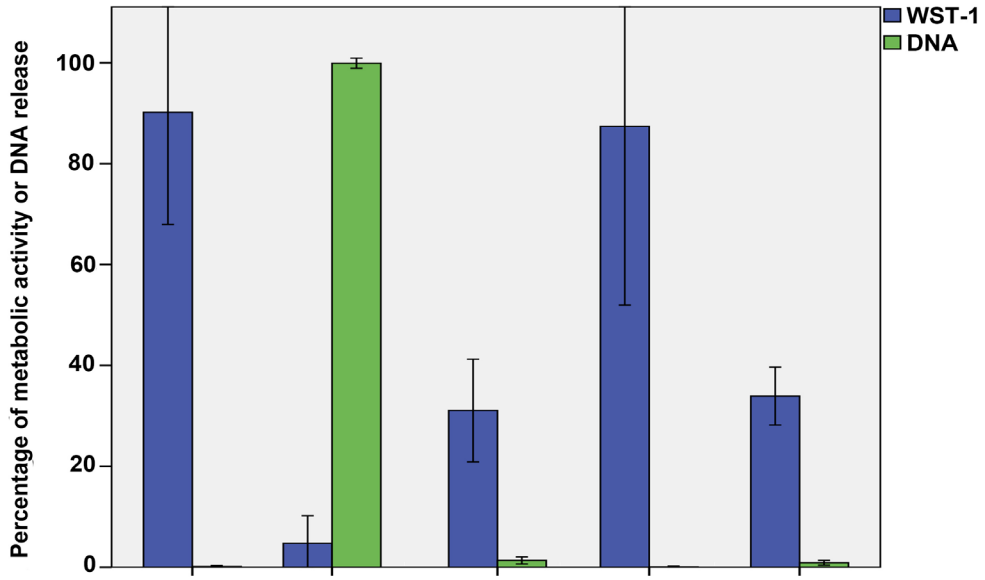
In vivo results

Functional results

Functional evaluation in animals with repaired tendon detected no limitation of movement in their cages: the injured legs were able to bear their full load during locomotion and showed normal sensitivity. In addition, examination of the walking tracks showed no significant functional impairment in any of the animals that underwent post-surgical treatment with the biomaterials and their tracks were very similar to those of animals used as controls and to healthy donors. Clinical evaluation confirmed correct wound closure with no post-surgical complications.

Macroscopical findings

The general appearance of the explanted repaired tendons showed a loss of the characteristic brightness of native tendons. In addition, a moderate increase in thickness was observed in all repaired tendons (Fig. 4). Slight signs of adhesions to the surrounding tissues were also observed, but macroscopical examination



		CTR +	CTR -	NFH	NFAH	NFCH
WST-1	*Mean ± SD*	90.20 ± 0.43	4.73 ± 0.11	31.07 ± 0.20	87.40 ± 0.69	33.93 ± 0.11
***p*-values**	*vs. CTR +*		< 0.00001*	0.85373	< 0.00001*	0.01230
	vs. CTR -			< 0.00001*	< 0.00001*	< 0.00001*
	vs. NFH				< 0.00001*	0.27287
	vs. NFAH					0.02905
DNA	*Mean ± SD*	0.13 ± 0.01	98.86 ± 0.19	1.33 ± 0.01	0.07 ± 0.01	0.87 ± 0.01
***p*-values**	*vs. CTR +*		< 0.00001*	< 0.00001*	< 0.00001*	< 0.00001*
	vs. CTR -			< 0.00001*	< 0.00001*	< 0.00001*
	vs. NFH				< 0.00001*	0.57551
	vs. NFAH					< 0.00001*

Fig. 3. WST-1 and DNA quantitative and statistical results. Graphical and numerical representation of normalised values for WST-1 and DNA release in 2D controls (CTR+ and CTR−), NFAH, NFH and NFCH. Results are shown as mean ± SD. Exact *p*-values for the Mann-Whitney non-parametric test are shown for each comparison. * Statistically significant *p*-values.

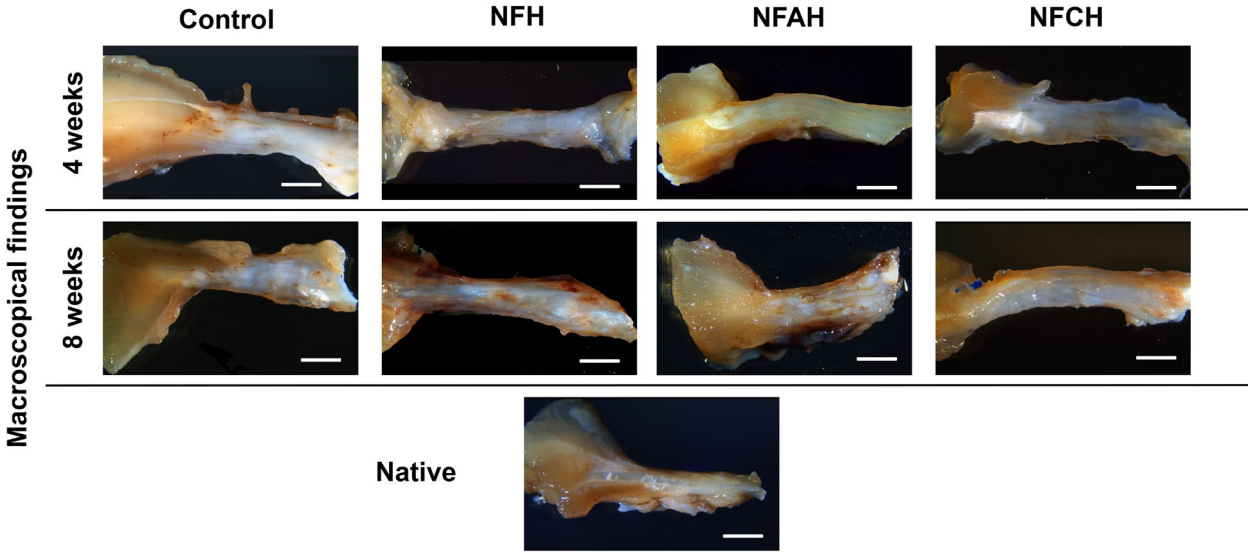


Fig. 4. Macroscopical findings in tendons from native and experimental groups. Native tendons appear less bright. In addition, all experimental conditions showed elongation and tissue oedema. All biomaterials were integrated with the tissue, with macroscopical signs of adverse reactions. Scale bar: 1 mm

did not disclose any evident signs of inflammation, tissue necrosis or tendon rupture (Fig. 4).

Macroscopical analysis confirmed that all biomaterials used were integrated or reabsorbed 4 weeks and especially 8 weeks post-surgery. There were no macroscopical signs of the presence of any of the biomaterials generated in the present study. A final observation regarding tendon dimensions and shape was that repaired tendons were slightly elongated compared to their native counterparts (Fig. 4).

Histological findings

Staining of repaired tendon sections with H&E revealed active tissue regeneration characterised by increased inflammatory cell content and ECM remodelling, attributable to a progressive decrease in vascularisation and increase in collagen orientation. Analyses conducted 4 weeks after surgery revealed the presence of blood vessels and a loss of the characteristic tendon tissue alignment in all operated animals compared to the native control group (Fig. 5). After 8 weeks, a decrease in cell content and an

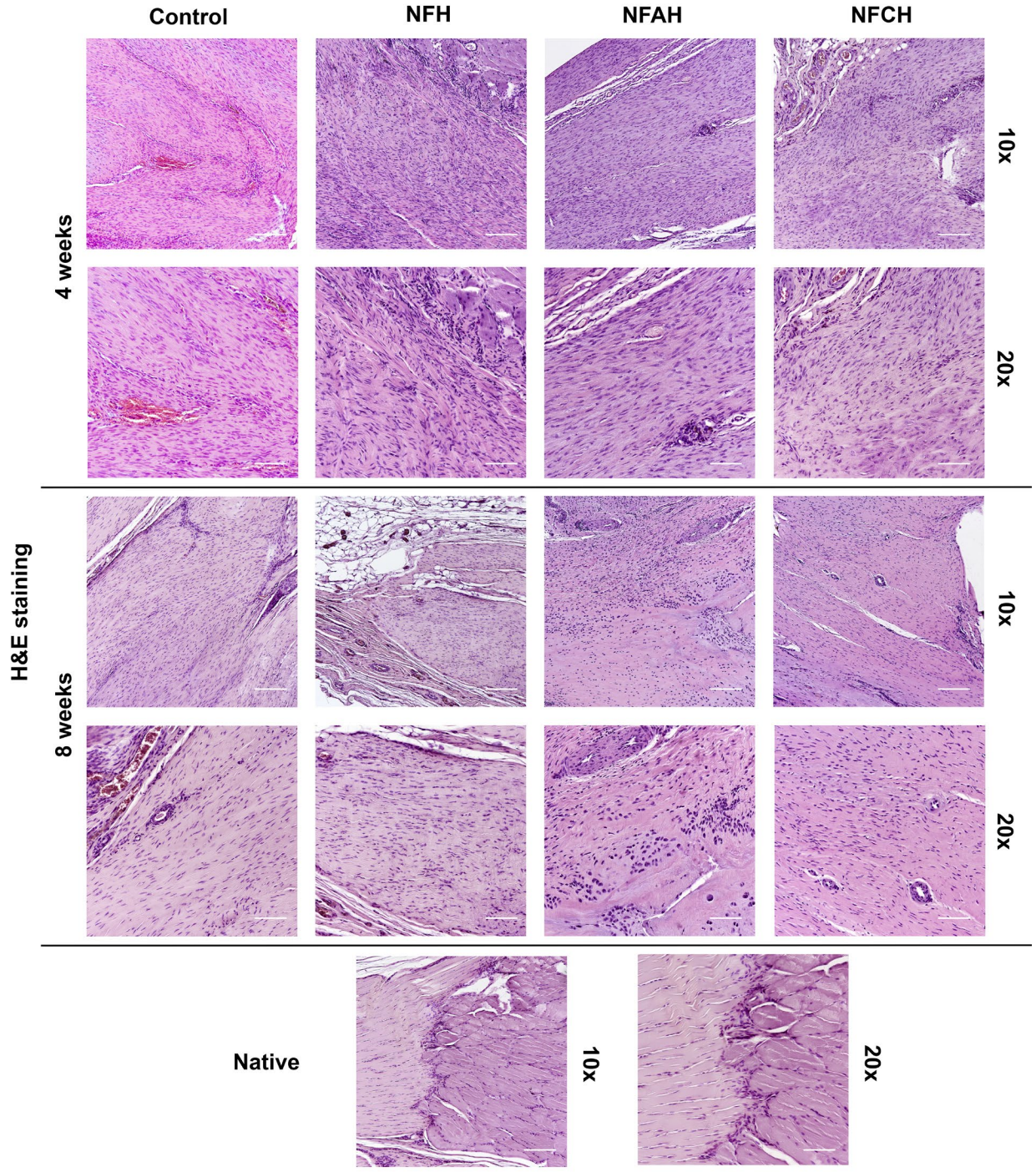


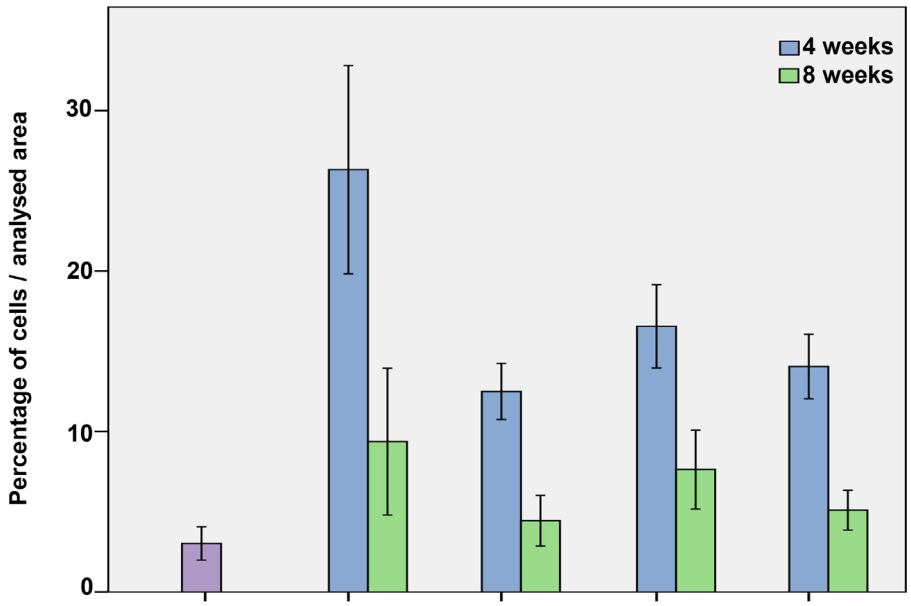
Fig. 5. H&E staining of explanted tendons. Explanted tendons showed an increase in cellular content. Under all experimental conditions, increased vascularisation and loss of tissue alignment were seen, especially in the control group. Analyses at 8 weeks showed an improvement in tissue alignment and vascularisation, mainly in the NFAH and NFCH groups. Scale bar: 200 μm (10×) and 100 μm (20×).

appreciable improvement in tissue alignment and vascularisation were observed and these processes were more evident when both NFAH and NFCH were used (Fig. 5). Importantly, conventional histological analysis showed a few localised inflammatory cells but no clear signs of acute or chronic inflammatory response, foreign body reaction, tissue necrosis or ECM degradation during the study period under all experimental conditions. However, slight signs of cartilaginous metaplasia were observed in the NFAH group (Fig. 5). Lastly, a tissue pattern similar to that in native tendons used as controls was not seen under any of the experimental conditions (Fig. 5).

The histochemical analysis of acid proteoglycan distribution documented by alcian blue staining showed a considerable increase in these non-fibrillar ECM molecules during tendon healing after 4 weeks. This histochemical reaction was similar in the control, NFH and NFCH groups, but proteoglycans were less widely distributed and more localised in the NFAH group (Fig. 6). In tissues analysed 8 weeks after surgery, this histochemical reaction was substantially decreased in repaired tendons and this finding was more evident in animals treated with the biomaterials (Fig. 6).

Histochemical evaluation of fibrillar collagen with Masson's trichrome and picrosirius staining confirmed deposition and remodelling of the fibrillar collagen network during tendon healing in all groups (Fig. 6). After 4 weeks, Masson's trichrome staining was clearly positive in all regenerating tendons, with signs of alignment and parallel organisation, as determined by picrosirius red staining (Fig. 6). Analyses at 8 weeks showed an evident increase in collagen content and organisation in all operated tendons, especially when the biomaterials were used. Organisation of the collagen network showed a tendency to recuperate the appearance of native tendons (Fig. 6).

The immunohistochemical analysis of CD45 confirmed the presence of localised leucocytes within the repaired tendons (Fig. 7). Lymphoid cells were observed in the connective tissue surrounding the blood supply, and especially around the suture



		Native	Control	NFH	NFAH	NFCH
Mean ± SD	*4 weeks*	3.03 ± 1.16	26.31 ± 5.62	12.49 ± 2.25	16.55 ± 2.25	14.04 ± 1.74
	8 weeks		9.37 ± 3.96	4.44 ± 1.36	7.63 ± 2.13	5.09 ± 1.07
4 weeks vs 8 weeks in each			0.04953	0.04953	0.04953	0.04953
***p*-values 4 weeks**	*vs. Native*		0.02535	0.02535	0.02535	0.02535
	vs. Control			0.04953	0.04953	0.04953
	vs. NFH				0.04953	0.27523
	vs. NFAH					0.27523
***p*-values 4 weeks**	*vs. Native*		0.02535	0.17971	0.02535	0.02535
	vs. Control			0.12663	0.27523	0.12663
	vs. NFH				0.12663	0.51269
	vs. NFAH					0.12663

Fig. 6. Graphical and numerical representation of cellularity in all groups. Results are shown as mean ± SD of the percentage area occupied by cell nuclei referred to the total histological area of analysis (area fraction) at 4 and 8 weeks. Exact *p*-values for the Mann-Whitney non-parametric test are shown for each comparison.

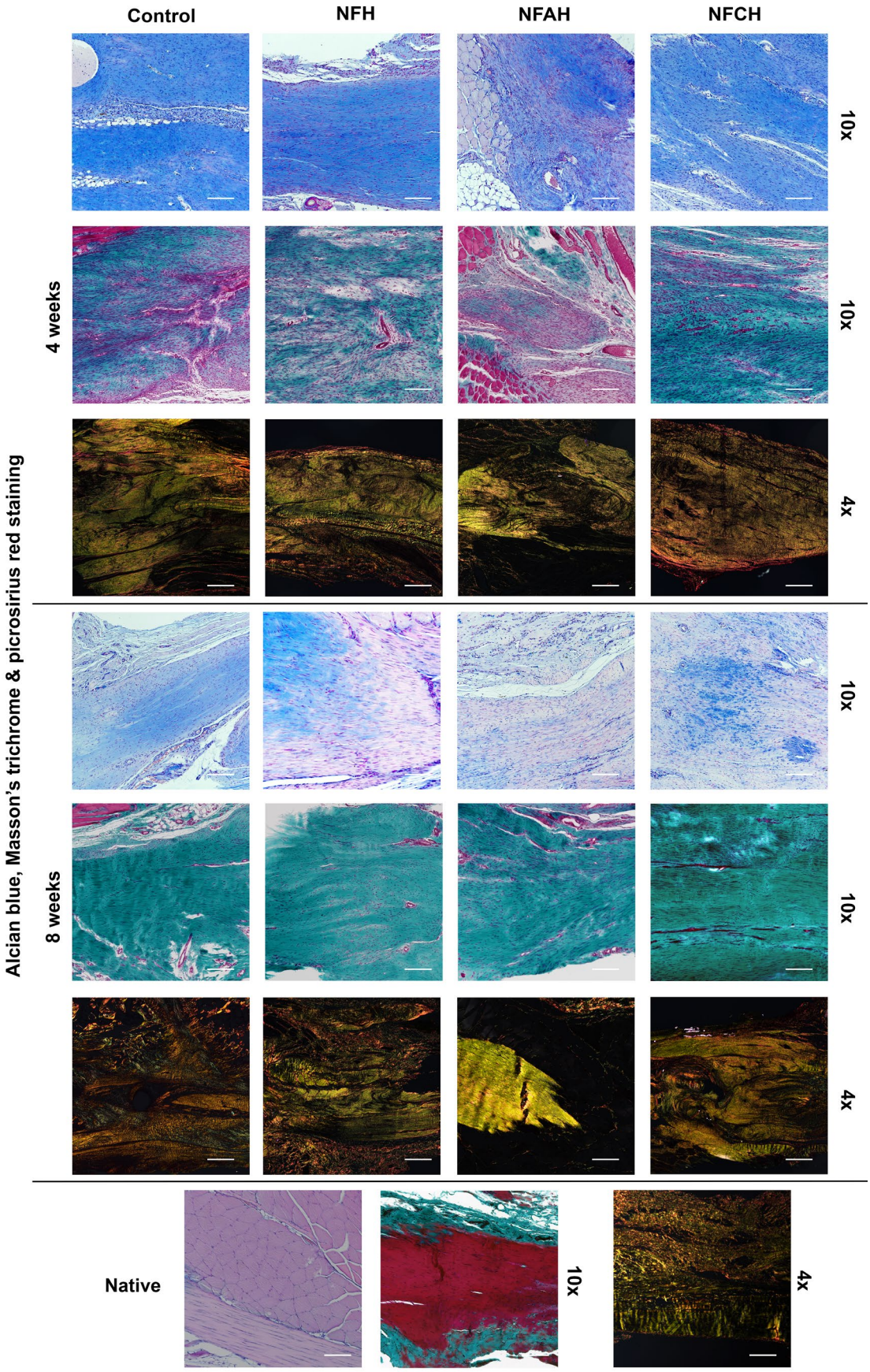


Fig. 7. Alcian blue, Masson's trichrome and picrosirius red staining of explanted tendons. Alcian blue staining showed an increase in proteoglycans and non-fibrillar molecules during tendon healing under all experimental conditions, with no significant differences between groups. These increases were smaller at 8 weeks. Masson's trichrome and picrosirius red staining confirmed the formation of collagen fibres that appeared to be better organised when biomaterials were used. Scale bar: 200 μm (10×) and 400 μm (4×).

material. These cells were less abundant in groups treated with a biomaterial. Overall, these results were consistent with the findings reported above for conventional histological analyses.

Quantitative analyses

The time-course quantitative histological evaluation of cell content or cellularity in H&E-stained sections confirmed the qualitative histological findings.

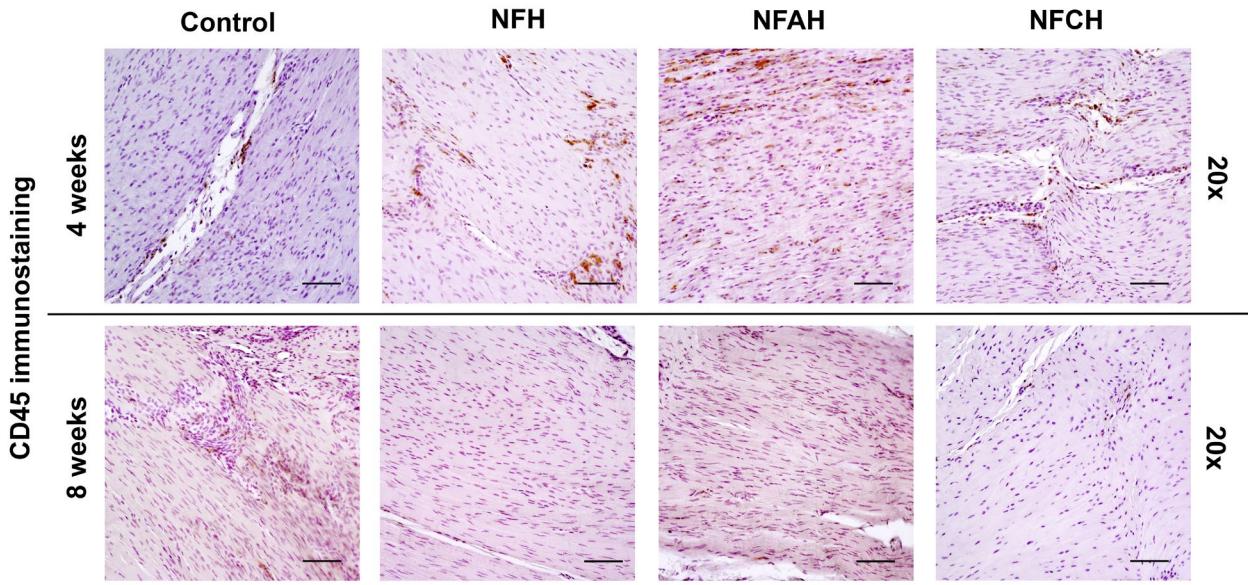
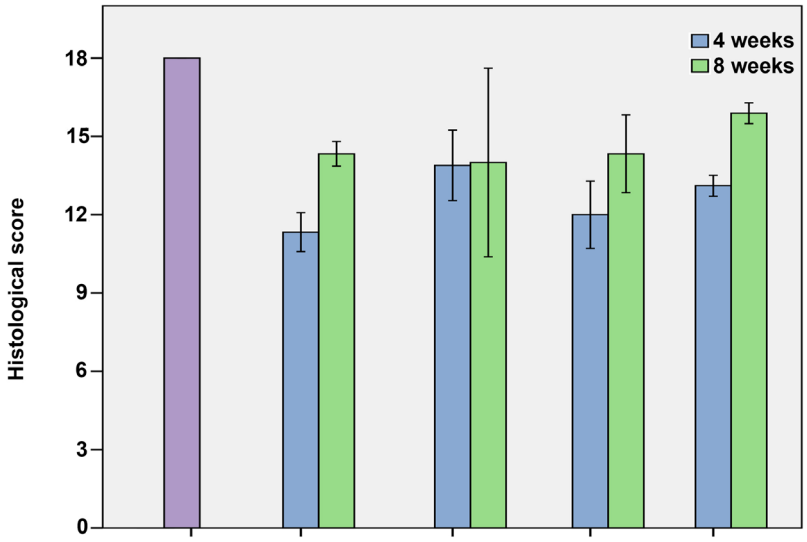


Fig. 8. CD45 expression in repaired tendons. Leucocytes (brown) were present, especially around vessels and suture materials. Scale bar: 200 μm.



		Native	Control	NFH	NFAH	NFCH
Mean ± SD	*4 weeks*	18.00 ± 0.00	11.67 ± 0.58	14.33 ± 2.08	12.00 ± 2.65	13.33 ± 0.58
	8 weeks		14.33 ± 1.16	13.00 ± 4.36	14.67 ± 2.08	16.33 ± 0.58
4 weeks vs 8 weeks in each			0.00023*	0.90508	0.05187	0.00020*
***p*-values 4 weeks**	*vs. Native*		< 0.00001*	0.00013*	0.00013*	< 0.00001*
	vs. Control			0.02510	0.30698	0.00050*
	vs. NFH				0.04952	0.23952
	vs. NFAH					0.28879
***p*-values 4 weeks**	*vs. Native*		0.00011*	0.02294	0.00012*	< 0.00001*
	vs. Control			1	0.71369	0.00077*
	vs. NFH				0.95246	1
	vs. NFAH					0.17380

Fig. 9. Graphical and numerical representation of histological score in native tendons and experimental groups. Results are shown as mean ± SD. Exact *p*-values for the Mann-Whitney non-parametric test are shown for each comparison. * Statistically significant *p*-values.

According to the Kruskal-Wallis test, cellularity was significantly increased (p = 0.00121, 99.9 % power) in all repaired tendons compared to native ones at both time periods analysed (Fig. 8). After 4 weeks, the differences were considerably greater in the control groups compared to the experimental biomaterial groups, although these differences were not statistically significant ($p > 0.05$). Interestingly, analyses of tissue sections obtained 8 weeks after surgery showed a decrease in cellularity in all operated tendons, with no significant differences compared to the control group (Fig. 8). Cellularity values were lowest with the use of NFH, followed by NFCH, but *post-hoc* comparative analyses did not detect any significant differences among different groups of animals that underwent surgery ($p > 0.05$) (Fig. 8).

These observations were in agreement with the results of the histological scoring system: significant differences were seen with the Kruskal-Wallis test (p = 0.0013, 89.3 % power) (Fig. 9). However, none of the experimental groups attained the histological score of the native condition. Compared to the control group, the histological score was higher when a biomaterial was used in tendon repair, with statistically significant differences for the NFCH group at 4 and 8 weeks but no significant differences for the NFH and NFAH groups in the *post-hoc* comparative analyses (Fig. 9).

Discussion

Novel fibrin hydrogel-based membranes were evaluated as an augmentation strategy in an animal model of Achilles tendon surgical repair. First, the *ex vivo* biomechanical and biological properties of the nanostructured biomaterials were investigated. Secondly, the clinical usefulness of NFH and NFCH was explored and compared with conventional direct repair and the use of NFAH as a biomaterial-based augmentation strategy.

In the biomechanical characterisation of the hydrogels, the Young's modulus was lower than in the human Achilles tendon, where it can be as high as 1.2 GPa (Maganaris *et al.*, 2008). Although the biomechanical properties of fibrin hydrogels, such as those of other hydrogels generated *ex vivo*, are not comparable to the properties of native tendons, they are nonetheless stable enough, especially when nanostructured, to be used as an augmentation strategy because the strength to keep the tendon ends together lies in the suture used and not in the biomaterials, which are not designed for this purpose. The tensile test of the biomaterials used in the present study confirmed that their biomechanical properties were suitable for use in TE protocols, as previously suggested (Scionti *et al.*, 2014). Furthermore, their use as an augmentation strategy in tendon surgical repair is supported by previous studies that used hydrogels to repair soft (Chato-Astrain *et al.*, 2018) and considerably dense tissues (González-Quevedo *et al.*, 2020). Previous research also found that hydrogels favour tissue regeneration without post-surgical complications (No *et al.*, 2020).

The *ex vivo* analysis of cell-biomaterial interactions demonstrated the high cytocompatibility of the generated biomaterials. In fact, TDF, the main cells involved in tendon healing, were functional and metabolically active when cultured with fibrin-based biomaterials. Fibrin is a natural biopolymer that is part of the clotting cascade and plays an active role in tissue healing processes, including tendon regeneration, together with binding proteins and growth factors. The generated hydrogels were associated with high viability of cultured TDF, a result in agreement with the high biocompatibility of fibrin-based biomaterials (Sproul *et al.*, 2018).

Cell metabolic activity, as documented by the WST-1 assay, showed better results in the NFAH group, accompanied by lower values of DNA release. These findings are in line with previous work in which nanostructured fibrin-agarose-based hydrogels showed very good biocompatibility in *ex vivo* assays (González-Quevedo *et al.*, 2020).

When the biomaterials were used as an augmentation strategy in tendon repair, none had any adverse effect or impact on limb function. Macroscopical examination confirmed adequate wound healing along with rapid, complete reabsorption of the biomaterials, thus preserving tendon integrity throughout the study period. No foreign body reactions, ruptures or undesirable effects were observed, although it should be noted that the appearance, thickness and length of all operated tendons slightly differed compared to healthy tendons. The increased length of repaired tendons may be related to the fact that the limbs were not immobilised after the operation – a factor described in previous research (Pelled *et al.*, 2012). Furthermore, the present study findings are consistent with the improvement in biomechanical and histological properties in a canine model of biological scaffolds as an augmentation strategy to repair partial rotator cuff tears (Smith *et al.*, 2020).

Histological analyses in the present study disclosed an improvement in the overall histological pattern associated with the use of biomaterials: not only did they help to efficiently reduce cellularity in the regenerating tendons compared to the surgical control group but they also increased proteoglycan content. This result can be attributed to the important role of these molecules during wound healing, in cell function and especially in collagen ECM remodelling and maturation (Screen *et al.*, 2015). The histochemical analysis of collagen confirmed a correlation between active proteoglycan synthesis and the maturation and progressive alignment of the collagen network during the study period, especially when NFAH and NFCH were used. These positive results can be explained by the roles of fibrin not only as a key component in coagulation but also because of its fundamental

role in tissue healing processes by promoting cell proliferation, migration, ECM reinforcement and angiogenesis (Sproul *et al.*, 2018). In addition, collagen is the main component of the tendon ECM, and similarly to fibrin, it supports cell function and tissue healing (Purcel *et al.*, 2016). Interestingly, there were no noticeable differences between the NFAH and NFCH groups in the wound healing characteristics studied and this can be explained by the natural biocompatibility of agarose and collagen.

The use of these hydrogels as an augmentation strategy enhanced the regeneration process, prevented inflammation and connective tissue infiltration and supported better tissue organisation and alignment, as shown by the similar histological scores in treated and native tendons. This result was consistent with a recent study in which fibrin-agarose hydrogel-based membranes were used successfully in tendon repair (González-Quevedo *et al.*, 2020). Thus, results suggested that the use of NFH-based membranes was a potentially safe and appropriate option in the repair of tendon injuries.

With regard to host responses to the biomaterials, immunohistochemical analysis disclosed the transitory presence of few lymphoid cells. This can be explained by the fact that collagen is the main component of native tendons and fibrin the main molecule involved in wound healing (Buschmann and Meier Bürgisser, 2017). Collagen is a fibrillar protein with excellent biocompatibility and biodegradability and low immunogenicity – characteristics that stimulated research aimed at improving its mechanical properties (Purcel *et al.*, 2016). Moreover, crosslinking can modify the molecular structure of collagen, minimising its degradation and improving its biomechanical properties (Gu *et al.*, 2019). A collagen-alginate-fibrin hydrogel has been developed with potential applications in musculoskeletal TE thanks to its biocompatibility *in vitro* (Montalbano *et al.*, 2018). Also, synthetic collagen could be used as a mediator in tendon repair and regeneration (Kew *et al.*, 2012). In addition, the use of a type I collagen sponge to repair tendon ruptures in a rat model resulted in histological and biomechanical properties similar to those of native tendons (Müller *et al.*, 2016). Lastly, preclinical results showed that fibrin-based hydrogels are potentially useful for tendon repair. In the context of tendon TE, biomimetic bioengineered tendon-like substitutes have produced promising improvements in the regenerative capacity of repaired tendons, although more research in this area is still needed (Anjana *et al.*, 2019). However, even with the development of promising TE strategies, the optimal treatment of tendon injuries remains controversial (Bhandari *et al.*, 2002) due to the limited knowledge of tendon pathology, function and healing (Snedeker and Foolen, 2017). Thus far, it is clear that more basic and time-course studies are needed to elucidate the complex biological processes involved in tendon healing with engineered biomaterials. Nevertheless, the promising results obtained to date in tendon TE research could lay the foundations for more efficient surgical treatments of injured tendons in the future (Turner and Badylak, 2013). In this regard, a recent meta-analysis of TE strategies for tendon repair in animal models showed that the use of different kinds of biomaterials can help to recover a histological pattern and biomechanical behaviour similar to those of native tendons (González-Quevedo *et al.*, 2018) and previous research has highlighted the use of hydrogels, which remain among the most versatile biomaterials in the field (Caló and Khutoryanskiy, 2015). One example is a photocrosslinkable magnetic hydrogel enriched with platelet lysate that was able to modulate the synthesis of a tendon-like matrix in co-cultures (Silva *et al.*, 2018). In addition, a chitosan hydrogel scaffold showed good cell-adhesive properties and was associated with tenocyte proliferation *in vitro* (Nivedhitha Sundaram *et al.*, 2019). The success of hydrogels in TE is further supported by their high hydration rate, which favours oxygen and nutrient exchange, and by their high biocompatibility with a wide range of cells, which makes them suitable for adaptation to specific structural and biomechanical needs (Campos *et al.*, 2018; Campos *et al.*, 2020; Chato-Astrain *et al.*, 2018).

To the best of the authors knowledge, this is the first study to report the potential value of a NFCH to enhance the surgical repair of ruptured tendons. Moreover, the study demonstrated that all strategies used in the *ex vivo* and *in vivo* experiments showed better outcomes in terms of histological healing than the control group and lacked any adverse effects. One limitation of the study was the lack of biomechanical and genetic analyses. Nonetheless, histological and histochemical evaluations showed clear improvements in tendon healing when using the NFH-based membranes compared to classical surgical repair. Additional experiments with these biomaterials should be designed to seek further evidence for their efficacy in the treatment of tendon injuries. Future studies should also evaluate the use of these hydrogels in other animal species that serve as appropriate models for the condition in humans.

Conclusions

The present *ex vivo* and *in vivo* study demonstrated a high degree of biocompatibility of nanostructured fibrin-based hydrogels for application in tendon TE. The use of these biomaterials as an augmentation strategy for tendon surgical repair resulted in a clear improvement in the tendon tissue regeneration profile and ECM remodelling with time. Compared to the NFH group, better functional, histological and histochemical results were obtained in animals 8 weeks after tendon injuries were repaired with both NFAH and NFCH. The hybrid biomaterials used in the present study offered a simple and inexpensive option than can be easily adapted for

use in tendon repair. Fibrin is a natural biomaterial that can be obtained from autologous sources and, then, combined under sterile condition with agarose or collagen prior to surgery, in a customised approach to treatment. However, further molecular and biomechanical preclinical studies, especially in large animal models, are warranted to determine the potential usefulness of this strategy for the treatment of tendon injuries.

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The authors declare that they have no conflict of interests.

Descriptions of individual author contributions are listed below. Each author has read and approved the final submitted manuscript. DGQ: data acquisition, data interpretation, statistical analysis, manuscript drafting, manuscript editing, funding acquisition. DSP: data acquisition, biomaterials generation. JCA: data acquisition, biomaterials generation. ODGG: data acquisition, biomaterials generation. MDR: data acquisition, biomaterials generation. FC: data acquisition, data interpretation, quantitative analyses, manuscript revision, supervision. AC: conception and design, manuscript revision. VC: conception and design, data interpretation, manuscript revision, manuscript editing, supervision, funding acquisition.

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Discussion with Reviewer

Reviewer: In which orthopaedic conditions could hybrid NFCHs be useful in the future? What are the potential implications of this research?
Authors: NFCHs and fibrin-collagen hydrogels could be useful in the future for both patients who need an optimal repair of acute tendon injuries (sport medicine) and to whom the traditional surgical repair offers worst results (larger tendon defects, surgeries after infections, *etc.*). The advantage offered by the described strategies lies in the speed with which it is possible to obtain one of these bioartificial membranes, which will have no adverse effect on the conventional repair but rather improve its results.

Reviewer: Why did the authors use a rat model?
Authors: This is a good and difficult question. Much of the understanding of tendon injury and healing derives from using animals as experimental injury models, being less expensive and easier to control than human clinical trials. The animal models used in each case depends on the similarities to humans and the cost-effectiveness in producing extrapolated results. Large-animal models, such as horses and sheep, indisputably have tendon sizes and dimensions more similar to human ones; nevertheless, they are not always the elected animal models because of the high cost and logistical activities required. In general, for smaller and delicate tendons, bigger animals are elected as only alternative. For instance, the canine forefoot, whose size and surrounding anatomy are similar to those of the human hand, is an excellent model to help the understanding of healing in the hand flexor tendons.

In this context, it was decided to work with rats, a cost-effective model, as they offer the possibility of working with a large number of animals, are easy to handle and allow for detection of differences among groups in short periods of time (few weeks). In addition, this is a good and acceptable animal model to test a wide range of bioartificial substitutes, being often used in tendon TE (Campos *et al.*, 2020; Carriel *et al.*, 2019; González-Quevedo *et al.*, 2020). In the present study, this model allowed for determining

the potential efficacy of the biomaterials in tendon repair, host response, tissue regeneration, functional recovery and/or fate of the biomaterials used under controlled experimental conditions. It is truth that these results are not comparable to human, but these preclinical results were useful to determine if certain therapies could be further developed.

Editor's note: The Guest Editor responsible for this paper was Manuela Gomes.