Trauma Monthly 2025; 30(4): 1530-1537

10.30491/tm.2025.522780.1835

Trauma Monthly

Research Article

Effectiveness of Autogenous L-PRF Membranes on Tendon Healing in A Rabbit Model

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Received 2025-01-08; **Accepted** 2025-02-25; **Online Published** 2025-04-29

Abstract

Introduction: Tendon injury is an important clinical issue for orthopedic surgeons. Tendon healing is a time-consuming process. This study aimed to investigate the effect of autologous Leukocyte-platelet-rich fibrin (L-PRF) on the healing of the superficial digital flexor tendon in a rabbit model.

Method: Ten healthy adult rabbits of mixed breed were randomly divided into Treatment and Control groups. After cutting the superficial digital flexor tendon and suturing it, the autologous L-PRF membrane was applied locally in the Treated group. The tendon was sutured with the Bunnell-Mayer pattern with no material implantation in the Control group. Five animals from each group were euthanized, and their tendons were collected for histopathological evaluation on the 30th day post-surgery.

Result: In morphological examination, a smaller tendon diameter and more adhesion were seen in the Treated group. There were significant differences in the total number of cells, cell maturation, the ratio of cellularity to matrix, and the alignment and order of cells between the Treated and Control groups at the histopathological level. The Treated group showed superiority over the Control group in tissue order, collagen fiber crimp pattern, inflammation, and vascularity; however, these differences were not significant.

Conclusion : This study found that L-PRF has a beneficial effect on tendon healing. Due to the short treatment period, additional research and evaluations are necessary in this field.

Keywords: Leukocyte-platelet rich fibrin; tendon injury; tissue engineering; tendon healing; rabbit

Introduction

Damage to the tendon is widespread and, in most instances, results in serious complications, which can worsen over time and eventually require surgery. Tendons have a low regenerative capacity and take a long time to repair [1]. This delay in the healing process is due to the special structure of tendons, which have low cell density, poor circulation due to a small number of blood vessels, and low metabolism. Although these features confer high resistance and improve the performance of healthy tendons, they are detrimental following tendon injury [2]. Many factors contribute to the onset and progression of tendinitis. Internal factors include age, sex, anatomical features, body weight, and systemic disease. Extrinsic factors include sports activities, physical loads, occupation, and environmental conditions, such as walking surfaces or shoes. In addition, genetic polymorphisms affecting collagen fiber formation or even blood type have been reported to be associated with tendon injury and tendinopathy [3]. The goal of surgical techniques is to repair ruptures in tendons. Surgery is often the only option in such cases of tendon rupture [4]. The application of growth factors is a suitable treatment option used together with tendon surgery [5].

The current study is an effort to improve tendon healing and increase the quality of its surgery. For this purpose, the effect of fibrin rich in leukocytes and platelets on the tendon repair process of the rabbit animal model has been investigated, and the anterior part of the superficial digital flexor tendon (SDFT) was used to operate due to

its availability. Leukocyte and platelet-rich fibrin or L-PRF is a blood product produced by a one-time centrifugation process that does not require the addition of any chemicals. Several investigations have studied the effects of PRP on the repair processes of tendon injuries and other tissues, but none have confirmed a significant impact [6]. To improve healing processes and increase the success rate of tendon surgery, we decided to use an L-PRF membrane, as it has been shown that its cytokines and growth factors accelerate bone healing and promote fibroblast proliferation. In addition, these factors increase tissue angiogenesis, accelerate collagen formation and mitosis of mesenchymal stem cells, endothelial cells, and osteoblasts [6,7].

The tendon repair process involves a complex and coordinated series of events. Recent evidence suggests that reducing the inflammatory process in the early stages of tendon repair can improve repair. Although many cells are involved in tendon healing, macrophages play an important role in regulating tissue repair by promoting angiogenesis and reducing inflammation [8]. The growth factors present in L-PRF enhance fibroblast division, proliferation, and differentiation and accelerate bone healing [8,9]. In addition, they increase tissue vascularization, collagen formation, and mitotic rate of mesenchymal stem cells, endothelial cells, and osteoblasts. Human clinical studies on the beneficial effects of using L-PRF growth factors in oral and maxillofacial reconstruction, including periodontal and sinus implant surgery, have been approved [9,10].

It has been shown that PRP and L-PRP can have adverse inhibitory effects on the proliferation and differentiation of tendon stem cells [11]. For this reason and other advantages of L-PRF over PRP, including greater stability of the L-PRF membrane in the environment and the release of more leukocytes, cytokines, and growth factors from L-PRF than from PRP, it was decided to use L-PRF for the healing of tendon injury [12]. L-PRF was first prepared by Choukroun et al. in 2001 using a cheap, easily accessible technique [13]. Platelet-rich fibrin (PRF) is the second generation of the platelet derivatives obtained from blood immediately after centrifugation, but the anticoagulants are not used in its preparation [14].

Methods

L-PRF preparation

For the preparation of autogenous L-PRF, 5 ml of blood was collected from the Jugular vein of a rabbit in the treatment group, and the sample was centrifuged in a small laboratory centrifuge for less than 1 min without adding any chemical materials. The centrifugation criteria were 2700 RPM for 12 minutes. The clot was pressed between two slides for 4 min, and an L-PRF membrane was produced.

Animals

Ten mix-breed rabbits, 12 months old, weighing 1.5±0.5 kg, were divided into Treated (n=5) and Untreated (n=5) groups. Before the experiment, the rabbits were kept in a room, and their diet consisted of standard rabbit food pellets, fresh vegetables, and water, available ad libitum. They were housed in a room maintained at a range of 18-22 °C with a 12-h light/dark cycle.

Ethics

All animals received humane care in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85–23, revised 1985). The study was approved by the local Ethics Committee, in accordance with the "Regulations for using animals in scientific procedures" of the School of Veterinary Medicine at Shiraz University.

Surgical procedures

After 15 days, the rabbits had become accustomed to the new environment and food. The rabbits were taken to the Clinical Science Department's operating room at our Veterinary School. The animals were anesthetized by intramuscular injection of 30 mg/kg ketamine and 0.2 mg/kg aspromazine, and the hair from the back of the thigh muscle to the tarsal joint of the leg was then shaved and prepared for the operation. An incision was made in the skin, in the area of the Achilles tendon. The superficial digital flexor tendon was exposed by blunt dissection. This tendon was cut with blade number 15, and the L-PRF membrane was placed in the tendon defect. The defect was sutured with 0.2 nylon thread by the Bunnell-Meyer pattern. The skin was sutured subcutaneously with 0.2 Vicryl thread.

Upon return from the anesthesia, the rabbits were released into the laboratory animal room without external fixation. Two days after surgery, the operated rabbits received daily penicillin injections at a dose of 40,000 IU and streptomycin at a dose of 12 mg/kg IM. During the 30 days' post-operation, the rabbits were examined daily for general body condition, illness, swelling at the surgical site, and lameness. In addition, symptoms of illness, loss of appetite, lethargy, and diarrhea were also checked in the operated animals.

Histopathological evaluation

For histopathological evaluation, the rabbits were euthanized 30 days after the operation. The animals were first anesthetized with 100 mg/kg ketamine and 8 mg/kg acepromazine based upon the ethical methods of euthanasia 15. They were analgesically euthanized by injecting 80 mg/kg of phenobarbital into the heart. The samples were taken from the defect site, fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin wax. Longitudinal sections 5 mm thick were prepared,

stained with hematoxylin and eosin and Masson's trichrome, and examined under an ordinary light microscope (Olympus, Tokyo, Japan). Three fields of each tissue section were photographed for histomorphometric analysis.

The total number of cells in three fields per section was counted at 400× magnification for histopathological assessment, and the average cell count was calculated. The mean values of the two groups were compared. In addition, the inflammatory cells include neutrophils. Macrophages, lymphocytes, plasma cells, and mesenchymal cells, including tenoblasts and tenocytes, from the three selected fields, were counted in each histopathology section, and their means were statistically compared.

The data were tabulated in Excel 2019 and evaluated in SPSS. Cellularity, cell alignment, cell distribution, nuclear morphology, vascularisation in the defect area, and pattern of inflammatory cell distribution were analysed and scored as follows 15:

Score 0 was considered when the collagen fibers, tenoblasts, tenocytes, and the newly regenerated blood vessels were irregular and showed a haphazard pattern in the tissue section. When these structures showed 50% irregularity and the next 50% were regularly organized, scores 1 and 2 were considered for the healing state of the injured tendon for sections that showed a regular pattern, were completely cohesive, and had wavy, compact collagen fibers. In evaluating the cellularity stage of each section, a score of zero was assigned when the cell level was generally elevated across all parts of the tissue section or when the section was highly cellular. Score 1 was assigned to sections with higher cell density in some parts, and score two was considered for sections that demonstrated a normal cellular pattern, almost comparable to a normal tendon.

Tissue orientation was scored 0 when more than 50% of the cells were not aligned in the same direction, 1 when 10 to 50% of the cells were irregularly arranged, and 2 when all the cells were uniaxial and aligned along the longitudinal axis. Cell distribution received a score of 0 in the case where focal areas of high cell density were observed and a score of 1 when the tissue section showed a homogeneous cell distribution. Score 0 was assigned when more than 30% of the nucleus morphology of cells were larger, oval, euchromatic or heterochromatic, score 1 when 10 to 30% of the nuclei were significantly oval, euchromatic or heterochromatic, and score two if most of the mesenchymal cells showed elongated, heterochromatic nuclei. Vascularization was scored 0 when four or more blood vessels were seen in the same tissue section and 1 when 0-3 vessels were seen in the same tissue section. The severity of inflammation was evaluated using a score of 0 when inflammatory cells were present in the tissue section and 1 when none were present.

Statistical analysis

The Comparison of the number of cells per surface unit, and the Comparison of the average number of cells counted in each section by the Student test. The histopathological data (semiquantitative) were compared using the Kruskal–Wallis test. When P-values were less than 0.05, pairwise group comparisons were performed using the Mann–Whitney U test. (SPSS version 21 for Windows, SPSS Inc., Chicago, USA).

Results

1. Clinical and Gross pathological findings

After surgery, the rabbits could walk easily, did not limp and could eat water and food. There was no mortality and the incisions were not infected. During the daily inspection, none of the rabbits showed signs of illness, anorexia, lethargy and diarrhea.

Pathological evaluations showed that the anatomical structure of the tendon was preserved in both groups, on the 30th day post-surgery. The injured tendons in untreated groups showed moderate adhesions and edema, and sutures were observed. The diameter of the injured area of the tendon increased compared to the normal tendon. The color of the tendons appeared darker due to the previous haemorrhages and hyperemia. Most tendons in the treated group were swollen and had brown spots. A suture thread was observed in one tendon in the treated group. Adhesion was also observed in two samples of the treatment group.

Histopathological findings

The histopathological sections of the treated tendons, showed proper alignment of the tenocytes, tenoblasts and collagen fibers along the longitudinal axis of the tendon, fewer but with higher calibre blood vessels, and enhanced maturation of tendon cells in comparison to the untreated tendons were evident (Fig. 1).

The cell counts for inflammatory cells including macrophages, lymphocytes and plasma cells were significantly higher in the untreated sections than the treated ones (Table 1). No neutrophil was found in the lesions of the treated and untreated animals at this stage of tendon healing. The tenoblast and tenocyte count was also lower in the treated lesions than the untreated ones (Table 2). Significant differences were noted between the treated and untreated lesions in terms of cell maturation, cell-to-matrix ratio, cells alignment and distribution (Table 3).

Staining by Masson-trichrome showed more collagen fiber formation in the treated lesions compared to the untreated ones. Masson's Trichrome staining also demonstrated improved alignment in the tenoblasts, tenocytes, collagen fibers and blood vessels, so that these structures were aligned along the longitudinal axis of the tendon. However, the tissue section in the untreated tendons were more vascular, hyper cellular and unorganised.

Figure 1. Gross pathological, 30 days after surgery. A, B, C) Control Group D, E, F) Treatment Group

Table-1- Comparison of the number of cells per surface unit

Groups	Mean±SEM	P value
L-PRF	118.33± 8.35	*0.03
Control	215.33±30.48	
*There are significant differences between L-LPRF group and Control group p≤0.05, the cell counts for inflammatory cells including macrophages, lymphocytes and plasma cells were significantly higher in the untreated sections than the treated ones		

Table-2-Comparison of the average number of cells counted in each section separately

	Control group	L-PRF group	P value
Cells			
Tenocytes/Tenoblasts	3.50±0.48*	1.04±1.54	0.003
Inflammatory cells	45.42±9.70*	24.24±4.88	0.004
*There are significant differences between L-LPRF group and Control group p≤0.05, the tenoblast and tenocyte count was lower in the treated lesions than the untreated ones			

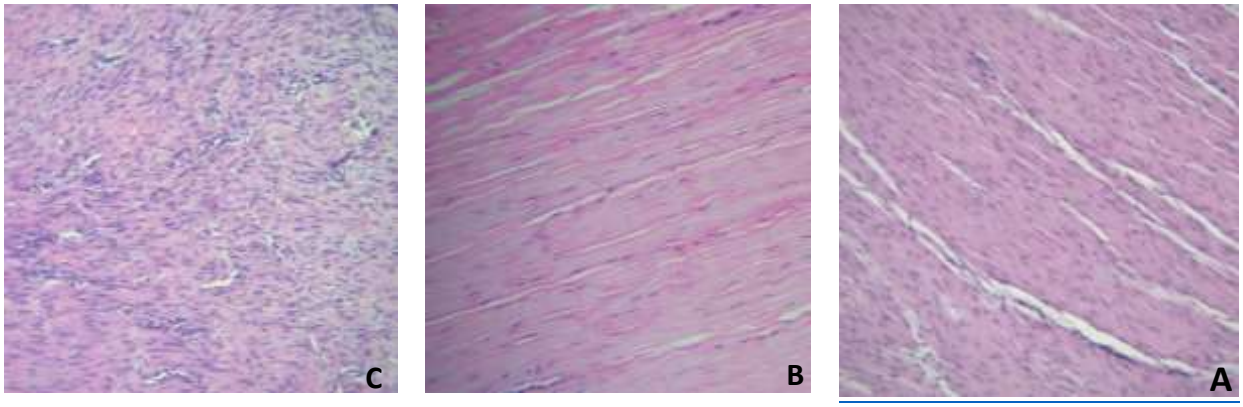

Figure 2. Histopathological sections, 30 days after surgery (100x, H&E): A) treatment group, B) healthy tendon, C) control group

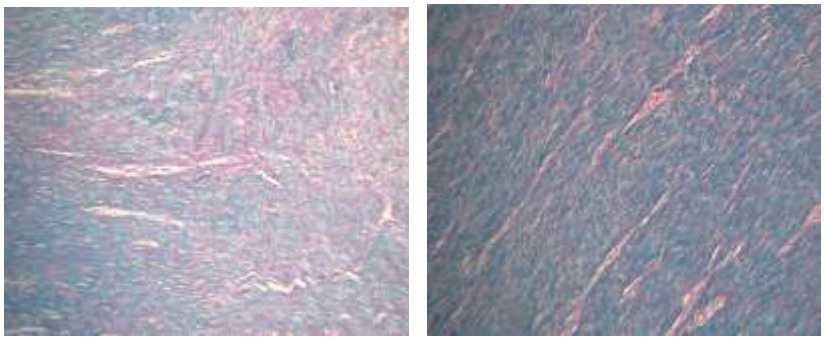

Figure 3. Histopathology sections, 30 days after surgery, (100x Masson's Trichrome): A) Treatment group, B) Control group

Table-3- Cases compared between two groups in terms of pathology evaluation

Topic	L-PRF	Control	P value
A)organization of whole tendon	2(0-2)	1(0-2)	0.45
B)Cellularity/cell matrix ratio	1(0-2)	0(0-1)	0.36
C)Cell alignment	2(1-2)	1(0-1)	0.04*
D)Cell distribution	1(0-1)	0(0-0)	0.02*
E)Cell necleus morphology	1(1-2)	0(0-1)	0.04*
F)Vascularisation in the defect area	0(0-1)	0(0-1)	0.63
G)Inflammation	1(0-1)	0(0-1)	0.24
*There are significant differences between L-LPRF group and Control group p≤0.05, Significant differences were noted between the treated and untreated lesions in terms of cell maturation, cell-to-matrix ratio, cells alignment and distribution in all of them criteria the LPRF group was significantly superior to control group			

Discussion

Based on the Wu et al. study, adhesion formation usually begins at about 1.5 weeks after tendon injury; it reaches its peak at about 4 weeks and becomes softer and more fragile about 7 weeks after surgery,[16,17]. In this way, the presence of an adhesion within 30 days after surgery is not as far off as expected.

Kobayashi and colleagues (2016) found that leukocyte- and platelet-rich fibrin attracts inflammatory cells, including platelets, neutrophils, B and T lymphocytes, and macrophages, to the lesion site within 10 days. The inflammatory cells then release various cytokines and growth factors, including PDGF-AA, PDGF-AB, PDGF-BB, transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (bFGF) and insulin-like growth factor (IGF)[18], These factors, especially IGF-1, PDGF, and bFGF, are essential in the

early stages of tissue healing and regeneration because they help fibroblasts divide, multiply, and synthesize extra cellular matrix (ECM) [19]. Insulin-like growth factor (IGF) is a positive regulator of proliferation and differentiation in most mesenchymal cells; it also acts as a protective agent. This cytokine mediates cell proliferation and is also the central regulator of programmed cell death (apoptosis) [20] .

It can be concluded that the stimulation of the production coming from this factor caused an upregulation and reduction of tendon tissue cell numbers during the healing process in the treated group. For this reason, we found that the histopathologic section of the treatment group had fewer cells, which were more mature than those in the control group. Besides, presences of fewer macrophages, lymphocytes and plasma cells and smaller number but more mature tenoblasts and tenocytes in the lesions of the treated animals indicate that the lesions in the treated animals are in the remodelling or maturation phase, while the haphazardly organized untreated lesions, which are still hyper cellular and highly vascular are still in the proliferative or fibroplasia phase of tendon healing. Oryan et al. (2017) studied the effects of gelatin scaffolds on tendon healing. They showed that the lesions in the treated tendons typically had fewer cells than those in the untreated tendons, but the difference was not significant. Compared with the control group, the cells in the treated group were more mature, and the cells and collagen fibers were more organised [21].

All types of collagen are composed of a few constant amino acids, such as glycine, proline, hydroxyproline, and hydroxylysine, and many non-fixed and variable amino acids, so that by measuring the content of some of the constant amino acids, such as hydroxyproline, the total collagen content can be calculated in a tissue [22]. Masson-trichrome staining of the lesions showed more collagen formation in the treated tendons compared to the untreated ones. Similar to our findings, treatment of tendon lesions with L-PRF and its derivatives increased the hydroxyproline content and total collagen content of the injured Achilles tendon in rabbits [23]. In the histopathology studies of the present research, tendon fibers in the treatment group were predominantly aligned and regular. The wave pattern was well-formed in these tissues. The collagen density Score was High, and the whole matrix structure was observed in sections. The scores of the treatment and control groups differed in terms of the organization of the whole matrix, but this difference was not significant.

Mesenchymal cells from the site of injury were divided into two groups based on nuclear size, cytoplasmic granules, and cell staining. Larger oval cells with granular cytoplasm and basophilic color were identified as immature fibroblasts (fibroblasts); Elongated and cigar-shaped cells with less granular cytoplasm. Eosinophils' cytoplasm was estimated to be tenocytes [24].

Using the same criteria, we scored cell nuclei morphology and found a significant difference between the control and treatment groups; this indicates cell maturation in the treatment group. During the regenerative phase, cells align with the direction of collagen fibers. The association and order of cells in the treatment group were apparent, and the difference in scores between the control and treatment groups was significant for this factor .

A research was published in 2020 in the journal Clinical Orthopedics and Related Research; and investigated the effects of PRP on the reduction of various tendon swellings; and unlike the present study, the results showed that the L-PRF group had a better improvement in swelling compared to the control group 25; However, in the control and treatment groups, there was no evidence of the accumulation of inflammatory cells and severe inflammation. Scores in the treatment group showed lower levels of inflammation markers, but this difference was not significant .

In Karimi et al.'s study (2015), the effect of xenologous and autologous platelet gel on the healing process was investigated in the rabbit animal model; The histological evaluations of this study showed that the xenologous and autologous groups had better scores for vascularization and inflammation compared to the control, although no significant difference was found in this study either [26].

The order of collagen deposition and the low number of tissue cells both indicate improved tendon repair in the treatment group. Although in the control group, the collagen fibers were not aligned. In the study by Oryan et al. (2017), which investigated the treatment of tendon rupture with a gelatin scaffold, it was found that 60 days after injury, the treated tendons had a better fibrillar alignment score than the untreated group.

Distribution and irregularity in the collagen fibers could be expected because, over time, the fibers will align and

the tissue will become more mature; at the same time, the parts of the Treated group showed greater order and scored higher, indicating that recovery is accelerating.
Regarding the therapeutic effects of PRF, the interaction of platelet-derived growth factors is known to be a key factor for enhancing and regulating tissue regeneration27-29 and the presence of growth factors in L-PRF has caused positive effects and better healing in the treated group.
In the control group with a tendon defect, due to the lack of therapeutic substance, tendon healing has occurred naturally, and since healing is a time-consuming process[1]. Histopathologically, the tendon tissue of the control group is far from healthy tendon tissue. It is also weaker compared to the tendon sections of the treatment group.

Conclusion

This study found that L-PRF has a beneficial effect on tendon healing. Due to the short treatment period, additional research and evaluations are necessary in this field.

Conflict of Interest Disclosures

There are no conflicting interests listed by the authors.

Funding Sources

Not applicable

Authors' Contributions

HK, ABS, AO an ATN contributed to the design and draft of the research and data collection, accomplished the data analysis, revised the all data and wrote the text. All authors approve and read the text version final

Ethical Statement

All animals received humane care in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85–23, revised 1985). The study was approved by the local Ethics Committee, in accordance with the "Regulations for using animals in scientific procedures" of the School of Veterinary Medicine at Shiraz University.

Declaration of Generative AI and AI-assisted technologies

None.

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