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Optimal Platelet Concentration for The Therapeutic Effect of Autologous Neutrophil-Reduced Platelet Rich Plasma in A Rat Model of Achilles Tendinopathy

M. Yoshida, H. Funasaki

Department of Orthopaedic Surgery,The Jikei University School of Medicine

CORRESPONDING AUTHOR:
Mamoru Yoshida
Department of Orthopaedic Surgery
The Jikei University School of Medicine
3-25-8 Nishi-shinbashi
Minato-ku, Tokyo
105-8461 Japan
E-mail: mamoru@jikei.ac.jp

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SUMMARY

Background. Autologous Platelet-Rich Plasma (PRP) therapy is used for treatment of tendinopathy. Efficacy, however, is variable, possibly caused by differences in PRP platelet concentrations. In an animal model of tendinopathy, we determined if there was an optimal PRP platelet concentration.

Methods. Tendinopathy was induced by collagenase injections into the Achilles tendons in rats. 10 days later, PRP at platelet concentrations of 50 (P50 group), 75 (P75 group), 100 (P100 group) and 150 × 10^4/μL (P150 group) or normal saline (control group) was injected into the tendons. To assess pain relief, spontaneous locomotor activity was measured for 12 hours at night. After 19 days, Achilles tendons were removed, and histological sections stained with hematoxylin-eosin or by TdT-mediated dUTP nick end labeling.

Results. Activities in the P75 and P100 groups were significantly greater than in the P50, P150 and control groups. The numbers of microtears, laminations and apoptotic cells in tendons in the P75 and P100 groups were significantly fewer than in the P50, P150 and control groups.

Conclusions. Pain relief and tendon repair were greatest in the P75 and P100 groups. In this rat model of tendinopathy, the optimal PRP platelet concentration in PRP was approximately a range of 75 to 100 × 10^4/μL.

KEY WORDS

Optimal concentration of platelets; platelets-rich plasma; tendinopathy; animal model; enthesopathy; Achilles tendinitis.

INTRODUCTION

Tendinopathy can occur in almost any tendon and is the most common tendon disorder. It is characterized by activity-related pain, focal tendon tenderness and decreased strength and movement, and can impair performance of workers in occupations that involve repetitive movements including various athletic pursuits (1).

Autologous Platelet-Rich Plasma (PRP) is now being used clinically for the treatment of several different tendinopathies, but effects have been variable. Several studies have shown PRP to be as effective or more effective than conventional therapies such as corticosteroid injections and focused shock-wave therapy for lateral or medial epicondylitis, jumper's knee and Achilles tendinopathy (2-12). In other studies, PRP had no significant therapeutic benefits in Achilles tendinopathy (13, 14). One possible factor contributing to different therapeutic outcomes is the difference in PRP platelet concentration in the different preparations. Since the PRP platelet concentration is dependent on the whole blood platelet concentration this is not unexpected, and has been noted in preparations from different institutions, and also from the same commercial preparation kit between different subjects. Platelets are the source of growth factors, cytokines and chemokines that regulate cellular anabolic metabolisms, and so different platelets concentrations might be expected to influence the tendon reparative reaction in the treatment of tendinopathies.

The optimal concentration of platelets in PRP for tendon healing is unclear. Several *in vitro* studies have shown that platelet concentrations up to approximately 1.0×10^6/μL had positive effects in tendons and tenocytes, including increasing growth factors and collagen synthesis, while higher concentrations had inhibitory effects including decreasing collagen synthesis and increasing inflammatory mediators (15-17). One animal study reported to date examined the therapeutic effects of neutrophil-reduced PRP at platelets concentrations of 5.0×10^5/μL and 10.0×10^5/μL in a model of patellar tendinopathy in rats (18), however the optimal concentration of platelets in the PRP was not determined.
The objective of the current study was to determine the optimal concentration of platelets in PRP for decreasing pain and improving tendon structure in a model of Achilles tendinopathy in rats. Tendinopathy was induced bilaterally by injections of collagenase, and starting 10 days later autologous neutrophil-reduced PRP at platelet concentrations from 5.0×10^5/μL to or 15.0×10^5/μL was administered. Efficacy was assessed by measuring a pain-relief by a locomotor activity score and the tendon tissue-repair by histology. There was also a different animal model of proximal patellar tendinopathy prepared using a repetitive running exercise on a rodent treadmill machine, in which Achilles tendinopathy was not induced, in rats (18).

MATERIALS AND METHODS

The study was conducted according to the journal's guidelines (19).

Reagents and materials

The autologous PRP preparation system, MyCells, was purchased from Kaylight, Tel Aviv, Israel. The normal saline for injections was purchased from Otsuka Pharmaceutical Factory, Inc., Naruto, Japan. The collagenase type I (185 IU/mg) derived from *Clostridium histolyticum* was purchased from Worthington Biochemical Corporation, Lakewood, NJ, USA. The Supermex system for measurement of spontaneous locomotor activity was purchased from Muromachi Kikai Company, Tokyo, Japan. The ApopTag Peroxidase *in Situ* Apoptosis Detection Kit was purchased from EMD Millipore Corporation, Billerica, MA, USA.

Animals

Sixty male Wistar rats (10 weeks of age, weighing 350-400 g) were purchased from Nippon SLC, Hamamatsu, Shizuoka, Japan. The rats were housed in an environmentally controlled animal facility on a 12:12 light/dark cycle. Food and water were available *ad libitum*.

Ten of these rats were untreated healthy controls. They were used for PRP preparation and for histological assessment of normal Achilles tendon structure. Fifty rats were used for the tendinopathy study. The general status of the animals was observed once a day from the day before collagenase injections to the end of the study. Body weight was measured every 5 days during the study period.

Collagenase-induced Achilles tendinopathy model

The study received Institutional approval (#29-003), and all experiments were conducted in accordance with the Institutional guidelines for the care and use of experimental animals. A rat model of collagenase-induced Achilles tendinopathy was prepared as previously described (20, 21). Collagenase type I powder was suspended in Phosphate Buffered Saline (PBS) at a concentration of 300 IU/mL and the solution was filtered through a sterile nylon syringe filter. Rats were anesthetized with 2.5% isoflurane. With the ankle flexed to 90 degrees to put tension the tendon, collagenase solution (50 μL) was injected into the distal insertional site of both Achilles tendons with 30-gauge needles. Spontaneous locomotor activity was measured with the Supermex system (see below) for 12 hours on the nights for 5 days before the collagenase injections and for 10 days after the collagenase injections. Rats with > 25% reduction in spontaneous locomotor activity 10 days after collagenase injections comparing with the average activities for 5 days before the collagenase injections were selected for the tendinopathy study. This decrease in activity was based on data from a preliminary study. To confirm the reproducibility and validity of the model, a preliminary study was performed in 30 rats. Consistent with reported observations, the values of locomotor activities or the reduction in spontaneous locomotor activity after collagenase injections was variable among rats (20, 21). In the preliminary study, locomotor activity decreased 21-31% 10 days post collagenase injections, with 26 rats (87%) having > 25% reduction. Histology of the Achilles tendons from 10 randomly selected rats with > 25% decrease in activity showed tendinopathy-related changes including microtears and laminations (*i.e.*, longitudinal disruptions between the fiber bundle layers at the insertion site of the Achilles tendon) (see later for details of the methods) (**figure 1**).

Treatment groups and injection of autologous platelet-rich plasma

The 40 rats with decreases in locomotor activity > 25% at 10 days post collagenase injections were randomized into five groups (n=8/group). The remaining three rats were

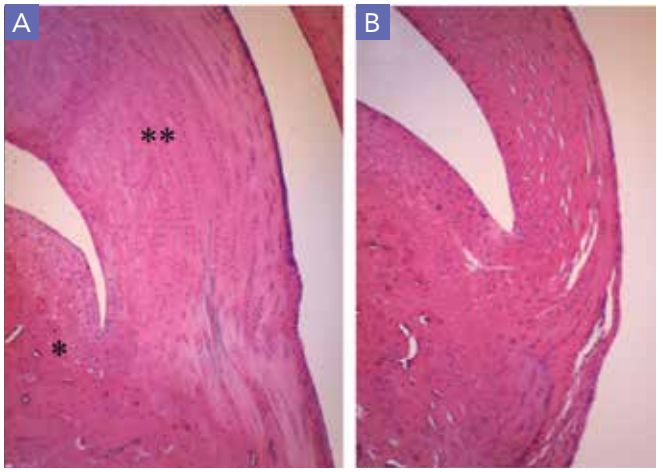

Figure 1. Histological sections of the insertion sites of Achilles tendons stained with Hematoxylin-Eosin (H & E) in a normal healthy rat **(a)** or in a collagenase-induced tendinopathy rat (a rat with > 25% decrease in locomotor activity 10 days after collagenase injections) **(b)** (×100). Two major pathological findings were found at the insertion site of Achilles tendons in the tendinopathy rats: 1) microtears in the body of the tendon and 2) several longitudinal disruptions between the layers of the fiber bundles of the tendon body, so-called laminations. An upper side is proximal. * Calcaneus bone, ** Achilles tendon.

reserved for late accidents. These groups had injections into the ankles bilaterally of normal saline (control group) or autologous neutrophil-reduced PRP at platelet concentrations of 50 × 10^4/μL (P50 group), 75 × 10^4/μL (P75 group), 100 × 10^4/μL (P100 group) and 150 × 10^4/μL (P150 group). For the injections, the rats were anesthetized with 2.5% isoflurane and the fur at the ankle joints was shaved. 50 μL of normal saline or PRP was injected into the distal insertional sites of the Achilles tendons bilaterally with a 0.5 mL injection syringe and a 30-gauge injection needle. All animals were sacrificed on day 19 after the PRP injections.

Preparation of autologous platelet-rich plasma

The autologous PRP preparation system, MyCells, was used to obtain a rat autologous neutrophil-reduced PRP. Twenty-two mL of whole blood was aspirated from the hearts of 10 normal healthy rats. From this whole blood, the PRP preparation system produced 4.0 mL of neutrophil-reduced PRP. Briefly, the whole blood was centrifuged for 7 minutes at 2000 × g. After aspiration of the supernatant plasma, the residual plasma of 4.0 mL was agitated to disperse the platelets, which were precipitated on the separation gel. This plasma was filtered to exclude the debris resulted in 4.0 mL of a filtered non-activated PRP (18). Analysis of a 100 μL sample of this rat autologous PRP showed a platelet concentration of 166 × 10^4/μL, and exclusion of approximately 90% of neutrophils and almost all erythrocytes. This PRP was classified as type 3 in Mishra's classification and as pure PRP in the PAW classification since the PRP was not activated before injection. This solution was diluted to produce the required platelet concentrations for the study.

Measurements of spontaneous locomotor activity

Spontaneous locomotor activity of each rat was measured with the Supermex system. Activity was measured for the 12 hours dark cycle the day before and after the collagenase injections, and then on alternate days through the end of the study (29 days after the collagenase injections and 19 days after the PRP injections). The locomotor activity has been shown to reflect the degree of the pain in limbs of rats, and so the degree of pain-relief in the ankle could be determined by the changes in locomotor activity. The Supermex system can quantify activity, and has been shown to be reliable compared to other methods. Details of spontaneous locomotor activity measurement have been described previously (18, 22, 23). Briefly, movement is detected from the infrared radiation emitted from the animal. Different lenses detect the radiation in different regions of the cage, and in this study, activity was measured as a single count when an animal moved from one region of the cage to another. The noise produced by grooming was filtered out. Total counts were calculated by summing up all counts of 10-min periods. Baseline locomotor activity is very variable among rats and so the post-PRP activity data are calculated and presented as % change in counts compared to the activity data at 10-day post-collagenase value. The absolute values for locomotor activities were different among rats, which were described in our previous literature (18).

Histological examinations

From the preliminary study, ankle tissue samples were obtained from 6 normal healthy rats and from 6 rats with > 25% decrease in activity 10 days post-collagenase injections. In the tendinopathy study, on day 19 post-PRP or normal saline injections, ankle tissue samples were obtained from all 40 rats in the P50, P75, P100, P150 and control groups. After anesthesia with 5% isoflurane, the rats were killed by an immediate blood draw. Whole ankle joints were dissected out and were fixed in 4% paraformaldehyde at pH 7.4 for 3 days, decalcified in 20% EDTA solution for 21 days at 4 °C, and then embedded in paraffin wax. Five μm thick

sections were taken in the sagittal plane and were stained with hematoxylin-eosin (H & E). A histopathological score was determined from the number of microtears and laminations, *i.e.*, several longitudinal disruptions between the fiber bundle layers seen at the insertion site of the Achilles tendon in a visual high-power (200×) field in each of five sections per rat. A score was doubled if the length of the tears or laminations were longer than half the diameter of the high-power field (18, 22). Histological sections were also prepared by TdT-mediated dUTP nick end labeling using the ApopTag Peroxidase *in Situ* Apoptosis Detection Kit according to the manufacturer's instructions. Apoptotic cells were quantified by counting the number of TUNEL-positive cells in high-power (400×) in each of five sections per rat. All H & E and TUNEL stained sections were analyzed by three researchers. The standard deviations of these scores or numbers among the three researchers were within 5%.

Statistical analysis

Comparative series were considered independent of each other. The % change in locomotor activity, the histopathological score, the number of apoptotic cells, and body weights are all presented as means and standard deviations for each group. The activity data, histopathological scores and apoptotic cell numbers were compared among groups with Mann-Whitney U Test. The body weight data was compared between groups with Student's t-Test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

General status and body weight

During the experimental period, no abnormalities were observed in any of the groups. All groups showed a similar increases in body weight compared to the control group and no significant differences were observed in body weight among groups.

Collagenase-induced Achilles tendinopathy model

In 50 rats injected with collagenase solutions, 43 rats had decreases in spontaneous locomotor activity at 10 days after injections of > 25% (overall reduction rate was in the range of 20% to 33% in 50 rats) and 40 rats (an average reduction rate was 28.7%) were selected for the PRP/tendinopathy study.

Spontaneous locomotor activity

In the P75 group, the counts for spontaneous locomotor activity increased by approximately 20% at day 2 after PRP injections compared to the activity immediately pre-PRP. Activity gradually increased to 30% at day 12 and was maintained until the end of the study (**figure 2**). The activities in the P75 group after day 12 recovered to a level similar with the activities before the collagenase injections, since after collagenase injections the activities were reduced to be in the range of 70% to 75% and the increased activity values of 30% were in the range of 91% to 100%. Similarly, in the P100 group locomotor activity increased by approximately 20% at day 2 after PRP injections and was maintained around this level until the end of the study. On the other hand, the spontaneous locomotor activities in the P50 and P150 groups increased by approximately 10% at day 2, were maintained until day 8 or day 12 and then decreased to the baseline level until the end of the study. The activity in the control group was around baseline level throughout the study. The spontaneous locomotor activities in the P75 or the P100 groups were significantly greater than in the P50, P150 and control groups from day 2 to day 18 ($p < 0.05$), and there was significantly greater spontaneous locomotor activity in the P75 group than in the P100 group from day 8 to day 14 ($p < 0.05$). Spontaneous locomotor activity in the P50 group was significantly greater than in the control group ($p < 0.05$) from day 2 to day 8, and locomotor activity in the P150 group was significantly greater than in the control group ($p < 0.05$) from day 2 to day 12 (**figure 2**).

Histological findings

In rats that exhibited tendinopathy (> 25% decrease in locomotor activity after collagenase injections), two major pathological features were observed at the insertion sites of Achilles tendons (**figure 1 b**). These were microtears in the tendon body and laminations, *i.e.*, several longitudinal disruptions between the fiber bundle layers. No tendinopathy-specific histopathological changes were observed in the six normal healthy rats (**figure 1 a**). In the P75 and P100 groups, the histopathological scores determined by the number and length of the microtears and laminations were significantly lower than those in the P50, P150 and control groups ($p < 0.05$). This indicates that the tendon repair processes in the P75 or P100 groups had progressed compared to those in the P50, P150 and control groups. There were no significant differences in the histopathological scores between the P75 and P100 groups, between the P50 and P150 groups, between the P50 and control groups or between the P150 and control groups (**figure 3, 4**).

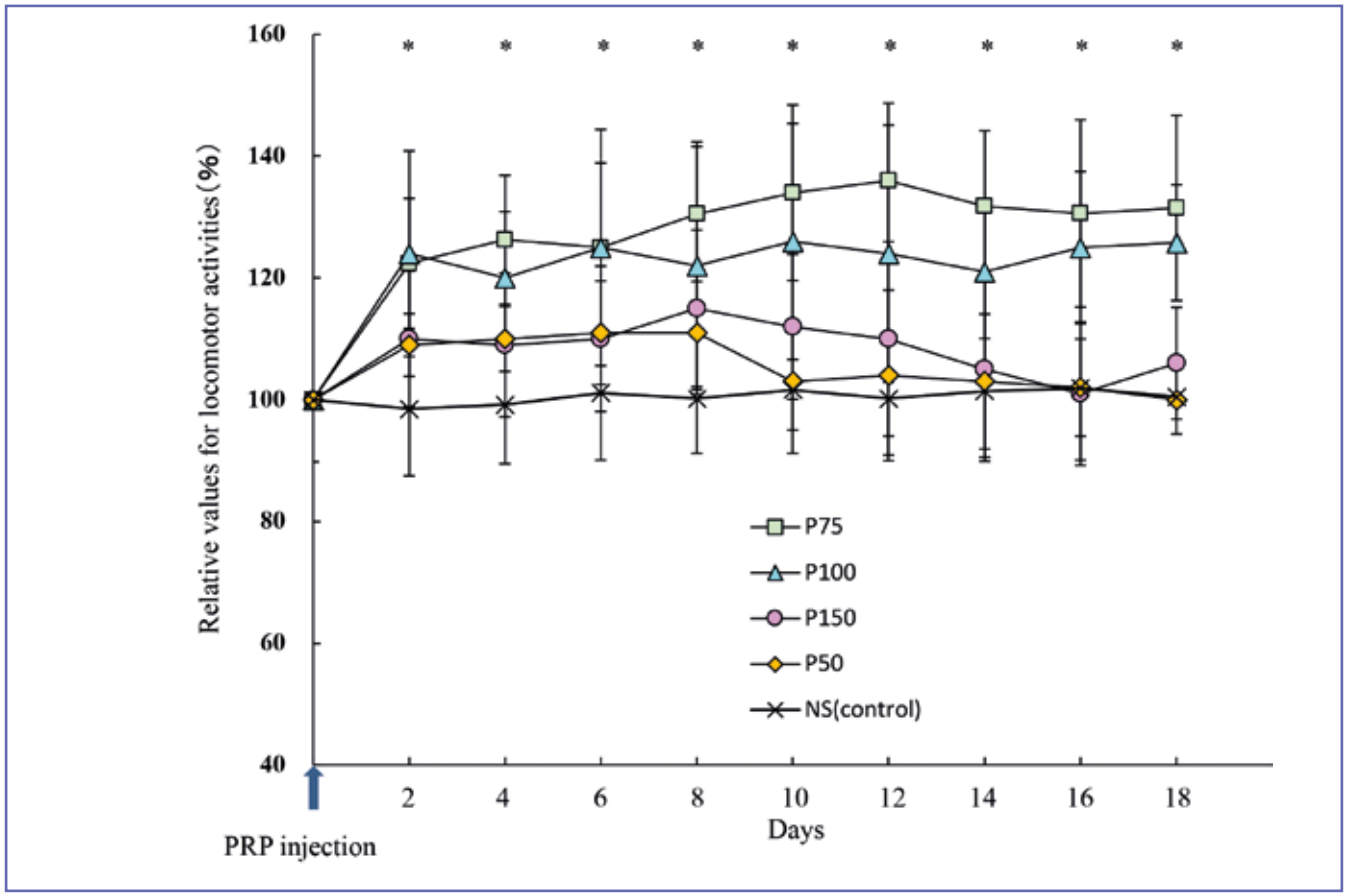

Figure 2. Relative values for spontaneous locomotor activities in the P50 (◊), P75 (□), P100 (O), P150 (Δ) and control groups (×). The spontaneous locomotor activities were measured using a Supermex system at night for 12 h on alternate days for 19 days after PRP injections. The relative counts of locomotor activities in each group were calculated from the post-collagenase pre-PRP baseline, and are shown as group means ± standard deviations.
*Significant differences between the P75 group and the P50, P150 and control groups ($p < 0.05$), and between the P100 group and the P50, P150 and control groups ($p < 0.05$).

In rats exhibiting tendinopathy, TUNEL-positive (apoptotic) cells were observed at the insertion site for Achilles tendon. In the P75 and P100 groups, the number of TUNEL-positive cells was significantly lower compared to the same tendon area in the P50, P150 and control groups ($p < 0.05$). There were no significant differences in the number of TUNEL-positive cells between the P75 and P100 groups, between the P50 and P150 groups, between the P50 and control groups or between the P150 and control groups (**figure 5, 6**).

DISCUSSION

In the present study, the following were the principal findings. In rats exhibiting collagenase-induced Achilles tendinopathy who were then treated with autologous neutrophil-reduced PRP at different platelet concentration or saline control: 1) the spontaneous locomotor activities in rats injected with PRP at platelet concentrations of $75 \times 10^4/\mu L$ and $100 \times 10^4/\mu L$ increased to pre-collagenase levels (similar to levels in normal, healthy rats) at day 2 after PRP injections and were then maintained for the following 17 days, the end of the study; 2) the spontaneous locomotor activities with PRP at platelet concentrations of $75 \times 10^4/\mu L$ and $100 \times 10^4/\mu L$ were significantly greater than those in the rats injected with PRP at platelet concentrations of $50 \times 10^4/\mu L$ and 150×10^4 µL or saline control at all times from day 2 until the end of the study; 3) the spontaneous locomotor activities in the rats injected with PRP at plate-

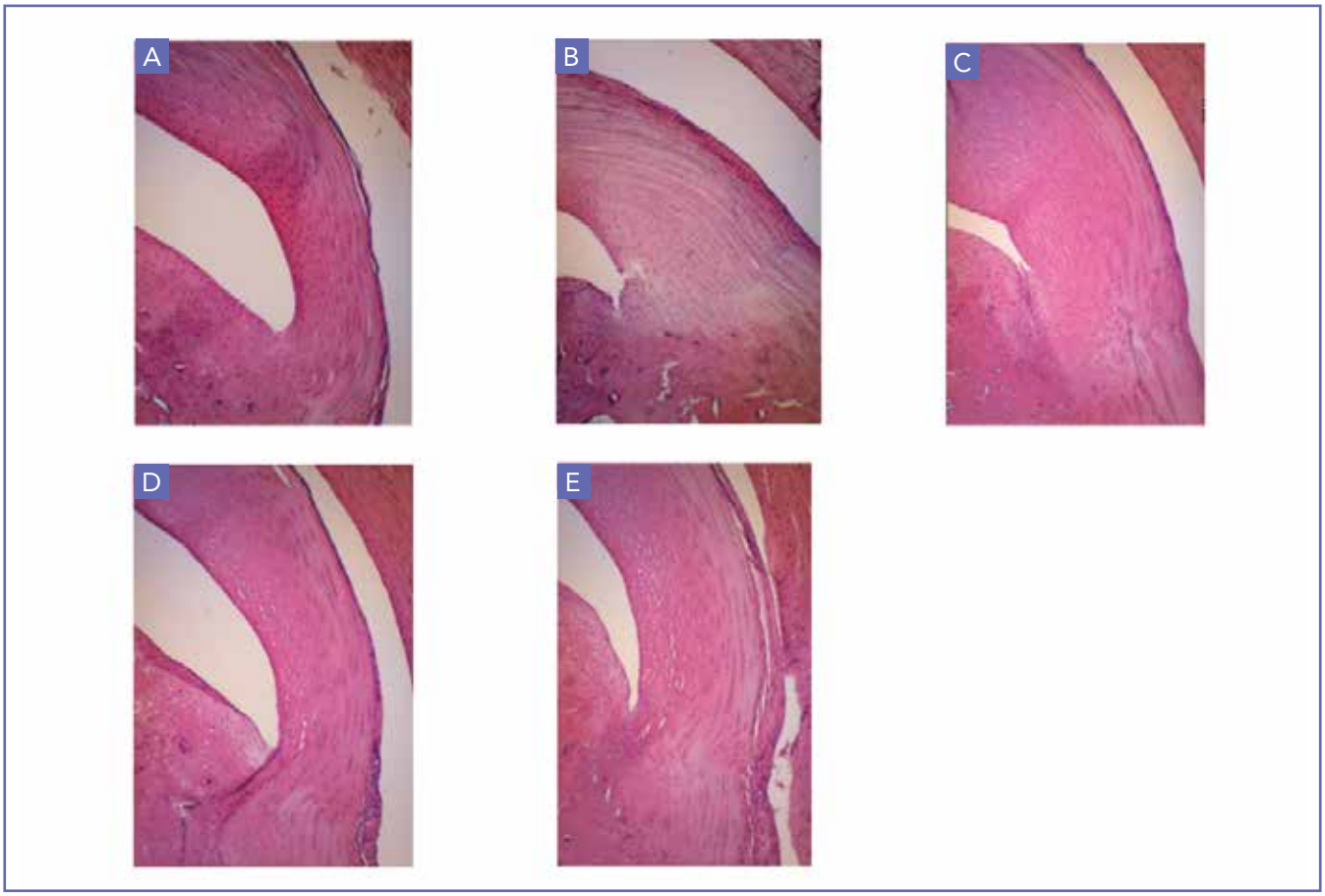

Figure 3. Histological sections of the insertion sites of Achilles tendons stained with Hematoxylin-Eosin (HE) in the P50 group **(a)**, P75 group **(b)**, P100 group **(c)**, P150 group **(d)** and control group **(e)** (×100). Ankle tissue samples were obtained from all 40 rats in all experimental groups on day 19 post-PRP or normal saline injections. Five μm thick sections were taken in the sagittal plane and were stained with Hematoxylin-Eosin (HE).

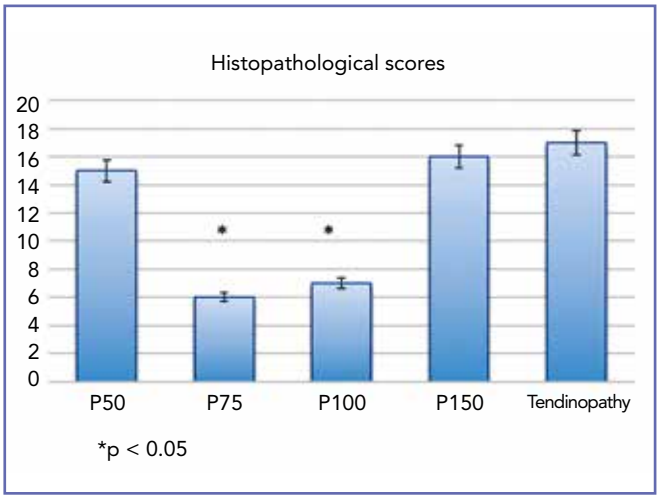

Figure 4. Histopathological scores for the HE-stained histological sections located at the insertion site of the Achilles tendon in the P50, P75, P100, P150 and control groups. A histopathological score was determined from the number of microtears and laminations, *i.e.*, several longitudinal disruptions between the fiber bundle layers seen at the insertion site of the Achilles tendon in a visual high-power (200×) field. A score was doubled if the length of the tears or laminations were longer than half the diameter of the high-power field. Scores are shown as group means ± standard deviations. The number of microtears and laminations counted in the P75 and P100 groups were significantly lower than those in the P50, P150 and control groups ($p < 0.05$). No significant difference was observed in the number of microtears or laminations between the P75 and P100 groups, between the P50 and P150 groups, between the P50 and control groups or between the P150 and control groups.

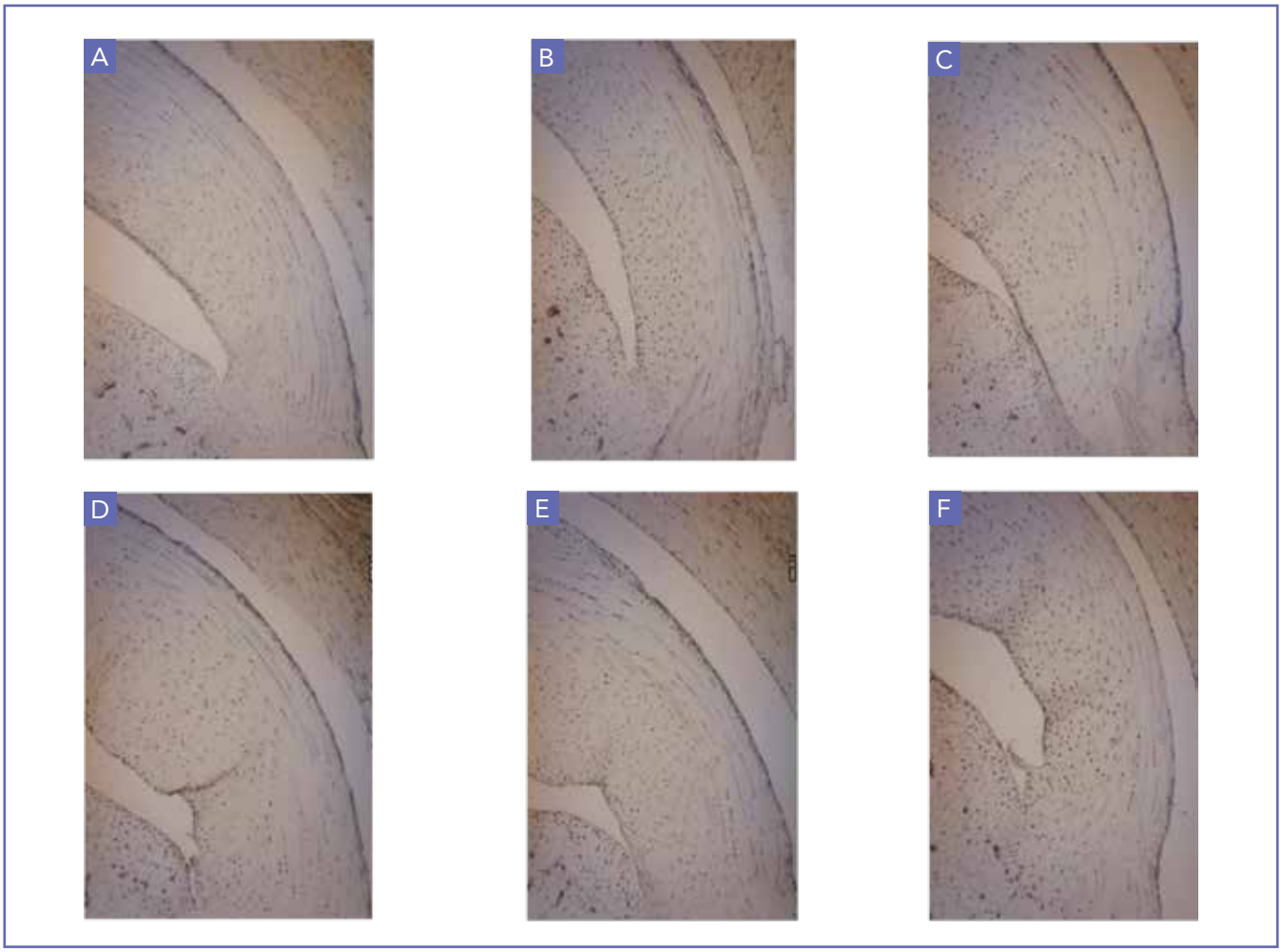

Figure 5. Histological sections of the insertion sites of Achilles tendons prepared by TdT-mediated dUTP nick end labeling (TUNEL) of healthy normal rats (**a**), and rats in the P50 group (**b**), P75 group (**c**), P100 group (**d**), P150 group (**e**) and control group (**f**) (×100). Ankle tissue samples were obtained from all 40 rats in all experimental groups on day 19 post-PRP or normal saline injections. Five μm thick sections were taken in the sagittal plane and were prepared by TUNEL using the ApopTag Peroxidase *in Situ* Apoptosis Detection Kit according to the manufacturer's instructions.

let concentrations of 50 × 10^4/μL and 150 × 10^4/μL were significantly greater than those in rats injected with saline control, although these activities were less than the pre-collagenase baseline; 4) in histological sections taken at the end of the study (day 19), the number of microtears and laminations, and the number of TUNEL-positive cells observed at the insertion site of the Achilles tendon in rats injected with PRP at platelet concentrations of 75 × 10^4/μL and 100 × 10^4/μL were significantly fewer than the numbers in rats injected with PRP at platelet concentration of 50 × 10^4/μL and 150 × 10^4/μL or with saline control; 5) there were no significant differences in the number of microtears and laminations, or in the number of TUNEL-positive cells between the P75 and P100 groups, or among the P50, P150 and saline control groups.

Taken together the findings indicate that in this model of Achilles tendinopathy the analgesic effect (from the increase in locomotor activity) and the reparation of the tendon (from the histological score and number of apoptotic cells) with PRP at platelet concentrations of 75 and 100 × 10^4/μL were greater than with PRP at platelet concentrations of 50 and 150 × 10^4/μL. The increase in locomotor activity to pre-collagenase levels suggest that the PRP at platelet concentration of 75 and 100 × 10^4/μL completely relieved the pain in these rats. From these data we propose that a platelet concentration of 75 to 100 × 10^4/μL in neutrophil-reduced PRP was

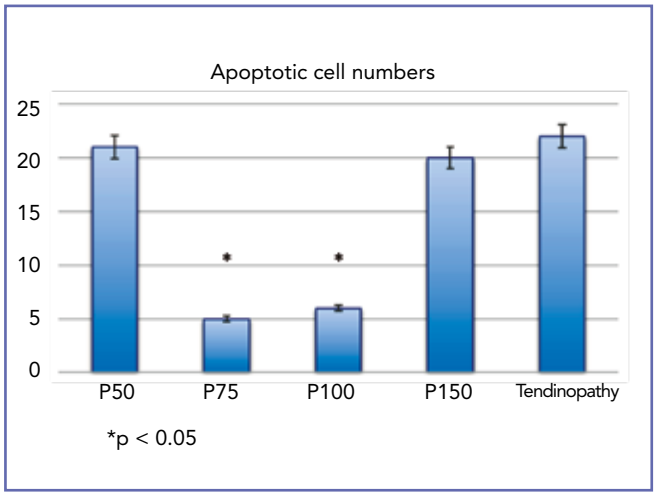

Figure 6. Cell number for apoptosis at the insertion site of the Achilles tendon of rats in the P50, P75, P100, P150 and control groups. Apoptotic cells were quantified by counting the number of TUNEL-positive cells in high-power (400×). The number of TUNEL-positive cells are shown as group means ± standard deviations. The number of TUNEL-positive cells in the P75 and P100 groups was significantly lower than those in the P50, P150 and control groups ($p < 0.05$). No significant difference was observed in the number of TUNEL-positive cells between the P75 and P100 groups, between the P50 and P150 groups, between the P50 and control groups or between the P150 and control groups.

the optimal range for effects of pain relief and tendon reparation in this rat model of Achilles tendinopathy. It is possible the optimal range extends to lower and higher concentrations, and this was not established. This is the first report to determine an optimal range of PRP platelet concentrations in an animal model (*i.e., in vivo*) of tendinopathy. The observation of platelet concentration dependent efficacy at low and medium concentrations and reduced efficacy at high concentrations is consistent with findings from *in vitro* cell culture studies on tendon metabolism (15-17).

Clinically, the platelet concentration in PRP isolated from commercial preparation kits is generally not adjusted before administration. It was suggested that different platelet concentrations in PRP might account for the different clinical outcomes of PRP therapy seen between institutions or between patients within an institution. Our data support this and suggest that for maximum therapeutic benefit in treatment of tendinopathy, the platelet concentration in PRP should be measured after isolation and be adjusted to an optimal concentration before administration. It is possible that the optimal concentration will be different for different tendinopathies, and this is an area for future study. It is hoped that eventually de novo PRP preparation kits will be available that can, automatically adjust the platelet concentration to the optimal range.

Locomotor activities in rats injected with PRP at platelet concentrations of 75 and 100 × 10^4/μL increased by approximately 20% at day 2 after PRP injections. Since the tendon tissue repair was unlikely to be complete at this stage, it was speculated that the mechanisms of PRP-induced pain relief and tissue restoration were different and separate. It has been suggested that pain generation in tendinopathy results from free nerve endings in abnormal capillary vessels with microshunts that form around the tendon (24-26), since focal embolization of these abnormal capillary vessels immediately relieved the tendinopathy pain (27). Therefore, we speculate that in this tendinopathy study a PRP-induced reaction degraded the abnormal capillary vessels and/or inhibited the free nerve endings to produce the initial pain relief. More detailed tendon histology is needed to confirm the presence of such capillary vessels and free nerve endings at the insertional sites of the Achilles tendons in this rat model of Achilles tendinopathy.

In general, an immobilization of the treatment sites after PRP therapy was recommended or ordered in order to escape the inhibition or the delay of the cellular reparative reactions in clinics for human (28). The present study demonstrated that tendinopathies recovered well in P75 or P100 groups without the immobilizations of the treatment sites after PRP therapy, suggesting that tendinopathies in human might also recover well by the PRP therapy under the condition that the concentrations of platelets in PRP were in the optimal range even though without immobilizations of the treatment sites.

It was found that locomotor activities of P50 or P150 groups increased up by approximately 10% for a week after PRP injections and then decreased down to baseline. Histopathological scores of these groups were significantly lower than those of P75 or 100 groups indicating the insufficient histological repair of the tendon tissues in P50 or P150 groups. For the reason of the finding it was suggested that cellular reparative reactions did not progress after the initial pain relieving affected in approximate half degree because of the non-optimal concentrations of platelets in PRP and no immobilizations for the ankles of rats in P50 or P150 groups.

Limitations of the present study were: small sample number, short experimental period and so reproducing early-stage but not late stage tendinopathy, lack of biomechanical evaluation, and different concentrations of leukocytes between experimental groups. In this study there was a maximal effect in terms of pain relief and tendon repair in the 75 to 100 × 10^4/μL platelet concentration range. A smaller

effect at a PRP platelet concentration of 50 × 10^4/μL can be explained by the lower number of platelets. A question is: why is the effect smaller at a platelet concentration of 150 × 10^4/μL? Neutrophils, a major source of inflammatory mediators that can impair healing and cause pain, were largely excluded from this PRP preparation. It has been suggested that the residual leukocytes other than neutrophils in PRP preparations have little or no effect on tendon metabolism. However, we cannot rule out that these residual leukocytes did contribute to the reduced efficacy in the P150 group. This requires further study.

CONCLUSIONS

In a rat model of Achilles tendinopathy, a single injection of autologous neutrophil-reduced PRP into the Achilles tendon reduced pain and initiated the tendon repair process. These effects of pain relief and tendon repair were greater at platelet concentrations of 75 and 100 × 10^4/μL than at platelet concentrations of 50 and 150 × 10^4/μL. These data suggest that there is an optimal PRP platelet concentration for maximal efficacy in the treatment of tendinopathy. Studies are needed to determine if there are similar optimal PRP platelet concentration ranges for maximal therapeutic efficacy in clinical tendinopathies.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

ACKNOWLEDGEMENT

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