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Restorative and pain-relieving effects of fibroin in preclinical models of tendinopathy

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ABSTRACT

The term tendinopathy indicates a wide spectrum of conditions characterized by alterations in tendon tissue homeostatic response and damage to the extracellular matrix. The current pharmacological approach involves the use of nonsteroidal anti-inflammatory drugs and corticosteroids often with unsatisfactory results, making essential the identification of new treatments. In this study, the pro-regenerative and protective effects of an aqueous fibroin solution (0.5–500 μg/mL) against glucose oxidase (GOx)-induced damage in rat tenocytes were investigated. Then, fibroin anti-hyperalgesic and protective actions were evaluated in two models of tendinopathy induced in rats by collagenase or carrageenan injection, respectively. *In vitro*, 5–10 μg/mL fibroin per se increased cell viability and reverted the morphological alterations caused by GOx (0.1 U/mL). Fibroin 10 μg/mL evoked proliferative signaling upregulating the expression of *decorin*, *scleraxin, tenomodulin* ($p < 0.001$), *FGF-2,* and *tenascin-C* ($p < 0.01$) genes. Fibroin enhanced the basal FGF-2 and MMP-9 protein concentrations and prevented their GOx-mediated decrease. Furthermore, fibroin positively modulated the production of collagen type I. *In vivo*, the peri-tendinous injection of fibroin (5 mg) reduced the development of spontaneous pain and hypersensitivity ($p < 0.01$) induced by the intra-tendinous injection of collagenase; the efficacy was comparable to that of triamcinolone. The pain-relieving action of fibroin (peri-tendinous) was confirmed in the model of tendinopathy induced by carrageenan (intra-tendinous) where this fibrous protein was also able to improve tendon matrix organization, normalizing the orientation of collagen fibers. In conclusion, the use of fibroin in tendinopathies is suggested taking advantage of its excellent mechanical properties, pain-relieving effects, and ability to promote tissue regeneration processes.

1. Introduction

Tendons are dense, highly structured tissues that connect muscle to bone; they are critical for the integrity, function, and locomotion of the musculoskeletal system [1]. Tendon tissue consists of tendon-resident cells and extracellular matrix (ECM). The most abundant cells are tenocytes, elongated fibroblast-like cells that are totally differentiated and non-self-renewable [2–4]. Fibroblasts are responsible for the synthesis of the ECM components, such as collagen, proteoglycans, and glycoproteins, and for the remodeling of the ECM in the healing tendon process [5]. Due to the increasing age of our society and due to the general escalation in the practice of extreme sports, there is a growing prevalence of tendinopathies [6]. The term “tendinopathy” defines the debilitating conditions due to tendon pathological alterations characterized by a combination of pain, swelling and functional limitation of the tendon itself and contiguous anatomical structures [7,8]. Tendinopathy represents one of the most frequent orthopedic diagnosis, accounting for over 30% of all musculoskeletal consultations [9]. Over 30

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million human tendon-related procedures take place annually worldwide, with significant socio-economic repercussions in terms of working hours and economic expenditure [10,11]. From the histopathological point of view, chronic tendinopathy causes disorganization of collagen fibers, with large changes in their structure and composition, damage to the ECM, cell death, loss of tissue functionality and integrity, and a predisposition to further injuries and breakages [12–15]. To the present, the etiology and epidemiology of tendinopathies are not yet clearly defined. The difficulty precisely lies in the extremely heterogeneous nature of tendinopathies and the variety of symptoms, such as pain, stiffness, tenderness, swelling, redness, heat, muscle weakness, myalgia, spasms, cramps, and even asymptomatic conditions [16]. Currently, the treatments offered for tendinopathies include non-steroidal anti-inflammatory drugs (NSAIDs) [17,18], only partially effective due to the reduced vascularity of tendons and the consequent low availability in the target tissue [19], glyceryl trinitrate patches [20,21], aprotinin injections [22], ultrasound [23] and platelet-rich plasma (PRP) injections [24,25]. In recent years, the protein fibroin has aroused interest in several biomedical applications [26,27]. Fibroin-based biomaterials are mainly prepared from the silk cocoons of *Bombyx mori*, a species of moth commonly known as "silkworm" [28]. Silk cocoons are composed of 60–80% fibroin, 15–35% sericin and, 1–5% non-sericin components [29], a particular composition that makes it unique giving characteristics of strength and elasticity [30,31]. Fibroin is easily transformed into gels, membranes, nanofibers, films, nanoparticles, scaffolds and, spongy forms [32] and, over the years, has found several applications in tissue engineering, wound healing, and drug delivery [33]. With favorable interactions in biological systems and minimal immunological responses, silk fibroin materials have demonstrated good biocompatibility with various cell types by supporting and promoting their adhesion, proliferation, growth and, differentiation, leading to tissue regeneration [33]. Due to its unique mechanical properties and structural integrity, fibroin is used as a three-dimensional scaffold in which implanted cells proliferate and organize to perform their function at the desired tissue level, to promote the healing process of ligaments and tendons [34,35]. The use of aqueous solutions of fibroin has also been shown to improve the adhesion and proliferation of human fibroblasts [36–38]. Therefore, assuming a potential protective and beneficial effect of fibroin in the treatment of tendinopathy, the aim of this work was focused to evaluate the effects of an aqueous solution of fibroin on primary cultures of rat tenocytes, alone or in the presence of oxidative damage induced by glucose oxidase (GOx). After evaluating its proliferative and protective effects *in vitro* and the underlying molecular mechanisms, the action of the same aqueous solution of fibroin was then studied in two *in vivo* models of tendinopathy induced in rats by the intra-tendon injection of collagenase type I or carrageenan.

2. Materials and methods

2.1. Primary culture of tenocytes

Achilles tendons were explanted from Sprague–Dawley rats (Envigo, Varese, Italy) weighing approximately 200–250 g after sacrifice. The explanted tendon was soaked in povidone-iodine for 3 min and washed twice in 1X PBS. Each tendon then was cut into small pieces of about 1.5–2.0 mm^3 (six pieces in total). These were individually placed in 6-multiwells. After 5 min of air drying for better adhesion, 0.5 mL of Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (Life Technologies Italia, Milan, Italy), supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, was added to each well (Sigma-Aldrich, Milan, Italy). The explants were then incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells migrated out from the explants, once reached confluence, were trypsinized and subcultured from passage 2–4 for the studies.

2.2. Tenocyte treatments

To evaluate the effectiveness of fibroin (Sigma-Aldrich, Milan, Italy) in the presence of damage, the cells were suspended in complete culture medium and plated to have a single cell colony per well. Forty-eight hours after plating, tenocytes were treated with the enzyme glucose oxidase (Gox; Sigma-Aldrich, Milan, Italy) at a concentration of 0.1 U/mL for 1 h. This first treatment was followed by 24 h of recovery during which tenocytes were incubated with fibroin at various concentrations (0.5 – 500 μg/mL).

For the evaluation of cell growth, 2 different concentrations of fibroin were used, 5 and 10 μg/mL. For the evaluation of the efficacy of fibroin, the cells were resuspended in a complete culture medium and plated in 6-multiwells, in order to have a single cell colony per well. 48 h after plating tenocytes were treated with fibroin and FGF-2 (5 and 10 ng/mL; Sigma-Aldrich, Milan, Italy) for 24 h.

2.3. Cell viability assay (MTT)

Cell viability was assessed by reduction of 3- (4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide (MTT), used as an index of mitochondrial function. Tenocytes were plated in 96-multiwells, cultured until confluence, and treated with GOx 0.1 U/mL for 1 h. GOx treatment was followed by 24 h of washout, during which the cells were treated with and without fibroin at various concentrations (0.5 – 500 μg/mL). After extensive washing, 1 mg/mL solution of MTT (Sigma-Aldrich, Milan, Italy) was added to each well and incubated for 30 min at 37 °C. After washing, formazan crystals were dissolved in 100 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milan, Italy). The absorbance was measured at 580 nm.

2.4. Immunofluorescence

Tenocytes were plated on appropriate slides. In each slide, 4 separate cell colonies were created by plating 4 drops of 20 μL each containing 2000 cells/drop. At the end of the treatment the cells were fixed in formalin 4% in PBS for 10 min at room temperature, washed with PBS (3 washes of 5 min each) and treated with Triton X-100 0.3% in PBS for 10 min at room temperature. After washing in 1X PBS and blocking with 0.5% BSA in PBS-0.3% Triton X-100 for 30 min, the cells were incubated with Alexa Fluor 488® phalloidin (1:40 in blocking solution) for 1 h at room temperature. Then the cells were washed with PBS, incubated with DAPI (Life Technologies, Milan, Italy) for the labeling of the nuclei and subsequently mounted with the ProLong mount (Life Technologies, Milan, Italy) and observed with a Leica DM6000B microscope equipped with a DFC350FX camera (Leica, Mannheim, Germany).

2.5. Biochemical analysis

Culture supernatants were collected and the concentrations of collagen type I, metalloprotease 9, and FGF-2 were assessed by enzyme-linked immunosorbent assay (ELISA Kit MyBioSource-MBS262647 for collagen type I; MyBioSource-MBS722532 for MMP-9; Abnova-KA5408 for FGF-2), according to manufacturer's instructions. The levels were normalized to cell protein concentrations.

2.6. RNA isolation and reverse transcription

Total RNA was extracted from cells by using the RNA isolation Nucleospin kit (Macherey-Nagel, Germany). The amount of RNA was determined by spectrophotometrically. One μg of total RNA from each sample was used to synthesize cDNA with the iScript cDNA Synthesis kit (Bio-Rad, Milan, Italy) according to the manufacturer's instructions.

2.7. Real-Time polymerase chain reaction (RT-PCR)

RT-PCR reactions were performed by using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Milan, Italy) and performed on the RotorGENE-Q instrument (Qiagen, Germany). Differential expression of transcripts of interest was calculated in relation to the 18 S house-keeping transcript for cDNA. The primers used were:

Primer	Forward	Reverse
Decorin	5'-GCA AGT CTC TTG GGC TGG ACC ATT TG-3'	5'-GTG TCA GGT GGA AAT TCC CAG GGT AC – 3'
Scleraxin	5'-TGG AAG CCA CTG AAG AGT CAT GGA GAG – 3'	5'-GAG CCA GCA TGG AAA GTT CCA GTG G – 3'
FGF-2	5'-TTC AAG GAT CCC AAG CGG CTC TAC TG-3'	5'-AGC AGC CGT CCA TCT TCC TTC ATA GC-3'
Tenascin	5'-GGC CTC TCT GAG ACC TGT TAT GTC C-3'	5'-CAG AAG CTG AAC CGG AAG TTG ACA AC – 3'
Tenomodulin	5'-ATG GCA CCG ATG AAA CAT TGG AAG TCC – 3'	5'-TTC TGC AGG AAC CCA AAT CAC TGA CTG – 3'
18S	5'-GGG GAA TCA GGG TTC GAT TCC G-3'	5'-GGC ACC AGA CTT GCC CTC CAA TG-3'

2.8. Animals

For all the experiments described below, male Sprague–Dawley rats (Envigo, Varese, Italy) weighing approximately 200–250 g at the beginning of the experimental procedure were used. Four rats were housed per cage (size 26 ×41 cm^2) in Ce.S.A.L. (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Animals were fed with standard laboratory diet and tap water ad libitum, kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance number: A5278–01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines [39]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.9. Induction of tendonitis and fibroin and triamcinolone treatments

The animals were anesthetized with isoflurane (4% for induction and 1.5% for maintenance of anesthesia). Tendon damage was induced near the osteotendinous junction of the rat's right Achilles tendon by a single percutaneous injection of 20 μL of collagenase type I (20 mg/mL) or 20 μL of carrageenan 0.8%, after having flexed the paw to form a 45° angle, using a 30 G needle. Collagenase and carrageenan were solubilized in physiological solution. Control animals were treated with saline solution [40,41]. Fibroin (100 μg, 1 and 5 mg in 20 μL) was alternatively injected by a single intra-tendon treatment (day 2 after damage) or by peri-tendon injections on days 1, 3, 5, and 7. A sum of both treatments was also used in the model of collagenase-induced tendinopathy. Triamcinolone acetonide (100 μg/20 μL or 250 μg/50 μL; Sigma-Aldrich, Milan, Italy) was administered following the same protocols.

2.10. Paw pressure test

Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical mechanical probe and recorded by an analgesimeter (Ugo Basile, Varese, Italy). Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. These limits assured a more precise determination of mechanical withdrawal threshold in experiments aimed to determine the effect of treatments. An arbitrary cut-off value of 100 g was adopted [42,43].

2.11. Incapacitance test

Weight-bearing changes were measured using an Incapacitance Apparatus (Linton Instrumentation, UK) detecting changes in postural equilibrium after a hind limb injury. Rats were trained to stand on their hind paws in a box with an inclined plane (65° from horizontal). This box was placed above the Incapacitance apparatus. This allowed us to independently measure the weight that the animal applied on each hind limb. The value reported for each animal is the mean of five consecutive measurements. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, indicating postural equilibrium, whereas an unequal distribution of weight on the hind limbs indicated a monolateral decreased pain threshold [44,45]. Data are expressed as the difference between the weight applied to the limb contralateral to the injury and the weight applied to the ipsilateral one (Δ weight) [46].

2.12. Rota-rod test

Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into four equal sections by five disks. Thus, up to four rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod for a maximum of 600 s. After a maximum of six falls from the rod, the test was suspended and the time was recorded [47].

2.13. Histological evaluations

Tendon samples were fixed in buffered 10% formalin, dehydrated and embedded in paraffin (Bio-Optica, Milan, Italy). Ten μm thickness longitudinal sections were processed for Hematoxylin-Eosin (Bio-Optica, Milan, Italy), and for Azan-Mallory staining (Diapath, Bergamo, Italy), as follows. After hydration through descending ethanol concentration, sections were incubated in 0.1% Azocarmine G for 45 min at 56 °C, and differentiated in 90% alcohol with 0.1% Aniline oil, checking periodically stain intensity under microscope. After rinsing in 1% acetic acid, slides were transferred to 5% Phosphotungstic acid for 1 h, stained in Aniline Blue-Orange G solution (1:3 dilution, stock solution: 0.5% Aniline Blue, 2% Orange G) for 1.5 h, and differentiated in 95% alcohol. Finally, sections were dehydrated and mounted in Canada balsam.

In addition to highlighting some structures in the damaged tendon, Azan-Mallory trichrome stain revealed differences in collagen fibers staining among treatments. These results are referred to as Azan-Mallory stain intensity. Five slides for each tendon were randomly selected and examined by two blinded investigators under optical microscope. Slides were interpreted using a modified histological grading score from Kihara and colleagues [48], composed by various parameters: extracellular matrix organization (0–2), tissue homogeneity (0–2), presence of degenerative changes (0–2), cell nucleus morphology (0–2), cell distribution (0–2) and alignment (0–2), vascularization (0–1), inflammation (0–1), Azan-Mallory red stain intensity (0–2). Total score for each animal ranged between 0 (most severe tendon impairment) and 16 points (control, normal tendon). Total score for each animal could range between 0 (most severe tendon impairment) and 16 points (control, normal tendon). Demonstrative images were acquired at 100X, 200X and 400X total magnification by Nikon light microscope (Nikon Olympus BX40).

2.14. Statistical analysis

The data were collected by observers blinded to the protocol. All experimental results are expressed as mean ± S.E.M. A one-way analysis of variance (ANOVA) was conducted, followed by the Bonferroni test to verify the significance between two means. The analysis of variance and the Bonferroni test were performed with the OriginPro9.1 statistical program. Values of $P < 0.05$, $P < 0.01$ or $P < 0.001$ were considered significant.

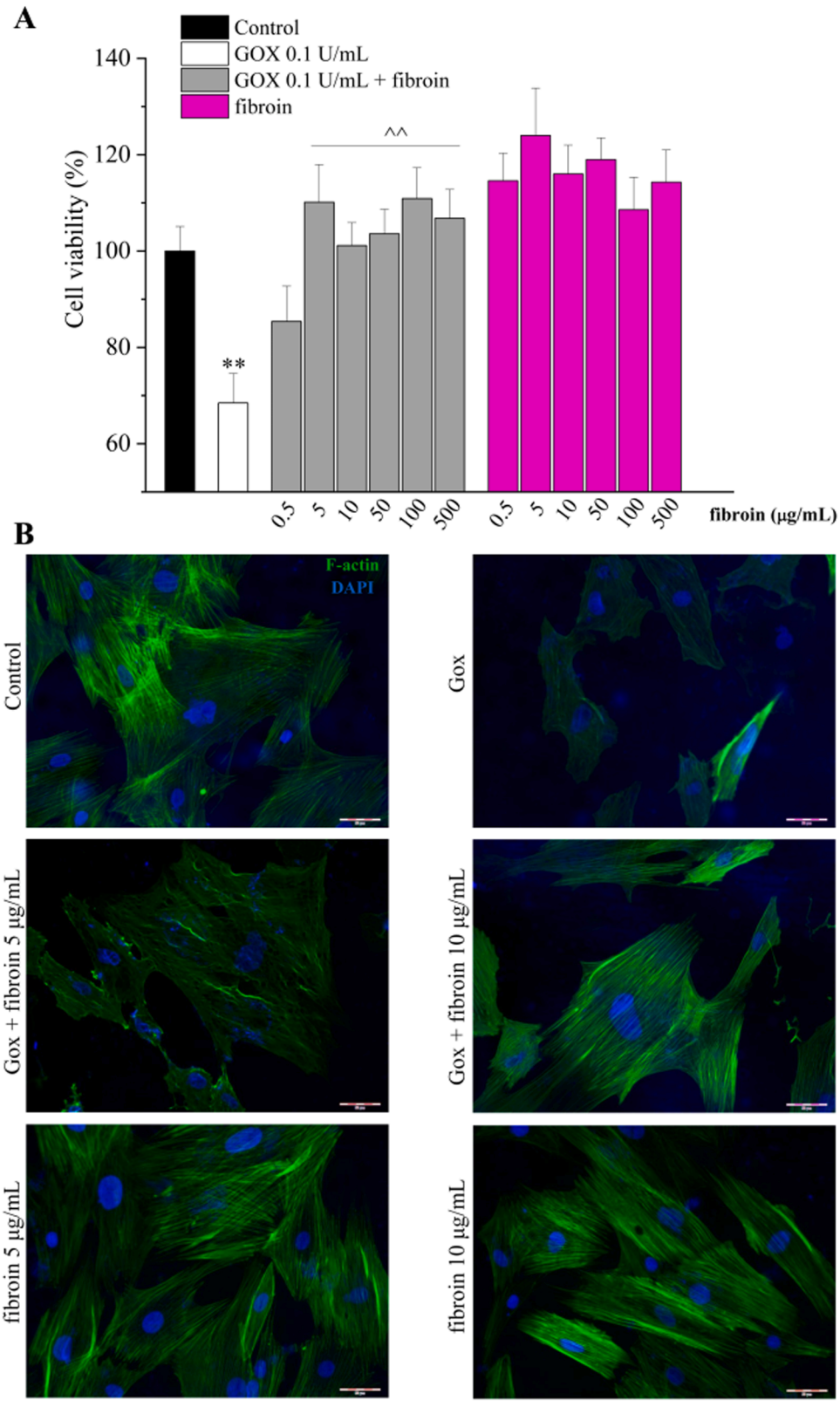

Fig. 1. Effect of fibroin on cell viability and morphology of tenocytes treated with GOx. (A) Cell viability of tenocytes. The primary culture of rat tenocytes was treated with increasing concentrations of fibroin (0.5 – 500 µg/mL) for 24 h, in the presence or absence of GOx (0.1 U/mL), previously incubated for 1 h. Cell viability was measured by analysis with 3- (4,5-dimethylthiozol-2-yl) – 2,5-diphenyltetrazolium bromide (MTT). Control condition was arbitrarily set as 100%, and the values were expressed as mean ± SEM. (B) Cytoskeletal organization of tenocytes. Primary rat tenocyte culture was treated with fibroin 5 and 10 µg/mL in the presence or absence of GOx (0.1 U/mL) for 24 h. The cells were then fixed and labeled with the phalloidin antibody conjugated to Alexa488 (in green) and with DAPI to label the nuclei (in blue). Scale: 50 µm. ** $P < 0.01$ compared to the control; ^^ $P < 0.01$ compared to treatment with GOx.

3. Results

3.1. Effect of fibroin on cell viability and morphology of tenocytes treated with GOx

In a primary culture of rat tenocytes, cell viability was measured in the presence of damage induced by glucose oxidase (GOx), following incubation with increasing concentrations of fibroin. Fig. 1 shows the results of cell viability obtained by MTT test after 1 h of treatment with GOx (0.1 U/mL) followed by 24 h incubation with fibroin (0.5 – 500 μg/mL); the effect of fibroin per se (in the absence of damage) was also studied. GOx reduced cell viability by 30% compared to the control ($p = 0.0054$), the treatment with fibroin fully restored cell viability up to the control values, starting from the concentration of 0.5 μg/mL ($P < 0.01$). This effect was not modified by higher concentrations (up to 500 μg/mL). Fibroin per se did not modify cell viability (Fig. 1A).

The cytoskeleton organization of tenocytes incubated with fibroin in the presence of GOx damage (0.1 U/mL) was evaluated by staining F-actin with fluorochrome-conjugated phalloidin. Fibroin was applied on healthy or previously damaged tenocytes, at the two lowest active concentrations (5 and 10 μg/mL) identified in the viability assay (Fig. 1B). The treatment with GOx led to cell disorganization, damage to cell membrane and loss of the physiological tenocyte morphology. Cells incubated for 24 h with fibroin at concentration of 10 μg/mL showed complete recovery from GOx damage for all the above-mentioned parameters. The lower fibroin concentration (5 μg/mL) was less effective in repairing GOx damage. The cells treated with fibroin alone (5 and 10 μg/mL) show, compared to the control, a better organization of the F-actin filaments resulting oriented in a unique direction (Fig. 1B).

3.2. Effect of fibroin on tenocyte proliferation pathways

Tenocytes were cultured for 24 h in the presence of fibroin (5 and 10 μg/mL) or FGF-2 (5 ng/mL and 10 ng/mL, used as a positive control), after that RT-PCR was performed to study specific marker genes related to tenocyte proliferation. The results shown in Fig. 2 represent the relative expression of the messenger with respect to the expression of 18 S. Fibroin (10 μg/mL) was able to positively modulate the expression of all the analyzed genes, in particular with significance $p < 0.001$ for decorin, scleraxin and tenomodulin, and with $p < 0.01$ for FGF-2 and tenascin-C. The lower concentration (5 μg/mL) induced an increase in FGF-2 mRNA expression ($p < 0.001$), while other messengers were unaltered. Similarly, the treatment with the standard stimulus FGF-2 growth factor (10 ng/mL) evoked an increase in the expression of all the genes investigated with a greater effect on scleraxin and tenomodulin genes ($p < 0.01$), and a minor, but still significant, effect on decorin, FGF-2 and tenascin-C genes ($p < 0.05$); the lower concentration of FGF-2 (5 ng/mL) did not induce changes in the expression of proliferation-related genes (Fig. 2).

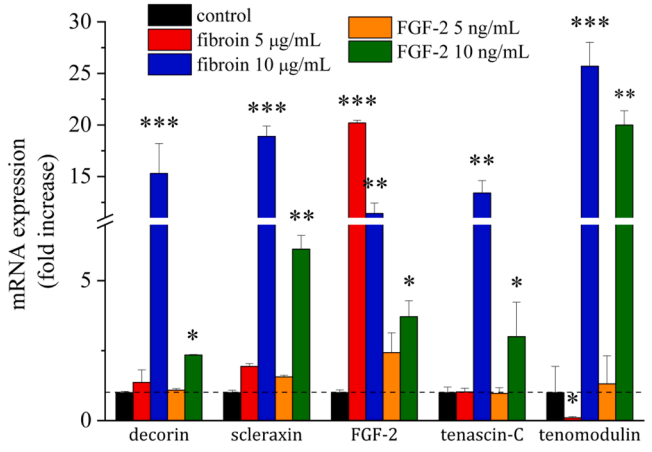

Fig. 2. Expression of genes involved in cell proliferation. Tenocytes were treated for 24 h with fibroin (5 and 10 μg/mL) and FGF-2 (5 and 10 ng/mL). The expression variation of the genes involved in tenocyte proliferation was evaluated by RT-PCR. The control was arbitrarily set to 1 and the mRNA levels were expressed as mean ± SEM (normalized on the expression of 18 S, chosen as housekeeping). Three biological and technical replicates were made per group. * ** $P < 0.001$, * * $P < 0.01$ and * $P < 0.05$ compared to the control.

3.3. Evaluation of the signaling mechanisms triggered by fibroin to preserve cells from damage

To deepen further the protective mechanisms by which fibroin acts, its ability to promote the production of type I collagen, the protein expression of the trophic factor FGF-2 and to prevent the degradation of the extracellular matrix, was assessed both in the presence and in the absence of the damage induced by GOx. Cell treatment with GOx (0.1 U/mL) for 1 h did not change the concentration of collagen protein, while it increased in a statistically significant manner when cells were incubated for 24 h with fibroin alone, at the concentration of 10 μg/mL (Fig. 3A; $p = 0.0072$). The same treatment increased the level of growth factor FGF-2 (Fig. 3B, $p = 0.0068$); furthermore, fibroin was able to prevent the reduction imposed by treatment with GOx ($p = 0.035$), in particular 5 μg/mL fibroin prevented the GOx-dependent alteration while the higher concentration increased FGF-2 up to 140% of the control (Fig. 3B).

Fig. 3C shows the concentration of MMP-9 in tenocytes treated with fibroin, in the presence and in the absence of GOx damage. In both experimental conditions, fibroin increased the levels of MMP-9 compared to the control and to the cells treated with GOx alone, in a statistically significant manner (Fig. 3C; $p < 0.001$).

3.4. Effect of intra-tendon injection of fibroin in a tendinopathy model induced by collagenase

Tendinopathy was induced by intra-tendon injection of 20 μL of collagenase type I (20 mg/mL) on day 1. The efficacy of a single intra-tendon injection of increasing doses of fibroin (100 μg, 1 and 5 mg in 20 μL solution) on day 2, was evaluated. The effect was compared with that obtained by the injection of triamcinolone acetonide (100 μg/20 μL). The behavioral assessments to evaluate the development of spontaneous pain (Incapacitance test- Fig. 4A), the pain threshold in response to a painful stimulus (Paw Pressure test- Fig. 4B) and the motor coordination of the animals (Rota Rod test- Fig. 4C), were carried out in time course (3, 7 and 10 days) after the induction of the damage.

Fig. 4A shows the efficacy of intra-tendon injection of fibroin in reducing the hind limb weight bearing alteration induced by the unilateral collagenase damage. Three days after damage, the difference between the weight placed by the animal on the contralateral and the ipsilateral paw (expressed as Δ weight) was significantly higher in animals treated with collagenase (49.0 ± 0.8 g; $p = 0.0047$, $n = 6$) than in animals treated with vehicle (−1.4 ± 1.8 g, $n = 6$). This difference remained significant even on days 7 ($p = 0.0056$, $n = 6$) and 10 ($p = 0.0088$, $n = 6$), although with a decrease in the painful state. Seven days after the induction of damage, the intra-tendon administration of fibroin, at all tested doses, was effective in reducing the Δ weight similarly to triamcinolone ($p = 0.0075$, $n = 6$). On day 10, only fibroin 5 mg was active ($p = 0.043$ $n = 6$) (Fig. 4A).

The administration of collagenase determined a lowering of the pain threshold to a mechanical painful stimulation starting 3 ($p = 0.0037$, $n = 6$) days after injection lasting until day 10 ($p = 0.0039$, $n = 6$) (Paw Pressure test, Fig. 4B). The single intra-tendon injection of the highest concentration (5 mg) of fibroin increased the weight borne by the animal on the ipsilateral paw on days 3 ($p = 0.040$, $n = 6$) and 7 ($p = 0.047$, $n = 6$) in a statistically significant manner, while it lost its effectiveness on day 10. The lowest concentrations were inactive.

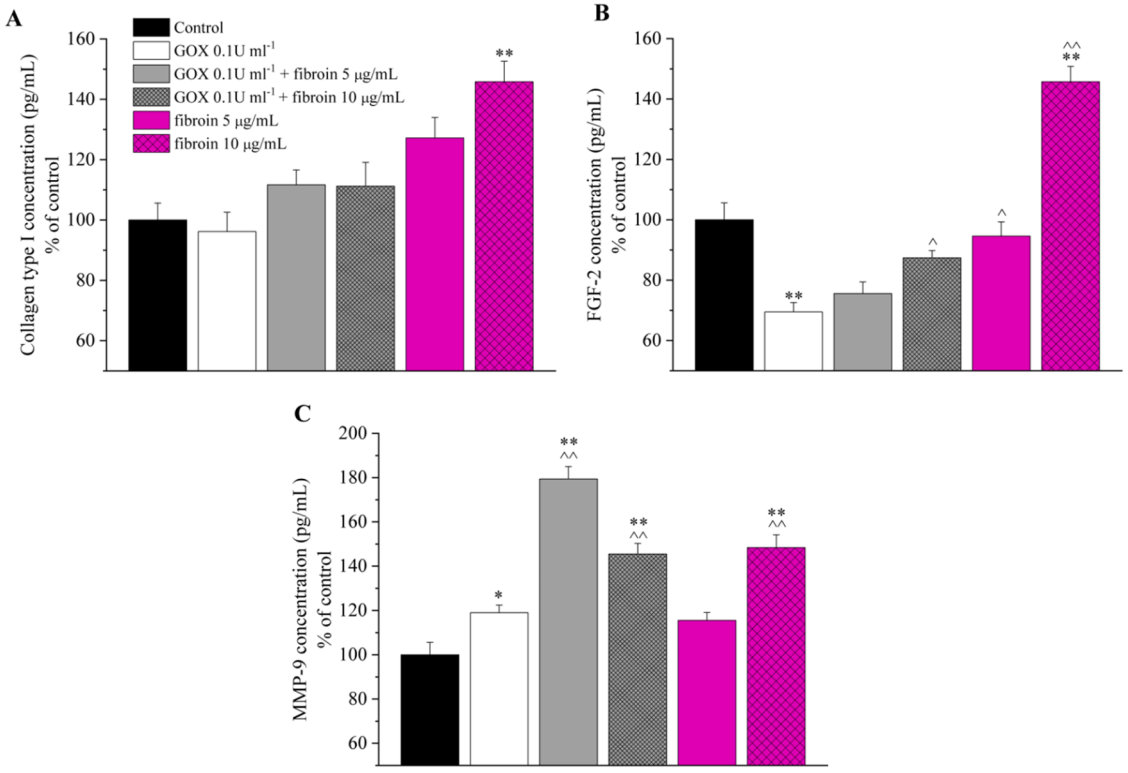

Fig. 3. Effect of fibroin on the release of proteins involved in regenerative processes. Rat tenocyte primary culture was treated with fibroin 5 and 10 µg/mL for 24 h in the presence or absence of GOx (0.1 U/mL), previously incubated for 1 h. The culture media were collected and the concentrations of (A) type I collagen, (B) FGF-2 and (C) MMP-9 released into the medium were detected by ELISA kit. The levels, expressed as mean ± SEM. have been normalized on the protein concentration. Three biological and technical replicates were made per group. * *P < 0.01and *P < 0.05 compared to the control; ^^ P < 0.01 and ^ P < 0.05 compared to treatment with GOx.

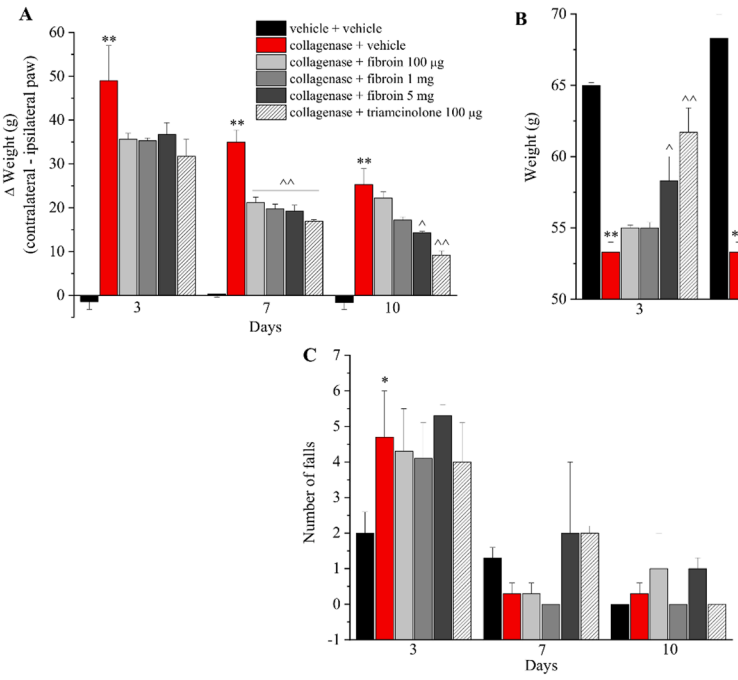

Fig. 4. Effect of intra-tendon injection of fibroin against collagenase-induced pain and motor impairment. Following intra-tendon injection of 20 µL of collagenase type I (20 mg/mL) on day 1, the efficacy of a single intra-tendon injection of fibroin (100 µg, 1 and 5 mg in 20 µL) was evaluated. The effect was compared with that obtained from the injection of triamcinolone acetonide (100 µg/20 µL). The development of spontaneous tendon pain (A - Incapacitance test), the pain threshold in response to a painful stimulus (B - Paw Pressure test) and the motor coordination of the animals (C - Rota Rod test), were carried out in time course (3, 7 and 10 days), after the induction of the damage. Control animals were treated with vehicle. The value represents the mean of 6 rats performed in two different experimental sets. * *P < 0.01and *P < 0.05 compared to the control; ^^P < 0.01and ^P < 0.05 compared to collagenase treatment.

Treatment with triamcinolone acetonide reversed the condition of hyperalgesia induced by collagenase at all considered times ($p = 0.0035$, $p = 0.0027$, $p = 0.0038$, respectively; $n = 6$) (Fig. 4B). Fig. 4C shows the effect of fibroin against the collagenase-induced alteration of motor coordination. The injection of collagenase increased the number of falls by a rotating rod compared to the control group (4.7 ± 1.3 falls and 2.0 ± 0.6 falls, respectively; $p = 0.045$, $n = 6$) on day 3 but not in the following days, treatment with both fibroin (at various concentrations) and with triamcinolone does not reduce the number of falls (Fig. 4C). The data relative to Incapacitance Test and Paw Pressure Test (Fig. 4A and B, respectively) were collected after a 10 min-exercise on Rota-rod apparatus, during which the joint was subjected to stress. The results obtained before performing the Rota-rod (data not shown) were not different with respect to those obtained after the exercise (Fig. 4A and B).

3.5. Effect of peri-tendon injection of fibroin in a tendinopathy model induced by collagenase

After inducing tendinopathy by intra-tendon injection of 20 μL of collagenase type I (20 mg/mL) on day 1, the efficacy of fibroin (100 μg, 1 and 5 mg in 20 μL) was also evaluated following 4 peri-tendon injections performed on days 1, 3, 5 and 7. The effect was compared with that obtained by the peri-tendon injections of triamcinolone acetonide (250 μg/50 μL). The behavioral assessments were carried out on days 3, 7 and 10 after the induction of the damage.

As shown before, collagenase induced spontaneous pain increasing by about 30 times the Δ weight between contralateral and ipsilateral paw compared to the control group (Fig. 5A). Peri-tendon treatments with fibroin (100 μg, 1 and 5 mg) were able to relieve pain starting from day 7 ($p = 0.0087$; $p = 0.0084$; $p = 0.0035$, $n = 6$; respectively), after three administrations of the protein. The reduction in spontaneous pain remained significant even on day 10. Treatments with triamcinolone have an efficacy comparable to that of 5 mg fibroin (Fig. 5A; $p = 0.0045$, $n = 6$).

Similar results were obtained by means of the Paw pressure test (measure of mechanical hyperalgesia, Fig. 5B). Fibroin reverted the state of pain induced by collagenase from day 7 to da day 10, at all three tested concentrations ($p < 0.01$, $n = 6$). Triamcinolone exhibited a comparable anti-hyperalgesic profile (Fig. 5B; $p < 0.01$, $n = 6$). The Rota rod test showed an increase in the number of falls on day 3 ($p = 0.041$, $n = 6$), both fibroin and triamcinolone were inactive (Fig. 5C). The data relative to Incapacitance Test and Paw Pressure Test (Fig. 5A and B, respectively) were collected after a 10 min-exercise on Rota-rod apparatus, during which the joint was subjected to stress. The results obtained before performing the Rota-rod (data not shown) were not different with respect to those obtained after the exercise (Fig. 5A and B).

3.6. Effect of intra-tendon, peri-tendon and intra/peri-tendon injections of fibroin in a tendinopathy model induced by carrageenan

Tendinopathy was induced by the intra-tendon injection of 0.8% carrageenan (20 μL), on day 1. The efficacy of fibroin (5 mg, the most effective and long-lasting dose in collagenase damage experiments) following intra-tendon, peri-tendon and intra/peri-tendon injections, was tested. Intra-tendon administration was performed on day 2, peri-tendon administration was carried out on days 1, 3, 5 and 7, while intra/peri-tendon administration is the union of the two protocols. Behavioral assessments were performed on days 3, 7 and 10 (Fig. 6).

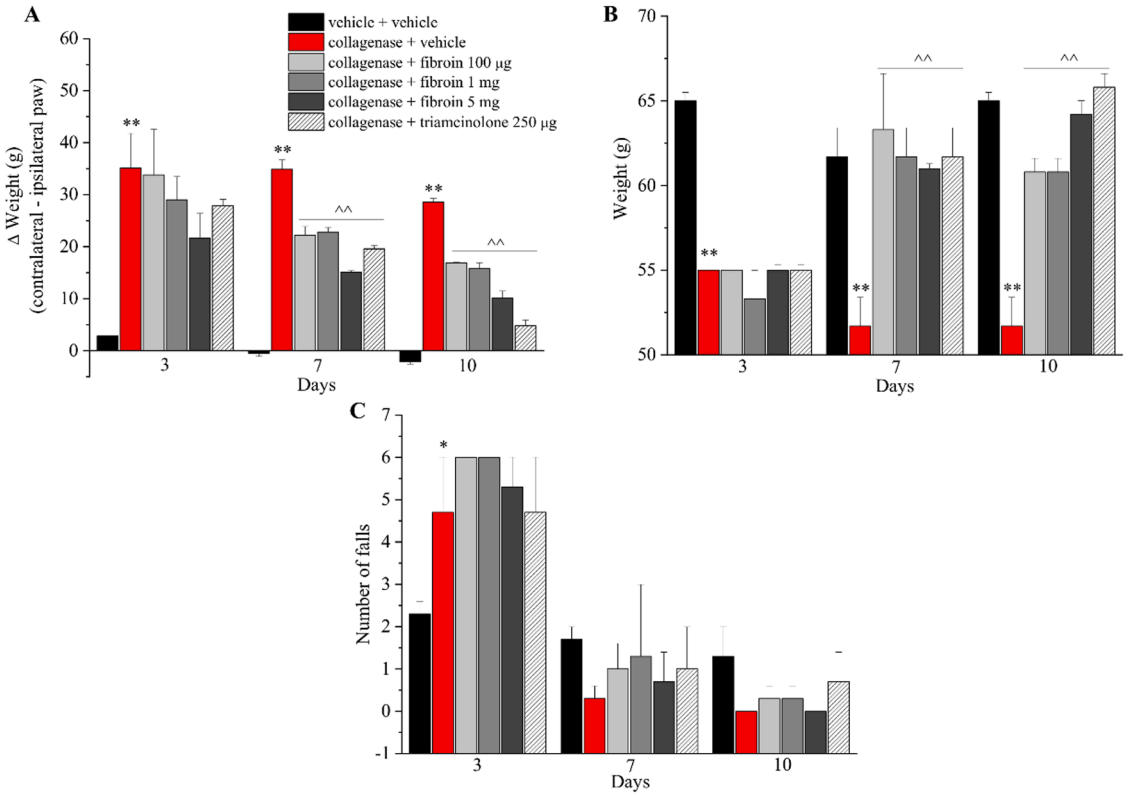

Fig. 5. Effect of peri-tendon injections of fibroin against collagenase-induced pain. Following intra-tendon injection of 20 μL of collagenase type I (20 mg/mL) on day 1, the efficacy of 4 peri-tendon injections of fibroin (100 μg, 1 and 5 mg in 20 μL) was evaluated. The effect was compared with that obtained from the injection of triamcinolone acetonide (250 μg/50 μL). The development of spontaneous tendon pain (A - Incapacitance test), the pain threshold in response to a painful stimulus (B - Paw Pressure Test) and the motor coordination of the animals (C - Rota Rod test), were carried out in time course (3, 7 and 10 days), after the induction of the damage. Control animals were treated with vehicle. The value represents the mean of 6 rats performed in two different experimental sets. * *P < 0.01and *P < 0.05 compared to the control; ^^P < 0.01and compared to collagenase treatment.

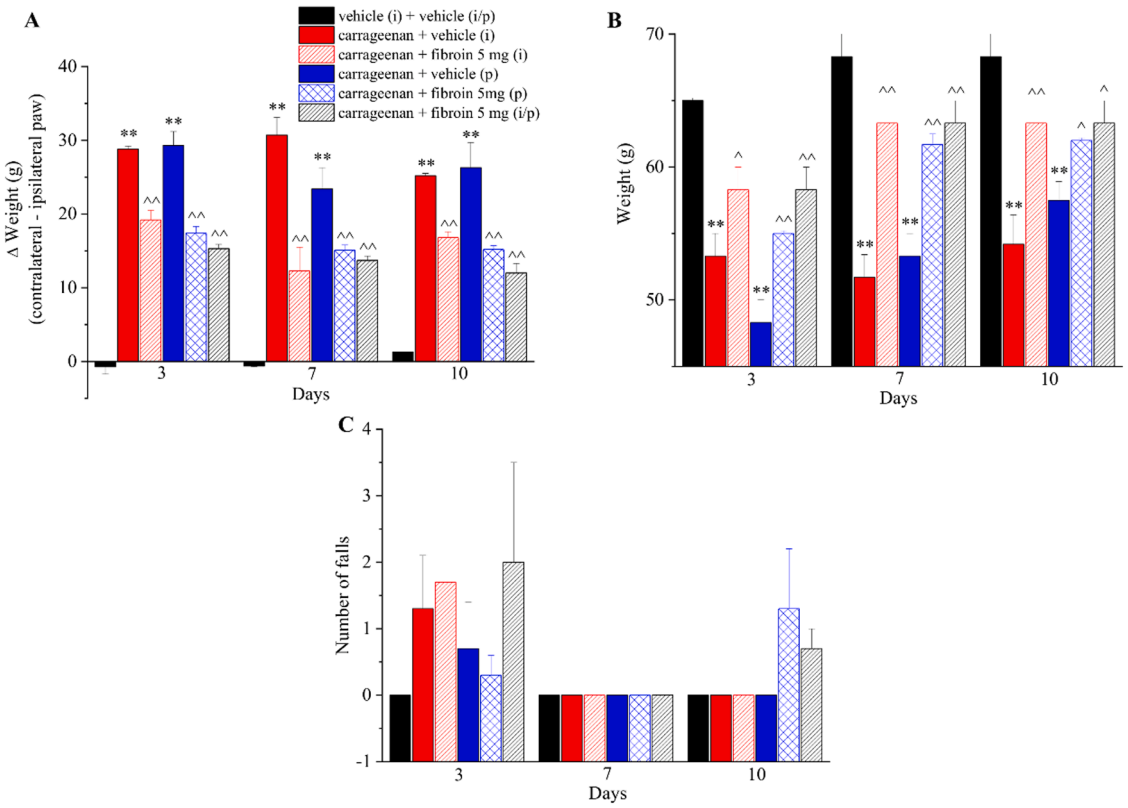

Fig. 6. Effect of intra-tendon, peri-tendon and intra/peri-tendon injections of fibroin against carrageenan-induced pain. Following intra-tendon injection of 20 μL of carrageenan 0.8% on day 1, the efficacy of intra-tendon, peri-tendon and intra/peri-tendon injections of fibroin (5 mg/20 μL) was evaluated. The development of spontaneous tendon pain (A - Incapacitance test), the pain threshold in response to a painful stimulus (B - Paw Pressure Test) and the motor coordination of the animals (C - Rota Rod test), were carried out in time course (3, 7 and 10 days), after the induction of the damage. Control animals were treated with vehicle. The value represents the mean of 6 rats performed in two different experimental sets. * *P < 0.01 compared to the control; ^^P < 0.01and compared to carrageenan treatment.

Fibroin reduced carrageenan-induced spontaneous pain (Incapacitance test) both after intra- and peri-tendon administration in a statistically significant manner (Fig. 6A; $p = 0.0065$ and $p = 0.0041$, respectively). The greatest effectiveness, however, was evident in the group of animals treated with both protocols ($P = 0.0037$, $n = 6$). The reduction in the weight difference occurred already on day 3 remaining constant for the entire duration of the experiment (day 10, Fig. 6A). The Paw pressure test showed an anti-hyperalgesic effect of fibroin given by all the treatments, with a peak of efficacy on day 7 when it reverted carrageenan-induced mechanical hyperalgesia (Fig. 6B, $p < 0.01$, $n = 6$). Regarding motor coordination, the injection of carrageenan did not significantly increase the number of falls compared to the control group (Fig. 6C). The data relative to Incapacitance Test and Paw Pressure Test (Fig. 6A and B, respectively) were collected after a 10 min-exercise on Rota-rod apparatus, during which the joint was subjected to stress. The results obtained before performing the Rota-rod (data not shown) were not different with respect of those obtained after the exercise (Fig. 6A and B).

3.7. Histological evaluation of 5 mg fibroin on collagenase- and carrageenan-induced tendinopathy

Based on behavioral assessments, the histological analysis was performed on tendons collected from animals treated with 5 mg peritendinous fibroin (on days 1, 3, 5 and 7) in the presence of collagenase damage (Fig. 7A), and intra-tendinous injection of 5 mg fibroin on day 2 after carrageenan damage (Fig. 7B).

Tendons of control animals appeared normal with well-aligned parallel and compact collagen fibers (Fig. 7A). Collagenase-treated animals showed a complete disorganization of the tendon matrix, referred to myxoid degeneration. Representative images demonstrate increased cell density with heterochromatic nuclei, inflammation and hypervascularization (Fig. 7A). Peri-tendon injection of fibroin 5 mg improved collagenase-induced damage, even if tendon matrix was still characterized by crimped collagen fibers and hypervascularization. Cell nucleus morphology was predominantly large and oval (Fig. 7A). A complete repair was achieved following triamcinolone treatment, collagen fibers appeared wavy, compact, and parallel arranged. Moreover, focal areas of elevated cell density, forming cell chains were detected (Fig. 7A).

Carrageenan induced a moderate disorganization of the tendon matrix represented by discontinuous, crimped and thinned collagen fibers, compared to control. Hypervascularity, represented by a moderate increased of smaller capillaries, and focal areas of irregular arranged cell, were also present. The intra-tendinous injections of fibroin were able to restore carrageenan-induced degenerative changes in the animals' tendons (Fig. 7B; $p = 0.0045$, $n = 6$).

4. Discussion

Our study highlights the protective properties of an aqueous solution of fibroin against *in vitro* and *in vivo* tendon damages. *In vitro*, fibroin counteracted the oxidative damage exerted on primary-cultured tenocytes by increasing cell viability, proliferation and improving cells morphology. *In vivo*, the local injection of fibroin in two rat models of tendinopathy (collagenase- or carrageenan-induced, respectively), relieved pain and improved tendon histology.

Classical therapeutic approaches in the treatment of tendinopathies

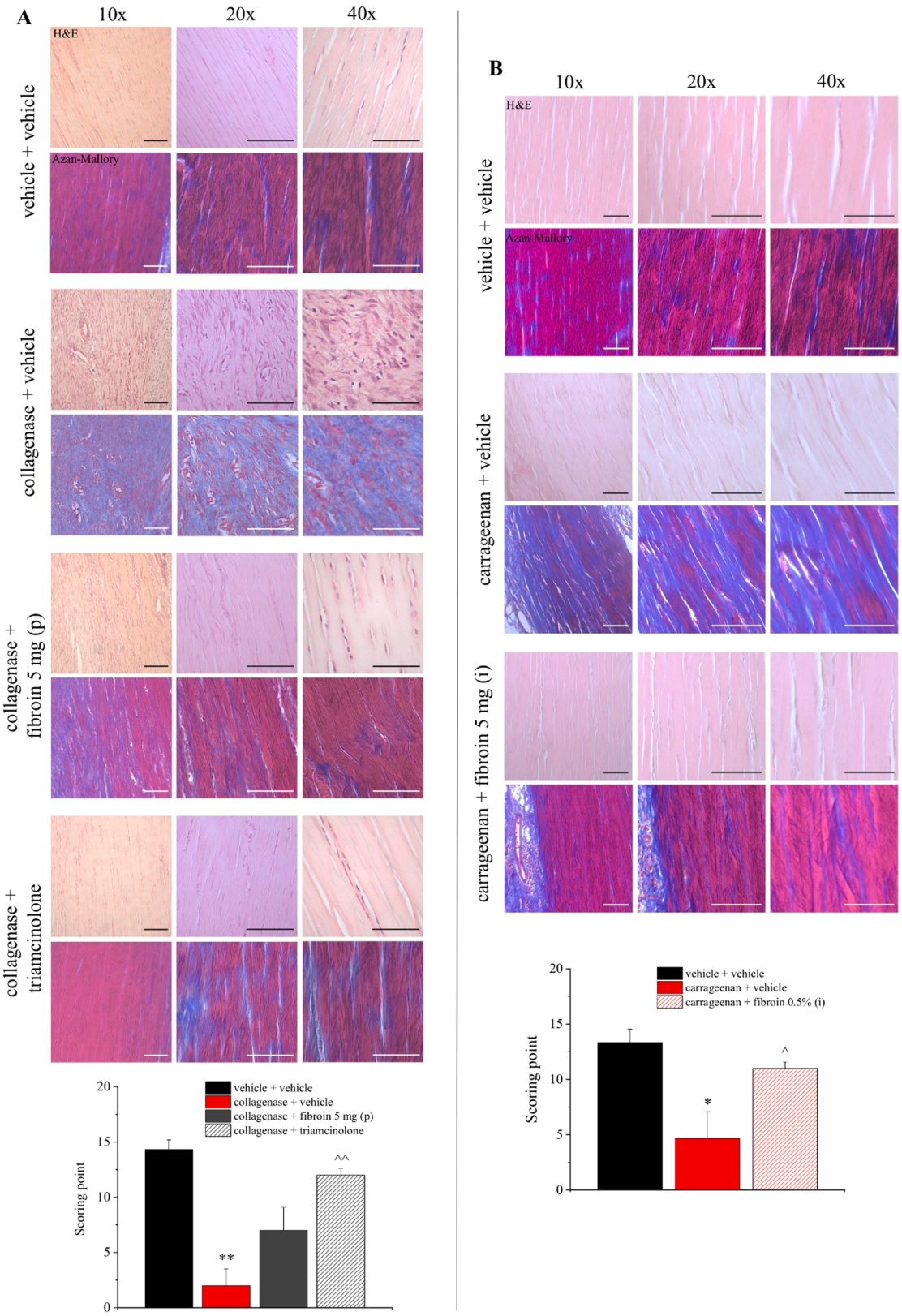

Fig. 7. Histological evaluation of fibroin injections on tendinopathy models. After (A) 4 peri-tendon treatments with fibroin (5 mg) on collagenase damage, and (B) intra-tendon injection of fibroin (5 mg on day 2) on carrageenan damage, tendon samples of the animals were collected. To make histological evaluation, Hematoxylin-Eosin and Azan-Mallory staining were performed. The histological score was calculated according to the following parameters: extracellular matrix organization (0–2), tissue homogeneity (0–2), presence of degenerative changes (0–2), cell nucleus morphology (0–2), cell distribution (0–2) and alignment (0–2), vascularization (0–1), inflammation (0–1), Azan-Mallory red stain intensity (0–2). Total score for each animal ranged between 0 (most severe tendon impairment) and 16 points (control, normal tendon). * *P < 0.01and *P < 0.05 compared to the control; ^^P < 0.01 and ^P < 0.05 compared to damage.

focuses on reducing inflammation and pain. However, many evidences suggest that the inflammatory component is reduced or absent in tendons unable to heal on their own [8]. Chronic pathological conditions involve alterations of biochemical processes, with a consequent loss of the mechanical integrity of the tendon [49]. Such conditions require treatments able to stimulate tissue regeneration. Local glucocorticoids injections are widely used for the treatment of tendinopathies, though positive results have been observed in some tendinopathies but not in others [50]. Indeed, corticosteroid injection is associated with beneficial effects in the short-term treatment of tendinopathy, while worsening of symptoms and spontaneous tendon ruptures have been reported after long-term treatments [51,52]. One of the primary outcomes of clinical efficacy is the pain score and corticosteroids turn out to be effective in tendinopathy mainly because they effectively relieve pain [53]. At the same time, masking painful symptoms, corticosteroids cause the individual to overexert a weakened tendon [50,54]. Biochemical investigations have shown that collagen synthesis can be decreased by corticosteroids [55–57], which can arrest cell proliferation and impair the viability of fibroblasts, thereby limiting tendon matrix regeneration [58,59]. I*n vitro* and *in vivo* evidence attest corticosteroids inhibit the differentiation of stem cells to tenocytes and inhibit Sirtuin 1 and the activation of p53/p21 pathway, thereby inducing irreversible senescence in human tenocytes [60–62]. In addition, corticosteroids treatment depletes the stem cell pool, which is the endogenous source for tendon repair and leads to the growth of non-tendinous tissues (e.g. adipose tissues) [63]. In the present work, by exploring the therapeutic potential of fibroin from different points of view, we observed that this protein can enhance tenocyte viability and proliferation, positively modulate gene expression, and enhance collagen production in primary culture of tenocytes, a combination of mechanisms that results in pain attenuation and tendon damage restoration in vivo. These effects might compensate for deficiencies in corticosteroid treatment. This is an aspect we will consider in future research aimed at maximizing the therapeutic efficacy of fibroin. The long-term beneficial effect provided by fibroin injection on tendon structure and tenocytes function, once combined with corticosteroids anti-inflammatory and pain-relieving efficacy, might empower the treatment of tendinopathy, and, besides, allow overcoming the therapeutic limits of corticosteroids-based approaches.

Silk fibroin, is considered a promising biomaterial for applications in tissue engineering and regeneration [64]. Compared to other biomaterials commonly used in tissue engineering, fibroin has exceptional mechanical strength and high thermal stability. It also contains the Arg-Gly-Asp (RGD) tripeptide sequences, which are capable of supporting cell adhesion, proliferation and migration of various cell types [65–68], including fibroblasts, keratinocytes, osteoblasts, epithelial, endothelial and glial cells [37,69,70]. Fibroin is often used as fiber to obtain scaffolds, but it can also be used as a powder, solution, hydrogel, film or sponge [64]. Our attention has been placed on the use of fibroin as aqueous solution, which in various studies has shown to improve the adhesion and proliferation of human fibroblasts [36–38].

Treatment of primary tenocytes with the enzyme glucose oxidase (GOx) was used to reproduce an oxidative damage *in vitro*. GOx catalyzes the glucose oxidation into gluconic acid and hydrogen in the presence of oxygen. Glucose depletion can block the energy supply to cells, while rising H_2O_2 levels can promote cell death. This process reduces oxygen levels, resulting in high acidity, hypoxia and oxidative stress [71]. This model was inspired by the evidence of a correlation between oxidative stress and degeneration of tendon tissue [72–74]. Indeed, a molecular link between the exaggerated dysfunctional repair response in tendinopathies and the subsequent orchestration of effective tendon healing is the control of the production and persistence of reactive oxygen species within the intracellular and extracellular milieu of the tendon tissue [75].

In vitro experiments allowed us to highlight the molecular effects exerted by fibroin directly on tenocytes, which are the main responsible for the production and the organization of the tendon matrix. Treatment with GOx (0.1 U/mL) reduced cell viability of tenocytes inducing loss of the physiological cell morphology, reduction in the organization of the filamentous structure of the cytoskeleton, and damage to the membrane. In our experiments, fibroin, employed at concentrations able to induce proliferative effects, resulted effective in restoring the normal organization of the cytoskeleton which was impaired following GOx damage. Furthermore, the cells treated with fibroin alone show a better organization of F-actin filaments, which appeared oriented in only one direction. Mechanical forces exerted by the ECM on the cell can cause signaling cascades that end with the reorganization of the cytoskeleton, changes in gene expression, protein synthesis, and cell differentiation. Conversely, signals from inside the cell can propagate outwards (for example through specific membrane proteins, such as integrins), regulating the cell-matrix interaction [2]. Therefore, the influence of fibroin on the orientation of the F-actin filaments in the cytoskeleton could be related to response mechanisms capable of influencing the composition and organization of the ECM.

Noteworthy, fibroin exerted a direct pro-proliferative action on tenocytes as attested by its ability to upregulate the expression of *scleraxin* (*Scx)* and tenomodulin (*TNMD)* genes, greater than that shown by FGF-2 (promoter of fibroblast-like cell proliferation). *Scx* is a basic helix-loop-helix (bHLH) transcription factor that plays a central role in promoting tenocyte proliferation and ECM synthesis during embryonic tendon development. The role of *Scx* in the growth and adaptation of adult tendons is not completely understood but has been hypothesized that *Scx* is involved in the growth of tendons promoting the differentiation of tendon progenitor cells into tenocytes [76]. *TNMD* is also a regulator of tenocyte proliferation and tendon development, but it acts in the late phase of cells differentiation [77]. The treatment with fibroin upregulated the expression of *FGF-2*, *tenascin* (*TNC),* and *decorin*. FGF-2 plays important roles in the development and functionality of numerous organs [78] and can improve the formation of tendon tissue by stimulating the proliferation of cells expressing Scx or TNMD [79]. TNC is a constitutive glycoprotein that contributes to the mechanical stability of the ECM and has a fundamental role in mediating cell-cell and cell-matrix interaction [2]. TNC can modulate either the cell adhesion and the cell signaling mediated by integrins [80–82] assuming a decisive role in the transduction of mechanical signals. On the other hand, *decorin* is a proteoglycan of the small leucine-rich proteoglycans (SLRPs) type involved in the growth and organization of collagen fibrils and in the assembly of ECM [83]. SLRPs are also known to impact many of the critical functions of fibroblasts during the wound healing process, including migration, proliferation, cell differentiation, and collagen synthesis [84]. The regulation of all these genes indicates that fibroin can modulate the signaling mechanisms responsible for the production of ECM components and attests to its regenerative and proliferative actions.

Collagen type I is the main component of both healthy and mature tendons. Collagen fibrils progressively assemble into a highly organized network that gives the tendon mechanical and structural integrity, thus allowing tissue functionality [85]. The increase in collagen production attests to the regenerative potential of fibroin. Indeed, stimulating the synthesis of ECM components is a fundamental property in the context of the regenerative therapeutic approach. During tendon healing, the cells surrounding the injury make a transition from a quiescent to a proliferative status, promoting ECM production and reorganization [86].

Fibroin increased metalloprotease-9 (MMP-9) expression, both in the presence and in the absence of oxidative damage. MMPs are a family of zinc-dependent endo-proteases with multiple roles in tissue remodeling and degradation of different ECM proteins. MMPs promote cell proliferation, migration, differentiation and are involved in cell apoptosis, angiogenesis, tissue repair, and immune response [87]. The increase in MMP-9 observed by treating tenocytes with fibroin alone could be explained by the structural changes observed in the immunofluorescence assay: variations in the organization of the tenocyte cytoskeleton might be responsible for the reorganization of tenocytes surrounding

matrix, allowing them to assume a more orderly arrangement.

The positive effects exerted by fibroin on cell viability, proliferation, and morphology predicted its ability to counteract tendon damage and to reduce pain associated with tendinopathy in animals. The intra-tendon injection of collagenase type I in rats induced a reproducible and consistent lesion, including fiber disruption, disordered collagen arrangement, and alteration in the ECM components [88]. As previously reported, collagenase injection into a tendon modifies its biochemical and biomechanical properties, resembling the main histopathologic features and dysfunctions observed in human tendinopathies [89–91]. Similarly, carrageenan injection caused the segregation of tendon fibers, the formation of intra-tendinous clefts, and a wave arrangement of tendon fibers with significant morphological changes [92,93]. Carrageenan induces the accumulation of neutrophils in the perivascular space [94], accompanied by the local release of chemical mediators such as glutamate, prostaglandins, histamine, and serotonin, which sensitize the primary afferents resulting in hyperalgesia [95–99]. The unilateral intra-tendinous injection of both collagenase and carrageenan-induced the development of spontaneous pain and alteration of the pain threshold in rats, starting 3 days after the injection and lasting the entire duration of the experiments.

We decided to compare the effect of a single intra or peri-tendinous injection of fibroin in both models. From our results, it emerged that both the routes of administration of fibroin allowed to reduce the pain induced by collagenase in rats; in particular, peri-tendon injection of fibroin reverted pain associated with collagenase-induced tendinopathy at all the concentrations tested. The higher concentration of fibroin was also able to improve collagenase-induced morphological damage on tendon tissue.

Peritendinous injection is typically used in the treatment of chronic tendinopathies [100]. This route of administration, in fact, avoids injecting the drug into tendon substance, a procedure that might contribute to tendon rupture. The anti-hyperalgesic profile of fibroin was comparable to that of triamcinolone, a corticosteroid used in the treatment of tendinopathies [101]. The restorative and pain-relieving effects of fibroin were also confirmed in the model of tendinopathy caused by carrageenan injection.

As mentioned above, previous findings in the literature reported fibroin as an extremely interesting biomaterial for applications in tissue engineering to trigger tissue damage repair responses by implanting scaffold-supported cells at the injury site [102–104]. Our results corroborated this evidence, demonstrating the proliferative and restorative effects exerted by fibroin on either tenocytes or tendons and demonstrated for the first time the fibroin anti-hyperalgesic efficacy against tendinopathy-related pain. Noteworthy, fibroin resulted able to directly regulate tendon regeneration, starting from the upregulation of proliferation-related genes in tenocytes to ECM production and remodeling, attesting a molecular signaling activity for fibroin behind its well-known biomechanical properties.

Silk fibroin biomaterials are also widely investigated for the support to nervous tissues healing [105] since silk fibroin is endowed with intrinsic properties, and the by-products derived from its degradation show anti-inflammatory and antioxidant properties [106]. Silk fibroin microparticles, injected into a brain damage area 1 day after the injury, were shown to exert neuroprotective effects by limiting brain damage and enhancing recovery of long-term neurological functions. Moreover, the cultivation of primary cell cultures of neurons and astrocytes on silk fibroin matrices demonstrated their higher viability under oxygen-glucose deprivation compared to 2D conditions on plastic plates [107]. Innervation of intact healthy tendons is localized in the surrounding structures, whereas the tendon proper is practically devoid of neuronal supply. After the injury and during the repair process, an extensive nerve ingrowth into the tendon proper is found, followed by a time-dependent emergence of different neuronal mediators, which amplify and fine-tune inflammatory and metabolic pathways in the tendon regeneration [108]. In this context, fibroin might also participate in the modulation of neuronal signaling and plasticity, though this hypothesis needs further investigations. These observations, in addition to providing novel knowledge, open new ways to the therapeutic employment of this natural protein.

5. Conclusions

The local injection of fibroin emerges as a valid strategy for the treatment of tendinopathies taking advantage not only of its excellent mechanical properties, but also of its ability to favor the tissue regenerative processes by triggering molecular pathways and, finally, of its beneficial effects against pain.

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CRediT authorship contribution statement

Laura Micheli: Investigation; Validation; Writing – original draft; Data curation. **Carmen Parisio:** Formal analysis; Writing – original Draft; Data curation. **Elena Lucarini:** Conceptualization; Methodology; Writing – review & editing; Visualization. **Donatello Carrino:** Software; Formal analysis; Writing – original draft. **Clara Ciampi:** Software; Data curation. **Alessandra Toti:** Investigation; Methodology; Writing – review & editing. **Valentina Ferrara:** Formal analysis; Methodology. **Alessandra Pacini:** Resources; Supervision; Visualization. **Carla Ghelardini:** Resources; Supervision; Funding acquisition. **Lorenzo Di Cesare Mannelli:** Conceptualization; Project administration; Writing – review & editing.

Conflict of interest statement

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability

Data will be made available on request.

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