



Biomechanical study of the effect of platelet rich plasma on the treatment of medial collateral ligament lesion in rabbits¹

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Abstract

Purpose: To evaluate the use of platelet-rich plasma in the early stages of healing of traumatic injury of the medial collateral ligament in the knee of rabbits.

Methods: Thirty rabbits were subjected to surgical lesion of the medial collateral ligament. Of these, 16 were treated with platelet-rich plasma and 14 with saline (control). After 3 and 6 weeks of treatment, 50% of the animals from each group were sacrificed, and biomechanical tests were performed on the injured ligament to compare the tensile strength between the two groups.

Results: Platelet-rich plasma significantly increased the tensile strength of the ligament in the groups treated after 3 and 6 weeks. In the group treated with platelet-rich plasma vs. saline, the tensile strength values were 3192.5 ± 189.7 g/f vs. 2851.1 ± 193.1 g/f at 3 weeks ($p = 0.005$) and 5915.6 ± 832.0 g/f vs. 4187.6 ± 512.9 g/f at 6 weeks ($p = 0.0001$).

Conclusion: The use of platelet-rich plasma at the injury site accelerated ligament healing in an animal model, demonstrated by an increase in the tensile strength of the medial collateral ligament.

Key words: Medial Collateral Ligament, Knee. Knee Injuries. Rabbits.

■ Introduction

Traumatic ligament ruptures are common knee injuries, particularly in young people and during sports practice¹. The medial collateral ligament (MCL) is the most commonly injured ligament in the knee, and its incidence appears to be higher because incomplete and mild injuries are not often diagnosed². The injuries occur when a valgus load is applied to a flexed knee. Other causes include forced external rotation, anterolateral trauma on the knee, and knee dislocations³. Although MCL injury is the most common, there are controversies regarding the best way to treat it, which includes conservative approaches and surgical procedures⁴.

An alternative to conservative and surgical treatment is a biological stimulus of ligament healing to accelerate the rehabilitation process and avoid the complications of surgical interventions. Among these options, the use of platelet-rich plasma (PRP) can expedite the physiological repair of the ligament⁵. PRP is a platelet concentrate with a platelet count higher than that found in the serum, and its basic mechanism involves the formation of a fibrin mold as a temporary matrix for cell growth and differentiation, helping repair the damaged tissue⁶.

Few studies have evaluated PRP in humans as an adjunct treatment for acute ligament injuries. Experimental studies have suggested that PRP stimulates ligament healing. In addition, PRP increases the synthesis of matrix and collagen, stimulates cell proliferation in the tendon, reduces pain, promotes bone integration, and reduces postoperative complications in MCL lesions and anterior cruciate ligament reconstructions⁷.

We aimed to evaluate the tensile strength of MCL of rabbits treated with platelet-rich plasma as compared to a control group.

■ Methods

This study was approved by the Animal Research Ethics Committee, UFMG under protocol nº354/2013.

We used 30 4-month-old male New Zealand White rabbits weighing $2,475.0 \pm 429.3$ g. The animals were kept in individual cages and received water and standard chow (NatureMultivita, SocilGyomarc'H) *ad libitum* throughout the study period.

The rabbits were randomly distributed into the following experimental groups:

I—control group treated with saline 3 weeks after MCL injury (n = 7)

II—study group treated with PRP 3 weeks after MCL injury (n = 8)

III—control group treated with saline 6 weeks after MCL injury (n = 7)

IV—study group treated with PRP 6 weeks after MCL injury (n = 8)

MCL injury

The animals were anesthetized with 100 mg/kg of ketamine (Ketalar, Sigma, St. Louis, MO, USA) and 8 mg/kg of xylazine (Rompun, Sigma, St. Louis, MO, USA), and treated with prophylactic antibiotic (cefazolin at 50 mg/kg; Kefazol, ABL, Cosmópolis, São Paulo, Brazil). Afterward, the animals were placed in supine position on the surgical table and trichotomy was performed on the medial side of the knee of the left posterior limb. MCL was identified by translucency. Then, the surgical site was sterilized, and a longitudinal parapatellar medial incision was performed on the knee. After isolating the superficial MCL and immobilization of the whole limb, a hemostat was positioned under the ligament and a force was exerted in the cephalic direction to rupture the ligament in mop-end lesion in its substance and femoral insertion (Figures 1 and 2)⁸.

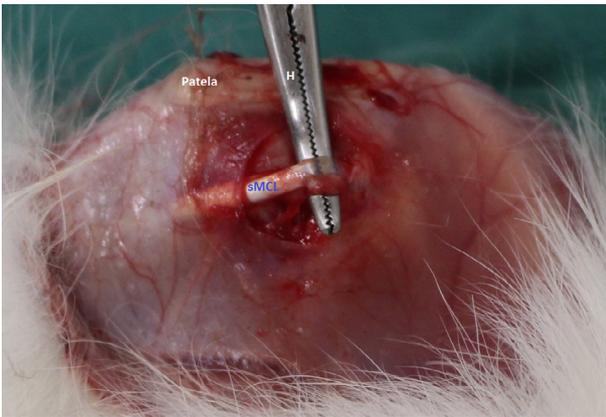


Figure 1 - Hemostat positioned under medial collateral ligament to rupture the ligament in tip swab. **Patela**: patella bone; **H**: hemostat; **sMCL**: superficial medial collateral ligament.

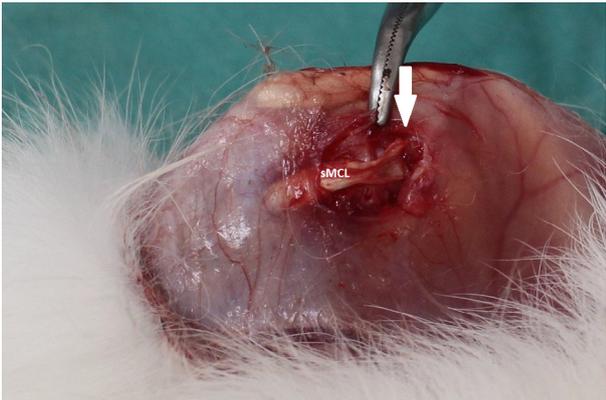


Figure 2 - Lesion in the femoral insertion of medial collateral ligament. **sMCL**: superficial medial collateral ligament; **Arrow**: rupture in mop-end lesion.

Subsequently, groups II and IV received 0.2 ml of a PRP infusion at the surgical site in the proximal region of the ligament, and 0.2 mL was applied via the femoral insertion. Groups I and III received the same volume of saline solution at 0.9% at the surgical site to simulate the application performed in groups II and IV. The rupture was closed in a single plane with mononylon 4-0 (Ethicon®) 4.0.

PRP preparation

The protocol used for the preparation of PRP was developed as described by Tavares

Junior⁹. Briefly, blood samples were obtained by punching the central auricular artery, and a 4-mL volume was collected in test tubes containing 0.3 ml of ACD-A-citrate dextrose as the anticoagulant. Immediately after collection, the samples were homogenized and processed using a Cell-DynRuby® device (Abbott) for automated platelet quantification².

After calculation of the baseline number of platelets, the samples were centrifuged in a HeraeusMegafuge 11® centrifuge (ThermoScientific) at a speed of 800 rpm for 8 minutes. The supernatant plasma was transferred to another test tube and subjected to a second centrifugation at a speed of 3200 rpm for 15 min. After the second centrifugation, the upper two-thirds of plasma were discarded, and the platelets present in the lower third of the tube were resuspended. In this step, platelets were quantified in PRP using a final volume between 0.4 ml and 0.5 ml, and PRP was inoculated into each animal.

Evaluation of healing

Three to 6 weeks after surgery, 50% of the animals in each group were euthanized with carbon dioxide. The left posterior limbs were disarticulated at the level of the hip joint, and the anatomical specimens were positioned in a linear tensiometer to measure the tensile strength with the knee extended to 0°. Tensile strength was measured for each ligament under constant tension. Data of each animal group were collected blindly by the examiner.

Statistical analysis

The Kolmogorov–Smirnov test was applied to assess normality in platelet count in the plasma of all animals and PRP in groups II and IV. The same test assessed the normal distribution of the tensile strength in each animal group. The results indicated that the distribution of platelet count and tensile

strength was normal for all study groups. After the confirmation of these parameters, the results were analyzed using Student's t-test for independent samples and Minitab software version 17, State College, PA at a level of significance of 5%.

■ Results

None of the animals evaluated presented local or systemic signs of infection 3 or 6 weeks after injury.

The platelet count per mm³ in the serum was 325.86 ± 79.98 in group I, 311.88 ± 81.67 in group II, 331.14 ± 75.54 in group III, and 323.87 ± 66.83 in group IV, without significant difference between the groups.

The platelet count after centrifugation and preparation of PRP in was 3.32 ± 0.89 times the baseline count in group II and 3.33 ± 0.79 times the baseline count in group IV (p = 0.48), and the count in PRP was on average 3 times higher than that in the blood, without significant difference between these two groups (Figure 3).

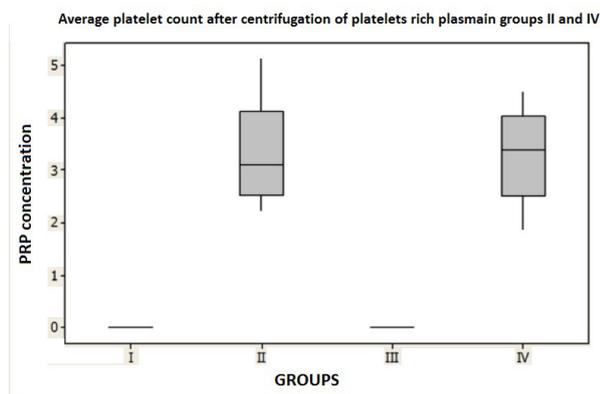


Figure 3 – Average platelet count after centrifugation of platelets rich plasma in groups II and IV (p = N.S.)

There was a non-significant increase in the tensile strength of the ligament in group II (3.192,5 ± 189.7 g/f) and a significant increase in this parameter in group IV (5.915,6 ± 832.0

g/f; p = 0.00001) compared with group I (2.851,1 ± 193.1 g/f) and III (4.187,6 ± 512.9 g/f).

The comparison between group I (2.851,1 ± 193.1 g/f) and group II (3.192,5 ± 189.7 g/f) treated 3 weeks after MCL injury indicated a significant increase in the tensile strength of the ligament after the use of PRP (p = 0.005). A significant increase was also observed (p=0.0001) in the comparison between group III (4.187.6 ± 512.9 g/f) and group IV (5.915.6 ± 832.0 g/f) (Figure 4).

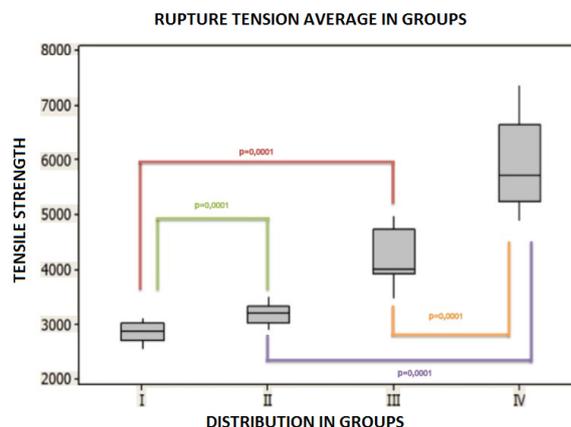


Figure 4 - Tensile strength of the medial collateral ligament (MCL) of rabbits treated with platelets rich plasma (PRP) 3 or 6 weeks after MCL injury as an adjunct to healing. I - saline 3 weeks; II - PRP 3 weeks; III - saline 6 weeks; IV - PRP 6 weeks; p = N.S.

■ Discussion

MCL is the most commonly injured knee ligament. Smaller lesions heal without intervention; however, full-thickness injuries and insertional lesions heal slowly because of tissue discontinuity¹⁰.

Several biological and non-biological therapies have been investigated alone or in combination to enhance the healing process and shorten the period of recovery. The

use of PRP may yield favorable outcomes in the treatment of these injuries owing to its easy of application, low cost, technical availability of production, potential benefits, probable lack of contraindications, and few adverse effects¹¹.

Different animal species have been used to study ligament healing. In this study, we chose to use MCL because this ligament is easy to manipulate, has a continuous cross section, may undergo primary healing, treatment options are controversial, and its healing process is reproducible. We chose to use rabbits because their anatomy and physiology of healing are similar to those of humans^{5,12}.

We used centrifugation because of its availability and easy handling and because this method yields concentrations similar to those of other methods^{13,14}. No platelet activation was performed before the application of PRP because activation can be associated with various intervening factors, including the need to use bovine thrombin, which can cause disseminated intravascular coagulation¹³. Moreover, the previous activation quickly releases the stored growth factors. Once the activation process is triggered, 70% of the growth factors are released in the first 10 minutes and the remainder within the first hour after application. Harrison *et al.*¹⁴ observed that these factors are endogenously activated by type I collagen, and activation occurs continuously over the first five days, allowing the joint release of platelet alpha-granules. The alpha granules are the most studied platelet structures (each platelet has 50–80 alpha units, and each granule has more than 30 bioactive proteins that can be released by a stimulus). There are different subpopulations of alpha granules, and an early release of these granules by activation with thrombin may promote an exacerbated reaction, leading to the antagonistic action of growth factors, and these factors should be released at different stages of the healing process¹⁵. In addition, the

previous activation can limit the percutaneous application, which is the preferred route used in conservative treatment, owing to the gelation PRP after activation.

The concentrates were prepared individually considering their clinical applicability. PRP was prepared with the blood of each study animal; this technique is preferably employed in humans, although it is more laborious. Therefore, this procedure will lead to more reliable results in future clinical studies.

In this study, we did not immobilize the knees in the immediate postoperative period, and the analgesic effect and potential acceleration of healing were credited to PRP. Furthermore, Frank *et al.*¹⁶ reported that the rabbit knee immobilized for a month was produced less extracellular matrix and collagen type I, resulting in as low recovery that lasted for one year.

The evaluation of biomechanical parameters alone indicated that none of the proposed treatments allowed full recovery of the tensile strength of MCL¹⁷. Therefore, PRP could have the potential to re-establish this strength by stimulating the healing process, with the production of extracellular matrix rich in type-I collagen. Therefore, we opted to use the biomechanical tensile strength test alone although we are aware that the disruption of the proximal osteoligamentar junction would limit the performance of histological and immunohistochemical evaluations and prevent the establishment of other inferences, including the likely mechanism associated with the gain of tensile strength in the scar tissue.

This investigation assumed that PRP could improve healing of experimental MCL lesions in rabbits, and this assumption was corroborated by the results. The comparison of the tensile strength of MCL in all animals after 3 or 6 weeks of injury indicated that PRP was effective in promoting early healing; however,

the effectiveness was superior after 6 weeks of treatment.

Few studies have evaluated the effect of PRP in ligament or tendon healing, and the studies focused on experimental research with small series. Therefore, there is insufficient data in the literature to allow the formulation of hypotheses to explain the action of PRP in these tissues. For this reason, assumptions need to be made on the basis of data from studies that evaluated other connective tissues, which, despite their specific characteristics, have similar healing processes.

Our results were consistent with those of Yoshioka *etal.*¹⁸, who used plasma rich in growth factors to MCL of rabbits and observed fibroblast proliferation and neovascularization in the scar tissue at the end of the study period. The tensile strength of these ligaments was superior 3 and 6 weeks after treatment. However, the authors used a growth factor concentrate obtained from a single donor. Ueshima *etal.*¹⁹ and Anaguchi *et al.*²⁰ applied growth factors in isolation to MCL and patellar tendon of rabbits. The first authors observed improvement of histological scores with increased neovascularization and improved the alignment of collagen fibers compared with the control group; however, no significant difference in tensile strength was observed between the groups. The second authors found a significant increase in tensile strength compared with the group treated with saline or the untreated group^{19, 20}. Other authors also obtained favorable results using PRP in ligament repair. Murray *et al.*²¹ applied a PRP mold to LCA rupture in dogs and observed significant improvement in biomechanical tests.

Van den Dolder *et al.*²² applied PRPs from humans, rats, and goats to cultures of bone marrow cells from mice and compared the osteogenic response and production of growth factors between these PRPs. They observed

that human PRP had a higher concentration of growth factors and stimulated cell proliferation in all cultures. This result may explain why a similar study in rats did not find similar results. Amar *et al.*²³ subjected rats to MCL transection, evaluated the animals 3 weeks after the lesion, and found no significant differences in the tensile strength of the ligaments and tissue maturity scores. However, there was a selection bias in relation to the animal model because the healing process in mice is known to be more efficient than that in other animals, including humans. Moreover, the authors analyzed the data only after 3 weeks of injury. The results of this and other studies indicate that the maximum effect of the application of PRP occurs after 6 weeks.

A possible beneficial effect of PRP is the stimulation of the expression of growth factors such as TGF- β , PDGF, and IGF, which are essential to the healing process. Growth factors are involved in proliferation, cell differentiation, and tissue morphogenesis of organs during embryogenesis, postnatal period, and adulthood²⁴. A traumatic injury is followed by the formation of a platelet-rich hematoma, which releases growth factors and cytokines, which in turn stimulate the healing process²⁵. Kajikawa *et al.*²⁶ applied PRP to rat patellar tendon and found an increase in the number of blood cells using labeled proteins.

PRP may promote the proliferation of fibroblasts. PRP stimulates the expression of the *COL 1* and *COL 3* genes and suppresses the genes that negatively affect the structure of the extracellular matrix²⁹; this suggests that PRP affects both mature and undifferentiated cells. In addition, it may influence the rate of collagen expression. The increased production of type-III collagen results in the formation of a fibrotic structure that is structurally inferior to that of the original¹⁷.

PRP stimulates early healing, which promotes the formation of healthier tissues

and better organization of fibroblasts and collagen. In such models, regression of granulation occurs, indicating an increase in the repair rate. For this reason, it is possible to observe an increase in scar stiffness³⁰.

All these factors together improve the clinical response to rehabilitation. Early onset of weight discharge and mobilization are associated with lower rates of fibrosis, improved range of motion, earlier return to activities of daily living, and higher patient satisfaction.

Therefore, our results suggest that the use of PRP as a conservative treatment for MCL lesions can be beneficial in acute lesions and accelerate the return to activities of daily living, particularly in elite athletes. However, other studies are necessary to corroborate these results.

■ Conclusion

The application of platelet-rich plasma to medial collateral ligament lesions in rabbits significantly increased the tensile strength in the groups treated 3 and 6 week safter lesion compared to the control group.

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