EFFECT OF AUTOLOGOUS PLATELET-RICH PLASMA ON ARRANGEMENT OF COLLAGEN FIBERS AT INJURED ACHILLES TENDON ENTHESES IN RABBITS

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ABSTRACT

Objective: To evaluate the effects of autologous platelet-rich plasma on arrangement of collagen fibers at injured Achilles tendon entheses in rabbits.

Study Design: Laboratory-based experimental study.

Place and Duration of Study: Department of Anatomy, Army Medical College, Rawalpindi, in collaboration with National Institute of Health, Islamabad, from March to May, 2018.

Material and Methods: Forty, healthy, male, "New Zealand white rabbits", 4-6 months of age, weighing 2000-2500g, were randomly divided into 4 equal groups (A, B, C and D). Group A served as control, while B, C and D were experimental groups. Injuries were surgically induced at Achilles tendon entheses of rabbits of experimental groups, which were treated with injections of autologous platelet-rich plasma, either at the time of inducing injury or two weeks after injury in groups C and D respectively while group B was left untreated. Animals were sacrificed after 12 weeks. Tissues were processed and stained for histological evaluation. Statistical analysis was done using SPSS version 22. A p-value of ≤ 0.05 was taken as significant.

Results: Experimental group B showed marked deterioration in Bonar's modified score for arrangement of collagen fibers as compared to the control group A. The scores were significantly improved in treatment groups C and D as compared to group B.

Conclusions: Injection of autologous platelet-rich plasma effectively improved the Bonar modified score for arrangement of collagen fibers at injured entheses, 12 weeks after injury as compared to non-treatment group.

Keywords: Achilles tendon enthesis, Collagen fibers, Platelet-rich plasma.

INTRODUCTION

The Achilles tendon or tendo calcaneus is the largest and strongest tendon in the body present on the back of the leg. It attaches the tendons of gastrocnemius and soleus muscles to the calcaneus bone of the heel and causes plantar flexion of foot at the ankle joint[1].

Enthesis is a transitional zone which attaches, tendons and ligaments to bones[2]. This zone has four distinct areas; Tendon, uncalcified fibro cartilage, calcified fibrocartilage and the bone[3,4]. Each zone has its own anatomical, mechanical and physiological characteristics which help in movement at joints and reduce mechanical stress. Collagen fibers are the major constituent of matrix and preserve the structural organization of tissue. Bundles of type 1 collagen fibers, arranged parallel to each other, are present in tendon and fibrocartilage areas of enthesis[3].

The Achilles tendon is the most commonly ruptured tendon in human body[5]. Surgical methods are often used to reconstruct tendon-bone interface[6] but, they fail to effectively recreate it and mostly end up in formation of scar tissue with poor mechanical properties associated with high risk of repeated injuries[2,7,8].

Thus, search for alternative methods for managing the tendon injuries and reconstruction of enthesis site is a topic of great clinical interest[9]. One of the latest methods, which has attained popularity, is the use of injection of platelet-rich plasma (PRP)[10].

PRP contains a number of growth factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), transforming growth

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factor (TGF), fibroblastic growth factor (FGF), hepatocyte growth factor (HGF) and insulin like growth factor-1 (IGF-1)[11]. These growth factors interact with the receptors present on the surface of cells and cause gene expression which promotes tissue healing by stimulating proliferation of cells, production of extracellular matrix and reducing inflammatory response[7,12].

Despite the fact that the a number of studies have been published in the recent years regarding the use of PRP, many important queries regarding appropriate dosage, frequency and timings of PRP injections, various modes of delivery, exact site of delivery and the most optimal physiological conditions for use still need to be investigated[13].

Most of the PRP researches revolve around the mid-tendon injuries rather than at enthesis. Researchers have focused on the use of PRP in acute phase of tendon injuries, in spite of the fact that in actual clinical practice patients with tendon injuries usually seek specialist medical attention in chronic phase of injury[8].

This study was conducted to evaluate the regenerative effects of early and delayed injections of autologous PRP, at surgically injured Achilles tendon enthesis (ATE) in rabbits, 12 weeks after injury, using arrangement of collagen fibers as the determining parameter.

MATERIAL AND METHODS

This research was carried out in the Department of Anatomy, Army Medical College, Rawalpindi, in collaboration with National Institute of Health (NIH), Islamabad, from March to May, 2018.The study was approved by Ethical Review Committee of the Army Medical College, Rawalpindi and National University of Medical Sciences, Islamabad.

It was a laboratory based experimental study and non-probability Convenient sampling technique was used. Forty, healthy, male, 4-6 months old, "New Zealand White" rabbits; Weighing 2000-2500gm were selected. Animals with any pathology in or around the ATE were excluded from the study. They were kept in separate cages at controlled room temperature of 20-25°C and 12 hours light and dark cycles were maintained. They were fed on standard NIH diet and water ad libitum.

Rabbits were randomly divided into four groups A, B, C and D having 10 animals in each group by lottery method. Rabbits in group A served as control. Rest of the animals were anesthetized by giving intramuscular injection of a mixture of xylazine-ketamine hydrochloride (xylazine: 5mg/kg and ketamine: 35mg/kg)[14]. A 3cm longitudinal incision was given on the skin, of left hind legs of all rabbits of experimental groups, at the region of ATE. A punch biopsy instrument (Sklar, 2mm Disposable Biopsy Punch) was used to induce an injury at the center of the ATE. Skin wounds were closed with disposable skin stapler (Advan). The injured rabbits were then randomly divided into three groups, B,C and D. No treatment was given to animals in group B. PRP was injected in ATE of rabbits of group C at the time of inducing injury while it was given 2 weeks after injury in group D. All animals were free to move and were examined daily for the development of any complications.

To prepare platelet-rich plasma, 10ml of whole blood was extracted from the marginal ear vein of each rabbit using a 21-gauge needle and was mixed with 1ml of 0.1M sodium citrate in a 15ml conical centrifuge tube. 1ml of whole blood was reserved for baseline platelet count[15]. First spin was done with a standard laboratory centrifuge (Hettich EBA 20) at 500g for 10min.

Three different layers were formed: first, supernatant containing platelet poor plasma (PPP), second, a buffy coat containing platelets and white blood cells, and third, residual layer containing red blood cells. The supernatant and buffy coat were collected by gentle aspiration and were transferred to another centrifuge tube. A second 10-min spin at 2200g was given to further concentrate PRP.

Supernatant (or PPP) was aspirated gently and was discarded. Then, pellet containing platelets, was resuspended by shaking gently and retrieved along with a little amount of PPP and was known as PRP. Platelets count was done in whole blood and prepared PRP, by automated cell counter. Concentration of platelets in PRP with almost 3 times over the baseline value of whole blood was used for tissue healing. PRP was activated by 500µl of 10% $CaCl_2$ solution[16]. All PRP injections were given within one hour of its preparation because concentration of growth factors is maximum during this period[12].

At the end of experimental period of 12 weeks, all rabbits were sacrificed by giving intravenous injection of pentobarbital (30mg/kg) followed by an intramuscular dose of 2% xylazine (5mg/kg)[17]. ATE of each animal was removed and fixed in 10% formaldehyde solution. After decalcification in 5% Nitric acid, the sagittal section of each sample was cut and was processed for histological study. Hematoxylin and Eosin (H&E) stains were used for staining the sections. For identification of collagen fibers, Masson's trichrome stain was used in separate sections.

Each slide was examined under light microscope, at 40X magnification. Area of most prominent change was selected for evaluation[18].

Bonar's modified semi score for tendon lesion determination was used to observe the arrangement of collagen fibers[19]. According to this score, tissue was graded as under:

0 = Collagen arranged in tightly cohesive well demarcated bundles.

1 = Separation of individual fibers with maintenance of demarcated bundles.

2 = Separation of fibers with loss of demarcation of bundles.

3 = Marked separation of fibers with complete loss of architecture.

The statistical package for social sciences (IBM-SPSS version 22) was used for data analysis. Chi square test was used for comparison. Results were expressed as frequency and percentages. A *p*-value of ≤0.05 was considered significant.

RESULTS

Total forty animals were selected and equally divided into four groups. All the animals stayed alive, healthy and active till the end of the study. Microscopic evaluation of all the sections stained with H&E and Masson's trichrome stains, was done at 40X magnification. Bonar's modified score was used to assess the arrangement of collagen fibers. Analysis was done on a 0-3 scale, 0 being completely normal and 3 being maximally abnormal. In all animals of group A, collagen fibers were arranged in tightly cohesive and well demarcated bundles, so they were placed in grade 0 (table, fig-1A). In experimental group B, 7 (70%) specimens showed separation of individual collagen fibers with loss of demarcation of bundles, 2 (20%) animals displayed marked separation of fibers with complete loss of architecture and 1 (10%) specimen had separation of collagen fibers but demarcation of bundles was maintained (table, fig-1B). In experimental group C which received PRP injection at the time of

Table: Frequency and percentages of different arrangements of collagen fibers of control group A and experimental groups B, C and D.

Parameter	Findings	Group A	Group B	Group C	Group D
Arrangement of collagen fibers	Grade 0 (completely normal)	10 (100%)	0 (0.0%)	6 (60%)	3 (30%)
	Grade 1 (mild abnormality)	0 (0.0%)	1 (10%)	4 (40%)	6 (60%)
	Grade 2 (moderate abnormality	0 (0.0%)	7 (70%)	0 (0.0%)	1 (10%)
	Grade 3 (maximum abnormality)	0 (0.0%)	2 (20%)	0 (0.0%)	0 (0.0%)

inducing injury, 6 (60%) animal showed completely normal arrangement of collagen fibers while 4 (40%) had mild abnormality in arrangement (table, fig-1C). In experimental group D, which received PRP injection, 2 weeks after injury, 3 (30%) specimens had collagen fibers with totally normal architecture, 6 (60%) had grade 1 abnormality while only 1 (10%) animal had moderate deterioration in arrangement of collagen fibers (table, fig-1D). A p-value was 0.001 which was found to be highly significant.

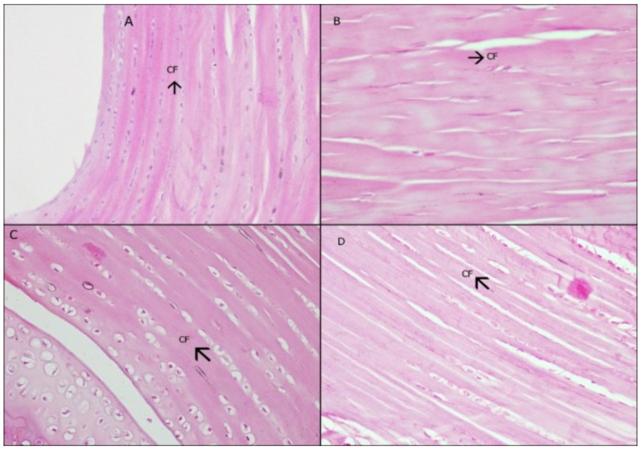

Figure-1: Photomicrographs showing arrangement of collagen fibers in control group A and experimental groups B, C and D, arrows indicating collagen fibers (CF), at 40X; H&E.

On intergroup comparison, highly significant difference was found between control group A and experimental group B (p-value =0.0001). Group A was also significantly different from experimental groups C and D (p-value =0.025 and 0.005, respectively). On comparison of group B with groups C and D, the p-value was 0.001 and 0.004 respectively. On intergroup comparison of groups C and D, p-value was statistically insignificant (p-value=0.301).

DISCUSSION

The Achilles tendon injuries are among the most commonly occurring musculoskeletal disorders[20]. Such injuries are followed by a long rehabilitation period and increased risk of re-injuries[21]. In case of injury at enthesis, this junctional complex is not completely regenerated in its natural shape because the zone of fibro-cartilage is difficult to heal[11].

One of the latest methods to regenerate tendons and damaged enthesis, is the use of PRP[10]. PRP is a natural product which is a constituent of the plasma portion of the blood, containing platelets in a concentration above the baseline value of the whole blood[21]. Numerous growth factors are present in platelets like PDGF, EGF, VEGF, IGF, HGF, which play a pivotal role in tissue healing[22].

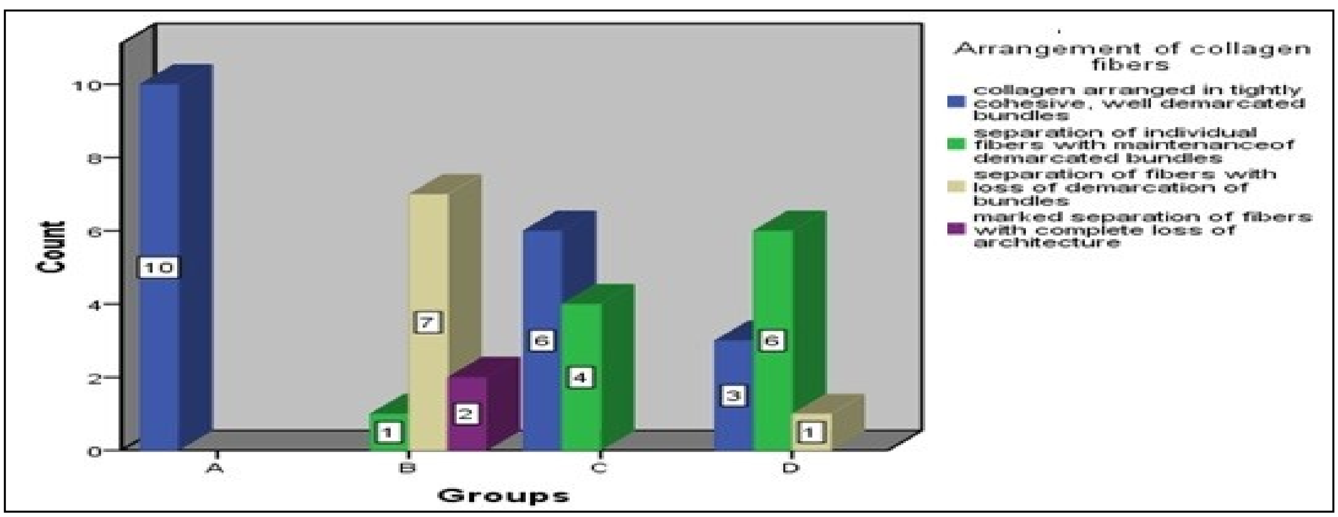

Figure-2: Cluster Bar chart showing frequency of different arrangements of collagen fibers among the control group A and experimental group B, C and D.

These growth factors exert therapeutic effect by stimulating proliferation of cells like fibroblasts, tenocytes, chondrocytes and mesenchymal stem cells, which in turn augment production of matrix. PRP also contains inflammatory mediators and modulators which reduce the inflammatory response after injury. Fibrinogen is another important constituent of PRP, which acts as a scaffold in initial phase of healing process[23].

The purpose of this research was to observe the effects of early and delayed injections of PRP on arrangement of collagen fibers, at injured ATE in rabbits. Production and subsequent arrangement of collagen fibers is one of the imperative parameter to assess healing of tendon injury. Grading for arrangement of collagen fibers was done according to Bonar's modified score, on a scale from 0-3.

In the experimental group B, 10% of the cases had mild abnormality, 70% had moderate abnormality while 20% showed severe abnormality. In this study arrangement of collagen fibers in group C, was close to group A in 60% of the cases while 40% had mild abnormality. It was observed in experimental group D that 30% rabbits displayed completely normal architecture, 60% had mild abnormality and 10% were moderately abnormal in regard to the arrangement of collagen fibers.

These effects showed improvement in Bonar's score, in experimental groups C and D, as compared to group B, which did not receive PRP injection. No statistically significant difference was found between groups C and D. Growth factors in PRP induced increase in production of extracellular matrix and are thus responsible for regeneration at the injured site. Fibroblasts and tenocytes are responsible for production of collagen fibers. Type III collagen produced during proliferative phase is replaced by type I collagen in remodeling phase[5].

The results were in accordance with the study done by Bagheri[24] who compared the effects of PRP and bone marrow derived mesenchymal stem cells and their combination on the healing of achilles tendon in rabbits. They observed increased collagen production and better organization in PRP treated group due to IGF in PRP. The findings were also supported by a study conducted in 2016, in which it was observed that PRP injection in acute phase of tendon injury improved healing by restoring orientation of collagen fibers, increased number of fibroblasts and better vascularity[10]. Oryan and co researchers emphasized the importance of collagen in healing of tendon injuries[5].

A study conducted by Sen and his colleagues demonstrated that autologous PRP had no histological effects on healing of achilles tendon 28 days after rupture. They used Tang's scale of tendon healing, which was although improved in treatment group but it was not statistically significant. These results are in contrast to the present study[25]. Zhang and his fellow researchers demonstrated that PRP moderately improved arrangement of collagen fibers at ATE and also increased tensile strength due to production of collagen fibers, when tested mechanically[11]. Guszczyn and coworkers also supported improved collagen production after administration of PRP in dermal fibroblasts[12].

Thus, it can be established by this study that regenerative ability of autologous PRP by virtue of multitude of growth factors present in it, the most important being IGF, PDGF and FGF, is responsible for increased production and better organization of collagen fibers in experimental models of injured ATE. PRP was found to be useful in both early and delayed treatment groups which were not found to be significantly different from each other.

CONCLUSION

Injection of autologous PRP effectively improved the Bonar's modified score for arrangement of collagen fibers at injured ATE, 12 weeks after injury, in both early and delayed treatment groups, as compared to non-treatment group.

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Disclosure

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CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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