Effects of platelet-rich plasma (PRP) on the healing of Achilles tendons of rats

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ABSTRACT

Platelet-Rich Plasma (PRP) contains growth factors involved in the tissular healing process. The aim of the study was to determine if an injection of PRP could improve the healing of sectioned Achilles tendons of rats. After surgery, rats received an injection of PRP (n=60) or a physiological solution (n=60) in situ. After 5, 15 and 30 days, 20 rats of both groups were euthanized and 15 collected tendons were submitted to a biomechanical test using cryo-jaws before performing transcriptomic analyses. Histological and biochemical analyses were performed on the 5 remaining tendons in each group. Tendons in the PRP group were more resistant to rupture at 15 and 30 days. The mechanical stress was significantly increased in tendons of the PRP group at day 30. Histological analysis showed a precocious deposition of fibrillar collagen at day 5 confirmed by a biochemical measurement. The expression of tenomodulin was significantly higher at day 5. The mRNA level of type III collage, matrix metalloproteinase 2, 3 and 9 was similar in the 2 groups at all time points whereas type I collagen was significantly increased at day 30 in the PRP group. In conclusion, an injection of PRP in sectioned rat Achilles tendon influences the early phase of tendons healing and results in an ultimate stronger mechanical resistance.

INTRODUCTION

Tendinous lesions are among the most frequent pathologies encountered in sportsmen and physical workers [1]. Improving the healing process and reducing the recovery time are the objectives of new treatments in development among which the use of growth factors. Injection of platelet-rich plasma (PRP) is a simple and little invasive procedure to deliver a natural mixture of autologous growth factors [2]. Some in vitro or animal studies have demonstrated that injection of concentrated blood platelets can initiate and stimulate tendon and ligament repair by locally releasing biological mediators [3-10]. PRP would enhance the type I and type III collagen synthesis by tendon cells as reported in some studies [5, 10-12]. It has also been suggested that PRP would limit matrix degradation by decreasing the matrix metalloproteinases (MMPs) [13]. Among them, MMP-9 is produced mainly by inflammatory cells and play a role in the inflammation-induced tissue remodelling associated with various physiological and pathological processes [14].

We therefore speculated that the healing process of sectioned Achilles tendons of rats and the mechanical resistance of the restored tissue could be improved by an injection of PRP. This hypothesis was tested by measuring the biomechanical properties of healing tendons during repair process. Histological and biochemical analyses were further performed to evaluate the extracellular matrix remodelling and collagen deposition in the newly formed matrix.

MATERIALS AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the University of Liège. The "Guide for the Care and Use of Laboratory Animals" was followed carefully [15]. One hundred and thirty-two Sprague Dawley male rats of 2 months (weight ± 320g) were used according to an experimental design summarized in Fig. 1.

1. Preparation of PRP: Whole blood was collected from 12 male Sprague Dawley. The donor rats (4 for one experiment on a group of 15 rats) were intraperitoneally (i.p.) anaesthetised with pentobarbital 60mg/kg and blood collected by cardiac puncture. One ml of anticoagulant, adenosine-citrate-dextrose-acid (ACD-A), per 4,5mL of blood was immediately added. We used a previously described technique designed to collect concentrated preparation of platelets inspired from the Plateltex® system (Prague, Czech Republic) [16]. This procedure allowed to obtain the highest platelet concentration in the smallest volume of PRP, by performing a first centrifugation of the blood, and a second of the isolated buffy coat with a little quantity of plasma to improve the platelet concentration [16]. Cell and platelets counts were made by an auto-analyser ABX Micros 60 (Horiba ABX, Kyoto, Japan). Platelets concentration was around 2.2 to 2.9 $x10^6/mm^3$ indicating a 3 to 4 fold increased density as compared to the peripheral blood in rats. We collected nearly no white nor red cells in the PRP; the white blood cells count was around $1x10^2/mm^3$ and red blood cells around $1x10^4/mm^3$.

2. Surgical procedure (Fig. 2): Rats were weighed and anaesthetised i.p. with pentobarbital (60m/kg of body weight), placed on a warm pad and the skin of the left hind limb shaved. Buprenorphine (0.05mg/kg of body weight) and tetracycline (15µg/kg of body weight) were directly given a priori by subcutaneous way. The complete surgical procedure was performed

under aseptic conditions under a dissecting microscope. The skin of the left Achilles tendon was incised laterally. The Achilles tendon complex was exposed after dissection of the surrounding fascia. The plantaris tendon was removed to avoid errors of measurement during the biomechanical testing. Subsequently, 5 mm proximal to its calcaneal insertion, the Achilles tendon was transversally cut and a 5 mm segment was removed. The tendon was left unsutured with a gap between the tendon stumps. The fascia and the skin were sutured with resorbable yarn Vicryl 6/0 (Ethicon, Johnson & Johnson, New Jersey, USA). The animals were placed in clean cages under heating lamp until awakening and there was no postoperative immobilisation. The rats were divided into "control" and "PRP" groups that received two hours postoperatively (after the clot formation was already initiated) a local injection (inside the defect) of 50µL of a physiological solution or fresh PRP after activation with 50µL $CaCl_2$ (0,55 M)/mL PRP. They were checked daily and followed for well-being and a global observation of walk and activity.

We previously controlled that the injection of physiological solution was not harmful for the healing process of tendon. Indeed, we did not observe any biomechanical difference between the natural healing process of sectioned Achilles tendons of rats (without injection) and the healing process of tendons injected with physiological solution (unpublished personal data). Furthermore, we measured the coagulation time (aPPT) of non-diluted rat plasma or plasma supplemented with 10% of physiological saline and we did not observe any difference between both groups suggesting that the addition of 10% of solute should not alter early phase of natural healing process.

3. Biomechanical evaluation: Five, 15 or 30 days post-surgery, 15 rats of both groups were weighed and euthanized with pentobarbital (i.p., 60mg/kg). The healing tendon, with the at-

tached calcaneal bone and a part of the triceps suralis, was dissected from surrounding tissues. Since rats were not immobilised after surgery, the healed tendon had very little adhesions, had the ability to glide smoothly in its sheath and a full range of motion of the joint was observed when dissecting tendons at the 3 time points. The mechanical testing was done using a traction – compression testing machine (106.2kN, TesT GmbH, Dusseldorf, Germany) and an original clamping device type "cryo-jaw" [17] shown to better preserve tendon integrity. The muscle-tendon-bone unit was fastened in the clamping device by freezing the muscular segment (triceps surae) with liquid nitrogen and clamping it between the "cryo-jaw" and by fixing the bony segment between the lower clamps. This device dramatically reduces the amount of pre-stress applied to the specimen before the actual loading takes place. It thus allows us to assume a stress free configuration at the beginning of each test. Furthermore, the risk of mechanical damage of the tissue is significantly lowered. Freezing the muscle with liquid nitrogen increases its stiffness and allows us to consider it completely rigid compared to the tendon.The cross-sectional area of the samples was calculated from pictures made with a set of 2 cameras positioned perpendicular to each other, assuming an elliptical shape. The tensile test was started as soon as the expansion of the freezing-zone reached the border of the metal clamp but did not extend into the tendon tissue. The displacement rate was set at a constant speed of 1mm/sec until rupture. Force vs displacement curves were recorded by a computer for subsequent data analysis. Ten non-injured rat Achilles tendons were similarly tested to establish normal basal values. The force at rupture or ultimate tensile strength (UTS) was expressed in Newton (N). To account for the difference in the cross-sectional area of the healing tendons, the value of UTS was normalised to a unit area (N/mm² or mega-Pascal MPa) and represents the mechanical stress of the tissue.

4. Histological evaluation: Five tendons of each group were fixed with 4% paraformaldehyde and embedded in paraffin. The specimens were sectioned (5µm) parallel to the longitudinal direction of the tendon. Only sections from the middle of the tendon callus were used and stained with Hematoxylin-Eosin (HE) and Masson's trichrome (MT). Some sections were stained with Light Green (LG) alone to visualise and quantify fibrillar collagen by computer assisted analysis. The sections were scanned by using Olympus BX51 (Olympus Corporation, Tokyo, Japan) and pictures were converted in nuances of grey using "IrfanView" software (Irfan Skiljan, Wiener Neustadt, Austria). Semi-quantification was made by using "Quantity One" software (BioRad Laboratories, Hercules, California, USA). Results were expressed as arbitrary units per unit surface.

5. Biochemical evaluation: Samples for biochemical analyses were taken directly after tendon rupture during the mechanical test, snap frozen in liquid nitrogen and stored at -80°C until use. Isolation of total RNA from the samples was performed using the RNeasy Total RNA Kit (Qiagen, Venlo, The Netherlands). The expression of type I (Col I) and type III collagen (Col III), matrix metalloproteinases 2 (MMP-2), 3 (MMP-3) and 9 (MMP-9) and tenomodulin (TNMD - a regulator of tenocyte proliferation further involved in collagen fibril maturation [18]) was measured by quantitative RT-PCR using an internal synthetic standard RNA as previously described [19]. Expression levels of mRNA were normalised to the levels of 28S. We also have measured the mRNA level of Col I, Col III, MMPs and TNMD in non-injured tendosn.

6. Histological sections of five tendons in each group were hydrolyzed in 6N HCl for 3 hours, and collagen content determined by measuring hydroxyproline (OHPRO - a specific aminoacid only found in collagen) [20], as an index of collagen concentration in 5µm un-

stained paraffin sections. Results were normalised by the section area measured by the "Quantity One" software and expressed in µg of collagen per unit surface.

7. Statistics: Results are expressed as the mean ± standard deviation of the mean. The bio-mechanical results were compared with analysis of variance using a two-way ANOVA and post-hoc test of Scheffé (parametric test). This analysis was done with statistical software Statistical Analysis System version 9.1 (SAS Institute, North Carolina, USA) and a level of significance set at $p<0.05$.

RESULTS

No significant difference in the body weight was observed between each group at the different times of the experiment. The UTS required to induce rupture of non-injured rat Achilles tendons was 42.0 ± 5.7 N (n=10). Five days after surgery, UTS was low in both control and PRP groups (Fig. 3a) and significantly increased with time in both groups ($p<0.0001$), above the basal normal values. At each time point, the UTS was higher in the PRP group, most significantly at day 15 and 30.

The cross-sectional area of non-injured tendon was 11.4 ± 5.5 mm^2 (n=10). Five days after injury, it was larger in the physiological saline-treated injured tendons and further significantly increased (+25%) in the PRP group (Fig. 3b). The section area of tendons in the control group continued to enlarge at day 15 and then levelled off at day 30. The cross-sections in the PRP-treated tendons were still significantly larger although more variable as compared to the control tendons at day 15 (+25%) and slightly decreased at day 30 to attain a size similar to that of controls.

As a consequence of the evolution of these parameters, the UTS per unit surface (mechanical stress) of the healing tissue was similar in both groups at day 5 and similarly increased at day 15 (Fig. 3c). However, at day 30, the PRP group displayed significantly higher mechanical stress values ($p=0.02$) than the control group.

The HE stained sections of the healing tendons at day 5 showed a high cellularity that decreased with time. No obvious difference with histological aspect was observed between the two groups. The MT staining revealed at day 5 a stronger green staining specific for fibrillar collagen in the PRP treated tendons (Fig. 4). A semi-quantitative evaluation was performed by

computer-assisted image analysis on tendon sections only stained with LG. Although not statistically significant, the intensity of the staining tended to be higher in the PRP group at day 5 ($p=0.06$) and also at day 15 but more variable. It was similar in both groups at day 30 (Fig. 5a). These data were objectivized by using another technique consisting in the measurement of the amount of hydroxyproline, an aminoacid specific to collagen (Fig. 5b). It was significantly increased in the PRP group at day 5 as compared to the control group ($p=0.03$) confirming the histological semi-quantifications (Fig. 5b). The concentration of collagen was quite variable in the PRP group as stated above, and levelled off at day 30 similarly in both groups. As the volume of the callus, as inferred from the transverse area measurements during the mechanical assays, is significantly higher in the PRP-treated animals in the early phases of the healing, these results suggest that PRP delivery in the injured tendon induced an increased and precocious deposition of fibrillar collagen in the healing tendon.

The expression level of selected molecules involved in the tendon healing process was measured by RT-PCR: collagen type III as one of the fibrillar collagens synthesised by tenocytes in vitro and during repair processes, while collagen type I is the predominant collagen found in the healthy tendons, MMP-9 as a matrix metalloproteinase produced by inflammatory cells and MMP-2 and 3 as enzymes involved in matrix remodelling, and TNMD, a marker of tenocyte differentiation [18, 21] (Fig. 6). Col III and TNMD were around 2.5-3.0 less expressed in non-injured tendons as compared to healing tendons at day 15. By contrast, Col I expression was almost similar in both types samples. As expected, MMPs were very little expressed in healthy tendons and around 12 times higher in the healing tendons whatever the time of healing.

A significantly increased expression of COL1A1 was observed in the PRP group at day 30 ($p=0.002$). Furthermore, a significant positive correlation was found between the COL1A1 expression and the UTS. The expression of Col III mRNA was high during the 2 first weeks and then decreased at day 30 similarly in both groups. The expression of mRNA coding for the MMP-9 was stable in both groups at the 3 time points. The expression of MMP-2 and MMP-3 was increasing with time during the healing process but are not influenced by the PRP. The expression of the mRNA coding for the TNMD was significantly increased in the PRP-treated animals at day 5 ($p=0.03$). It strongly increased at day 15 followed by a reduction at day 30, similarly in both groups.

DISCUSSION

Platelets are critical cells during tissue injury particularly during the early inflammatory phase. Platelets degranulation as seen during exposure to clotting factors or by contact with connective tissues structures releases growth factors stored in α granules such as PDGF, TGF-β, bFGF, VEGF, EGF and other soluble mediators [22]. All together, these factors stimulate the synthesis of extracellular matrix macromolecules and mesenchymal cells proliferation, exert chemotactic activity towards circulating progenitor cells and promote angiogenesis and cell differentiation [8, 22]. In this study, we used complementary experimental approaches to evaluate the influence of a single post-operative injection of PRP on the repair of ruptured Achilles tendon by following mechanical, histological and biochemical parameters as a function of time during the healing process.

The mechanical studies were performed by using a high performance clamping device specially designed for ex vivo tensile testing of the Achilles tendon in rats [17]. The “cryo-jaw” device consists in snap freezing the upper extremity of the specimen (part of the triceps surae) by liquid nitrogen, the lower extremity being fixed by the calcaneal bone. It is a simple, secure and non-compressive clamping method, avoiding tissue damage and preserving mechanical integrity. By using this procedure on healthy Achilles tendons, the UTS was 42 N, a value close to that previously reported [17].

Our tensile data (Fig. 3) on injured tendons showed that a PRP injection significantly improved the UTS of the healing Achilles tendons 15 and 30 days after surgery and the mechanical stress in the late phase (30 days) of the repair. The UTS increases also with time in the control group in relation with the normal healing process, which is however slower as compared to the PRP group. A most significant observation was the increased transverse area of

the PRP-treated tendons already 5 days post-surgery that persisted until day 15 to return to the control values at day 30. These results suggest that PRP delivery at the time of surgery improved the ultimate mechanical resistance of the healing tendons without however attaining the mechanical stress value of healthy tendons (4.4 ± 2.0 MPa, n=10). The increase of UTS measured at day 15 coincides with a significant increase in cross sectional area that can be explained by the inflammation in the early phase of healing. Both UTS and cross-sectional area are higher in the PRP-treated tendons as compared to the physiological saline-treated tendons. This results in a similar mechanical stress of the tissue. By contrast, at day 30, the cross-sectional area of the tendon is stabilized at a similar value for both groups of animals likely related to a decrease in inflammation and the organization of a cicatricial matrix but the UTS continues to increase in the PRP-treated tendons. This leads to a significantly higher mechanical stress in the PRP group indicating a stronger resistance of the newly formed tissue. This might be related to the higher expression of type I collagen observed at day 30.

To support our mechanical results, we further investigated the organisation of the healing tissue by histology and by measuring various metabolic parameters. The HE staining of the tendon sections 5 days post-injury showed a highly cellularized tissue with no obvious differences between the two groups (Fig. 4). However, a MT and LG staining suggested an increased deposition of structured collagen fibres already at day 5 in the PRP treated group that was semi-quantified by computer-assisted image analysis. It was further objectivized by measuring collagen concentration in tendon sections (Fig. 5). It was indeed significantly increased in the PRP-treated group at this early stage of healing. At day 15, a great variability was observed for PRP samples that could be explained by the inter-individual variation of responses to PRP. As the transverse area and thus the size of the callus are significantly enhanced in the PRP-treated tendons, the absolute amount of collagen deposited in the callus is

therefore increased. Although we did not observe altered level of collagen III mRNA in the PRP-treated tendons (Fig. 6), an increased level of Col I mRNA was measured in the PRP-treated tendons at day 30 [13]. Furthermore, it has been previously shown that PRP inhibits proliferation of macrophages and IL-1 [23] and could prevent an excessive inflammation during the early phase of healing [7], and promote proliferation, metabolic activity and differentiation of the mesenchymal cells into active tenocytes [11]. The higher expression level of TNMD in the PRP-treated tendons further suggests that the locally injected biological mediators may recruit circulating cells [12] and induce their differentiation towards tenocyte phenotype. Altogether, these results clearly demonstrate that a single post-operative injection of PRP in ruptured Achilles tendon influences the early phase of healing and result in an ultimate stronger resistance. These data support previous observations [4, 9, 24], that the very early improvement of the quality of the callus allow cells to perceive and respond to mechanical loading, a potent mechanical regulatory process of the metabolic activity of mesenchymal cells [11, 25]. Indeed, Aspenberg and his team observed that platelets injection could improve the mechanical properties of tendons as soon as 3 and 5 days [9]. They demonstrated that mechanical loading is important for growth of the callus but not its mechanical quality. The main effect of loading during healing might thereby be sought among growth stimulators [24], confirming our results for the mechanical stress. However, in the late phase of healing, tendon-specific genes as TNMD and scleraxis, were upregulated with loading, and the healing tissue might to some extent represent a regeneration of tendon rather than a scar [24]. Our results reinforce also the in vitro observations of de Mos et al [5] and Schnabel et al [26] about the increased synthesis of collagen after injection of PRP.

Further studies are now needed to confirm these positive effects in human tendinopathies. Indeed, many controversies exist related to the use of this treatment in practice in human tendon lesions [27, 28], perhaps due to a lack of mobilisation after injection, as Aspenberg goup suggested in their rat models [9, 24]. Indeed, they demonstrated that the healing process of sectioned Achilles tendon after an injection of PRP was better in rats freely moving than in rats immobilised by an injection of botulinum toxin A in the injured leg. As we also observed, platelets improve the early phases of the healing process of tendons allowing tendon cells to perceive and respond to mechanical loading more rapidly than in untreated tendons. This concept of mechanical stimulation after PRP treatment could be perhaps beneficial to human tendon pathologies such as ruptures or tendinopathies.

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The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1: Chart of the experimental design.

Fig. 2: Surgical procedure. a) The Achilles (✦) and plantaris (✻) tendons were dissected. b) The plantaris tendon was removed. c) The Achilles tendon was transected and a 5mm defect was made. d) The samples of the whole plantaris tendon and 5mm of the Achilles tendon. e) The fascia and after the skin were each surgically closed.

Fig. 3: a- Breaking strength expressed in Newton (N) recorded in control groups (n=15 at each time point) and PRP groups (n=15 at each time point) (mean ± SD) at increasing time after surgery (5, 15 and 30 days). The dotted line represents the mean value measuring for healthy Achilles tendons of rats (42N, n=10). A significant increase of UTS occurred in both groups with time, the PRP group showing significantly higher values than the control group at day 15 and 30. * p-value < 0.05. b- Evolution of the transverse area of the tendons (mm^2) with time (mean ± SD, n=15 at each time point). The section area for healthy tendons is 11 mm^2. Tendons of the PRP group were characterized by significantly larger cross-sectional area during the 15 first days. After 1 month, tendons of both groups were similar. c- Calculated ratio between UTS and the surface area of the section of the tendon (in MPa) at the three time points (mean ± SD). The mechanical stress values for healthy tendons is 4,4MPa. Mechanical stress of both groups were similar after 5 and 15 days and significantly higher in the PRP group at day 30.

Fig. 4 Representative longitudinal sections of Achilles tendon of rats from control (C) and PRP groups, stained with HE and MT. Scale bar = 100µm. Note the stronger green staining with MT in the tendon of PRP group at day 5.

Fig. 5: a- Semi-quantification of the LG staining (mean ± SD) on the tendons sections of control group (n=4 at each time point) and PRP group (n=4 at each time point), at increasing time after surgery (5, 15 and 30 days). The values are expressed in arbitrary units per unit surface. b- Collagen concentration (µg) per unit surface was calculated from OHPRO measurements performed on unstained paraffin sections as described in Materials and Methods. * p-value < 0.05

Fig. 6: Steady state level (mean ±SD) of mRNA coding for a- Collagen type I, b- Collagen type III, c- MMP 9 and d- tenomodulin in tendons of the control group (n=5 at each time point) and the PRP group (n=5 at each time point) at increasing time after surgery. The results are expressed in arbitrary units per unit of 28S. * p-value < 0.05

FIGURES

Fig. 1

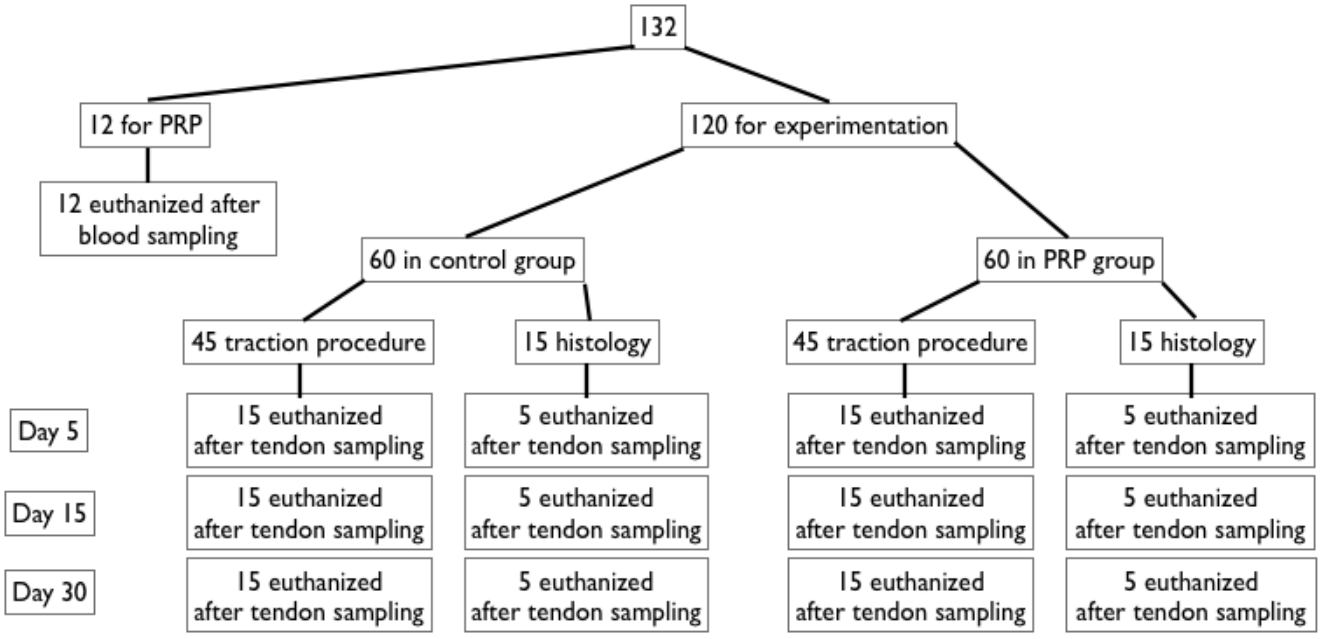


Fig. 2

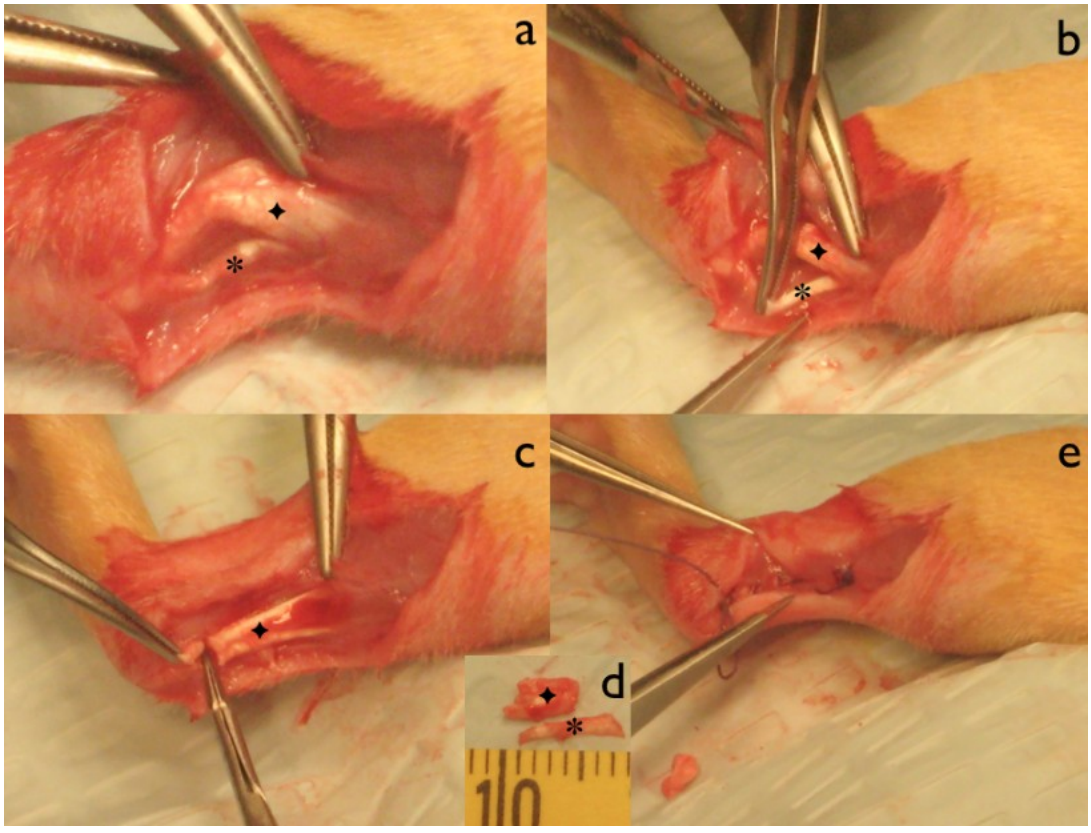


Fig. 3

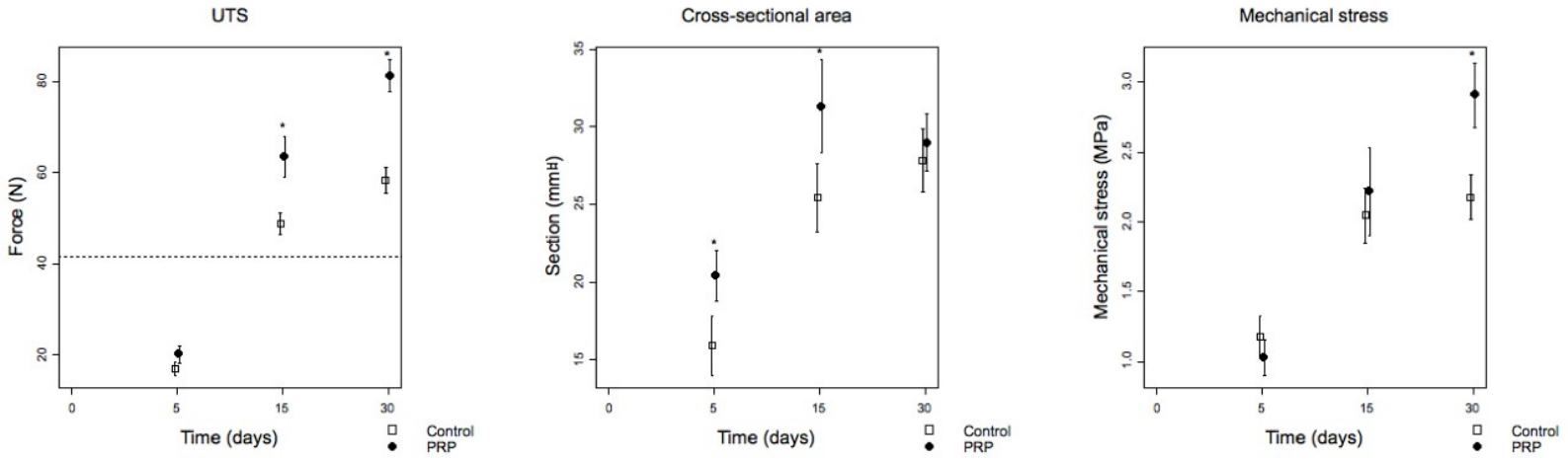

UTS
Force (N)
Time (days)
Control
PRP
Cross-sectional area
Section (mm²)
Time (days)
Control
PRP
Mechanical stress
Mechanical stress (MPa)
Time (days)
Control
PRP

Fig. 4

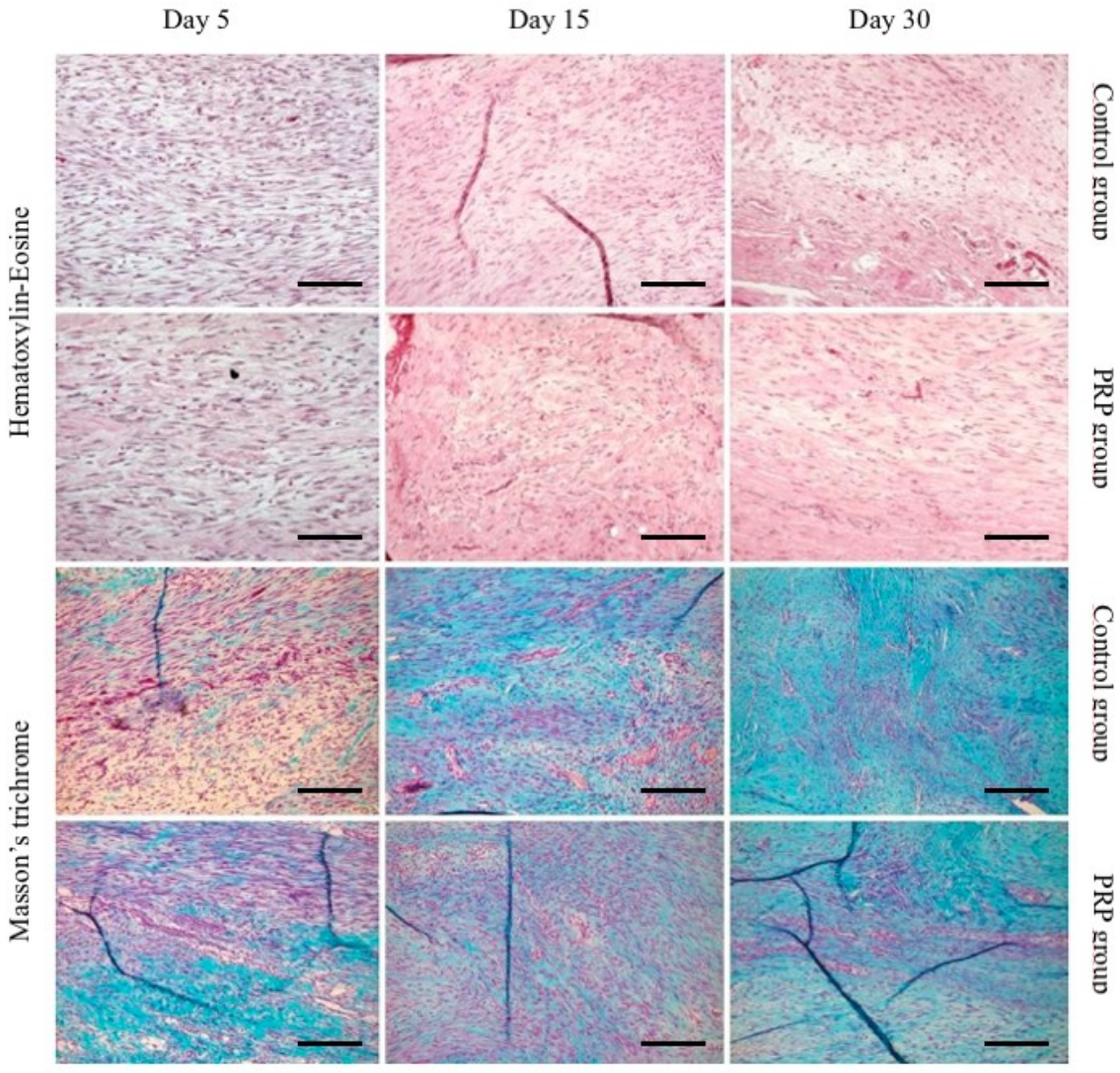

Day 5
Day 15
Day 30
Hematoxylin-Eosine
Masson's trichrome
Control group
PRP group
Control group
PRP group

Fig. 5

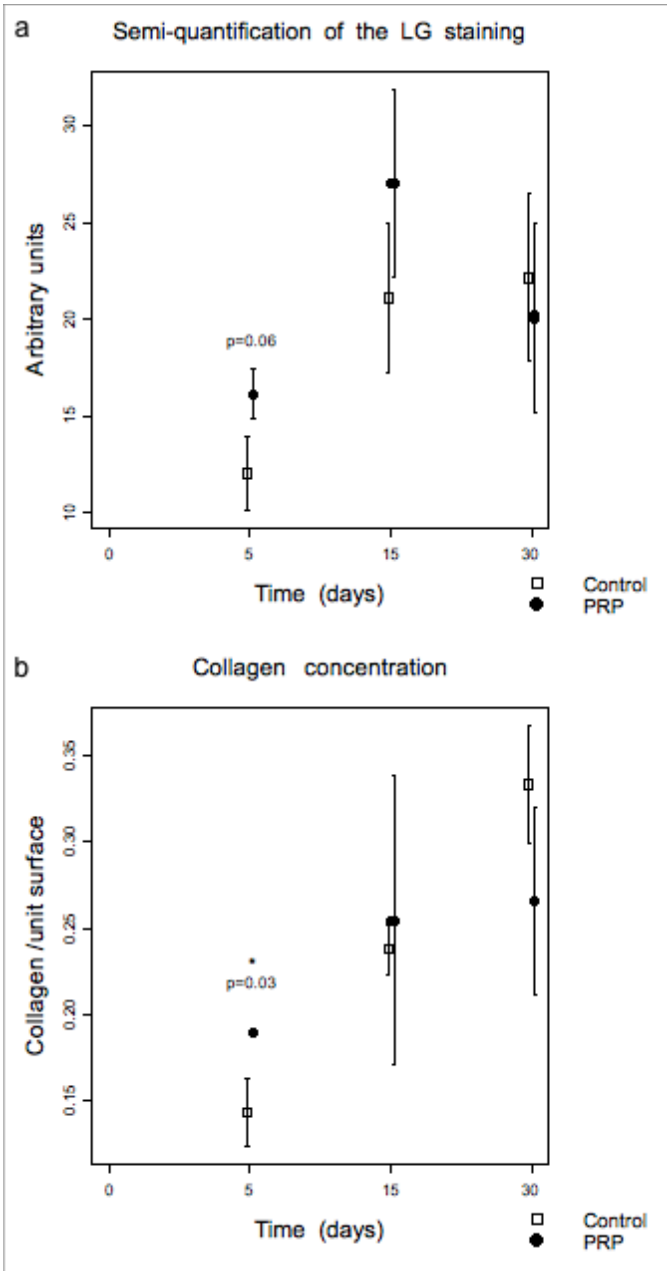
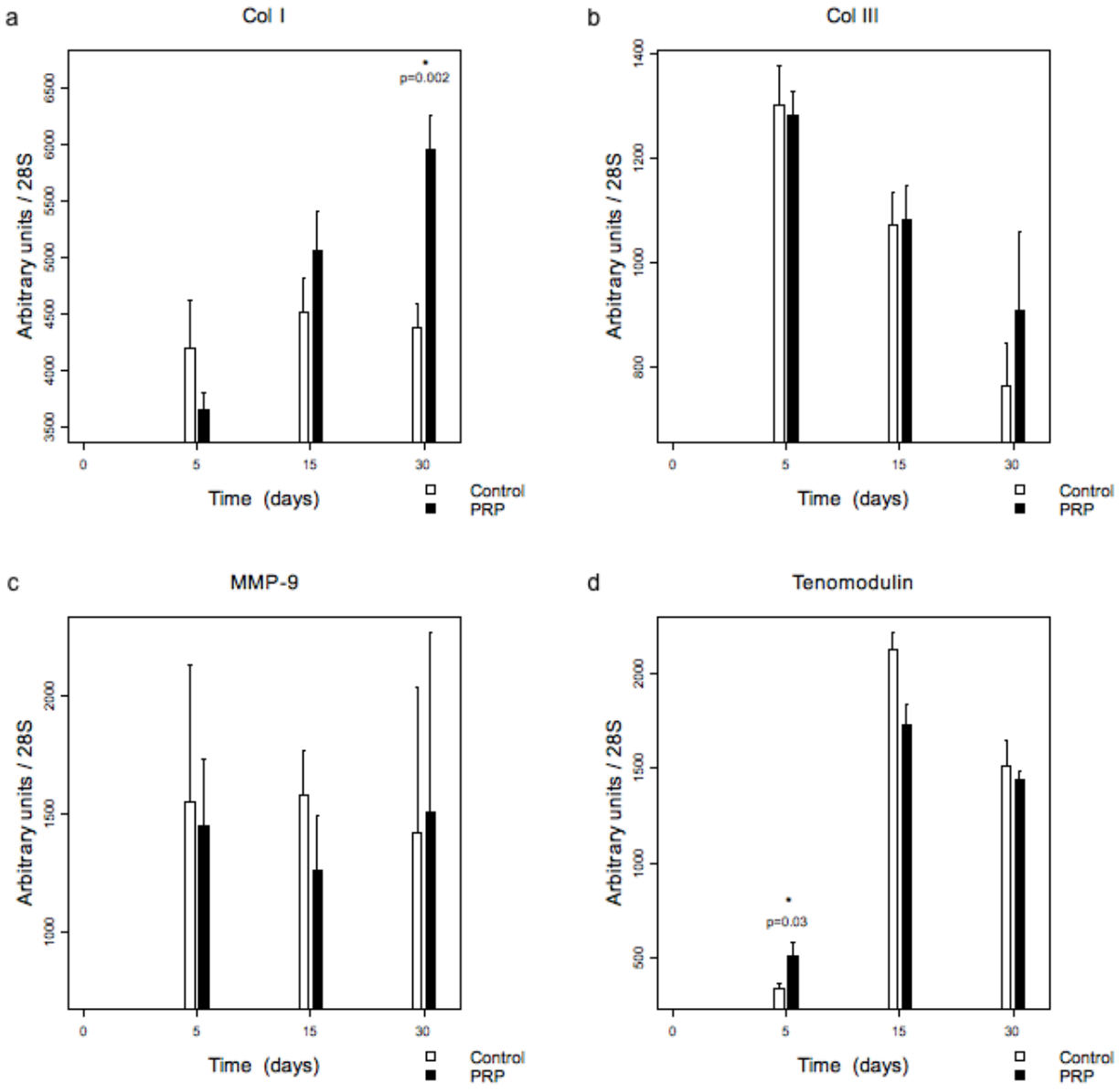

a
Semi-quantification of the LG staining
Arbitrary units
10
15
20
25
30
p=0.06
0
5
15
30
Time (days)
Control
PRP
b
Collagen concentration
Collagen /unit surface
0.15
0.20
0.25
0.30
0.35
*
p=0.03
0
5
15
30
Time (days)
Control
PRP

Fig. 6


a
Col I
p=0.002
Arbitrary units / 28S
Time (days)
Control
PRP
b
Col III
Arbitrary units / 28S
Time (days)
Control
PRP
c
MMP-9
Arbitrary units / 28S
Time (days)
Control
PRP
d
Tenomodulin
p=0.03
Arbitrary units / 28S
Time (days)
Control
PRP