Effects of Repetitive Platelet-rich Plasma Application on Human Tenocyte Proliferation

AUGUSTUS D. MAZZOCCA, MS, MD; MICHAEL O’MALLEY, MS, MD; KNUt BEITZEL, MD; MARY BETH R. MCCARTHY, BS; DAVID M. CHOWANIEC, BS; MARK P. COTE, DPT; JAMES P. BRADLEY, MD; ANTHONY ROMEO, MD; ROBERT A. ARCIERO, MD

Abstract

Current clinical application of platelet-rich plasma is showing a trend toward multiple treatments. The goal of this study was to show the benefit of interval platelet-rich plasma application in the healing and recovery of human tenocytes using an in vitro cell model. Eight volunteers (6 men and 2 women) were included in this study (mean±SD age, 31.6±10.9 years). Venous blood was collected from new blood draws at 3 different times. Two blood products were prepared on each day of treatment: platelet-rich plasma derived from a single-spin process (PRP$_{SS}$) and platelet-rich plasma derived from a double-spin process (PRP$_{DS}$). The study had 2 limbs: 2-day and 4-day intervals. Cell proliferation, measured as disintegrations per minute, was then examined via a radioactive thymidine assay. In the 2-day-interval group, the difference in disintegrations per minute between days 0 and 2 in the PRP$_{SS}$ group reached statistical significance ($P=.006$). In the PRP$_{DS}$ group, statistical difference was seen between days 0 and 4 ($P=.001$) and between days 2 and 4 ($P=.030$). In the 4-day-interval group, the difference in disintegrations per minute between days 4 and 8 in the PRP$_{SS}$ group reached statistical significance, showing a decrease in cell proliferation ($P=.013$). In the PRP$_{DS}$ group, a statistical difference was seen between days 0 and 8 ($P=.021$), also showing a decrease in cell proliferation. The greatest effect of platelet-rich plasma, which has a positive effect on tenocyte proliferation and recovery, is seen on initial application. Its effect is diminished with repetitive application, and this finding leads to questioning of the efficacy of interval platelet-rich plasma dosing. [Orthopedics. 2015; 38(1):e19-e24.]
Improvements in clinical outcome after administration of platelet-rich plasma to treat soft tissue injury have been inconsistent and controversial.1-5 Concurrent in vitro data have highlighted the positive effects of in vitro and in vivo application of platelet-rich plasma on animal and human cells as well as its effectiveness in the treatment of various injuries.1,3,6-8 Most studies have evaluated single-dose treatments.7,9-12

Few studies have examined the effects of platelet-rich plasma exclusively on human tendon cells. Anitua et al13 reported that tenocytes treated with platelet-rich plasma showed increased cell proliferation as well as increased production of endogenous growth factors, vascular endothelial growth factor, and human growth factor-2. More recently, Fallouh et al14 showed increased overall cell proliferation and collagen production in anterior cruciate ligament cells treated with platelet-rich plasma produced in a 2-step procedure.6 The optimum timing and method of application of platelet-rich plasma has been discussed, but there is no consensus.8,11,14

In clinical practice, however, the effect of platelet-rich plasma may be improved with multiple applications.8,12 Team physicians advocated a second application 2 days after the initial treatment, whereas others advocated an interval of 4 days. Therefore, the effectiveness of multiple platelet-rich plasma treatments remains in question because information on its effect, provided in this manner, in current studies is limited.12 Using a conventional in vitro cell culture model, the authors investigated the potential benefit of interval platelet-rich plasma treatment on human tenocytes.

The goal of this study was to evaluate the effects of platelet-rich plasma application on the proliferation of human tenocytes administered over 2 intervals. The authors hypothesized that human tenocytes treated with repeated applications of platelet-rich plasma at specific intervals would show an increasing proliferation response.

**MATERIALS AND METHODS**

**Patients**

The current study included 8 volunteers (6 men and 2 women; mean±SD age, 31.6±10.9 years). Formal institutional review board approval to carry out the study was obtained (institutional review board #10-204-2). The study included healthy adult subjects who agreed to participate. Those with a history of blood-derived illness or use of medications known to affect platelet function or concentration for a minimum of 2 weeks before testing were excluded.

Venous blood (approximately 125 mL venous blood+5 mL acid citrate dextrose) was collected from new blood draws. Approximately 125 mL peripheral blood was drawn from each subject at 3 different times (at 2-day and 4-day intervals, as explained later) to allow sufficient platelet recovery. A 60-mL syringe prefilled with 5 mL acid citrate dextrose was used for the standardized blood draw. Acid citrate dextrose binds calcium and prevents blood clotting without affecting platelet function.

Platelet-rich plasma was isolated using 2 methods, a single-spin process (PRP$_{ss}$) and a double-spin process (PRP$_{ds}$). Both PRP$_{ss}$ and PRP$_{ds}$ were performed on each day of treatment. Two systems were examined: 1 that produced a high concentration of platelets and another that had lower platelet values. The single-spin process is quicker, but typically yields a lower platelet concentration.15 Marx16 recommended that the goal of a platelet-rich plasma product should be a platelet concentration 3 to 5 times greater than the baseline platelet count.17 Under a laminar flow hood and using sterile technique, 10 mL blood was transferred into the platelet-rich plasma preparation system or Arthrex Double Syringe (Arthrex Inc, Naples, Florida) to produce 3 mL platelet-rich plasma. The double syringe is a device used to isolate platelet-rich plasma from peripheral blood. According to the manufacturer’s recommendations, blood is drawn or loaded into the outside area of the double syringe, then centrifuged at 2000×g for 5 minutes. The resulting top plasma layer is drawn into the inner syringe to retrieve the platelet-rich plasma product. Syringes were centrifuged at 1500 rpm for 5 minutes. This separated the blood into 3 layers: plasma, erythrocytes, and theuffy coat containing the platelets. The plasma anduffy coat were then isolated with the inner syringe and used as the platelet-rich plasma separation.

In preparing the PRP$_{ds}$, after a first centrifugation at 1500 rpm for 5 minutes, the buffy coat was aspirated and centrifuged a second time (20 minutes at 6300 rpm). Finally, half of the superficial plasma layer was removed and the platelet pellet was suspended in the remaining half of the plasma volume.8

The composition of the treatment preparations may ultimately affect the outcome. The authors analyzed 3 specific components of these different treatments: platelet concentration, white blood cell concentration, and red blood cell concentration.

Human tenocytes were isolated from specimens of proximal biceps tendon extracted in the process of tenodesis during shoulder surgery. This portion of the study was carried out under a second institutional review board approval (institutional review board #07-22-4). Specimens were included if they appeared to have no gross degenerative changes. These changes were proven in previous studies to correlate with the histologic appearance.18 Tendons were cut and placed into a 2% collagenase solution. Tendon cells were successfully obtained via a primary digest at a concentration of 8 million cells per sample. The resulting tenocyte cell suspension was filtered, collagenase was removed, and the tenocytes were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, New York) with 10% fetal bovine serum and 10% FBS.
penicillin-streptomycin. The tenocyte genotype was confirmed by quantitative real-time polymerase chain reaction for the tenocyte markers tenasin-C, decorin, and collagen types I and III. Normal tenocyte morphologic findings were confirmed by microscopy. For all experiments, only cells from passage 1 were used.

For this study, the authors referred to tendon cells as tenocytes, but previous work based on phenotypic criteria showed the presence of collagen type I. Additional proteins were assessed as well, such as collagen type III, decorin, biglycan, and tenasin-C, which have been found to be expressed in tenocytes.

The study had 2 limbs (Figure 1). The first limb included tenocytes exposed to experimental treatments at 2-day intervals. Three separate treatments of PRP$_{SS}$, PRP$_{DS}$, and media were given at 0, 2, and 4 days. The second limb included tenocytes exposed to experimental treatments at 4-day intervals. Three separate treatments of PRP$_{SS}$, PRP$_{DS}$, and media were given at 0, 4, and 8 days. On the day of each treatment, fresh blood draws were obtained from the 8 subjects. The PRP$_{SS}$ and PRP$_{DS}$ products were then prepared accordingly, as described earlier, for a total of 24 samples (8 blood draws each for PRP$_{DS}$, PRP$_{SS}$, and media) in both the 2-day and 4-day interval groups. Cultured human tenocytes underwent interval PRP$_{SS}$, PRP$_{DS}$ (10% v/v), or control media (10% fetal bovine serum) treatments. Before experimentation, fetal bovine serum was reduced to 2% in all platelet-rich plasma derived from a double-spin process; PRP$_{ss}$, platelet-rich plasma derived from a single-spin process.

**Figure 1:** Experimental setup. Abbreviations: Dpm, disintegrations per minute; PRP$_{ss}$, platelet-rich plasma derived from a double-spin process; PRP$_{ds}$, platelet-rich plasma derived from a single-spin process.

**RESULTS**

Preparation for both PRP$_{SS}$ and PRP$_{DS}$ for both the 2-day-interval and 4-day-interval limbs of the study, are shown in the Table. More variability was seen in the PRP$_{DS}$ preparation. Compared with PRP$_{SS}$, higher platelet concentrations were seen in PRP$_{DS}$ for the 2-day-interval preparation vs the 4-day-interval preparation.

**Figure 2** shows tenocyte proliferation for the 2-day-interval limb of the study. Both PRP$_{SS}$ and PRP$_{DS}$ showed less tenocyte proliferation between days 0 and 2 and between days 2 and 4. In the 2-day-interval group, the difference in disintegrations per minute between days 0 and 2 in the PRP$_{SS}$ group reached statistical significance ($P=.006$). The difference in disintegrations per minute between days 2 and 4 ($P=.290$) and days 0 to 4 ($P=.319$) in the PRP$_{SS}$ group did not reach statistical significance. In the PRP$_{DS}$ group, a statistically significant difference was seen between days 0 and 4 ($P=.001$) and days 2 and 4 ($P=.030$). The difference seen between days 0 and 2, however, did not reach statistical significance in the PRP$_{DS}$ group ($P=.710$). For both PRP$_{SS}$ and PRP$_{DS}$, a positive effect compared with the control group was seen, but did not reach statistical significance.

The effect seen in the 4-day-interval limb of the study is shown in Figure 3.
The difference in disintegrations per minute in the PRP<sub>SS</sub> group between days 4 and 8 reached statistical significance, showing a decrease in cell proliferation ($P=.013$). In contrast, in the PRP<sub>DS</sub> group, an increase in mean tenocyte proliferation occurred between days 0 and 4. The difference, however, between days 0 and 4 ($P=1.00$) and the effect seen between days 4 and 8 ($P=.054$) did not reach statistical significance for this group. In the PRP<sub>DS</sub> group, statistical difference was seen in the interval between days 4 and 8 ($P=.021$), showing less cell proliferation than in the interval between days 0 and 4. The difference in the interval between days 0 and 4 ($P=.771$) and the interval between days 4 and 8 ($P=.039$) did not reach statistical significance for this group.

**DISCUSSION**

The current study evaluated the effects of 2 different platelet-rich plasma preparations administered at 3 time points, but applied at different intervals. Human tenocytes treated with repeated applications of platelet-rich plasma at specific intervals showed a positive cell proliferation response at each interval in both 2-day-interval and 4-day-interval treatment groups. Initial application showed the greatest effect, and the effect decreased with subsequent interval treatments. Both PRP<sub>SS</sub> and PRP<sub>DS</sub> increased cell proliferation more than in control subjects, but showed no increase in effect with interval treatment.

The most recent literature includes no studies of interval testing of platelet-rich plasma products on human tenocytes. However, as stated in previous single-application studies, Anitua et al<sup>13</sup> reported increased tenocyte proliferation with 20% platelet-rich plasma product and Fallouh et al<sup>8</sup> showed encouraging results with a 2-step procedure, with increased overall cell proliferation and collagen production in anterior cruciate ligament cells treated with platelet-rich plasma. The first treatment (at day 0) of both limbs of the study was the most effective, with one exception being the interval between days 0 and 4 in the PRP<sub>SS</sub> group of the 4-day-interval limb. This can explain the finding that the greatest effect on tenocyte proliferation was seen on the first application and did not occur with subsequent applications. Funding was not available to use enzyme-linked immunosorbent assay to determine growth factors in platelet-rich plasma and native blood. Previous studies showed that the biologic effect of platelet-rich plasma depends on platelet production of various growth and differentiation factors (eg, transforming growth factor-beta, insulin-like growth factor-1, platelet-derived endothelial growth factor, vascular endothelial growth factor).<sup>1</sup>

Previous studies showed that platelet-rich plasma may stimulate both cell proliferation and total collagen production, promote angiogenesis and the formation of fibrovascular callus, improve tenocyte migration capacity, and promote tendon stem cell differentiation into tenocytes.
rather than nontenocytes.\textsuperscript{5,8,18,22} In the current study, the authors could not control the effects on vascularization or injured tendon. Previous studies showed a similar positive effect of PRP\textsubscript{SS} and PRP\textsubscript{DS} as in the authors’ study, as indicated by the initial increase in tendon cell proliferation after administration of the product.\textsuperscript{22}

The authors reported a positive effect in subsequent intervals for both study limbs; however, this effect was decreased. Other studies raised the question of optimal timing and application of platelet-rich plasma, as noted by Lopez-Vidriero et al.,\textsuperscript{9} but without formal investigation. The current results suggest that even though repeated applications of platelet-rich plasma provide an additive effect on human tenocyte proliferation, the effect remains positive. However, it is uncertain whether these in vitro results are true in vivo. Reasons for the lack of additive effect on human tenocyte proliferation with repeated applications of platelet-rich plasma are unclear and require further study. Possible explanations include reduced expression of receptors of a certain growth factor and the possibility that repeated applications of platelet-rich plasma could enhance the synthesis of an antagonist of a certain growth factor.

One limitation of the current study is the inherent weakness of an in vitro study when applied to the in vivo clinical setting. Such investigations would require application of platelet-rich plasma to be matched to the donor tissue to meet the criteria for autologous treatment. This can be challenging because it would require coordination of intraoperative tissue harvesting, time needed for cell isolation and culture, and fresh plasma preparations at the time of application. As a result, previous investigators used homologous platelet-rich plasma in comparable experimental designs.\textsuperscript{14,17,19} As in these studies, the term platelet-rich plasma in the current study refers to homologous platelet concentrates. Further, the current study involves evaluation of platelet-rich plasma treatments at only 2 time points. Thus, no conclusions based on other treatment intervals can be made. Further investigation is required to determine the most advantageous number and interval of platelet-rich plasma treatments in maximizing cell proliferation. The effects seen in this study are not based on an injury model, which is typically the setting in which platelet-rich plasma injection is used. Therefore, the authors cannot report the effects of platelet-rich plasma on damaged tendon cells after injury.

**CONCLUSION**

The current study showed that the use of PRP\textsubscript{SS} and PRP\textsubscript{DS} had a positive effect on tenocyte cells. Human tenocytes treated with repeated applications of platelet-rich plasma at specific intervals showed a positive cell proliferation response in both the 2-day-interval and 4-day-interval treatment groups. Both PRP\textsubscript{SS} and PRP\textsubscript{DS} increase cell proliferation more than occurred in control subjects. Initial application showed the greatest effect, and the effect decreased with subsequent treatments.

**REFERENCES**


