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Comparison of the Effects of Platelet Rich Fibrin and Platelet Rich Plasma on Experimentally Induced Tendon Injury in Rabbits Treated with Methylprednisolone

Comparación de los efectos de la plasma rico en plaquetas y la fibrina rico en plaquetas sobre la lesión tendinosa inducida experimentalmente en conejos tratados con metilprednisolona

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ABSTRACT

The aim of this study is to compare the effects of PRP and PRF on tendon injuries treated with methylprednisolone. In this context, the histopathological and immunohistochemical effects of PRP and PRF on tendon healing were evaluated. The study included 30 New Zealand rabbits (15 females and 15 males) with varying weights (1.5-3 kg) and ages (1-3 years). The experimental groups were designated as the steroid control group (Group B, n=10), the steroid+PRP group (Group C, n=10), and the steroid+PRF group (Group D, n=10). Additionally, the control tissues from a simultaneous study served as the empty control group (Group A). On the 14th postoperative day, a section encompassing the tenotomy site was excised from the area via incision. Healing was observed to be better in Groups C and D compared to Groups A and B. In Group A, inflammatory cell infiltration, primarily consisting of macrophages and polymorphonuclear leukocytes, along with a small number of lymphocytes and foreign-body giant cells, was higher than in all other groups. Group B exhibited less inflammatory cell infiltration compared to Group A. Additionally, hyperemia, hemorrhage, necrosis, new vessel formation, and fibroblasts of varying shapes and orientations were noted in Groups A and B. In contrast, cell infiltration was significantly lower in Groups C and D, while vascularization, fibroblast activity, and collagen density were higher. Collagen fibers were observed more regular bundles forms in Groups C and D compared to the control groups. The findings suggest that the combination of PRP or PRF with systemic methylprednisolone can effectively enhance tendon healing by modulating inflammatory responses, reducing adhesions, and supporting the formation of a well-organized extracellular matrix.

Key words: Glucocorticoid; PRP; PRF; tendon healing; rabbit

RESUMEN

El objetivo de este estudio es comparar los efectos de PRP y PRF en lesiones tendinosas tratadas con metilprednisolona. En este contexto, se evaluaron los efectos histopatológicos e inmunohistoquímicos de PRP y PRF en la curación de tendones. El estudio incluyó 30 conejos de Nueva Zelanda (15 hembras y 15 machos) con diferentes pesos (1,5-3 kg) y edades (1-3 años). Los grupos experimentales se designaron como el grupo de control con esteroides (Grupo B, n = 10), el grupo esteroides + PRP (Grupo C, n = 10) y el grupo esteroides + PRF (Grupo D, n = 10). Además, los tejidos de control de un estudio simultáneo sirvieron como grupo de control vacío (Grupo A). El día 14 del posoperatorio, se extirpó una sección que abarcaba el sitio de la tenotomía del área a través de una incisión. Se observó que la curación fue mejor en los grupos C y D en comparación con los grupos A y B. En el grupo A, la infiltración de células inflamatorias, que consistía principalmente en macrófagos y leucocitos polimorfonucleares, junto con una pequeña cantidad de linfocitos y células gigantes de cuerpo extraño, fue mayor que en todos los demás grupos. El grupo B mostró una menor infiltración de células inflamatorias en comparación con el grupo A. Además, se observaron hiperemia, hemorragia, necrosis, formación de nuevos vasos y fibroblastos de diferentes formas y orientaciones en los grupos A y B. En contraste, la infiltración celular fue significativamente menor en los grupos C y D, mientras que la vascularización, la actividad de los fibroblastos y la densidad de colágeno fueron mayores. Se observó que las fibras de colágeno formaban haces más regulares en los grupos C y D en comparación con los grupos de control. Los hallazgos sugieren que la combinación de PRP o PRF con metilprednisolona sistémica puede mejorar efectivamente la curación del tendón al modular las respuestas inflamatorias, reducir las adherencias y apoyar la formación de una matriz extracelular bien organizada.

Palabras clave: Glucocorticoide; PRP; PRF; curación del tendón; conejo

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INTRODUCTION

Tendon injuries can occur for various reasons, such as sports injuries and chronic diseases. Since traditional treatment methods provide limited healing, new treatment approaches are being investigated. Autologous blood products such as Platelet Rich Plasma (PRP) and Platelet Rich Fibrin (PRF) have emerged as promising treatment methods for tendon and ligament healing [1 , 2 , 3].

The idea of healing bone and tendon tissue damages in a shorter time and more robustly has directed researchers towards biomaterials. PRF [4 , 5 , 6] and PRP [7 , 8 , 9 , 10] have been used for this purpose. Corticosteroids have the potential to modulate tendon healing through various immunological effects, whether used as systemic or local treatments. However, this therapeutic approach may adversely impact tendon health due to potential side effects, sometimes even leading to tendon ruptures [11].

Following injury, the inflammatory phase initiates the healing process, which is subsequently followed by the proliferative and remodeling phases [12]. Corticosteroids exert their effects primarily by promoting the resolution of inflammation [13]. While they can suppress the inflammatory response [14], they also influence connective tissue granulation and the formation of a more organized extracellular matrix [15]. Resolution of inflammation plays a vital role in the tendon healing process, as prolonged inflammation can hinder effective recovery [16]. Thus, the timely resolution of inflammation is crucial to facilitate progression to subsequent repair stages, including proliferation and remodeling. This resolution process involves a complex interplay of mediators such as lipoxins and resolvins, as well as various cellular mechanisms [17]. Additionally, anti-inflammatory cytokines like interleukin-10 (IL-10) contribute to inflammation resolution by inhibiting pro-inflammatory signaling pathways and promoting macrophage transition from the M1 to the M2 phenotype [18]. Corticosteroids not only aid in the resolution of inflammation but may also support tendon repair by reducing fibrotic healing [19].

During this process, reductions in extracellular matrix-associated components such as fibronectin and tenascin C have been observed. Fibronectin rapidly increases post-injury and plays a role in fibrosis [20], while tenascin C facilitates the recruitment, migration, and differentiation of myofibroblasts, contributing to early stages of myocardial repair [21]. However, prolonged corticosteroid use may have serious systemic effects on the cardiovascular, gastrointestinal, endocrine, and immune systems. Metabolic effects, such as glucose intolerance and hypertension, are frequently reported with long-term corticosteroid therapy [22]. Their immunosuppressive effects can suppress immune responses and increase susceptibility to infections [23]. Furthermore, an association between corticosteroid use and an elevated risk of peptic ulcers and gastrointestinal bleeding has been identified [24]. Prolonged corticosteroid use may also lead to degenerative changes in tendons and ligaments, alterations in stress levels, and disruptions in homeostasis [25].

The aim of this study was to compare the effects of PRP and PRF on experimentally induced tendon damage in rabbits (*Oryctolagus cuniculus domesticus*) treated with methylprednisolone. In this context, the histopathological and immunohistochemical effects of PRP and PRF on tendon healing were evaluated. The hypothesis of the study was that PRP and PRF would accelerate the healing of tendon damage in animals treated with methylprednisolone and that this effect may differ depending on the treatment method. Although the healing effects of PRP and PRF on various tissue types have been demonstrated, the comparison of their effectiveness in tendon damage during methylprednisolone treatment has been the subject of a limited number of studies in the literature. Therefore, this study will make significant contributions to the existing literature and will guide clinical applications.

MATERIALS AND METHODS

Animals and study design

The study was carried out with the ethical permission of Erciyes University Animal Experiments Local Ethics Committee (EUHADYEK, Number: 17/018).

The study included 30 New Zealand rabbits *(Oryctolagus cuniculus)* (15 females and 15 males) with varying weights (1.5-3 kg) and ages (1-3 years). The rabbits were divided into three equal groups, each consisting of 10 rabbits (5 males and 5 females). Prior to surgery, all animals underwent routine clinical examination, and only healthy rabbits were included in the study. The experimental design consisted of a steroid control group (Group B), a steroid+PRP group (Group C), and a steroid+PRF group (Group D). Additionally, control tissues from a simultaneous project conducted at Balikesir University Scientific Research Projects (Project number: 2017/132) were used as the empty control group (Group A).

In all animals, IV catheters were inserted into the ear vein to facilitate intravenous medical applications when necessary and to obtain blood samples for PRP and PRF preparation. Prior to the surgical procedure, 5 mL of blood was collected from the PRP group for PRP preparation, while 8 mL of blood was collected from the PRF group for PRF preparation.

Preparation of PRP

PRP was prepared using a specialized PRP kit (EasyPRP® Kit 10, Neotec Biotechnology, Türkiye). Initially, 0.5 mL of sodium citrate as an anticoagulant (PPS Natrium Citricum 3.13%, Medi-Pac, Germany) was drawn into the kit's 10mL syringe. Subsequently, 4.5 mL of blood was collected from each rabbit into the syringe, bringing the total volume to 5 mL. The lower chamber of the syringe was inserted, and the syringe was gently shaken to mix the anticoagulant with the blood. The syringe was then placed in a centrifuge (DMO412, Onilab, USA) and centrifuged at 1200G for 5 min. After the first centrifugation, the red blood cells that had accumulated in the lower chamber were removed, and a second centrifugation was performed at 1200G for 10 min. Following the second centrifugation, three distinct layers were formed in the syringe: PRP, PPP (Platelet-Poor Plasma), and the buffy coat at the bottom. The stopper of the syringe was then removed, and the PRP application syringe (3 mL) with a luer lock tip provided in the kit was attached. The buffy coat and PRP portion were drawn into the 3 mL application syringe, and the PRP was prepared for use.

Preparation of PRF

Blood collected in tubes without anticoagulant was immediately centrifuged at 400G for 10 min [26]. After centrifugation, three layers formed in the tube: red blood cells at the bottom, a

fibrin clot (PRF) in the middle, and acellular plasma (PPP) at the top. The PRF clot was carefully removed and placed on sterile gauze. Gentle pressure was applied to flatten the clot, making it ready for use (FIG. 1).

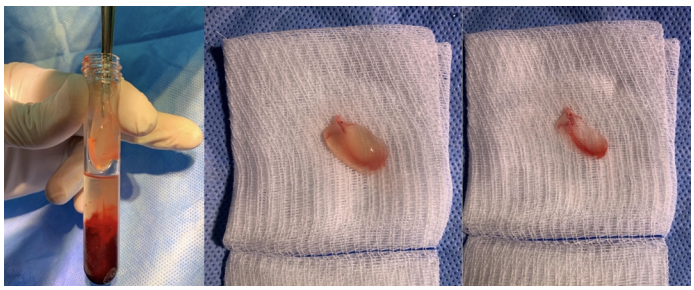

FIGURE 1. The preparation of Platelet Rich Fibrin (PRF) for use in the experiment

Surgical Procedure

In all groups, anesthesia was induced with medetomidine HCl (0.3 mg/kg, IM, Domitor, Pfizer, Germany) and ketamine HCl (30 mg/kg, IM, Alfamine, Alfasan, Netherlands). Anesthesia was then maintained using sevoflurane (Sevorane, Abbvie, USA) at a concentration of 4% in 100% oxygen.

Under anesthesia, the right Achilles tendon region was prepared for surgery. A skin incision was made to access the Achilles tendon and the paratenon membrane by dissecting the subcutaneous connective tissue. The tendon was exposed by incising the paratenon membrane. The Achilles tendon of the gastrocnemius muscle was carefully isolated, and a tenotomy was performed. Following the tenotomy, the tendon ends were approximated and sutured using the modified Kessler technique [27]. The paratenon membrane was closed with 4-0 monofilament absorbable suture material (PDSII, Ethicon®, USA). In the PRP group, 0.5-1 mL of prepared PRP was injected between the paratenon and the tendon. In the PRF group, the prepared PRF was wrapped around the tendon to cover the incision site. After the paratenon membrane was closed, the subcutaneous connective tissue and skin incision were routinely sutured using 3-0 absorbable multifilament suture material (Vicryl, Ethicon®, USA), completing the procedure.

Post-operative Procedure

Following the surgery, all rabbits received antibiotic (Penicillin, 11,000 IU/kg, IM, Ieciline, IE Ulagay, Türkiye) for 5 days (d) and methylprednisolone (4 mg/kg, IM, Prednol-L amp, Mustafa Nevzat, Türkiye) for 14 d. Additionally, a protective bandage was applied to the surgical site for 7 d. At the end of this period, the bandage was removed, the wounds were inspected, and the animals were returned to their cages without reapplication of the bandage.

Collection of Tissue Samples

On the 14th postoperative d, all rabbits underwent a second surgery following the same anesthesia and operative protocols. A tissue section encompassing the previous tenotomy site was excised and fixed in 10% buffered formalin solution. The tendon ends were then reattached using the modified Kessler method, and the skin and subcutaneous connective tissue were routinely sutured.

Histopathological and Immunohistochemical Evaluation

Tendon tissues were fixed in 10% neutral buffered formalin solution, routinely processed, and embedded in paraffin blocks. Five-micron sections were cut from the paraffin blocks using a microtome (RM2245, Leica, Germany), and stained with hematoxylin-eosin and Masson's Trichrome for histopathological analysis. Tendon healing was assessed using a light microscope (Eclipse N*i*, Nikon, Japan).

Immunostaining was performed using the Avidin-Biotin-Immunoperoxidase complex method to assess factors influencing fibroblast activation and collagen production during wound healing. Five-micron thick sections from paraffin-embedded tendon tissues were mounted on poly-L-lysine coated slides and dried overnight in an oven. Following deparaffinization, the sections were washed in PBS (Phosphate Buffer Solution) three times, with 5-min intervals between washes, and incubated (WiseVen, Wisd, France) with 0.001% trypsin at 37˚C for 30 min to unmask antigenic receptors. After washing in PBS three times, the sections were incubated in 3% hydrogen peroxide-methanol solution for 20 min to inhibit endogenous peroxidase activity. The sections were washed again in PBS and incubated with non-immune goat serum for 30 min at room temperature to block non-specific staining. Without further washing, sections were incubated with primary antibodies for 1 h at 37°C, targeting inducible nitric oxide synthase (iNOS, 1:100, ThermoFisher, PA5-16855), nitrotyrosine (AB5411, 1:500, Millipore, USA), βFGF (rabbit polyclonal; 1:200, AB8880, Abcam, Cambridge, UK), VEGF (Mouse monoclonal; 1:200, Abcam, AB1316, Cambridge, UK), and TGFβ1 (AB190503, 1:200, Abcam).

Following the incubation, the sections were washed three times in PBS and incubated with a biotinylated secondary antibody for 30 min. After further washing in PBS, the sections were treated with peroxidase-coupled streptavidin for 30 min. The sections were then washed three times in PBS and incubated with 3.3-diaminobenzidine tetrahydrochloride-H2O2 (DAB) solution as the chromogen. After rinsing in distilled water, the sections were counterstained with Mayer's Hematoxylin. Immunostaining intensity was evaluated in five distinct areas of each section under high magnification using a light microscope (Eclipse N*i*, Nikon, Japan), with staining scored as none (-), low (+), moderate (++), and intense (+++).

RESULTS AND DISCUSSIONS

In the study, healing was found to be superior in Groups C and D compared to Groups A and B. In Group A, inflammatory cell infiltration, predominantly comprising macrophages and polymorphonuclear leukocytes, along with a small number of lymphocytes and foreign-body giant cells, was greater than in all other groups (FIG. 2a). Group B exhibited reduced inflammatory cell infiltration compared to Group A (FIG. 2b). Additionally, hyperemia, hemorrhage, necrosis, neovascularization, and fibroblasts of varying shapes and orientations were observed in both Groups A and B. In contrast, Groups C and D showed significantly lower levels of cell infiltration, with increased vascularization, fibroblast activity, and collagen density. Collagen fibers in these groups formed more regular bundles compared to the control groups (FIGS. 2c and 2d).

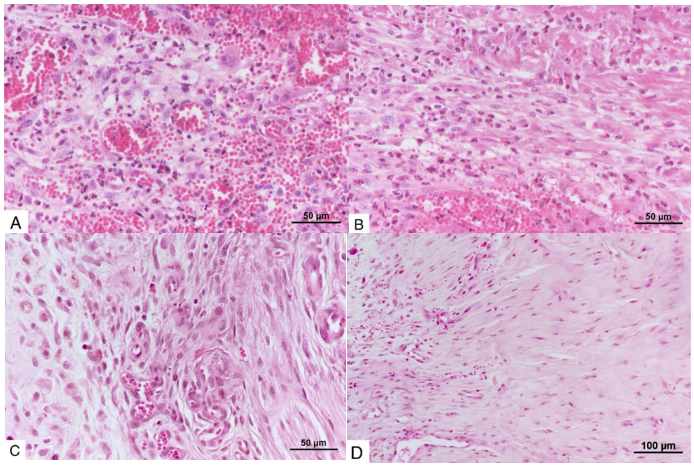

FIGURE 2. a: Inflammatory cell infiltration predominantly consisting of macrophages, neutrophils, a small number of lymphocytes, foreign-body giant cells, and areas of hemorrhage (Group A), b: Hemorrhage accompanied by macrophage and neutrophil infiltration (Group B), c: Increased connective tissue and new vessel formation (Group C), d: Regularly arranged connective tissue bundles (Group D) (Hematoxylin-Eosin stain)

In Masson's Trichrome staining, used to visualize connective tissue, collagen structures of varying thickness and irregular orientation were observed in the control groups. In contrast, the study groups exhibited collagen fibers that were more regularly aligned and parallel to one another (FIG. 3).

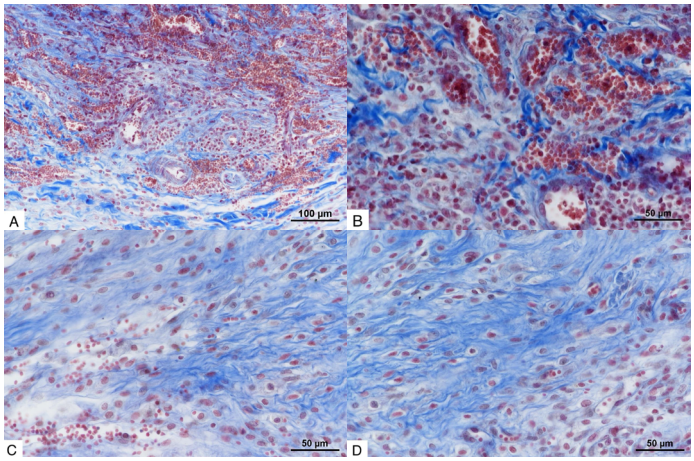

FIGURE 3. a: Hemorrhage and irregular collagen fibers (Group A), b: Hemorrhage and irregular collagen fibers (Group B), c: Regular connective tissue cells and collagen (Group C), d: Regular connective tissue bundles and collagen (Group D) (Hematoxylin-Eosin stain)

The immunohistochemical staining scores for TGF, VEGF, FGF-β, iNOS, and nitrotyrosine observed in the study are presented in TABLE I. While the immunoreactivity of TGF and VEGF was similar in the study groups, their intensity was found to be less severe compared to the control group. TGF immunostaining was particularly intense in macrophages and was present in the cytoplasm of neutrophils, fibroblasts, and vascular endothelial cells (FIG. 4). Cells staining positively for VEGF were primarily vascular endothelial cells, macrophages, and fibroblasts. Intense FGF-β immunostaining was observed across all groups (FIG. 5), predominantly in fibroblasts, with weaker staining in the cytoplasm of macrophages (FIG. 6). iNOS and nitrotyrosine immunostaining were most intense in Group A, moderate in Group B, and similar in the study groups. In Group A, both iNOS and nitrotyrosine staining were strongly present in macrophages and neutrophils, with moderate staining in connective tissue. In the other three groups, staining was moderate in the cytoplasm of macrophages, fibroblasts, and vascular endothelial cells (FIGS. 7 and 8).

TABLE I.
Immunohistochemical staining scores according to groups

	TGF	VEGF	FGF-β	iNOS	Nitrotyrosine
Grup A (Control)	+++	+++	+++	+++	+++
Grup B (Steroid)	+++	+++	+++	++	++
Grup C (Steroid+PRP)	++	++	+++	++	++
Grup D (Steroid+PRF)	++	++	+++	++	++

low (+), moderate (++), intense (+++)

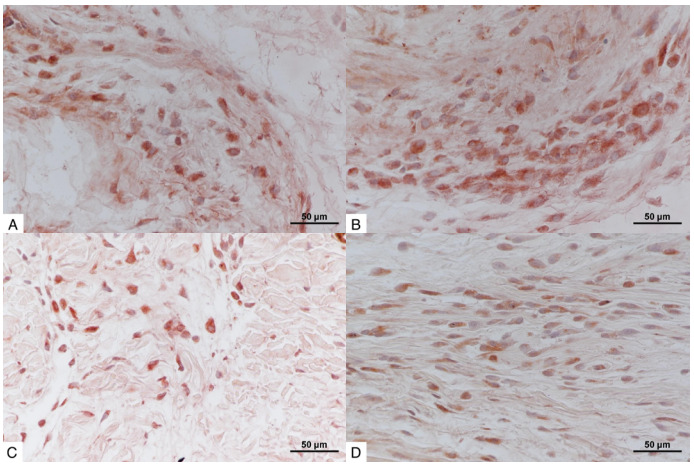

FIGURE 4. Severe (a, b) and moderate (c, d) TGF-β immunoreactivity in the cytoplasm of fibroblasts and macrophages (Immunohistochemistry, IHC)

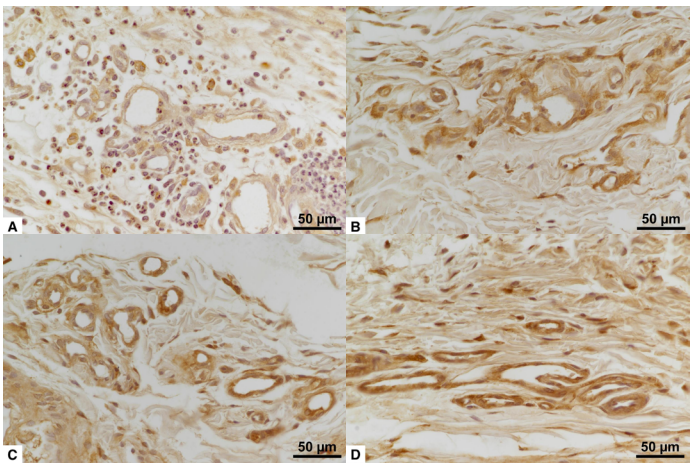

FIGURE 5. Severe (a, b) and moderate (c, d) VEGF immunoreactivity in the cytoplasm of fibroblasts, macrophages, and vascular endothelial cells (IHC)

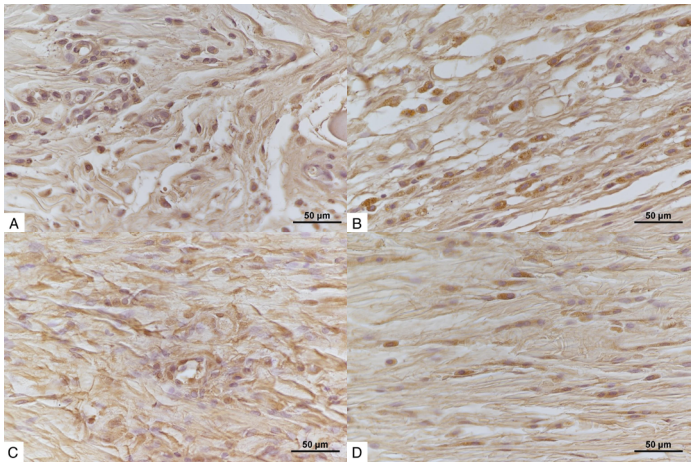

FIGURE 6. Severe FGF-β immunoreactivity in the cytoplasm of fibroblasts and macrophages (a, b, c, d) (IHC)

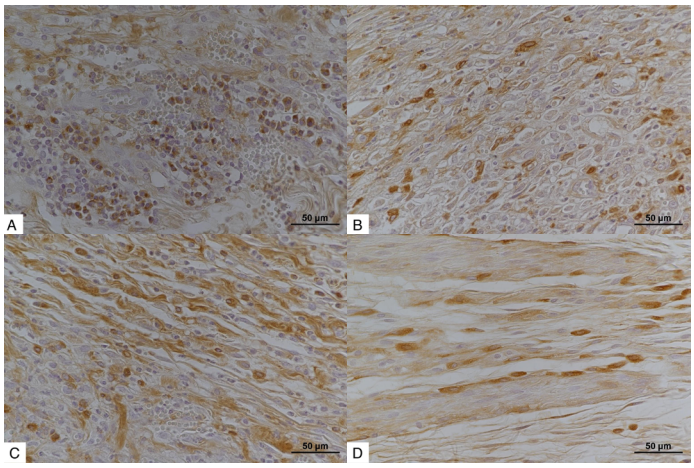

FIGURE 7. Severe (a) and moderate (b, c, d) iNOS immunoreactivity in the cytoplasm of fibroblasts, neutrophils, and macrophages (IHC)

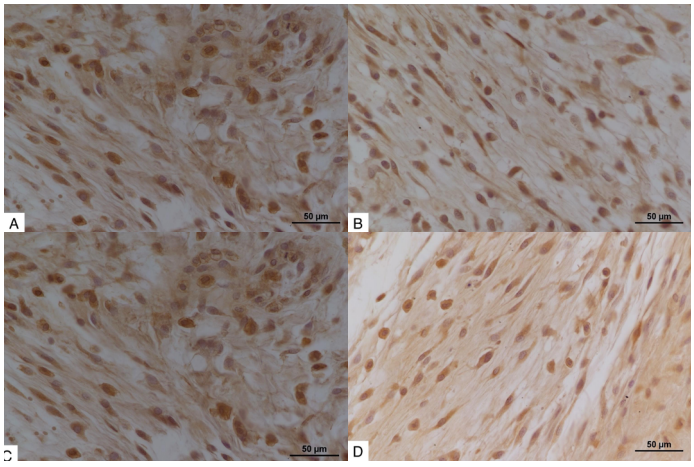

FIGURE 8. Severe (a) and moderate (b, c, d) Nitrotyrosine immunoreaction in the cytoplasm of fibroblasts, neutrophils, and macrophages (IHC)

Rabbits serve as an effective model due to their availability, appropriately sized tendons that allow for surgical manipulation, and the ease of sample examination. Furthermore, rabbit flexor tendons are similar to human tendons in terms of diameter and the presence of a synovial sheath [28]. In the present study, rabbits were selected to enable more accurate evaluation of the interventions and collected samples, which proved advantageous and consistent with the literature.

Systemic corticosteroids have been studied in rats with Achilles tendon incisions, with the timing of administration being evaluated. When steroids were administered between the 5th and 9th postoperative days, they suppressed inflammation during this period and positively impacted tendon healing by the 12th d [29]. Another study conducted on rats also demonstrated that systemic dexamethasone treatment administered between d 7 and 11 could enhance Achilles tendon healing [30]. Moreover, when the treatment was extended into the proliferative/early remodeling phase, there was a more pronounced improvement in material properties. Although late administration of systemic corticosteroids is recommended in studies, it has been reported that additional research is needed to obtain further insights into mechanisms potentially mediated by systemic effects as well as local effects specific to tendon cells and matrix. In the present study, steroids were applied for 14 days postoperatively and evaluated. Histopathological healing was superior in the steroid-only group compared to the control group, with a notable decrease in inflammatory cell infiltration. Consistent with the literature, steroid use reduced inflammatory cell infiltration and showed positive effects on tendon healing. However, the extent to which the timing of steroid administration influenced these results remains unclear. While the steroid's positive effects on healing throughout the process were evident, further studies are needed to determine the optimal timing and duration of steroid application to maximize its benefits.

The use of local corticosteroids is controversial [31], as they can have both positive [32] and negative [33 , 34 , 35 , 36] effects on tendons when used in isolation. In the present study, corticosteroids were administered systemically, and no literature was found addressing their systemic use in combination with PRP or PRF. It was thought that PRP or PRF application could provide positive outcomes in patients requiring systemic corticosteroids while mitigating the negative effects of local corticosteroid use on tenocyte viability.

The effectiveness of PRF in healing Achilles tendon injuries in rabbits, reported that the PRF group exhibited more organized collagen fibers and reduced vascularity compared to the control group [37]. Similarly, a significant increase was found in fibroblast density in groups treated with PRF or saline two weeks after tenotomy [38]. They also noted reduced inflammatory cell infiltration in the PRF-treated tenotomy group, though collagen fibrils were of similar density and irregularly distributed. In contrast, Sen et al. observed no statistically significant difference in healing between the PRP-treated and untreated groups in rabbits' Achilles tendons 28 days post-tenotomy [39]. Dietrich et al. reported that the PRP and control groups showed similar cellularity on day 14, with the PRP group exhibiting active granulation tissue and large hemorrhagic areas [40]. Vascularization was more developed in the PRF group, with reduced inflammatory cell infiltration and no granuloma formation, whereas the PRP group displayed dense inflammatory infiltration rich in polymorphonuclear and mononuclear cells. Due to containing a proportional combination of multiple growth factors, PRP and PRF can accelerate healing through synergistic effects by promoting cell proliferation and differentiation [41].

Inducible nitric oxide synthase has been shown to play an important role in tendon healing [29]. In the present study, iNOS expression was severe in the control group and moderate in the other groups. Some studies have reported that iNOS levels peak between the 4th and 14th d, followed by a decline after the 14th d [29 , 30]. In the present study, it was suggested that healing progressed more rapidly in the PRP and PRF groups, while the moderate iNOS expression observed in the steroid group was likely due to immunosuppression.

Synthesized iNOS rapidly reacts with superoxide to form peroxynitrite, a potent oxidant. Due to its short half-life, measuring peroxynitrite levels in tissues is challenging. Consequently, nitrotyrosine is used as a marker for nitrosative stress. In the present study, nitrotyrosine expression was moderate in the PRP, PRF, and steroid-treated groups, but severe in the control group. This indicates that the application of PRP and PRF reduced nitrosative stress.

Previous studies have reported the overexpression of iNOS, nNOS, and eNOS mRNAs in a rat tendon degeneration model [30 , 31]. In the present study, nitrosative tissue damage was assessed by measuring nitrotyrosine expression. However, as only iNOS immunostaining was performed to assess NO-related damage, it was not possible to determine which NOS isoform was responsible. Further detailed studies are required to clarify this issue.

CONCLUSION

This present study, which evaluated the effects of systemic methylprednisolone combined with PRP or PRF applications on tendon healing in a controlled rabbit model. The findings suggest that the combination of PRP or PRF with systemic corticosteroids can effectively enhance tendon healing by modulating inflammatory responses, reducing adhesions, and supporting the formation of a well-organized extracellular matrix. Systemic corticosteroid administration may provide a balanced anti-inflammatory effect, counteracting the adverse impacts observed with local steroid applications, such as decreased collagen synthesis and cellular activity. Overall, the results indicate that systemic corticosteroids in conjunction with PRP or PRF could be a viable therapeutic strategy for tendon injuries. However, further research is necessary to determine the optimal timing, dosage, and concentration for maximizing therapeutic efficacy of corticosteroid.

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Conflict of interest

The authors declare there is no conflict of interest.

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