Evaluation of the Effect of Platelet Rich Plasma (PRP) in Tendon Gap Healing by Measuring Collagen Synthesis in Guinea Pig

Fatemeh Dehghani Nazhvani , Abotorab Tabatabaei Naeini and Seifollah Dehghani

Abstract

Objectives- Hydroxyproline is a specific amino acid of collagen which is widely used to estimate the collagen content in biological specimens. The purpose of this study was to compare the effect of the Platelet Rich Plasma (PRP) on tendon gap healing using two different scaffold made from subcutaneous fascia and polypropylene tube.

Design- Experimental study

Animals - 16 young adult Guinea pigs

Procedures- The animals were anesthetized and 3 cm incision was made on the caudal surface of the right rear limb. The deep digital flexure (DDF) tendon was severed to induce a 0.7 cm long gap. The animals were divided into 4 groups. Group 1: A tube scaffold was made from the subcutaneous fascia of the back of animal, and the two ends of severed tendon were sutured into the tube scaffold. No PPR was injected. Group 2: Similar to the first group but PRP was injected into the scaffold. Group 3: a 2.0 cm long polypropylene tube was used as scaffold and the two ends of severed tendon were sutured into this tube. No PRP was injected. Group 4: Similar to the third group but PRP was injected into this tube scaffold. After 6 weeks the animals were euthanized and tendon samples were prepared for measurement of hydroxyproline content.

Results- The group 2 and 4 that received PRP, showed significant statistical difference in both Hydroxyproline and collagen content compared to their control groups 1 and 3 respectively (P<0.05). The polypropylene scaffold (group 3) was significantly better on tendon gap healing, compared to subcutaneous fascia scaffold (group 1) (P<0.05). The group 4 had a better effect on tendon gap healing significantly (P<0.05) compared to the group 2.

Conclusion and Clinical Relevance - PRP in two different scaffolds improved the healing significantly compared to the groups without PRP, due to the effect of growth factor rich granules of the platelets. In group 4 synergistic effects of both PRP and polypropylene tube scaffold was observed. So the PRP was effective to enhance the healing process of tendon gap in Guinea pigs.

Keywords- Guinea Pig, Tendon gap healing, PRP, Fascia scaffold, Polypropylen tube scaffold.

Introduction

Orthopedic problems including tendon laceration is an important challenge in medicine. Often lower parts of the limbs have been injured in racing horses. Athletes often develop tendon problems. Burning accidents lead to limb's tendon injures. Sometimes tendon gaps could not be sutured together, therefore in order to save the function of the limb, using prosthesis or tendon graft is inevitable. Therefore new technologies have to be developed in order to save the injured or shortened tendons. Platelets alpha granules containpolypeptide growth factors that stimulates the

replication of normal connective tissue cells,[1] all of which help initiate and accelerate the inflammatory response by the host.[2] They cause formation of granulation tissue by facilitating synthesis of new extracellular matrix (ECM) and neoangiogenesis.[2] Several alpha granule proteins released from platelets may affect wound healing by causing directed fibroblast migration.[3] Hydroxyproline is a specific amino acid of collagen that is widely used to estimate the collagen content in biological specimens.[4] Collagen is one of the few proteins that contain the amino acid hydroxyproline. In fact most of the hydroxyproline in vertebrates is present in this protein.[5] The total hydroxyproline content is measured by summing the contribution due to collagen and that due to elastin, assuming average values of 12.5 g and 2.0 g hydroxyproline/100 g protein for collagen, and elastin, respectively.[6] The purpose of this study was to compare the effect of the PRP in tendon gap healing using two different

Department of Veterinary Surgery, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
Address all correspondence to Dr. Fatemeh Dehghani Nazhvani
E-mail: fatidn135@gmail.com

scaffold made from subcutaneous fascia and polypropylene tube.

Materials and method

Surgery

This study was approved by institutional ethical committee of the Shiraz University. Sixteen mature Guinea pigs of two genders, weighing 750.7± 54.6 gm were used in this study. After one week of adaptation period, they were prepared for an aseptic surgery. They were anesthetized by Ketamine (40mg/kg) and xylazine (5mg/kg). Three centimeter long incision was made on the caudal surface of the right rear limb of each animal and the skin and connective tissue was separated and the tendons were separated and identified. The deep digital flexure (DDF) tendon was severed to induce a 0.7 cm gap. They were divided into 4 groups.

Group 1: in this group the subcutaneous fascia of the back of animal was dissected (1cm x2cm), separated and sutured around a catheter (no.4) to form a 2.0 cm long tube scaffold. The two ends of severed tendon were sutured into the tube scaffold to maintain the 0.7 cm gap between the two ends of tendon inside the scaffold. No PPR was injected.

Group 2: in this group the tube scaffold was prepared similar to the first group and PRP was injected into the scaffold.

Group 3: in this group the pyrogen free sterile polypropylene tube (2.0 cm long) was used as scaffold and the two ends of severed tendon was sutured into this tube to maintain 0.7cm gap. No PRP was injected.

Group 4: in this group the polypropylene tube scaffold was used similar to the previous group. PRP was injected into this tube scaffold.

The skin was sutured and the limb was protected by a temporary splint.

PRP preparation

Following the anesthesia and prior to operation, 2 ml blood was collected from the heart of each animal in a citrated dextrose tube. The tubes were centrifuged at 1240 rpm for two minutes. The top layer which was plasma had three distinct layers in ratio of 2:1:1 from the top. The first top layer was Platelet poor plasma (PPP), the middle plasma average platelet (PAP) and the lower platelet rich plasma (PRP). The first (PPP) and the second (PAP) layers were removed by pipette. The third (PRP) layer was carefully separated by pipette and centrifuged again for 5 minutes at the same rate. Then the first layer (plasma) was discarded and the second layer (PRP) was collected for intra scaffold injection.[7]

Sampling

After 6 weeks the animals were euthanized by thiopental (30 mg/kg) and the tendon specimens were dissected (5mm+7mm+5mm) for further study. In 4 animals the normal intact tendon of the left rear limb were removed for comparison. The samples were frozen (-20°c) immediately following sampling in saline rinsed sponges before being tested.[8]

Biochemical analysis

 For the process of hydrolyzing of the specimens, 50.0 mg of each tendon specimen was placed in a universal tube, 5 ml 9 molar HCL was added, thawed in a hot air oven at 105 °c overnight (14-16 h). After cooling the hydrolyzed sample, the volume was added by distilled water to 100 ml, then 1 ml of the diluted sample was transfused to another tube and 50 ml of 1% alcoholic phenolphthalein indicator was added and left to shake by vortex shaker. The diluted sample solution turned to purple and by gradual adding of 1 molar HCl, the dye of the solution was disappeared. Then 0.5 ml chloramine-T reagent was added for oxidation. After adding 1 ml Ehrlich indicator and shaking the solution, the tubes were thawed at 60°c (for 20 to 25 minutes) till a red dye solution was achieved. Then an acid-base clearance used to remove the distributer dye materials, that for this purpose 0.5 ml 6 molar NaOH was added to make the basic solution. After shaking by vortex 2 ml Toluene was added to the solution as preservative and rotated for 10 min by rotary mixer (60 rpm). It was centrifuged for 5 minutes (1000 rpm), 2 ml of toluene phase removed and added to a 3 ml 0.03 molar HCl in another tube and shaked for 30 seconds. It was centrifuged for the second time for 5 minutes (1000 rpm), toluene phase extracted by suction pump and acidic phase (dye product) into a cuvette and the absorbance of dye product was read by spectrophotometer at 543 nm.[8]

For preparation of Chloramine-T reagent, 0.14 g chloramines T dissolved in 2 ml distilled water and 8 ml acetate citrate buffer added to it. For preparation of acetate citrate buffer (PH=6.0), 5.7 g sodium acetate, 3.75 g Trisodium citrate and 0.55 g citric acid poured into a volumetric flask (100 ml) and 25 ml distilled water was added. Following the mixture by 38.5 ml Isoprpoanol, distilled water was added to the volume of flask. This solution was stable for few weeks.[8]

For preparation of Ehrlich indicator, 2.5 g Para-dimethyl-amino-benzaldehyde dissolved in 2.7 ml HCl 96%, then 16 ml Isopropanol was added to that. This solution lacks stability and must be prepared immediately before use.[8]

Standard calibration curve of Hydroxyproline (5-40 µg/ml) was made by different standard concentration that had passed all above step along with the tendon specimen except hydrolyzation. According to the standard calibration curve (graph 1) and by considering the concentration coefficient, the

amount of Hydroxyproline was evaluated in the tendon specimens.[8]
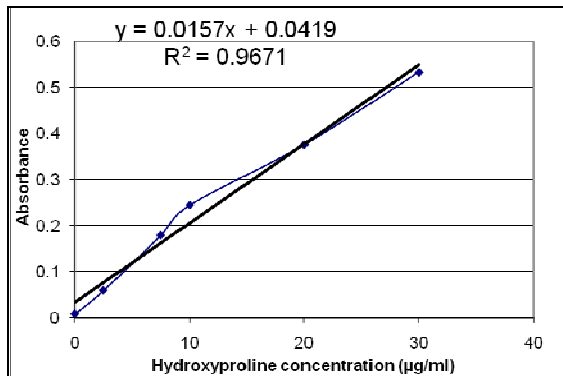
Graph 1- The standard calibration curve

In order to measure the weight of the dry matter, 50-100 mg of the specimens was placed on a slide, weighted, dried in a hot air oven (100 °c) for 3 hours, till a stable dried mater weight achieved. By measuring the percent of humidity of the specimens the quantity of Hydroxyproline in dry mater of tendon was recorded.[8]

Calculations

The formula used for calculation is:[9]

H (g/100 g) = (h×2.5)/ (m×v)

{H = hydroxyproline content (g/100 g DM), h = hydroxyproline concentration which was read based on photo absorbance (μg/ 2 ml), m = weight of the specimen (g), v = volume of diluted hydrolyzed specimen}

Since hydroxyproline content assuming average values of 12.5 g hydroxyproline/100 g (12.5%) protein for collagen:

C (g/100g) =H×8

{C = collagen content (g/100 g DM), H = hydroxyproline content (g/100 g DM)}

Statistical analysis

The data was analyzed by ANOVA and Duncan test using SPSS soft ware. The level of significance was assumed to be $P<0.05$.

Results

The group 2 (fascia tube scaffold) and the group 4 (polypropylene tube scaffold) which received PRP, showed significant statistical difference in both Hydroxyproline and collagen content compared to their control groups 1 and 3

respectively ($P<0.05$). The group 4 (polypropylene tube scaffold) had a better effect on tendon gap healing significantly ($P<0.05$) compared to the group 2 (fascia tube scaffold). Also the group 3 (polypropylene tube scaffold) had significantly ($P<0.05$) better healing properties compared to the group 1 (fascia tube scaffold) without any PRP supplement. All 4 treatment group had a wide significant statistical difference ($P<0.05$) with the intact tendon group.

Table 1- The mean ± SD of the hydroxyproline and collagen contents (g/100 g DM) of the specimens in the different groups of study. Group 5 was the intact tendon group.

Groups	Hydroxyproline	Collagen
1	0.15±0.01	1.17±0.05
2	0.23±0.01	1.85±0.09
3	0.34±0.02	2.74±0.14
4	0.51±0.06	4.09±0.46
5	1.06±0.05	8.48±0.39
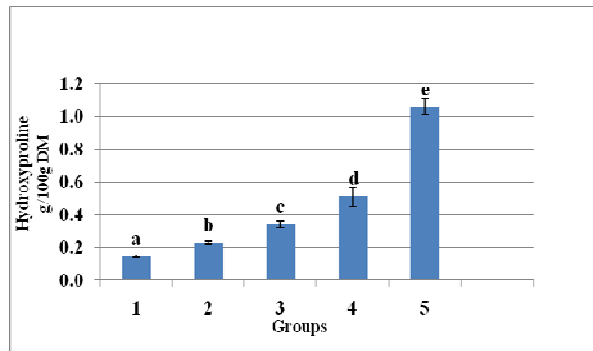
Histogram No 1- Hydroxyproline contents in g/100 g DM of tendon in treatment groups 1 to 4. The group 5 demonstrate intact tendon group. The different letters show the significant statistical difference between groups ($P<0.05$).
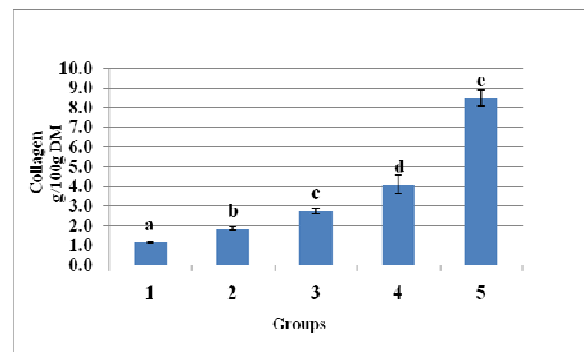
Histogram No 2- Collagen contents in g/100 g DM of tendon in treatment groups. Group 1 to 4. The group 5 demonstrate intact tendon group. The different letters show the significant statistical difference between groups ($P<0.05$).

25

Discussion

Tendon is a hypocellular and hypovascular fibrous collagen tissue with slow healing rate.[10] Since tendons require less blood supply than muscles to function, they take more time to heal.[11] Nearly 30% of tendon weight and over 70% of tendon dry matter weight is collagen.[12] The spectacular presence of Hydroxyproline amino acid in collagen protein makes it as an indicator to measure collagen level in tissue.[13] Biochemical analysis showed increased Hydroxyproline content to be a reflection of increased collagen synthesis.[14] Collagen is one of the most dominant extracellular matrix proteins in the granulation tissue.[15] For instance, inflammation can lead to degradation of intact collagen and to viable cell, thereby increasing the functional deficit and recovery period. Paradoxically, many cellular and sub cellular events occurring during the inflammatory response lead to the release of a plethora of growth factors that trigger the healing phase.[10]

Platelets are produced in bone marrow and have growth factors that are the main factor in healing and tissue return. Growth factors are stored in alpha granules and once released; induce cellular growth, proliferation and differentiation in different cells. Thereby this event leads to increase in collagen, elastin, intracellular matrix, vascularity and finally thickening and tissue healing.

Platelet activation and aggregation, in addition to accelerating coagulation, provide a bolus of secreted proteins and α-granule contents to the immediate area, all of which help initiate and accelerate the inflammatory response by the host. Examples of such secreted proteins include arachodonic acid metabolites, heparin, serotonin, thrombin, coagulation factors (factor V), adhesive proteins (fibrinogen and von Willebrand factor), plasma proteins (immunoglobulin-γ and albumin), cell growth factors (platelet-derived growth factor (PDGF), platelet-derived angiogenesis factor, transforming growth factor-α (TGF-α), TGF-β and basic fibroblast growth factor (bFGF)), enzymes (heparinase and factor XIII) and protease inhibitors (plasminogen activator inhibitor-1, α2-macroglobulin and α2-antiplasmin). Following platelet-induced haemostasis and release of TGF-β1 and PDGF, formation of granulation tissue is facilitated by chemotaxis of neutrophils, monocytes, fibroblasts and myofibroblasts, as well as by synthesis of new extracellular matrix (ECM) and neoangiogenesis.[2]

In response to wounding, the fibroblasts migrate into the wound bed and initially secrete collagen type III, which is later replaced by collagen type I. Synthesis and deposition of these collagens by fibroblasts is stimulated by factors including TGF-β1, -β2 and -β3, PDGF, IL-1α, -1β and -4, and mast cell tryptase. Once sufficient collagen has been generated, its synthesis is stopped; thus, during tissue repair, production as well as the degradation of collagens is under precise spatial and temporal control.[2]

In this study, PRP significantly had better improvement on tendon gap healing connecting the two severed ends together. Different scaffolds such as fascia and polypropylene scaffold had significantly different effect on healing of the gap produced in the tendon. The polypropylene scaffold had better improvement on healing compared to the fascia scaffold. After 6 weeks the amount of collagen synthesis was so much in group 4 that the tendon shape was surprisingly thicker in this group on the time of sampling, compared to the other groups; and this could be due to the synergistic effect of both PRP and polypropylene tube scaffold. PRP in two different scaffolds improved the healing significantly compared to treatment without PRP, and that is the effect of growth factor rich granules of the platelets.

All treatment groups (4 groups) were significantly different compared to each other and also compared to the intact tendon group, showing that the healing process after 6 weeks in the 4 groups was not yet completed and requires longer study period.

References

1. Kaplan DR, Chao FC, Stiles CD, et al. Platelet alpha granules contain a growth factor for fibroblasts. *PubMed Blood* 1979; 53(6):1043-52.

2. Lisa M, Coussens, Zena Werb, Inflammation and cancer. *Nature* 2002;420:860-867.

3. Senior RM, Griffin GL, Huang JS, et al. Chemotactic activity of platelet alpha granule proteins for fibroblasts. *PubMed J Cell Biol* 1983;96(2):382-385.

4. Lin YK, Kuan CY, Development of 4-hydroxyproline Analysis Kit and its Application to Collagen Quantification. *Food Chemistry* 2010;119:1271-1277.

5. Miller EJ, Chemistry, Structure and Function of Collagen. In: Menako L, eds. *Biological Bases of Wound Healing. New York* 1975;243-345.

6. Edwards CA, WD O'Brien JR, Modified Assay for Determination of Hydroxyproline in a Tissue Hydrolyzate. *Clinica Chemica Acta* 1980;104:161-167.

7. Ahmad Z, Howard D, Brooks RA, et al. The role of platelet rich plasma in musculoskeletal science, *Journal of the Royal Society of Medicine* 2012;3(6):40-45.

8. Rassouli A, Shams GR, Sharifi D, Determination of Hydroxyproline in Equine Tendon Using a Modified Spectrophotometric Method. *J. Pajouhesh and Sazandegi No* 2007;73:114-119.

9. Falahi E, Ghazi N, Evaluation of Hydroxyproline in Sausage Products of Khorramabad. *Yafteh*, 2011;13(3):21-26.

10. Marsolais D, Frenette J, Inflammation and Tendon Healing. *PubMed Med Sci (Paris)* 2005;21(2):181-6.

11. Cook MS, Healing Soft Tissue Injuries: Muscles, Tendons, Fascia and More. *Pro Health* 2004;17:112-120.

12. Harkness RD, Mechanical properties of collagenous tissues. In: Gould BS eds. *Treatise on collagen, Vol. 2, Biology of collagen, Part A,* London:Academic Press Inc,1968;254-257.

13. Eastoe JE, Composition of collagen and allied protein. In: Ramachandran GN eds. *Treatise on collagen, Vol. 1, Chemistry of collagen*, London: *Academic Press Inc,*1968:1-55.

14. Nayak BS, Udupa AL, Udupa SL, Effect of Ixora coccinea Flowers on Dead Space Wound Healing in Rats. *Fitoterapia* 1999;70(3):233-236.

15. Grillo HC, Advances in Biology of Skin. *Journal of Medical Education* 1964;39(5): 526.

چکیده

بررسی نقش پلاسمای غنی از پلاکت در ترمیم پارگی و نواقص تاندون در خوکچه‌ی هندی

دکتر فاطمه دهقانی نازوانی، دکتر ابوتراب طباطبایی نائینی، دکتر سیف الله دهقانی

گروه آموزشی علوم درمانگاهی، دانشکده دامپزشکی دانشگاه شیراز، شیراز، ایران

هدف– هیدروکسی‌پرولین یک اسیدآمینه‌ی اختصاصی کلاژن است که به صورت گسترده به عنوان فاکتوری برای تخمین میزان کلاژن در نمونه‌های بیولوژیک استفاده می‌شود. هدف از این مطالعه، مقایسه اثر پلاسمای غنی از پلاکت در ترمیم نقیصه‌ی تاندونی با استفاده از دو داربست متفاوت، فاسیای زیرپوستی و لوله‌ی پلی‌پروپیلن، می‌باشد.

نوع مطالعه– مطالعه تجربی

حیوانات– ۱۶ خوکچه هندی بالغ جوان

روش کار– ابتدا حیوانات بیهوش شدند، سپس برشی به طول ۳ سانتی‌متر در سطح خلفی اندام عقبی راست ایجاد شد. تاندون خم کننده‌ی عمقی انگشتان آزاد و نقیصه‌ای ۷ میلیمتری در آن ایجاد شد. حیوانات به ۴ گروه تقسیم شدند. گروه ۱: در این گروه از فاسیای زیرپوستی پشت حیوان به عنوان داربست استفاده شد ودو انتهای قطع شده تاندون به داربست بخیه شد، ولی پلاسمای غنی از پلاکت تزریق نگردید. گروه ۲: در این گروه شبیه به گروه ۱ عمل شد اما پلاسمای غنی از پلاکت داخل داربست تزریق گردید. گروه ۳: در این گروه از لوله‌ی پلی‌پروپیلن به عنوان داربست استفاده شد و دو انتهای قطع شده تاندون به به لوله بخیه شد، ولی پلاسمای غنی از پلاکت تزریق نگردید. گروه ۴: در این گروه شبیه به گروه ۳ عمل شد اما پلاسمای غنی از پلاکت داخل داربست تزریق شد.

نتایج– گروه ۲ و ۴ که پلاسمای غنی از پلاکت دریافت کرده بودند، اختلاف آماری معنی دار هم در میزان هیدروکسی‌پرولین و هم کلاژن، در مقایسه با گروه‌های کنترل خود (گروه ۱و۳) داشتند ($p<0.05$). داربست لوله‌ی پلی‌پروپیلن در مقایسه با داربست فاسیای زیرپوستی، اثر بهتری در ترمیم نقیصه‌ی تاندونی داشته است($p<0.05$). مداخله‌ی انجام شده در گروه ۴ به صورت معنی دار نسبت به گروه ۲ در ترمیم نقیصه‌ی تاندونی مؤثرتر بوده است.

بحث– پلاسمای غنی از پلاکت در هر ۲ داربست متفاوت، به صورت معنی داری از نظر آماری، سبب بهبود ترمیم نقیصه‌ی تاندونی در مقایسه با گروه‌های بدون پلاسمای غنی از پلاکت شده است. که این امر به دلیل اثر گرانول‌های غنی از فاکتورهای رشد در پلاکت‌ها می‌باشد. آنچه در گروه ۴ مشاهده می‌شود به دلیل اثر همگرایی پلاسمای غنی از پلاکت وداربست لوله‌ی پلی‌پروپیلن با یکدیگر می‌باشد.

نتیجه گیری و کاربرد بالینی– پلاسمای غنی از پلاکت در پیشبرد روند ترمیم در نقیصه تانونی در خوکچه‌های هندی، مؤثر بوده است.

کلید واژگان– خوکچه هندی، ترمیم نقیصه‌ی تاندونی، پلاسمای غنی از پلاکت، داربست فاسیا، داربست لوله‌ی پلی‌پروپیلن