THE EFFECT OF CaCl2 ADDED PRP (Platelet Rich Plasma) TO THE HEALING TIME, TENSILE STRENGTH AND ADHESION DEGREE OF THE RUPTURED TENDINOPATHIC ACHILLES TENDON OF RATS (*Rattus norvegicus*)

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Abstract: Achilles tendinopathy produce morbidity, long-lasting disability in athletes and non-athletes and remain a challenge for clinician. Tendinopathy may lead to reduced tensile strength and a predisposition to rupture.The aim of our study was to evaluate the effects of activated and non-activated PRP on the healing process of ruptured tendinopathic achilles tendon in rat. Tendinopathy achilles condition induced by injecting collagenase bacterial type-1 locally. Right achilles tendon in 48 rats ruptured by transecting it transversely and repaired it by using a Kessler technique. Further, the wound immobilized with PRP and injecting activated PRP (5% CaCl2 added) in one group, non-activated PRP on the other group and saline on control group. Tendons from each group were collected at the 1st and 2nd week postoperatively also assessed for biomechanical test. Tendons were also evaluated histologically by using hematoxylin-eosin to know adhesion degree based on Tang criteria. The significant differences was found between intervention group and control ($p<0.05$) at the 1st week but there was not any significant differences at the 2nd week in tensile strength test ($p>0.05$). Adhesion degree of the intervention group also reduce better rather than the control at the 1st and 2nd week ($p<0.05$). PRP have a positive effect on healing tendons by improving healing time, mechanical strength and decreasing adhesion degree.

Keywords: achilles tendinopathy, PRP, healing time, tensile strength, adhesion

INTRODUCTION

The tendon is a musculoskeletal tissue functional unit that channel the energy from the muscles to the bone which has poor vascularization, especially on big Achilles tendon. Laceration, rupture and inflammation of the tendons cause morbidity in patients and could interfere their work and daily activities.[1,2] Achilles tendon rupture is still a challenging problem, with incidence report 18 cases per 100,000 population per year.[1] Achilles tendinopathy associated with degenerative process in the tendon causes the pathophysiological and multi factorial pathogenesis yet still unclear. Many theories mention that tendinopathy is failed tendon healing process, qualitatively and quantitatively and not an inflammatory process.[2,3] Arner et al reported 70 people were examined with an Achilles tendon rupture has occurred degenerative changes in the tendon and believed there were intrinsic abnormality prior the injury.[4]

Generally, the healing process has been slow and the Achilles tendon is aggravated by scar formation which is different in quality and quantity.[5]

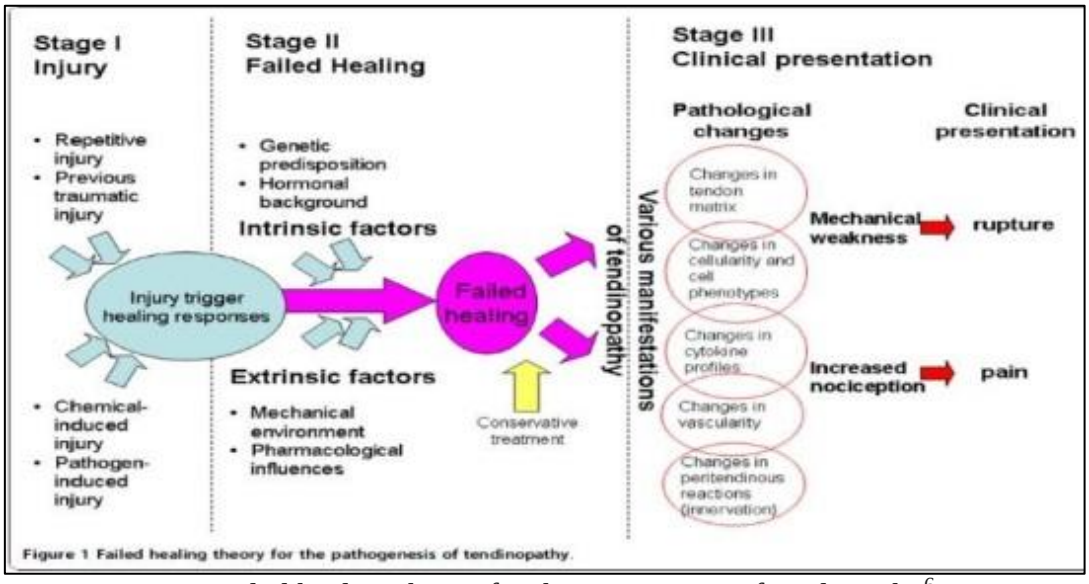

Figure 1 Failed healing theory for the pathogenesis of tendinopathy.

Figure.1 Failed healing theory for the patogenesis of tendiopathy[6]

Complications such was rerupture and adhesion incidence occurred after therapy, which happen due to less than optimal of the healing process and tissue that forms scar tissue which is structurally different from the original.[2] For excellent healing, two aspects must be accomplished: should be able to achieve its original strength and able to glide within surrounding tissue.[7] Various ways attempted to avoid this complication with the aim to restore the anatomy and function as much as possible.[8]

PRP is an autologous blood with platelet concentration above baseline values in blood platelets (whole blood). Platelets contain alpha granules, dense granules and various cytokines wherein the alpha granules contain a variety of growth factors such as PDGF, EGF, VEGF, TGF-β1, IGF-1 and dense granules contain serotonin, bradykinin, histamine which play an important role in the process of wound hea ling.[2,9-12]

Standarized preparation of optimum PRP is not established.[13-15] For optimal

function, the platelet must be activated to release growth factor, cytokine, signalling molecule, integrin, coagulation protein, adhesion molecule. Activated PRP could be induced by addition of thrombin, calsium, etc or platelets can be slowly activated by exposure to tendon derived collagen alone. The use of non-activator added PRP diverse effects on the tissue under study as research conducted by Bo Hanet al[20], Thomas et al[16] and added activator researched by Smith[12], Fenwick et al.[15,16] Bovine thrombin has been widely used in studies in vitro and in vivo, but reportedly also gives immunity reaction rarely.[17-19] The use of calcium chloride (CaCl2) without thrombin as an activator of PRP are also allowed and used in research. Five percent calcium chloride concentration is optimal in turn PRP.[20] Yet how much influence on platelet activation still requires further research.[12,21,22] Comparative studies of the PRP added activator and the activator added to the process cause by tendinopathy healing of tendon rupture has not been done. Our objective are to observe the effect of PRP injection on the early healing of rat's Achilles tendon rupture so as to provide the experimental basis for clinical practice.

RESEARCH METHOD

The study was performed in Orthopaedic and Traumatology Laboratory and Pathological Anatomy Laboratory Dr. Saiful Anwar Hospital Malang from June 2013 – September 2013. (No:400/XC/K.3/302/2013). The study population was the tendon Achiles of adult male Wistar rats aged 2 months, weight 190-240g.

Experimental laboratory studies were by comparing the degree of adhesion formed by histopathological examination, biomechanical test and to evaluate the speed of tendon healing process between the given PRP with addition of activator CaCl 2 and PRP without CaCl2 addition on tendinopathic achilles tendon of Wistar strain rats (*Rattus norvegicus*) at week 1 and week 2. Each group consists of 4 rats minimal, requiring 65 Wistar rats (2 healthy rats, 48 rats in conditions tendinopathy,15 rats were sacrificed and blood drawn to make PRP donor rats where 3 to 10 rats as recipient[4]).

The inclusion criterias were healthy adult male wistar rats, with active movement and normal movement of the lower limb. Exclusion crterias were xtremities defects and extremities infection.

The rats were placed in a hutch for adaptation and fed for 2 weeks. On the Achilles tendon were injected bacterial collagenase type-1 250 units or 30 mL (0.015 mg/mL) in 0.9% saline at osteotendinous junction using a 30 G needle. After that the rats were allowed to move and observed for infection signs in the area of injections for 2 weeks.

The 15 Wistar male were anaesthetized with ketamine 15ml/kg, 5cc syringe aspiration in the heart of wistar then collected in 1 glass tube which previously 10% anticoagulant citrate dextrose phosphonate (CPD) buffer (12:15 mg/mL) were given with a ratio of 1 mL of citrate to 5 mL blood in a blood glass tube, put into centrifuge with speed 220 G for 20 minutes.[23] Supernatant layer containing platelet rich plasma (PRP) was taken with a pipette for the second centrifuge with 480 rpm speed for 20 minutes. Pelets from the second centrifuge supernatant was taken and diluted with platelets obtained over 1-1.5 × 1012 platelets/L. The PRP result was divided into 2 parts, 1 part ac- tivator substance is added in the form of 5% CaCl2, while part 2 the activator substance was not added.

Surgery procedures started with 50 Wistar male (2 healthy mice, 48 rats tendinopathy) anesthetized with ketamine 15 ml/ kg bw. Wistar positioned prone, all 4 extremities were fixed. The operating field on lower left extrimity were shaved and cleaned with savlon solution,

alcohol 70% and povidone iodine, covered with sterile cloth. Tendon Achilles ruptures made by transverse cutting at 5 mm proximal to its insertion on the calcaneus with blade no.15. The ruptured Achilles tendon reconnected and sutured with non-absorbable thread (prolene) 5.0 with Kessler techniques for each group. Group 1 had normal saline administered at the site of the Achilles tendon suture in rats with tendinopathy (negative control); group 2 had platelet rich plasma (PRP) without addi- tion of activator 5% CaCl2 given to the repaired area of the Achilles tendinopathy; group 3 had platelet rich plasma (PRP) plus 5% CaCl2 activator given to the repaired area of Achilles tendinopathy in rats. Two healthy Wistar treated samely, normal saline was given to the repaired of the Achilles tendon and used as a positive control. The skin were closed and were immobilized with POP, the feet were in plantar flexion position and then allowed to move freely, then they were placed back to the hutch.

On day 7 and 14 post-operative treatment, 50 rats euthanasized by injecting phenobarbital intraperitoneally 100mg/kgBW, for 4 rats each group a longitudinal skin incision were performed over the Achilles tendon, macroscopically the signs of adhesion was visible. The specimen then collected parallel to the end of the wound stump to the subcutan, tendons and surrounding tissue of bone then placed in a container of 10% formalin and labeled, then sent to a laboratory for PA histology preparations. Afterward stained with hematoxylineosin and label, examined under the microscope to see the degree and rate of tendon healing and adhesion that occurs based on the method of Tang.[24]

Table. 1 His topathological evaluation criteria by Tang 24

Histopathological Evaluation Criteria		
Ahesion	**Histological**	**Degree**
No adhesion	Normal	0
Light degree adhesion (good)	Few filaments. Fine, long filament structure	1
Moderate adhesion	Average number of filaments. Large, thick filament structure	2
Severe adhesion	Loss of filament structure. Dense fibrosis	3

Achilles tendon from 4 rats of the each groups were taken in calcaneus insertion proximally (musculocutaneous junction), and then stored in a physiological saline solution for later assessment of tensile strength by IMADA machine for loading test. The tendon was pulled up by 0.1mm/sin and the graph will be resulted in a computer.

Figure 2: IM ADA Biomechanical Testing Machine Parameters That Were Measured In Biomechanical Testing Are Tensile Load (N) and Tensile Strength (N/mm2)

Non parametric data was used to determine the adhesion degree and used ruskal Wallis by SPSS 17. While the data used to determine the strength of the connection using one-way ANOVA.

The animal samples were used in this study and approved by the Research Ethics Committee of the Medical Faculty, University of Brawijaya. (No:400/XC/K.3/302/2013)

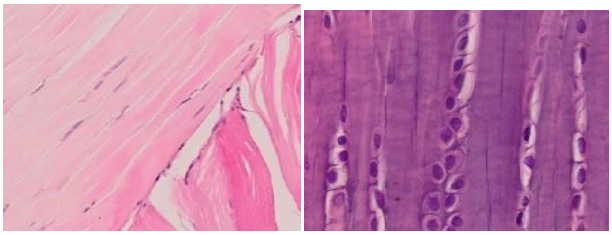

Figure 3. A.Normal tendon sliced longitudinally
B. Tendinopathy

RESULTS AND DISCUSSION

Tendinopathic condition were created by injecting bacterial collagenase type I to the achilles tendon with prior histological examination 2 weeks after injection and initial study. The tendinopathic suspected tendon sample were cut transversely and longitudinally to make histologic preparation. The normal tendon sample was also obtained and made into histological preparation to be compared. The histological preparation then evaluated by a pathologist in the pathological anatomy laboratory of dr. Saiful Anwar Hospital. In tendinopathic suspected histological preparation the collagenous fibre was smaller, widespreaded onto random orientation, there were vacuole/patches between collagenous fibres, large round-shaped tenocytes surrounded the tendon fibre. The histological pattern of normal tendon was tight, parallel to the tenocyetes with flat and slightly waved nucleus between collagenous fibres.

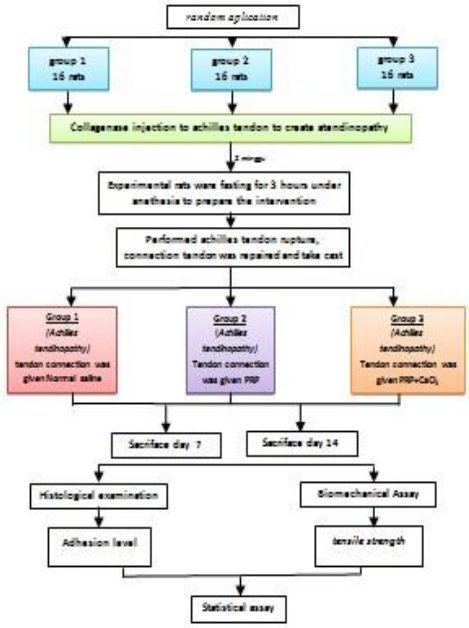

Figure 4. A Macroscopic tendon without scar formation. B Mild adhesion. C Moderate adhesion. D Severe adhesion

The result of sample measurement of tendon in normal condition (control (+)), tendinopathy (control (-)), group that have PRP and group that have PRP+5%CaC12 in 1st week and 2nd are presented in the following table :

Table 2: Sample Measurement Result

	No	1st Week		2nd Week	
		Tensile load (N)	Tensile Strengt (N/m)²	Tensile Load (N)	Tensile Strengt (N/mm)²
Normal	1	17,43	1,687	29,19	2,878
	2	16,90	1,496	28,48	3,140
Tendinopathy	3	16,52	1,932	22,81	2,955
	4	18,87	1,772	23,59	2,228
	5	17,29	2,287	24,01	2,311
	6	15,71	1,071	24,92	2,460
Tendinopathy + PRP	7	20,18	1,920	28,59	2,950
	8	19,07	137	34,89	3,492
	9	21,31	3,49	25,24	2,484
	10	20,93	1,894	27,48	2,478
Tendinopathy + PRP + CaCl2	11	27,58	2,476	24,39	2,809
	12	26,53	2,519	21,51	2,132
	13	24,29	1,842	43,93	3,873
	14	24,08	2,261	27,58	3,131

Figure 5. Mean value tens ile load in 1st week and 2nd week.

Tensile load of intervention groups at 1st week were higher than control group (+/-) with significant difference (p=0.000) but not significant difference (p= 0.544) at 2nd week although tensile load of intervention groups were as high as control (+) but higher than control (-).

Tensile strength of intervention groups at 1st week and 2nd week were not significant difference than control group (+/-) statistically (p=0.478 and p=0.542).

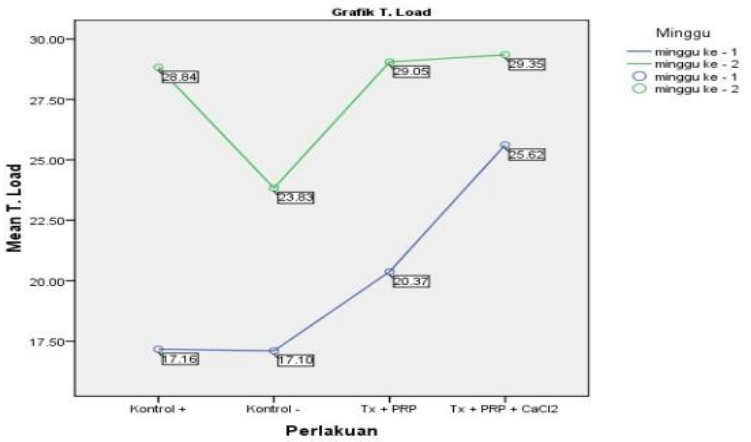

Figure 6. Mean value of tensile strenght in 1st and 2nd week.

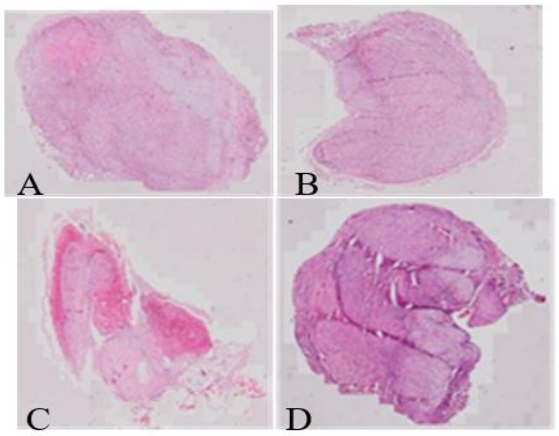

Figure 6. Transversal slice of t endon in 1 st week after intervention(100x magnificat ion) A. normal tendon B. Tendinopathy C. Achilles tendinopathy was treat ed byP RP D. Achilles tendinopathy was treated by PRP +5% CaCl2which appeared a gap between tendon sheath and tendon.

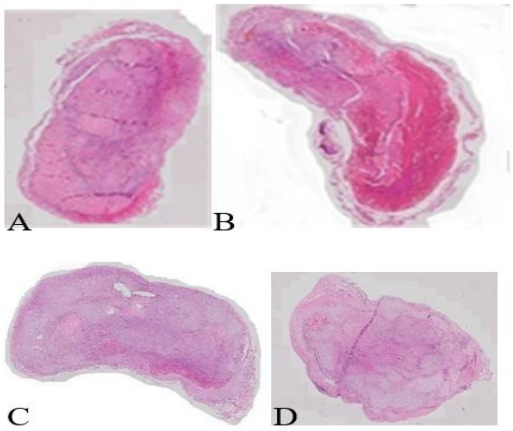

Figure 7. Transversal slice tendon in 2nd week after intervention (100x magnification) A. normal tendon. B. Tendinop athy . C. Achilles tendinopathy treated by PRP. D. Achilles tendinopathy treated by PRP+5% CaCl2

Tendinopathic tendon was brighter colored than the normal one, only the group which received PRP + 5%CaCl2 formed gap between the tendon and tendon sheath at 1st week. The gap of tendon sheath was seen clearly at 2nd week, but not in the tendinopahty group that did not receive PRP.

Table 4. Adhes ion level

Adhes Ion		none	mild		moderate		Severe	
		0	1	2	3	4	5	6
Normal	Week	2						
T .pathy	1			1		3		
PRP			1	2	1			
PRP+CaCl2			1	3				
Normal	Week		1	1				
T .pathy	2					3		1
PRP					2	2		
PRP+CaCl2					2	2		

There were significant differences between PRP groups and CaCl2 added PRP groups in 1st (p=0.014) and 2nd weeks (p=0.044). The adhesion degree of intervention groups were lower than the tendinopathy groups at 1st and 2nd weeks but still higher than normal groups.

Tensile load of intervention groups at 1st week were higher than control group (+/-) those shown healing time of intervention groups were faster (early healing in proferation stage). Tensile load of intervention groups at 2nd week were as high as control (+) but higher than control (-), which may be at 2nd week the tendon are already healed which almost have optimal strength. Tensile strength of intervention groups at 1st week and 2nd week were not significant difference than control group (+/-) statistically.

Activated PRP and non-activated PRP accelerate the return of tensile power of the tendon faster/earlier than normal tendon is visible even in the 1st week biomechanical test. The addition of 5% CaCl2 at PRP proved to boost the tendon tensile strength higher than PRP administration are significantly different at 1st week alone, but not significantly different at 2nd week. Achilles tendon tensile strength in conditions of tendinopathy is at low value so that the elasticity could be reduced which facilitates partial tear or total rupture.[2] In biomechanical testing at 1st and 2nd weeks shows that the achievement of tensile strength of the tendinopathy tendon is at low value which can be assumed that had longer time of healing or healed in suboptimal condition.

Only 2 weeks period to assess the effects of PRP in tendon healing by assessing biomechanical and histopathologic of adhesion degree were not able to describe the process of healing of tendon injuries completely considering the overall healing process through three overlapping phases in a long time (phase remodeling/maturation continues until 6-9 months). We realized it as limitation in this study. Further research is necessary to investigate other doseresponse curves and effective PRP with longer time periods assuming it can be obtained in the final value tendon healing process.

Tendon biomechanical strength is determined by the presence of collagen fibers.[1,5,25] Biomechanical strength will increase as a result of completion of fibers of collagen fibers in the direction of pull force and an increase in the intermolecular between collagen fibers. Collagen formed by fibroblasts derived from outside the tendon tissue (extrinsic mechanism) or from the tendon itself from epitenon and endotenon which will form new collagen fibers (intrinsic mechanism).[13,25] Fibroblasts derived from undifferentiated mesenchymal cells, which are activated during the inflammatory phase. Tensile strength in healing tendon at 3rd to get to

7th day (inflammatory phase) is the period of the weakest, because the lesion area is only maintained by the fibrin clot as a "provisional matrix" that will turn into granulation tissue which is the arrangement of collagen fibers. Arrangement of collagen fibers corresponding longitudinal axis will have a tensile strength that is close to normal. Tendon healing process is a biological process that takes place over a long period and overlap phase. This process is composed of the stages of restoring the function and structure of the network. In fibroblastic phase, ie between day 7 to the 21, there is accumulation of collagen in the injured area which aims to improve the biomechanical strength of the network.[25,26]

The rapid healing of the tendon having optimal biomechanical forces at the beginning of the healing period allows early rehabilitation performed on the injured leg. The increased tensile strength gives the assumption that the tendon will be more resistant to loads that would be more convincing to be done for early rehabilitation.[12,18]

Early rehabilitation is useful to prevent muscle atrophy, increase nutrition and lubrication to the joints, reducing the occurrence of adhesions and prevent contractures.[2,18]

CONCLUSIONS

Activated PRP was better than non-activated PRP in tensile strength test at the 1st week. Unfortunately, there was not any significant differences in tensile strength test at 2nd week and adhesion degree test at the 1st and 2nd week. This study showed that administration of PRP and 5% CaCl2 added on PRP in achilles tendinopathic injury improvee arly tensile strength and reduce adhesion formation effectively however furthers research is needed such as dose-response, with longer time periods, larger sample, etc. The rapid tendon healing with optimal biomechanical forces allow early rehabilitation.

Specific resarch topics are suggested here: (1)the growth factor receptors in the achilles tendon and (2)which receptors are activated by administration of PRP.

This study has several limitations , among others: measurement of tendon diameter has difficulty due to macroscopic shape of the tendon which is not a sym- metrical tube, therefore we did measurements at the seam area to reduce bias; another problem is the nature of the tendons which is tender and may be influnced by the pressure perform for measurements using calipers, so the bias may occur in the length and width measurements to calculate the cross-sectional area of the tendon; in biomechanical testing it is difficult to create a con- stant traction force with a certain speed per given time because IMADA machines are used manually actuated thereby affecting the results of tensile strength of the tendon.

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