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Original Article

Assessment of Hydroxyproline Content in Rabbit Achilles Tendon Treated with Platelet Rich Fibrin (PRF)

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Abstract

Tendon is similar to rope and consists of strong, flexible, dense connective tissue. Tendon disorder healing is challenging as it is an avascular tissue. The repaired tissue appears scar-like, and its biomechanical properties never ultimately return to their pre-injury state. This study aimed to evaluate the effects of platelet-rich fibrin (PRF) on the hydroxyproline content of the Achilles tendon after injury. For this purpose, 24 adult rabbits weighing 1.5-2 kg were used in this study. The animals were divided into three groups of eight, including the advanced-PRF (A-PRF) group in which the tendon defect was treated with xenogeneic A-PRF, the leukocyte-PRF (L-PRF) group in which xenogeneic L-PRF was used for tendon defect treatment, and a control group which was treated with normal saline. Hydroxyproline concentration was measured 1 and 2 months after the operation. Clinically, lameness was improved in the A-PRF group, compared to the L-PRF and control groups at the end of the third week after the surgery. Hydroxyproline level was significantly increased in the A-PRF group (50.33±1.44), compared to the L-PRF (44.70±1.12) and control (35.97±1.05) groups 2 months after the surgery ($P<0.05$). Moreover, the L-PRF group showed an increase in hydroxyproline content, compared to the control at the same period. The results of the current study demonstrated that A-PRF could enhance the hydroxyproline content of rabbit Achilles tendon after injury. Xenogenic PRF can be used as an alternative biomaterial to accelerate and regenerate tendon tissue.

Keywords: Hydroxyproline, PRF, Rabbit Achilles tendon, Tendon healing

1. Introduction

Tendon is a fibrous connective tissue with the main function of connecting muscle to the bone, and it is composed of collagen and a range of non-collagenous proteins. The major structural protein of the tendon is collagen type 1 which accounts for approximately 90% of the total collagen content and 60% of the dry mass of the tendon (1). Mechanical properties of tendons are primarily derived from type 1 collagen fibers arranged in dense and parallel arrays (2). The hierarchical architecture of the tendons and their high organization can bear large tensile forces and prevent muscular damage. However, these properties cannot affect the high susceptibility of the tendon to damage, microtrauma, and rupture caused by constant and high mechanical load activities (3).

Tendon injury treatment is challenging, and its repair requires long rehabilitation periods, especially in older patients. Medical and surgical treatments of tendon disorders often fail to help the patients regain full tendon function. Laser therapy and bone marrow have accelerated animal tendon healing (4). The current strategies used for the enhancement of tendon repair are based on the application of stem cells, growth factors, and natural and artificial biomaterials alone or in combination (5).

One of the significant challenges of clinical research is the development of bioactive surgical additives, which have been used to regulate inflammation and increase the speed of the healing process (6). Platelet concentrates have been used as a surgical adjunctive to regenerate different tissue types, like growth plates and wounds (7). Platelet-rich fibrin (PRF) is one of the most important bioactive materials used alone or with other biomaterials. It is the second generation of platelet concentrate and consists of a clot of fibrin enriched with platelet, leukocytes, immune cytokines, and circulating stem cells (8).

Hydroxyproline is the amino acid found in collagen molecules, and its quantification is used indirectly to quantify the total collagen content of tissue (9). Quantification of hydroxyproline can be indirectly used to calculate the collagen quantity at the site of tissue wound healing (10, 11). Moreover, this quantification can reflect the level of new cell regeneration at wound sites. The present study aimed to investigate the effect of PRF on hydroxyproline content in the Achilles tendon of rabbits after injury and evaluate the efficacy of xenogenic advanced-PRF (A-PRF) on hydroxyproline level and compare it with the effect of leukocyte-PRF (L-PRF).

2. Materials and Methods

In total, 20 rabbits weighing 1.5-2 kg were used in the present study. The animals were acclimatized in individual cages and divided into three groups of eight. The control group was treated with normal saline, the L-PRF group was treated with L-PRF, and the A-PRF group was treated with A-PRF. The animal anesthetic protocol was accomplished by intramuscular injection of a combination of ketamine HCL 10 mg /kg and xylazine 3 mg/kg. In addition, for the maintenance of anesthesia, only one-third of the ketamine dose could be used (12).

2.1. Surgical Procedure

The right hind limb was surgically prepared by being shaved and cleaned with iodine. The Achilles tendon was exposed by a midline skin, subcutis, and fascia incision at about 1 cm distal to the gastrocnemius muscle and 1 cm above the calcaneus (Figure 1A). To mark the proposed tendon defect, stay sutures were placed on both the proximal and distal ends of the tendon (Figure 1B). Core lesion defect was created by perforation of the Achilles tendon using a needle gauge of 16 in (Figure 1C). In the control group, the tendon defect was locally treated (Figure 1D) using normal saline, while in the L-PRF group, the tendon defect was locally treated with L-PRF. Furthermore, in the A-PRF group, the tendon defect was locally treated with A-PRF. Finally, the skin was closed routinely, and the rabbits were euthanized 1 and 2 months after the surgery.

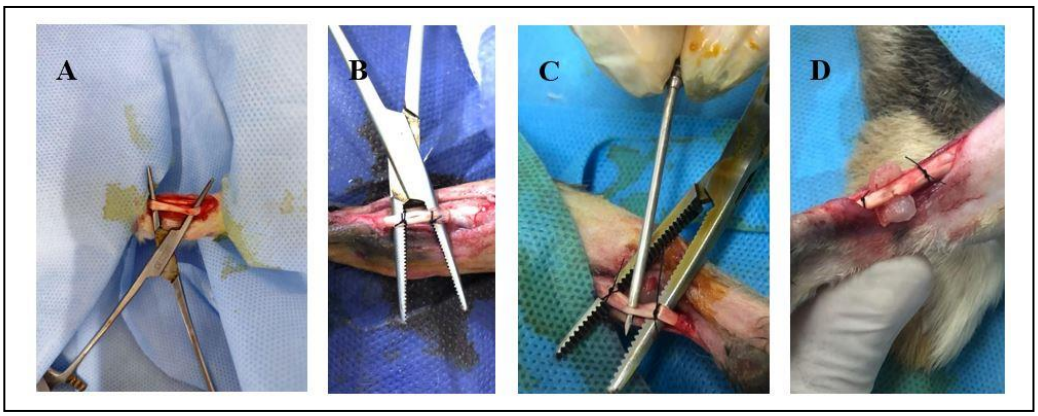

Figure 1. The surgical procedure involved (**A**): Exposure of Achilles tendon; (**B**): Stay suture; (**C**): Core lesion; and (**D**): Treated locally by PRF

2.2. Preparation of L-PRF and A-PRF

The L-PRF was prepared by collecting 10 ml of peripheral blood sample directly from the jugular vein. The blood sample was collected without anticoagulant in a unique PRF tube (Figure 2) and immediately subjected to centrifugation at 2,700 rpm (around 400 g) for 12 min. It should be noted that the obtained PRF is called L-PRF (13). A-PRF was prepared the same way as L-PRF, but the centrifugal force was 1,500 rpm for 14 min (14).

2.3. Clinical Observation

All animals were evaluated clinically during the study. The rabbits were observed daily for gait, lameness, body temperature, and local signs of inflammation at the operative site as well as weight-bearing based on the scoring system of lameness (Table 1).

2.4. Hydroxyproline Content Measurement

The rabbits were euthanized 1 and 2 months after the surgery, and Achilles tendons were harvested to determine hydroxyproline levels. Moroever, high-performance liquid chromatography with a reverse-phase column (4.6 mm internal diameter×250 mm length; TSK-gel, ODS80TM) was used to determine hydroxyproline content. The column was eluted with 0.4% ammonium acetate (pH 7.4) and 75% acetonitrile at a flow rate of 2 mL/min through an isocratic pump. The concentration of hydroxyproline in each specimen was detected using a fluorescence detector and integrated from the retention time and area under the eluting peak. The results were normalized with protein concentration in each sample.

Figure 2. PRF tube

Table 1. Pain scale for rabbits after femoral orthopedic surgery

Criteria / Score	0	1	2	3
Standing	Continuous weight-bearing	Intermittent weight-bearing	Completely non-weight-bearing	N/A
Gait with movement	Continuous weight-bearing	Intermittent weight-bearing	Toe touches Non-weight bearing	Non weighs bearing
Swelling	None	Mild	Obvious	N/A
Pain on palpation of the operated limb	None	Mild (occasional vocalization)	Moderate (frequent vocalization)	Severe (vociferous vocalization withdraws limb, bites, struggles)
Behavior	Normal cage exploration food and water consumption, animal calm in cage	Minimal exploration, food, and water consumption	No cage exploration, hunched posture, movement when stimulated anorexic for 24 hours	No cage exploration, hunched posture, piloerection, no movement, anorexic, increased respiratory or labored breathing
Body temperature	Normal	>39.4ºC and a lameness score of 5; or 40ºC	> 40ºC for 24 hours post-treatment (analgesia) and anorexic	> 40ºC for 48 hours post-treatment (analgesia) and anorexic
Appearance of incision	Clean, no chewing, no redness	Mild chewing, redness, suture intact	Severe chewing, incision open	Incision infected (redness, swelling, purulent drainage)

3. Results

Clinical observation of the treated animal groups showed redness and swelling at the operation site, and the lameness was evident in all rabbits. The lameness was improved in the A-PRF group at the end of the third week post-surgery, compared to the L-PRF and control groups. Evaluation of hydroxyproline level at 1 month post-operatively showed a significant increase of hydroxyproline content in the Achilles tendon of the A-PRF group (40.48±3.26) in comparison with the L-PRF group (31.69±1.93) and control group (22.57±3.22) (Figure 3, Table 2).

Two months after the surgery, given the normal level of dry matter (53.08±1.89 mg/g), a positive result was observed in the A-PRF group with 50.33±1.44 mg/g dry matter, compared to the L-PRF (44.70±1.12 mg/g) and control (35.97±1.05 mg/g) groups. It should be noted that the hydroxyproline level in the A-PRF group was close to the normal level. Moreover, in the L-PRF group, the hydroxyproline content was higher, compared to the control group (Figure 4, Table 3).

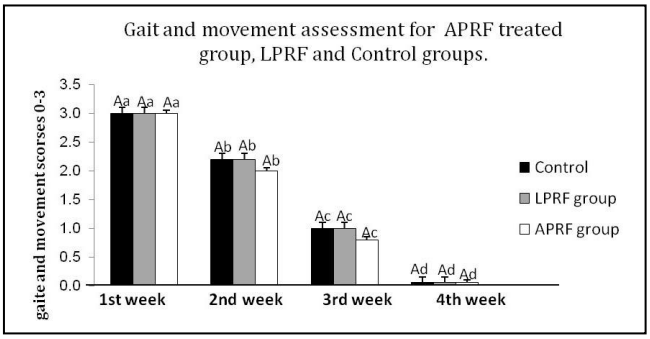

Figure 3. The clinical study showed the gait and movement of control, LPRF, and APRF groups during different periods (7, 14, 21, and 28 days post-surgery). [ABC] Different letters among groups indicates significant differences (P< 0.05). [abc] Different letters within a group indicates significant differences (P<0.05)

Table 2. The gait and movement scores for control, LPRF, and APRF groups during different periods (7, 14, 21, and 28 days post-surgery) (means and standard errors)

Groups	7 days	14 days	21 days	28 days
Control	3.00± 0.00^{Aa}	2.20±0.20^{Ab}	1.00±0.00^{Ac}	0.00±0.00^{Ad}
LPRF	3.00± 0.00^{Aa}	2.20±0.20^{Ab}	1.00±0.00^{Ac}	0.00±0.00^{Ad}
APRF	3.00± 0.00^{Aa}	2.00±0.00^{Ab}	0.80±0.20^{Ac}	0.00±0.00^{Ad}

[ABC] Different letters within each column indicate significant differences (P<0.05). [abc] Different letters within each row indicates significant differences (P<0.05)

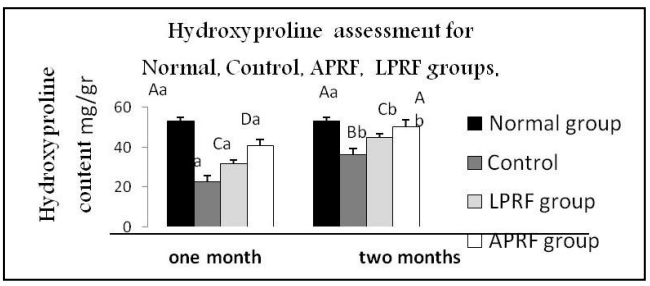

Figure 4. Biochemical study showed the hydroxyproline content (mg/g) dry matter of normal tendon and tendon of Control, LPRF, and APRF treated groups after 1 month and 2 months post-surgery. [ABC] Different letters among groups indicates significant differences (P< 0.05). abc Different letters within a group indicates significant differences (P<0.05)

Table 3. Amount of hydroxyproline content (mg/g) dry matter of normal tendon and tendon of Control, LPRF, and APRF treated groups after 1 month and 2 months post-surgery (means and standard errors)

Groups	1 month	2 months
Normal	53.08 ± 1.89^{Aa}	53.08 ± 1.89^{Aa}
Control	22.57 ± 3.22^{Ba}	35.97 ± 1.05^{Bb}
LPRF	31.69 ± 1.93^{Ca}	44.70 ± 1.12^{Cb}
APRF	40.48 ± 3.26^{Da}	50.33 ± 1.44^{Ab}

ABC Different letters within each column indicates significant differences ($P<0.05$). abc Different letters within each row indicates significant differences ($P<0.05$)

4. Discussion

In the current study, tendon hydroxyproline concentration was measured to determine the collagen content of the tendon. Hydroxyproline is one of the main amino acids in collagen molecules, and its quantification is used as an indirect quantification of the total collagen content of tissue (9). Clinical observations of the current study revealed an improvement in lameness in the A-PRF group, compared to the L-PRF and control groups. This result may be due to the local effects of growth factors (present in the PRF) on tendon healing which improve and facilitate the healing process.

This result is consistent with those of a study performed by Wanstrath, Hettlich (15) who showed that a platelet-derived product, a self-protein solution, reduced pain and lameness, compared to client-owned dog saline. Moreover, in another study, there was a reduction of lameness in horses that received single intralesional treatment with platelet-rich plasma for superficial finger flexor tendon disorders. According to the aforementioned study, platelet-rich plasma treatment may increase the number of horses returning to previous performance levels (16).

In the present study, A-PRF increased the hydroxyproline concentration, compared to L-PRF and control groups. Cumulatively, increasing hydroxyproline content leads to a significant increase in collagen fibers synthesis resulting in improved biomechanical functionality and collagen alignment. There is a strong correlation between hydroxyproline content and the ability of the Achilles tendon to sustain mechanical stress suggesting that hydroxyproline may be a good tendon function indicator (17).

Alkhalifa, Sharifi (18) investigated the efficacy of the mesenchymal cells and platelet-rich plasma on the hydroxyproline content of frozen allograft tendons after grafting in lamb and found a significant increase in hydroxyproline concentration. Moghaddam and Kazemi (19) evaluated the feasibility of using PRF membrane as a novel on-lay patching biomaterial in canine esophagotomy and its effects on esophageal wound healing. They assessed collagen production by measuring the amount of tissue hydroxyproline, and their results revealed an increased level of hydroxyproline due to PRF use.

The results of the present study showed that PRF could positively affect Achilles tendon healing. Moreover, xenogenic A-PRF can increase hydroxyproline levels in the Achilles tendon after injury without adverse effects. Nevertheless, further investigations are needed to explore the effects of xenogenic PRF on the complete tearing of the tendon.

Authors' Contribution

Study concept and design: S. S. A. A.
Acquisition of data: A. A. I. A. D.
Analysis and interpretation of data: R. M. N. A.
Drafting of the manuscript: S. S. A. A.
Critical revision of the manuscript for important intellectual content: S. S. A. A.
Statistical analysis: R. M. N. A.
Administrative, technical, and material support: A. A. I. A. D.

Ethics

The present study was conducted after approval of the Research Committee of the Faculty of Veterinary Medicine, University of Basrah, Basrah, Iraq. Moreover, all animal procedures were conducted

according to the guidelines provided by the Faculty of Veterinary Medicine, University of Basrah.

Conflict of Interest

The authors declare that they have no conflict of interest.

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