

Platelet concentrate injection improves Achilles tendon repair in rats

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Background Blood platelets release a cocktail of growth factors when activated, some of which are thought to initiate and stimulate repair.

Experiment and findings We studied whether a platelet concentrate injection would improve Achilles tendon repair in an established rat model. The Achilles tendon was transected and a 3 mm segment removed. After 6h, a platelet concentrate was injected percutaneously into the hematoma. This increased tendon callus strength and stiffness by about 30% after 1 week, which persisted for as long as 3 weeks after the injection. At this time, the mechanical testing indicated an improvement in material characteristics—i.e., greater maturation of the tendon callus. This was confirmed by blinded histological scoring.

Interpretation Platelet concentrate may prove useful for the treatment of Achilles tendon ruptures.

In previous experiments, we have noted an increase in bone formation in response to a blood platelet concentrate (Siebrecht et al. 2002). Since we regarded this as a general stimulation of repair rather than a specific increase in bone formation, we now studied whether an injection of platelet concentrate could also improve repair of the Achilles tendon. This possibility was further suggested by the observation that healing of the Achilles tendon can be accelerated by a single injection of a growth factor from the cartilage derived morphogenetic protein (CDMP) family (Forslund and Aspenberg 2001, 2002), and that another growth factor, platelet-derived growth factor (PDGF), can stimulate ligament repair in rats (Batten et al. 1996, Hildebrand et al. 1998). PDGF is included in the cocktail of growth factors and other

molecules released by activated blood platelets. In this study, we therefore hypothesized that the healing of Achilles tendon ruptures can be improved by the injection of a platelet concentrate (PC).

Experiment

Survey

296 Sprague-Dawley rats were used. The platelet concentrate was prepared from the blood of 30 rats that had been killed. We started with an experiment in which 20 rats were randomized to receive the platelet concentrate or control buffer injections. The findings were evaluated mechanically at 8 days. This experiment showed a significant improvement in tendon callus strength and was followed by a repeat experiment that did not reach statistical significance. Therefore, a series of similar experiments was performed, each time with 10 rats receiving platelet concentrate and 10 rats receiving control treatment (Table 1). After 5 such experiments, we performed experiments with a healing time of 11, 14, 21 or 28 days. In the final analysis, data from the various experiments were pooled into larger groups with identical treatment and follow-up times.

Preparation of platelet concentrate

Whole blood was collected from female Sprague-Dawley rats (200 g., M&B, Ry, Denmark). Normally, 3 donor rats were sufficient for an experiment on 10 recipient rats. The donor

Table 1. Experimental groups

Experiment	Treatment	Animals, n	Days	Note
1	PC	10	8	
	Control	10	8	
2	PC	10	8	
	PPP	10	8	
3	Control	10	8	
	PC	10	8	
4	PC	9	8	
	Control	8	8	
5	PC	10	8	
	Control	10	8	
6	PC	20	11	^a
	Control	20	11	^a
7	PC	10	14	
	Control	10	14	
8	PC	20	21	^a
	Control	20	21	^a
9	PC	10	28	
	Control	10	28	
10	Unoperated	29		

PPP Platelet-poor plasma
^a 10 for histology

rats were anesthetized with a mixture of Rompun and Ketalar (xylazine and ketamine 1 mL/kg and 1.5 mL/kg), but in the later experiments, we changed to isoflurane as described below. The blood was collected by cardiac puncture. The animals were then killed by an intracardiac injection of an overdose of pentobarbital sodium. The blood was added immediately to an anticoagulant, citrate phosphonate dextrose (CPD) buffer (0.15 mg CPD/mL) in a ratio of 1 mL of CPD buffer to 5 mL of blood. The blood was then centrifuged at 220 g for 20 minutes. The supernatant, containing platelet-rich plasma, was used for a second centrifugation at 480 g for 20 minutes. The pellet from the second centrifugation was saved and diluted with supernatant until the platelet PC concentration became 1.5×10^{12} platelets/L, measured in a Bürkner chamber. The PC was then activated by adding thrombin 20 U per mL PC (USP Thrombostat Parke-Davis, Morris Plains, USA, 200 units/mL). To reduce the risk of a graft-versus-host reaction, the PC was irradiated at 25 Gy according to international blood banking standards to inactivate the white blood cells. Up until the operation, the platelet concentrate was stored at +4 °C for a maximum of 24 h. Control solutions

consisted of 1 part CPD buffer as above, added to 9 parts saline.

One of the first experiments included another group that received the supernatant from the second centrifugation—i.e., platelet-poor plasma, as an extra control.

Surgical procedure and treatment

The rats were anesthetized with 5% isoflurane in an anesthetic induction chamber and then 3.5% in a mask. The skin was shaved and the operation performed under aseptic conditions. A 3 mm transverse incision was made in the skin lateral to the right Achilles tendon. The surrounding fascia was cut longitudinally and the Achilles tendon exposed. The plantaris tendon was removed. The Achilles tendon was cut transversely 3 mm proximal to its calcaneal insertion and a 3 mm long segment was removed to enlarge the defect. The wound was closed. Preoperatively, the rats were given tetracycline 15 µg and buprenorphine 0.03 mg. After the operation, the rats were randomized for treatment by taking colored marbles out of a hat, randomizing groups of 10 rats at a time. The rats were given a local injection 6 h postoperatively with 50 µL of the PC or control solution.

Evaluation

After 8, 11, 14, 21 or 28 days, the rats were anesthetized again with isoflurane and killed by an intracardiac injection of pentobarbital sodium. The tendon with the attached calcaneal bone was removed and dissected free from other tissues, wrapped in gauze soaked with saline pending mechanical testing, that was performed within a few hours. In 29 rats, the tendon from the unoperated leg was taken for comparison. Mechanical testing was done using a material testing machine (100 R, DDL Inc. Eden Prairie, MN, USA). The tendon was fixed between two metal clamps and pulled at a constant speed of 1 mm/sec until failure. The angle between the calcaneus and Achilles tendon during testing corresponded to 30° dorsiflexion of the foot. We recorded the peak force, stiffness and energy uptake (until the force had fallen to 90% of maximum).

The tendons for histological examination were decalcified in EDTA, prepared with routine methods for paraffin sections and stained with

Ehrlich's hematoxylin and eosin. The specimens were sectioned parallel to the longitudinal direction of the tendon. Only sections from the middle of the tendon callus were made. 3–5 glass slides per specimen comprising the entire length of the tendon callus were prepared, blinded and labeled with a specimen-related code number, so that all slides from one specimen could be evaluated together, without knowledge of the treatment and duration of the follow-up. The specimens were examined in random order, with a microscope, mainly using a 12× objective. They were classified according to an arbitrary scoring system from 1 to 5, where 1 represented an immature loose callus and 5 a dense organized fibrous tissue with mostly parallel fibers.

Statistics

Biomechanical results were analyzed by two-way ANOVA. Only buffer controls were included. In the analysis of the 5 experiments with an 8-day follow-up, the independent variables were treatment (experiment vs control) and experiment (1–5). In the analysis of the entire set of experiments with all follow-up times, the independent variables were treatment (experiment vs control) and duration of follow-up (8, 11, 14 or 28 days). Confidence intervals for comparisons between subgroups are based on t-statistics. Histological scores were compared using Kruskal-Wallis non-parametric ANOVA, followed by the Mann-Whitney test for comparison of controls versus PC at 11 and 21 days, respectively.

Results

No rat was lost from mechanical or histological follow-up, but sometimes 1 or 2 rats had to be taken from the original experimental groups of 10 for the production of more PC. Thus, tendons from 223 rats were used for mechanical analysis and 40 for histological examination.

Mechanical results at 8 days (Table 2)

Of 122 tested specimens, 112 ruptures occurred in the tendon callus, 7 at the proximal clamp and 3 at the calcaneus.

Table 2. Mechanical results of experiments with 8-day follow-up

Experiment	n	PC		Control		
		mean	SD	n	mean	SD
<i>Force at failure (N)</i>						
1	10	14	5.3	10	9.7	5.0
2	9	13	4.0	8	11	3.5
3	9	15	7.0	10	12	2.6
4	9	21	5.0	8	15	4.0
5	10	12	3.2	10	11	2.1
All	47	15	5.8	46	12	3.8
<i>Stiffness (N/mm)</i>						
1	10	2.3	0.7	10	1.5	0.7
2	9	1.3	0.5	8	2.0	0.6
3	9	1.3	0.5	10	1.2	0.4
4	9	2.4	0.9	8	1.6	0.6
5	10	1.5	0.4	10	1.3	0.3
All	47	2.0	0.9	46	1.5	0.6
<i>Energy uptake (Nmm)</i>						
1	10	33	18	10	19	14
2	9	30	7.7	8	25	12
3	9	48	16	10	47	18
4	9	66	24	8	63	24
5	10	44	31	10	35	16
All	47	44	24	46	37	23

The force at failure was increased by PC treatment ($p = 0.001$) by an average of 27% (95% confidence interval (CI) 10–44%). There was also a difference between the different experiments ($p = 0.002$). This difference was mainly caused by differing responses to PC treatment, whereas the controls did not differ significantly between experiments.

The transverse area of the tendon callus differed between the experiments ($p = 0.006$), but it was also increased an average of 18% by PC (95% CI 5–31%) ($p = 0.004$).

The maximum stress was similar in the experiments and was not significantly affected by PC treatment (95% CI decrease by 20% to increase by 20%).

The stiffness differed between the various experiments ($p < 0.001$), but it was also increased an average of 35% by PC ($p < 0.001$) (95% CI 14–56%) ($p < 0.001$).

The energy uptake differed between the experiments, but no effect of treatment could be shown (95% CI decrease by 8% to increase by 43%).

The effect of platelet-poor plasma (supernatant from the second centrifugation) was about the same as that of the control solution and yielded

Table 3. Mechanical results in all groups

Follow-up, days	n	PC		n	Control	
		mean	SD		mean	SD
<i>Force at failure (N)</i>						
8	47	15	5.8	46	12	3.8
11	10	29	7.7	10	25	7.8
14	10	38	9.3	10	30	6.9
21	10	47	10	10	35	14
28	10	47	12	10	39	12
<i>Stiffness (N/mm)</i>						
8	47	4.3	1.9	46	3.2	1.3
11	10	9.7	2.2	10	6.9	2.2
14	10	12	2.8	10	8.9	1.7
21	10	16	3.5	10	13	3.9
28	10	18	2.2	10	17	3.7
<i>Energy uptake (Nmm)</i>						
8	47	44	24	46	37	23
11	10	70	31	10	67	31
14	10	92	26	10	79	26
21	10	95	24	10	80	31
28	10	95	33	10	66	27
<i>Area (mm²)</i>						
8	37	5.7	1.4	36	4.9	1.4
11	10	5.8	1.3	10	4.7	1.2
14	20	6.1	1.2	10	5.8	1.1
21	10	6.8	1.3	10	6.9	1.4
28	10	6.2	1.0	10	6.2	0.8
<i>Stress (MPa)</i>						
8	37	2.7	1.2	36	2.7	1.1
11	10	5.1	1.3	10	5.7	2.3
14	20	6.0	2.2	10	5.5	1.6
21	10	7.3	2.2	10	5.5	3.4
28	10	7.8	2.1	10	6.4	1.8

Table 4. Site of rupture

Days	M	PC			M	C	P
		M	C	P			
8	47	1	1	44	1	3	
11	10	0	0	10	0	0	
14	10	0	0	9	0	1	
21	8	0	2	6	1	3	
28	8	0	2	7	0	3	
Unoperated	–	–	–	20	0	9	

M – Midsubstance; C – Calcaneus; P – Proximal clamp

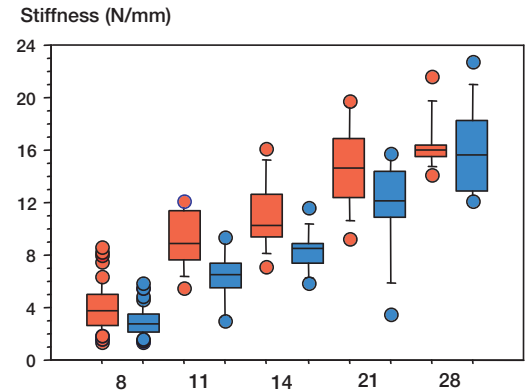
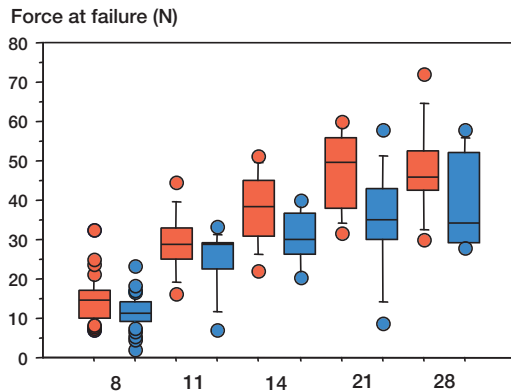
Mechanical results at all times (Tables 3 and 4)

With time, the number of tendons that failed at the clamp or at the calcaneus increased so that at 28 days, 5 of 20 tendons had not ruptured in the midsubstance. Of these, 3 were untreated tendons. Exclusion of all tendons that did not rupture in the midsubstance had little effect on the results and did not change any of the conclusions based on the statistical analysis. The PC-treated tendons reached 84% of the force at failure of unoperated tendons at 21 days. The controls had reached 63% at 21 days, and 70% at 28 days.

When the data at all times were analyzed together, the force at failure was increased by PC ($p < 0.001$). The average difference between PC-treated specimens and controls was largest at 21 days after surgery (increase by 36%), but the 95% CI at 21 days was large (5–67% increase).

The transverse area was increased by PC treatment at 8 and 11 days after surgery, but after 14

less force at failure than PC ($p = 0.01$; one-way ANOVA of all data during 8 days, Fisher’s post hoc test).



Force at failure and stiffness of PC (red) and control (blue) at 8, 11, 14, 21 and 28 days. Boxes indicate interquartile range, whiskers 10–90 percentile range, and dots extreme values.

Table 5. Histological findings

Treatment	Days	n	Mean	Min	Median	Max
Control	11	10	1.7	1	1.5	3
PC	11	10	2.0	1	2	3
Control	21	10	3.2	2	3	4
PC	21	10	4.2	3	4	5

days, the areas were similar in the PC-treated and control groups. Therefore, the maximum stress was increased on days 14, 21 and 28. At 21 days, the maximum stress was increased by 31% (95% CI decrease by 14% to increase by 76%), and even at 28 days by 23% (95% CI decrease by 4% to increase by 57%). In the analysis of all groups together, the maximum stress was increased by PC treatment ($p = 0.04$).

The stiffness in all groups increased with time ($p < 0.001$) and PC ($p < 0.001$). The effect of PC seemed to be largest at 11 days (increase by 41%; 95% CI 12–70%), and had disappeared at 28 days. At this time, the stiffness was 57% of the unoperated tendons.

The energy uptake in all groups was affected by time ($p < 0.001$) and was increased by PC ($p = 0.02$). The effect appeared to be largest at 21 days (increase by 18%; 95% CI decrease by 12% to increase by 48%). The energy uptake exceeded that of the unoperated tendons from 14 days and onwards.

Unoperated tendons

Half of the unoperated tendons showed load deformation curves that indicated slipping of the clamps or other problems (this did not occur in operated tendons). The remainder ($n = 14$) had a force at failure of 56 N, sd 9.7, stiffness of 14 N/mm, sd 5.7 and energy uptake of 78 Nmm, sd 30.

Histological findings (Table 5)

All specimens contained an almost homogeneous mass of fibrous callus, with a few inclusions of fat cells. The histological scores were higher at 21 days, and differed between the 4 groups ($p < 0.001$). PC had no detectable effect at 11 days ($p = 0.4$), but at 21 days, the tendon calluses were more mature ($p = 0.02$).

Discussion

Our results show that a single injection of platelet concentrate can improve tendon repair in rats. This effect is probably due to the growth factors that are released from the platelets during activation. We have previously found an improvement in tendon repair in the same model, using recombinant cartilage-derived morphogenetic proteins (CDMPs) (Aspenberg and Forslund 1999, Forslund and Aspenberg 2001, Forslund et al. 2003), but similar experiments using TGF β 1 or FGF2, have been unsuccessful (data not shown). The present results seem to differ in several respects from those obtained with CDMPs. With CDMP1, 2 or 3, we mainly studied the findings at 8 days. At that time, CDMPs increase the transverse area by 250%, but with platelet concentrate, we found only an 18% increase. The force at failure was increased 40% by CDMPs and 27% by the platelet concentrate. Thus, the CDMPs caused a dramatic proliferative response, making the tendon callus larger, while the platelet concentrate mainly affected maturation. We also noted an improvement in the maximum stress with platelet concentrate as late as 21 and 28 days.

We were surprised that a single injection had an effect even 4 weeks later, and it remains to be seen whether the effects of CDMPs are similarly long lasting. Recent results from a rabbit experiment indicate that the initial increase in the size of the tendon callus induced by CDMP treatment lasts during remodeling and therefore increases the maximum strength at later times as well (Forslund and Aspenberg 2002). Although CDMPs seem to be more potent, they are not available for clinical use, but the patient's own platelets can be easily prepared during routine care. We also believe that our platelet concentrates were not always optimally prepared. The effects of PC varied significantly between the groups with an 8-day follow-up. This difference in response was probably due to differences in the platelet concentrate preparation. The platelets were obtained by percutaneous heart puncture and it seems likely that trauma to the endothelium caused premature platelet activation in some of our batches, so that the growth factors were lost in the supernatant. We plan to improve our methods of preparation in other experiments.

Moreover, the dose injected into the rat tendon defects could be increased, for example, by simply doubling the injected volume. Thus, it might be possible to increase the effect in this rat model.

We had no controls which did not receive injections. In previous studies, we have repeatedly found that injection of an acidic buffer does not affect healing in this model (Forslund et al. 2003, unpublished data). The only difference between PC and platelet-poor plasma preparations in this study was the presence of platelets, and the effects of PC differed from those of platelet-poor plasma controls, but the effects of the latter were about the same as those of buffer controls.

The tendon specimens were mounted so that rupture usually occurred in the tendon substance and not at its attachments. The unoperated tendons also usually ruptured in the midsubstance. The uninjured tendon may be more sensitive to uneven loading during testing, so that a few of the well-organized fiber bundles may have taken most of the initial load in an unphysiological way, which would cause premature initiation of rupture. With the more diffuse arrangement of fibers in the tendon calluses, this effect should be less in the operated tendons. Therefore, we believe that the strength of the intact tendon in the physiological situation is greater than our measurements had indicated. The comparisons with unoperated tendons in the results section are intended to give a general impression and should be interpreted with caution.

Platelet concentrate has been used for many purposes in orthopedics, but to our knowledge only one controlled clinical study has evaluated its effects. Marx et al. (1998) implanted maxillofacial bone grafts enhanced with PC and found a higher rate of bone formation and more bone in the PC-treated grafts than in controls. Human PC improved bone ingrowth into porous hydroxyapatite in nude rats (Siebrecht et al. 2002). Muscle regeneration in rabbits was improved by rabbit PC (Jodczyk et al. 1986), and in skin wounds, PC increased granulation tissue and fibrous tissue formation (Ksander et al. 1990). One of the growth factors in platelets is platelet-derived growth factor (PDGF). This factor has improved regeneration in rabbit experiments on ligament repair (Batten et al. 1996).

Many uncontrolled clinical studies have reported that PC has a good effect on bone and soft tissue

repair (Lowery et al. 1999, Tischler 2002). However, clinical applications of methods developed in experimental animals have often been disappointing, and it remains unclear whether PC can improve tendon repair in humans. If the incidence of rerupture were to be the effect variable, many patients would have to be included in any study aimed at showing a beneficial effect. On the other hand, the risks of injecting an autologous platelet concentrate into, —e.g., the hematoma of an Achilles tendon rupture— would be minimal.

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