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PLATELET-RICH PLASMA (PRP) AND TENDON HEALING: COMPARISON BETWEEN FRESH AND FROZEN-THAWED PRP

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Abstract

Platelet-rich plasma (PRP) is increasingly used in the treatment of musculoskeletal diseases. Its preservation by freezing it for the realization of multiple injections in clinical use has never been discussed. Calcaneal tendons of rats were surgically sectioned. Platelet concentration of the PRP was 2.5 x 10^6/µl with autologous plasma of rats. Frozen-thawed PRP was prepared by performing two cycles of freezing and thawing on PRP aliquots. Both platelet preparations were injected in the lesion. Biomechanical and histological evaluations were carried out after 7, 20 or 40 days post surgery. After 7 and 40 days, no significant difference was observed between the PRP and the frozen-thawed PRP group. There is however a difference 20 days after surgery: the ultimate tensile strength (UTS) was greater in the fresh PRP group. No obvious difference with histological aspect was observed between the two groups. In conclusion, fresh PRP and frozen-thawed PRP injections can lead to similar results in the healing process of section calcaneal tendons of rats. Improvements with fresh PRP are slight. PRP could thus be frozen to be preserved if multiple injections are needed (e.g. osteoarthritis).

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Introduction

Platelet-rich plasma (PRP) is increasingly used in the treatment of musculoskeletal diseases, even if the proof of their effectiveness has not been clearly demonstrated yet [1–3]. However, in the case of knee osteoarthritis, multiple PRP injections seem to be more effective than the same number of hyaluronic acid (HA) injections [4,5], which is up to now recommended by international scientific societies [6,7]. In both cases, several injections are required to significantly improve the patients' condition. As PRP treatment can be expensive, and since only a fraction of the collected PRP is injected to the patient, it would be time- and cost-effective to freeze it for the subsequent injections [8]. Even if the PRP preparation techniques and composition are often discussed [5,9,10], its preservation for clinical use has never been discussed. It has been experimentally shown that the freezing-thawing process does not alter the growth factors effectiveness of platelets [11– 13]. The aim of this study was to confirm that the healing process of sectioned calcaneal tendon of rats is similar to fresh and frozen-thawed PRP.

Materials and Methods

All experimental procedures and protocols used in this investigation have been reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the University of Liège (Belgium). The Guide for the Care and Use of Laboratory Animals has been carefully followed.

Thirty-eight Sprague-Dawley male rats of 2 months (weight 350 g) were used according to our experimental design. Our protocol was described in the previous test series [14–18].

(1) Preparation of platelet-rich plasma (PRP): Whole blood was collected from eight males Sprague-Dawley. The donor rats (four for one experiment on a group of 15 rats) were intraperitoneally (i.p.) anesthetized with pentobarbital (60 mg/kg) and blood was collected by cardiac puncture (platelet concentration of 578 ± 27 x 10^3/µl, red blood cell concentration of 8.41 ± 0.12 x 10^6/µl and white blood cell concentration of 13.63 ± 1.49 x 10^3/µl). One milliliter of anticoagulant, adenosinecitrate-dextrose acid, per 4.5 ml of blood was immediately added. PRP was prepared as described previously [19]. Briefly, blood samples were centrifuged at 150 g for 10 min at room temperature. The upper phase consisting of platelet-rich plasma was gently collected, and platelet count was measured on a Sysmex XS 800i hematology analyzer. Platelet concentration was then adjusted to 2.5 x 10^6/µl (i.e., a 4.3-fold increase in platelet concentration as compared to whole blood) with autologous plasma. Contaminating red and white blood cells were respectively below 1 x 10^4/mm^3 and 1 x 10^3/mm^3.

(2) Frozen-thawed PRP was prepared from PRP by performing two cycles of freezing and thawing on PRP aliquots (10 min at −80°C followed by 5 min at 37°C). The samples were homogenized between cycles (15-s vortex). Finally, platelet lysate was centrifuged for 5 min at 2000 g to remove membrane debris. Platelet lysates were used within 1 h.

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(3) Surgical procedure: Rats were weighed and anesthetized i.p. with pentobarbital (60 mg/kg of body weight), placed on a warm pad, and the skin of the left hind limb shaved [16]. Buprenorphine (0.05 mg/kg of body weight) and tetracycline (15 mg/kg of body weight) were directly given a priori by the subcutaneous way. The complete surgical procedure was performed under aseptic conditions under a dissecting microscope. The skin of the left calcaneal tendon was incised laterally. The calcaneal tendon complex was exposed after dissection of the surrounding fascia. The plantaris tendon was removed to avoid measurement errors during the biomechanical testing. Subsequently, 5 mm proximal to its calcaneal insertion, the calcaneal 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 Brunswick, NJ). The animals were placed in clean cages under a heating lamp until awakening and there was no postoperative immobilization. The rats were randomized into PRP and frozen-thawed PRP groups that received 2hours postoperatively (after the clot formation was already initiated) a local injection (inside the defect) of 50 µL of fresh PRP after activation with 50 µl $CaCl_2$ (0.55 M)/ml PRP, or frozen-thawed PRP. They were checked on a daily basis and followed for well-being and a global observation of walk and activity.

(4) Biomechanical evaluation: 7, 20 or 40 days after surgery, eight rats of each group were weighed and euthanized with pentobarbital (i.p., 60 mg/kg). The healing tendon, with the attached calcaneal bone and a part of the triceps suralis, was dissected from surrounding tissues [16]. Because rats were not immobilized after surgery, the healed tendon had very little adhesions and 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 three time points [19]. The mechanical testing was done using a traction–compression testing machine (106.2 kN, TesT GmbH, Düsseldorf, Germany) and an original clamping device type cryo-jaw shown to better preserve tendon integrity [20]. 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 prestress 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 to the tissue is significantly lowered. Freezing the muscle with liquid nitrogen increases its stiffness and allows us to consider it completely rigid compared with the tendon. The cross-sectional area of the samples was calculated from pictures made with a set of two 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 1 mm/second until rupture. Force versus displacement curves were recorded by a computer for subsequent data analysis. Ten noninjured rat calcaneal tendons were similarly tested to establish normal basal values. The force at rupture or ultimate tensile strength (UTS) was expressed in Newtons (N). To account for the difference in the cross-sectional area of the healing tendons, the value of UTS was normalized to a unit area (N per square millimeter or megapascal) and represents the mechanical stress of the tissue.

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(5) Histological evaluation: Two tendons of each group were fixed with 4% paraformaldehyde and embedded in paraffin. The specimens were sectioned (5 mm) 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).

(6) Growth factors dosage: Fresh PRP concentrated at a platelet density of 2.5 x 10^6/µl was incubated for 5 min with a dihydrate $CaCl_2$ solution (Calciclo Sterop) (50 µl/ml PRP) prior to centrifugation at 2000 g for 5 min. Growth factors were measured in freshly collected supernatants or in platelet lysates from frozen/thawed PRP prepared as described above. Levels of VEGF, FGF-basic, and IGF-1 were determined by luminex mouse assay (R&D Systems), while active TGF-β1 levels were measured by ELISA (Quantikine ELISA kit, R&D Systems) according to manufacturer's instructions.

(7) Statistics: Results are expressed as mean values and standard deviations. For each group, a Shapiro–Wilk test was performed to ensure that the data were normally distributed and a Welsch test was used to compare the biomechanical results of both groups. The analysis was performed using RStudio (RStudio, Boston, MA, USA) and the level of significance set at $p < 0.05$.

Results

No significant difference in the body weight was observed between each group at different times of the experiment.

Table I presents the ultimate tensile strength (UTS) values for both fresh and frozen PRP groups at different times and UTS time evolution. After 7 and 40 days, no significant difference was observed between the fresh PRP and the frozen-thawed PRP groups. There was however a difference at 20 days ($p = 0.039$): the UTS was 68.3 N ± 7.11 for the fresh PRP group and 59.5 N ± 7.14 for the frozen-thawed PRP group. It appeared that the maximum UTS could be reached quicker using fresh PRP even though final UTS was similar after 40 days. This suggests that for a longer duration of treatment, frozen PRP preparation can be as efficient as fresh PRP.

The HE stained sections of the healing tendons after day 7 showed a high cellularity that slightly decreased with time (**Figure 1**). No obvious difference with histological aspect was observed between the two groups after 20 and 40 days.

Several growth factors known to be released by platelets and to be highly relevant to the healing processes were quantified for both types of PRP preparation (**Table II**). Significantly higher concentrations of all growth factors tested were reproducibly measured in the frozen-thawed PRP group, except for IGF-1 levels. It can be assumed that cell lysis induced by this preparation process most likely results in a massive release of total platelet content, whereas with fresh calcium-activated PRP, a more progressive, controlled, secretion of cytokines/growth factors is expected.

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Table 1. Ultimate tensile strength (mean value and standard deviation) by group and euthanize time.

	UTS – frozen-thawed PRP (N)	UTS – fresh PRP (N)	*p*-Values
T7	18.85 ± 9.95	15.32 ± 2.78	0.380
T20	59.50 ± 7.14	68.30 ± 7.11	0.039
T40	61.71 ± 23.17	67.53 ± 9.12	0.520

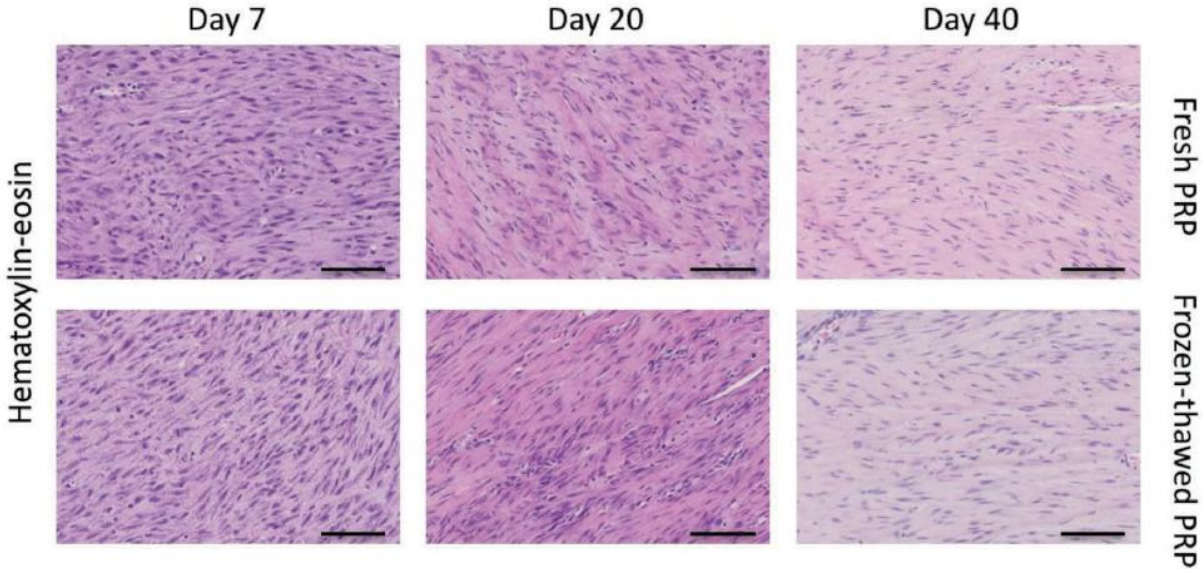

Figure 1. Representative longitudinal sections of calcaneal tendon of rats from fresh PRP and Frozen-thawed PRP groups at day 7, 20 and 40, stained with hematoxylin-eosin. Scale bar = 100 µm. PRP, platelet-rich plasma.

Table II. Dosage of growth factors in frozen-thawed PRP and fresh PRPs.

	Frozen-thawed PRP	Fresh PRP	*p*-Values
VEGF (pg/ml)	6.37 ± 0.63	3.37 ± 1.30	>0.001
FGF-b (pg/ml)	87.00 ± 19.70	36.46 ± 28.45	0.006
IGF-1 (pg/ml)	22472 ± 57475	147042 ± 21732	>0.001
TGF-B1 (pg/ml)	109130 ± 46361	3776 ± 2806	>0.001

Discussion

PRP injection is an increasingly used therapy in regenerative medicine, for example, in sports medicine, orthopedics, dermatology, ophthalmology… [3,21–24]. Moreover, nearly no side effects have been described [25]. Indeed, by the release of the platelet growth factors, it was demonstrated that PRP can stimulate the healing process of various tissues: tendons, cartilage, bone, skin, cornea… [4,24,26,27]

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Even if one injection of PRP can be effective to treat patients with tendon lesions for example [28], multiples injections can be necessary for other pathologies (e.g. osteoarthritis…) [4,5]. To avoid the multiplication of platelet samples, it could be useful to preserve them. Freezing could be an interesting way to preserve PRP for several months since it is well established that freezing/thawing does not alter the efficacy of the growth factors released by platelets, as demonstrated on keratinocytes, chondrocytes, synoviocytes or tumor of epithelial and mesodermal origin for example [11,13,29,30].

Using our procedure, concentrations of growth factors present in the plasma were mainly significantly higher in the frozen-thawed PRP group. Total platelet content is indeed released following platelet lysis by the freezing-thawing process, while growth factors are progressively and likely differentially secreted by platelet degranulation as in the case of fresh PRP. So far only few studies have addressed this issue [31–34] and major differences in study design and experimental settings preclude direct comparisons. Most of the available results available were obtained on different species, using various methods of PRP preparation. Some reports do not even provide crucial information, like platelet concentration.

Considering studies conducted in rats, our results are in line with the findings of van den Dolder et al. [35], reporting growth factor levels in a similar order of magnitude.

Data regarding tendon healing in this series are similar to those observed in our previous studies on the healing process of sectioned rat calcaneal tendon with PRP or growth factors compared to control group (saline) [14–16]. The final results obtained in the fresh PRP and frozen-thawed PRP groups are similar, but a quicker improvement of the tendon strength was observed in the fresh PRP group, especially after 20 days. We propose the following explanation for the healing dynamics. After 7 days, no significant difference was observed between the fresh PRP and the frozen-thawed PRP groups since the treatment did not have enough time to modify the mechanical resistance of the repairing tendon. At the contrary, at 40 days, tendons are completely healed with the two treatments, explaining the similar observed values [14,19]. We can infer that, while the final quality of the repaired tendon is similar after treatment with fresh or frozen PRP, the healing process is however faster with fresh PRP. In light of our observation, fresh PRP should be preferred for the treatment of high-level sportsmen who need a quick recovery, while frozen-thawed PRP could be conveniently used for multiple injections as in the case of chronic pathologies such as osteoarthritis where injections have to be carried out more regularly. However, human series are needed to confirm these therapeutic options.

Conclusion

Fresh PRP and frozen-thawed PRP injections can lead to similar results in the healing process of sectioned calcaneal tendons of rats, even if results with fresh PRP are slightly quicker than with frozen-thawed PRP. PRP could thus be frozen to be conserved if multiple injections are needed (e.g., osteoarthritis).

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Disclosure Statement

The authors did not have any conflict of interest related to this study.

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