Title

Effects of plasma rich in growth factors (PRGF) on biomechanical properties of Achilles tendon repair in sheep

ABSTRACT

Purpose

To assess the biomechanical effects of intra-tendinous injections of PRGF on the healing Achilles tendon after repair in a sheep model.

Methods

Thirty sheep were randomly assigned into one of the six groups depending on the type of treatment received (PRGF or placebo) and survival time (2, 4 and 8 weeks). The Achilles tendon injury was repaired by suturing the tendinous edges employing a three-loop pulley pattern. A trans-articular external fixation system was then used for immobilization. The PRGF or placebo was administered on a weekly basis completing a maximum of 3 infiltrations. The force, section and tension values were compared between the operated and healthy Achilles tendons across all groups.

Results

The PRGF-treated tendons had higher force at eight weeks compared with the placebo group ($p=0.007$). Between two and four weeks, a significant increase in force in both the PRGF-treated tendon ($p=0.0027$) and placebo group ($p=0.0095$) occurred. No significant differences were found for section ratio between PRGF-treated tendons and the placebo group for any of the time periods evaluated. At 2 weeks PRGF-treated tendons had higher tension ratio compared to placebo group tendons ($p=0.0143$). Both PRGF and placebo treatments significantly improved the force ($p<0.001$ and $p=0.0095$, respectively) and tension ($p=0.009$ and $p=0.0039$, respectively) ratios at 8 compared to 2 weeks.

Conclusions

The application of PRGF increases Achilles tendon repair strength at 8 weeks compared to the use of placebo. The use of PRGF does not modify section and tension ratios compared to placebo at 8 weeks.

The tension ratio progressively increases between two and eight weeks compared with the placebo. PRGF can be used in the clinical setting as a complementary therapy to improve the repair strength of acute Achilles tendon ruptures.

Level of Evidence

Not applicable. Controlled laboratory study.

Key Terms

Plasma rich growth factors; PRGF; PRP; Achilles tendon repair; Biomechanical properties

INTRODUCTION

Tendon injuries are very common and the Achilles is one of the most commonly involved [2,16]. The Achilles tendon supports loads 10 times higher than the body weight [25]. Due to its viscoelastic properties the tendon has high resistance to elongation before the breaking point [20,31]. However, when the tendon ruptures the healing potential is low because of poor vascularization and low cell metabolic rate [13]. Therefore, an attempt to improve tendon healing and biomechanical properties seems timely.

Platelets are known to have a critical role in the initiation and maintenance of the repair and regeneration of tissues [3,10]. A large number of growth factors and other biologically active proteins that play important roles in the process of tendon repair are found inside the alpha granules [3]. The use of both platelet-rich plasma (PRP) and plasma rich in growth factors (PRGF) has demonstrated to improve the healing of Achilles tendon injuries [14,17,23,30,32]. PRGF is a specific type of PRP poor in leukocytes. The main difference with PRP is the method used for obtaining the PRGF and its final composition [6,14].

From a histological standpoint, the use of PRGF on the healing Achilles tendon produced better nuclei orientation of the tendon fibroblasts, better organization of the collagen fibers, lower inflammatory cell infiltration, faster vascular regression, and lower density of fibroblasts compared to a placebo group [14]. From a biomechanical standpoint, previous studies related to Achilles tendon repair have used PRP

preparation in a rat or rabbit model [17,32,7,18]. In addition to using a different preparation than PRGF, the rat or rabbit model has obvious differences in tendon size (length and thickness) compared to the human specie. Despite it has been demonstrated that PRGF produce improvements in healing of Achilles tendon repairs after histologic analysis, there is little evidence investigating the biomechanical effects of PRGF on the healing Achilles tendon in an animal model with tendon characteristics more similar to the human specie.

The purpose of this study was to assess whether the application of PRGF can improve the biomechanical properties of the Achilles tendon repair in sheep. It was hypothesized that the intra-tendinous injection of PRGF would improve force, section and tensile strength of the Achilles tendon repair compared to the injection of saline solution (placebo group).

MATERIALS AND METHODS

Procedures

This study was approved by the Bioethics Committee for animal research at the XXXXXX. The animal model was the young healthy adult merino female sheep between 45 Kg and 55 Kg of body weight without orthopedic disorders. A total of 30 animals were randomly assigned into 1 of the 6 groups (5 animals per group) depending on the type of treatment received (PRGF or placebo) and survival time (2, 4 and 8 weeks). After sectioning and suturing the Achilles tendon, half of the animals were treated with intra-tendinous injection of PRGF and half with intra-tendinous injection of saline solution. Animals were sacrificed at weeks 2, 4 or 8. For each study period, the tendon force, section, and tension were measured in the operated and contralateral healthy Achilles tendons. These biomechanical variables were then compared among all groups. The sheep model employed in this study is considered suitable to study Achilles tendon repairs because of easy surgical access, similar length and thickness compared to human Achilles tendons, and adequacy for biomechanical testing [4,22].

Treatment groups

The animals were placed under general anesthesia and had their right hind limbs aseptically prepared for surgery. An approach was made to the Achilles tendon by accessing its lateral face at approximately 2 cm from the calcaneal tuberosity and was extended 8 cm approximately. The surrounding fascia was released and the paratenon was cut longitudinally, clearly exposing the Achilles tendon (Figure 1A). Then, with the help of a ruler, a mark was made 5cm from the insertion of the tendon into the calcaneus tuberosity. Before performing the tenotomy, a pre-suture was placed using a triple pulley pattern (Figure 1B). This triple pulley pattern suture was done in a standardized manner using a non-absorbable monofilament (polypropylene) (Premilene USP 1, B Braun Aesculap, Melsungen, Germany). The tenotomy was then performed 5 cm proximal to the Achilles tendon insertion at the level of the mark previously done. The suture was carefully tied and knotted ensuring that tendon edges were well positioned (Figure 1C). Subsequently, an intra-operative, intra-tendinous PRGF or saline solution injection was applied using a 23G gauge needle. A total of 1 ml was administered in each tendon edge. Finally, the wounds were closed using a simple discontinuous pattern. An external trans-articular fixator was placed connecting the tibia and the tarsus setting the ankle joint to be locked at an angle of 140° (Figure 1D) to ensure enough immobilization and to not compromise the tendon repair.

PRGF Endoret ® system (Biotechnology Institute [BTI], Vitoria, Spain) was used for obtaining PRGF. Four 5ml blood tubes containing 0.5 mL of 3.8% sodium citrate as anticoagulant were extracted. The blood was then centrifuged at 630G for a period of 8 minutes. After centrifugation, three different blood fractions were separated according to density: a plasma fraction, an intermediate line corresponding to leukocyte, and an erythrocyte sediment. A total of 0.5 ml of PRGF from the plasma portion that lies just above the leukocytic line was used. To obtain this portion, a process of pipetting fractionated in laminar flow was used and a total of 2 ml of PRGF (0.5 ml from each of the 4 tubes) was obtained. PRGF were then loaded into a syringe, and prior to intra-tendinous application the platelets were activated by addition of calcium chloride [5,6].

In addition to the first intra-operative injection, all sheep received two (the animals sacrificed at 2 weeks) or three (the rest of the animals) ultrasound-guided injections of PRGF or saline solution during the post-operative period on a weekly basis. Weekly physical and orthopedic examinations were performed and the degree of lameness was evaluated.

Measurement methods

Once the animals were sacrificed at weeks 2, 4 or 8, the external fixators were removed and both operated and healthy tendons (control) were extracted including the bone and all proximal muscular tissue. All tendons were placed in plastic bags with their own identification number, and each pair of tendons of the same animal was placed in a re-sealable numbered bag. The samples were frozen at -20^{o}C and transported in an insulated container with dry ice (solid CO2) to the XXXXXXXXX, where they were stored at -80^{o}C until biomechanical testing was to be carried out.

A total of 60 tendons (20 for each study period) were obtained. Additionally, four more tendons from surplus sheep from other studies were used for calibration and preliminary biomechanical testing. These 4 extra tendons were used to identify limitations in the biomechanical measurement methods. The two limitations observed were the inclusion of bone structures and the tissue slippage. A decision was taken to remove the calcaneus from all samples. To avoid slippage, the samples were fixed to the testing machine using low profile knurled-surface clamps with deposits for the freezing fluid so that the whole tissue-clamp system can be frozen (Figure 2) [8].

Samples were thawed during the day before the test and placed in a refrigerator at +5°C. The calcaneus, excess muscle and suture used during the healing phase were removed. Each clamp was placed at 1’5cm from the tendon repair area (3 cm of space in between clamps). The samples were placed between the clamps wrapped with a surgical gauze and cotton cloth to prevent tissue contact with the metal. Then the clamps were fixed by uneven tightening of the screws and placed in the universal testing machine. System freezing began using laboratory grade acetone poured into the clamp deposits and adding solid CO2 to gradually freeze the soft tissue-clamp system avoiding freezing of the tested area (Figure 2). The freezing process was monitored by a laser thermometer to prevent freezing of the tested area. After obtaining the freezing of the tissue–clamp system, the test was started at a tensile speed of 0.4mm/s (Figure 3).

The biomechanical outcomes measured in the present study were the ratio of force, section, and tension between the operated and healthy tendons (expressed in %). The force ratio was obtained by dividing the maximum force in Newtons (N) of the operated tendon by the maximum force of the healthy tendon, and multiplied by 100. The section ratio was obtained by dividing the cross-sectional area in mm^2 of the operated tendon by the cross-sectional area of the healthy tendon, and multiplied by 100. The

tension ratio was obtained by dividing the maximum tension in megapascals (MPa) of the operated tendon by the maximum tension of the healthy tendon, and multiplied by 100. The tension was calculated by dividing the maximum breaking force recorded during the test by the section of the tested tendon.

Statistical analysis

Descriptive statistics were used to summarize values of force, section, and tension ratios in all groups. Before the inter-group statistical comparison for the biomechanical outcomes, tests for normality (using the Shapiro-Wilk test), and homogeneity of variances (using the Levene test) were conducted. The Dixon Q test was also used to identify and reject outlier values. Given the type of study, data independence could be assumed. A 2x3 (treatment by time) Analysis of Variance (ANOVA) was conducted to compare biomechanical variables with normal distribution among all groups. For non-normal distribution variables, a Kruskal-Wallis test was used for the inter-group comparison of biomechanical data. All statistical analyses were conducted with the software R (R Foundation for Statistical Computing, Vienna, Austria) The alpha level was set at 0.05.

RESULTS

No animals had to be discarded during the study. The mean (SD) duration of surgery was 51 minutes. The external fixation system remained intact throughout the postoperative period. Eight animals had clean discharge at the pins site without any sign of infection.

The mechanical anchorage with clamps and the freezing system was adequate to assure accurate biomechanical testing. No tissue slippage or failures in the tissue-clamp system occurred. The 3 cm distance between clamps during the test proved to be wide enough to prevent freezing of the test area and permitted getting close enough to analyze the scar area.

Table 1 summarizes the descriptive statistics for all biomechanical variables evaluated in the 3 groups. The comparison among all groups for force ratio is shown on Figure 4. Two values were

considered outliers according to the Dixon Q test. At the 8-week period, the force ratio in the PRGF group was significantly higher compared to the placebo group ($p=0.007$). PRGF and placebo treatments demonstrated significant increase in force ratio from 2 weeks to 8 weeks ($p<0.001$ in both cases).

The comparison of section ratio among all groups is shown on Figure 5. None of the values were considered outliers after applying the Dixon Q test. There were no significant differences in section ratio between the use of PRGF and placebo for any of the study periods. The use of PRGF produced a significant increase in section ratio at 4 weeks compared to 2 weeks, and a significant reduction of section ratio at 8 weeks compared to 4 weeks.

The tension parameter had 2 outlier values according to the Dixon Q test. These values were kept to avoid a too low number of samples available for the analysis. At 2 weeks, the PRGF tension ratio was significantly higher ($p=0.014$) compared to the placebo group (Figure 6). The treatment with PRGF produced a significant increase in tension from weeks 2 to 8, but also from weeks 2 to 4 and 4 to 8 (Figure 6). PRGF and placebo treatments demonstrated higher tension ratio values at 8 weeks compared to 2 weeks ($p=0.009$ and $p=0.039$, respectively) (Figure 6).

DISCUSSION

The principal finding of this study was the between-treatment differences for force ratio after Achilles tendon repair. The PRGF-treated tendons were biomechanically stronger compared to placebo-treated tendons. Section and tension ratios were essentially similar between treatment groups, except a higher tension ratio in the PRGF compared to the placebo group at 2 weeks.

The use of sheep is an adequate model for basic science investigations of Achilles tendon injuries. The dimensions of the Achilles tendon of the sheep provide enough tissue to perform biomechanical testing, and it is similar in size (length and thickness) to the human Achilles tendon [22,26]. In addition, the weight of the sheep is closer to humans, as compared to the use of smaller animals previously employed (i.e., rats). The methods employed in this study in terms of tissue fixation, freezing and testing velocity share some similarities but also differences from previous studies. The

present study used external clamps for tissue fixation into the testing machine. Similar methods have been successfully employed in several other orthopedic experiments [12,14,28]. Previous studies with rat model have kept the tendon attachment to bone [17,32]. Given that the mechanically weakest point in preliminary testing was the tendon-to-bone attachment, a decision was made to remove the calcaneus. The freezing method employed in the present study differs from previous investigations using liquid nitrogen into the tanks of the clamp [8], or immersing clamp in liquid nitrogen [11,17,23,32]. Use of acetone and solid CO2 was adequate to ensure an adequate tissue grip and avoid the freezing of the tested area. Liquid nitrogen provides a very fast freezing which may increase the risk of soft tissue freezing between the clamp. The testing velocity was based on previous studies [8]. Mechanical properties of tendons are affected by the testing velocity due to its viscoelastic characteristics [31]. Too high velocity testing was avoided because water is not removed from the tissue, increasing its rigidity [27].

Biomechanical studies usually involve force measurements through load cells of universal testing machines [15,21]. The decision to report the outcomes as ratio (in %) between operated and healthy tendons decreases the effect that variability between animals may have on the results [19]. The inclusion of the tension variable (force/section) makes the difference with other published studies, but also greatly complicates the analysis and interpretation of results. This variable is used in studies with all kinds of materials in which the section is maintained during the test [1,24]. However, tendons change their section as the tissue is stretched. Therefore, changes in section without changes in force may elicit different tension values. At the beginning of the test, tension is low as small force is applied over a large section, but as the tendon is stretched the section is reduced and the tension increases. Tension should be measured just before the tendon ruptures, giving the maximum tension value for a given section. The tension parameter was included in the present study because provides more information on the quality of the scar tissue that forms in the healing tendon.

The results of the present study show that tendons treated with PRGF are stronger at 8 weeks compared to placebo-treated tendons. This provides a biomechanical basis for the results obtained by Fernández-Sarmiento et al. [14], who observed a more elongated silhouette, more type-I collagen, and better core orientation of tendon fibroblasts with PRGF compared to placebo after 8 weeks (better histologic characteristics). In addition, the results observed in the present study for force ratio also support the results observed in other studies [4,26,31]. It was found that the use of PRP in Achilles tendon produced a faster maturity of the scar tissue in rats [17]. In fact, rats treated with PRP or placebo had

increased tendon resistance in the first weeks and later stabilized regardless of treatment [17], which corresponds to the findings of the present investigation. Interestingly, Kaux et al. also observed significantly higher force for PRP-treated animals compared to placebo at 8 weeks [17]. In the rat model, it has been concluded that the application of PRP improves the strength of the tendons until 42 days [29]. However, caution must be taken when comparing the results of the present study with previous studies, as differences in the animal model or PRGF preparation may explain different results [14,6,29].

Overall, section ratios of the samples showed no significant differences among groups. However, section ratio in PRGF-treated tendons at 4 weeks was significantly higher compared to 2 and 8 weeks. It has been demonstrated that sections evidently change in the early days in the PRP-treated rats, and evened out after a longer time in the placebo-matched group [17]. This result means that the sizes of healing scars in the tenotomy areas do not vary significantly between week 2 and 8 for the placebo group. Our findings for section ratio changes were similar than the one observed by Kaux et al. in their rat model [17]. With PRGF, repair scars significantly increase from week 2 to 4 and then significantly decrease in size from weeks 4 to 8, whereas the placebo treatment shows no significant section changes throughout the study period. Time-related changes in section ratio in PRGF-treated tendons may be explained by an earlier initiation of the inflammatory and reparative processes with lower inflammatory infiltration at weeks 4 and 8 leading to early thickening [14].

The PRGF-treated tendons demonstrated significantly higher tension ratio at week 2 compared to placebo-treated tendons. Therefore, it may be interpreted that the scar tissue of tendons treated with PRGF withstands more force per unit area. This may indicate a higher quality of the scar tissue in tendons treated with PRGF compared to those treated with placebo. This would coincide with the results of Fernández-Sarmiento et al. [14], where tendons treated with PRGF proved to have an earlier period of maturity and matured faster than those treated with saline solution, despite histologic studies at week 2. Although histologic analysis was not carried out in the present investigation, it is reasonable to expect that better collagen fibers and structure would confer an increased resistance of the scar tissue [14]. It has also been shown that PRGF-treated supraspinatus tendons in rats produce an early improved resistance of the tendon and a greater infiltration of fibroblast and greater alignment of fibroblast in the longitudinal axis of the tendon [9]. The placebo-treated tendons exhibited a significant increase in tension ratio from week 2 to 4, but not from 4 to 8 weeks, whereas the PRGF-treated tendons significantly increased tension between all time periods. This can be interpreted as an improvement in the tissue quality of tendons

treated with PRGF, causing the tension ratio to gradually progress from 2 to 8 weeks. This could correspond with previous histological studies on the effects of PRGF using the same animal model [14].

This study has some limitations. First, the sample size was small. This may explain the absence of significant between-treatment differences at some time periods where a trend towards significance was observed (force ratio at 2 weeks). Second, the presence of abnormal high or low values (highly dispersed values) may explain the absence of significant differences in studies with small sample size. Although the Dixon Q test was employed to rule out outliers, other values not classified as outliers but obviously high or low were not discarded as to not further decrease the sample size. Third, tension was a calculated rather than a measured parameter. Therefore, abnormal force and section values affected the final tension ratio values. However, tension was relevant to calculate because provides further information on how a certain section resists forces, which may be related to the quality of the scar tissue. On the other hand, this study provides valuable information on the biomechanical effects of PRGF on Achilles tendon repair in an in-vivo model. In addition, this study uses an animal model closer to humans and evaluates both early (2 weeks) and late (8 weeks) biomechanical effects.

Conclusion

The application of PRGF improves the strength of the Achilles tendon repair at eight weeks compared to application of saline solution. PRGF can be used in the clinical setting as a complementary therapy to improve the treatment of acute Achilles tendon ruptures. The clinical relevance of this study is that PRGF can be used in the clinical setting as a complementary therapy to improve the repair strength of acute Achilles tendon ruptures.

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PERIOD (weeks)	GROUP	N	Mean (SD), in %	Range, in %
FORCE RATIO				
2	PCB	4	2.5 (0.3)	2.1-2.9
2	PRGF	5	5.2 (2.8)	2.7-9.6
4	PCB	5	18.2 (8.8)	4.7-28.4
4	PRGF	5	18.7 (6.5)	9.5-24.8
8	PCB	4	15.1 (1.4)	13.5-16.6
8	PRGF	5	26.3 (5.8)	19.1-32.6
SECTION RATIO				
2	PCB	4	216.1 (24.9)	191.3-245
2	PRGF	5	227.9 (74.1)	140.3-328.6
4	PCB	5	276.1 (91.1)	124.3-352.6
4	PRGF	5	416.7 (113.5)	258.5-528.3
8	PCB	4	249.2 (90.2)	166.7-375.5
8	PRGF	5	286.6 (52.2)	208.2-341
TENSION RATIO				
2	PCB	4	1.2 (0.2)	1-1.4
2	PRGF	5	2.3 (0.9)	1.6-3.9
4	PCB	5	6.2 (1.6)	3.8-8.1
4	PRGF	5	4.8 (1.7)	1.8-5.8
8	PCB	4	6.6 (2.3)	3.8-9.4
8	PRGF	5	9.7 (3.8)	6.4-15.7

359 **Table 1.** Descriptive statistics for force, section, and tension ratios between operated and healthy tendons
360 across all groups

PCB, placebo; PRGF, platelet-rich growth factors

Figure legends

Figure 1. Procedure for the section, suture and immobilization of operated tendon.

Panel A. Dissection of the fascia overlying the Achilles tendon.

Panel B. Pre-tendon suture. The various loops that make up the pattern in triple pulley are made. Each of the loops is placed in a flat 120º from the previous loop, so that they are will never be in the same plane.

Panel C. Knotted suture demonstrating the adequate apposition of the tendon edges.

Panel D. Implantation of the external fixator. Pins at the tibia and metatarsus are connected together at an angle of 140º.

Figure 2. Freezing of the system using laboratory grade acetone poured into the clamp deposits and adding solid CO2.

Figure 3. Final assembly of the clamp jaws in the testing machine.

Figure 4. Force ratio values between operated tendon and healthy tendon across all groups.

Figure 5. Section ratio values between operated tendon and healthy tendon across all groups.

Figure 6. Tension ratio between operated tendons and healthy tendons across all groups.