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Clinical and Histopathological Studies on the Efficacy of Multiple Injections of I-PRF Versus the Single Use of A-PRF in Repair of Achilles Tendon Rupture in Dogs: An Experimental Study

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ABSTRACT

Platelets concentrates (PC) proved in many studies to enhance the healing process in different tissues including tendon healing as they produce large number of GFs and cytokines so the aim of the current study was to evaluate the efficacy of both Injectable-platelets rich fibrin (I-PRF) and Advanced-platelets rich fibrin (A-PRF) in enhancement of the healing of surgically treated AT rupture in dogs and compare the efficacy of the single administration of the A-PRF membranes with multiple injections of the I-PRF.

Study design: Eight adult male mongrel dogs with average weight 18 kg ± 3 and with average age 2 years ± 1.5 were used in this study and were divided into two groups, A-PRF group: A-PRF membranes were applied during surgery only. I-PRF group: I-PRFwas injected during the surgery with 2 more booster injections with 3 weeks intervals after surgery. During observation period, dogs were observed clinically at 3,6, 8, 10- and 12-weeks post-operation and histologically at 6- and 12-weeks post-operation.Results: the clinical observation revealed a similar effect of the two groups at the early stages of the healing process while the I-PRF group showed significantly better healing of the tendon with decreased thickness and histological examination revealed significantly higher healing, arrangement of the fibers and collagen deposition in the I-PRF group.Conclusion: The present study revealed that A-PRF and I-PRF have the ability to enhance and accelerate the healing and regeneration processes of the Achilles tendon while the multiple injection of the I-PRF add a great value over the A-PRF group.

Keywords: Achilles tendon, A-PRF, I-PRF, PC, Platelets GFs and Tendon healing.

INTRODUCTION

Recently in the veterinary practice, Achilles tendon rupture became one of the most common affections of the Musculoskeletal system in dogs.it is usually seen in Mature middle-aged, middle to large-breed dogs and more

frequent in athletic dogs (Kramer et al. 2001). It also became an important field of research in the human sports medicine because the healing of the tendon usually takes several months for complete recovery and regaining the normal mechanical and functional strength(Sánchez et al. 2007).

Tendons have the ability to heal naturally after injury, but with significant fibrosis that reduces mechanical properties and alters their functional performance, also making them more susceptible to further damage (Mast 1997). Also, Healing of the tendon is a slow process due to its limited blood supply and slow cell turnover that's why several studies evolved for the development of techniques to enhance the tendon healing, providing earlier return to more normal structure and function with higher strength of the tendon and minimal adhesion to the surroundings in human and veterinary practice. (Walsh et al. 2004)

As for other tissues, Healing of tendon injury is characterized by the onset of inflammation, cell proliferation, reparation by collagen deposition and Extra cellular matrix (ECM) production up to remodeling; these events are stimulated by the release of growth Factors (GFs) and cytokines by platelets at the repair site after surgery. Interleukins(IL), tumor necrosis factor (TNF), connective tissue growth factor (CTGF), Vascular endothelial growth factor (VEGF), Platelet derived growth factor (PDGF), Fibroblast growth factor (FGF), Transforming growth factor beta-1(TGF-β1), Epidermal growth factor (EGF) and Insulin like growth factor-1(IGF-1) are the most common types of GFs and cytokines released by platelets. Each of them participates in different stages of the healing process(Molloy et al. 2003, Nourissat et al. 2015).

Considering their function, GFs support/control different stages of healing. IGF-1, PDGF and FGF are essential during the early and intermediate stages of tissue regeneration as they aid fibroblasts in migration, proliferation and in synthetizing ECM. Regarding TGF-β1 and VEGF, they have some role in these processes too, but they are mainly involved in angiogenesis of the injured area where they regulate tissue remodeling. However, embryological and genetic studies proved that TGF-β1 and FGF are the main GFs with a role in tendon development (Tozer and Duprez 2005, Schweitzer et al. 2010)The eminent role of IGF-1 in tenogenesis is toaid the migration and proliferation of fibroblasts, increase collagen expression in tenocytes, protein and extracellular matrix synthesis and fibril diameter in tendon constructs by enhancing the metabolic response of tendon fibroblasts (Herchenhan et al. 2015)and in vitro (Bissell et al. 2015).

Platelets concentrates (PC) proved in many studies to enhance the healing process in different tissues including tendon healing as they produce large number of GFs and cytokines. Platelets Rich Plasma (PRP) as a first generation of PC has been successfully used to enhance the healing process in different tissues with some drawbacks as its need for activation by thrombin or calcium and the presence of anticoagulant that interferes with the natural healing process despite containing a number of growth factors participated in tissue repair(Del Corso et al. 2012). For this reason a second generation of platelets concentrates (PRF) was developed for further improvement of wound healing in comparison to PRP but still has its advantage of being injectable(Ghanaati et al. 2014).

PRF is prepared by single centrifugation of the whole blood without the addition of anticoagulant with the formation of a fibrin clot rich in platelets without addition of thrombin or calcium during preparation as

the activation of platelets occurs by endogenous coagulation cascade. The autologous thrombin contributes to the direct polymerization of fibrinogen into fibrin, resulting in a 3D flexible fibrin network, in which platelets and WBC are entrapped. PRF membranes can release a high quantity of critical GFs with a very significant slow continuous ratefor a prolonged period (2-4 weeks) acting as a space filler due to its scaffold-like function and GFs temporal release that provides a great potential for wound healing (He et al. 2009, Masoudi et al. 2016).

A new protocol for production of PRF membranewas evolved by Ghanaati, Booms et al. (2014) through modification of the centrifugation process that resulted in production of Advenced PRF membranes (A-PRF) that was higher in platelet cell numbers and monocytes/macrophages behavior thus better wound healing. That was proved by (Kobayashi et al. 2016).

The preparation of A-PRF as membranes limits its use in the Musculoskeletal system affections as joint and tendon affections except for the open surgery problems.that's why a novel technique for preparation of PRF evolved by Joseph Choukron and his co-workers in 2014 depending on the low-speed centrifugation concept by centrifugation of the whole blood in low speed (700 rpm) for shorter period (3 minutes) without the use of anticoagulant that resulted in PRF for use in liquid (injectable) form (I-PRF) that coagulates within few minutes after the injection forming the 3d fibrin matrix inside the tissue. Thus, gathering the both advantages of PRP and PRF, being injectable form with no need for anticoagulant and the formation of the 3d fibrin matrix that acts as a scaffold for the tissue regeneration(Choukroun et al. 2017).

Recent studies proved the efficacy of I-PRF for enhancement of the healing process in different tissues(Wang et al. 2018). Abd El Raouf et al. (2019)demonstrated for the first time the superior cartilage regeneration potential of I-PRF using the low-speed centrifugation concept in the early stages of cartilage repair while Shevchenko and Rublenko (2020) proved the higher concentration of platelets and leukocytes of the I-PRF in their study.

This study aimed for evaluation of the efficacy of both I-PRF and A-PRF in enhancement of the healing of surgically treated AT rupture in dogs and compare the efficacy of the single administration of the A-PRF membranes with multiple injections of the I-PRF.

MATERIAL AND METHODES:

Animals:

The present study was performed ontwelve adult male mongrel dogswith average body weight18 kg±3 and with average age 2 years ± 1.5. Clinical and physical examination were done to all animals to ensure that they are physically normal and free from any Musculoskeletal disorders. Special examinationof the area of the hock and Achilles tendonwas done to ensure the integrity and healthiness of the AT before the beginning of the study. Also, Chemistry profile and complete blood count was done to all animals. During the study period, dogs were kept in their Kennel, fed balanced food and allowed free access to water. Throughout the study, all efforts were exerted to minimize any stress factors on the dogs throughout the study period.This study was approved by the Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, University of Sadat city.

Animal groups:

Two equal groups of animals were obtained;each group consisted of 6 animals; the 1st group was defined as A-PRF group while the second group was defined as I-

PRF group. Both groups were treated as following:

- **A-PRF group**: Animals of this group received Advanced PRF membranes as an additional treatment after experimental cutting and surgical repair of the Achilles tendon of the right hind limb of each animal.Before closure of the SC and skin wound,the A-PRF membranes were applied between and around the cut ends of the tendon.
- **I-PRF group**: Animals of this group received Injectable PRF as an additional treatment after experimental cutting and surgical repair of the Achilles tendon of the right hind limb of each animal.Before closure of the SC and skin wound, The I-PRF wasinjected between the cut ends and at the proximal and distal stumps of the tendon.

Preparation and application of the A-PRF membranes(Figure 1):

A-PRFmembranes were prepared just before the surgery, 10ml of venous blood was aspirated from the jugular vein using 20-gauge sterile needle and divided in 2 sterile glass tubes without addition of anticoagulant.According to (Kobayashi, et al., 2016), A-PRF membranes were obtained by the centrifugation of the whole blood at 1500 rpm for 14 minutes at room temperature. Fibrin clot formed above the RBCs layer below the acellular serum. Then the fibrin clot was separated from the RBCs layer by a sterile tissue forceps and scissors and squeezed from the acellular serum in a sterile gauze**(Fig. 1)**. The prepared A-PRF membranes were applied between and around the cut ends of the tendonbefore closure of the SC and skin wound.

Preparation and application of the I-PRF(Figure 2):

I-PRF was prepared during the surgery, 10ml of venous blood was aspirated form the jaguar vein using 20-gaugesterile needle and divided in 2 sterile glass tubes without addition of anticoagulant. According to (Abd El Raouf, Wang et al. 2019), the whole blood was centrifuged at 700 rpm for 3minutes in a laboratory centrifuge. The I-PRF appeared as the upper most 1 ml of plasma that was aspirated by a sterile syringe with 21G needle and became ready for injection(Fig. 2). The prepared I-PRF was injected between the cut ends and at the proximal and distal stumps of the tendon before closure of the SC and skin wound. The preparation and injection repeated 2 more times at the 3^{rd} and 6^{th} week but the injection was performed through the skin within and around the tendon at the site of the cut.

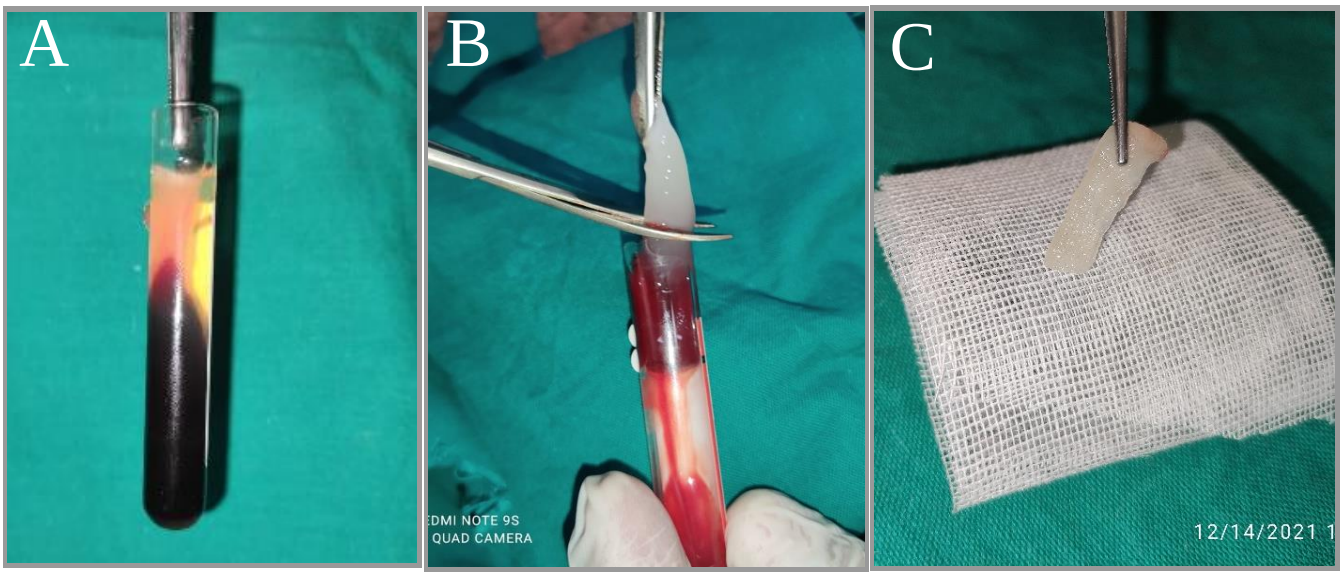

Figure (1): Preparation of A-PRF membrane; **A:** showing blood sample after centrifugation with A-PRF in the upper part of the tube above the RBCs layer at the bottom, **B:** showing separation A-PRF membrane red corpuscle layer by scissors, **C:** showing A-PRF membrane after squeezing of acellular serum.

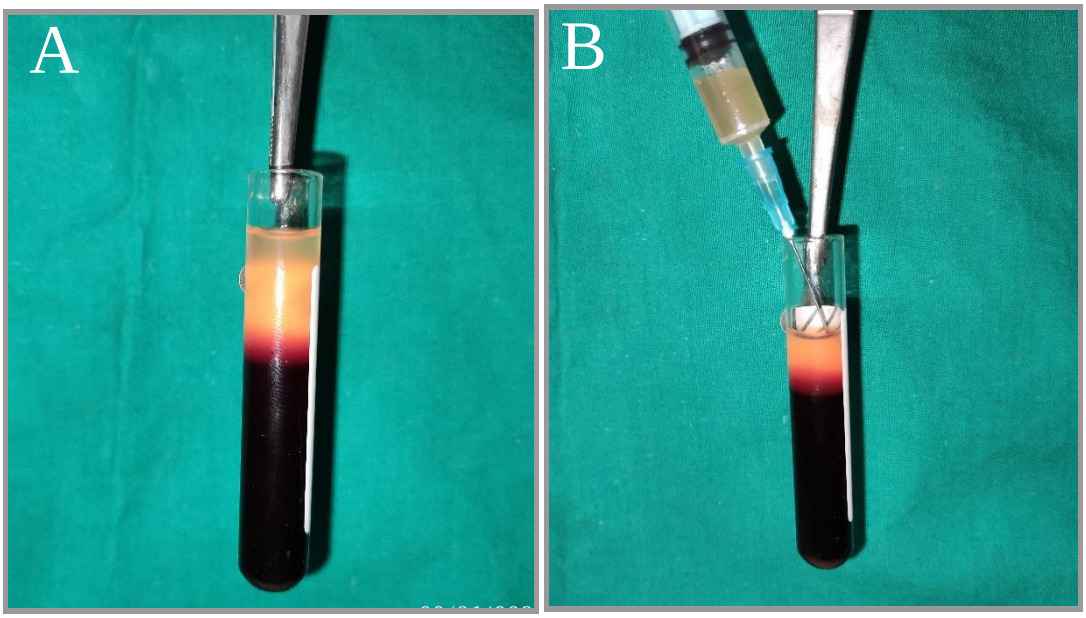

Figure (2): Preparation of I-PRF; **A:** showing the liquid I-PRF after centrifugation above RBCs layer, **B:** showing aspiration of I-PRF for injection.

Anesthetic regimen:

Before the surgery, animals were premedicated with Xylazine HCL (Xylaject® 2% sol. ADWIA Co., A.R.E.) at dose of 0.5 mg/kg bw I.V injection. Diazepam at dose rate 0.4 mg/Kg BW (Neuril® Memphis Co., A.R.E.). and ketamine HCL (KETALITE®, ELITEPHARMA Co., Pakistan) 5 mg/kg BW were used to induce general anesthesia while the surgical anesthesia was maintained using propofol at dose rate 1 mg/Kg BW (Propofol® 1% FRESENIUS KABI Co., Germany) with I.V injection as intermittent boluses until the end of the surgery. Meloxicam (Melocam®, AMOUN Co., A.R.E.) at dose rate 0.2 mg/Kg BW was injected subcutaneously during the induction to control postoperative pain**.**

Surgical induction and repair of AT rupture(Figure 3):

- Aseptic preparation of the tarsal and Kannon regions was performed.
- The operation was done with the animal in lateral recumbency with the right hind limb was the upper side.

- 10 cm longitudinal skin incision was performed on the lateral aspect of the AT just above the point of the hock, blunt dissection of the S.C till visualization and separation of the AT.
- Complete sharp transverse cut of the AT was done 5cm above its calcaneal insertion then repaired using Modifiedkessler's tenorrhaphy using poly propylene usp 2. the skin and S.C tissue closed using poly galctinusp 0.
- Tarsal immobilization for 3 weeks using complete Fiber glass cast (Tomato® cast Korea.) was done then replaced by cranial half cast for 3 more weeks.
- after the operation (Cefotax®: EPICO. Co., A.R.E) at dose rate 20 mg/kg b.wtas a broad spectrum antibiotic course for five days.

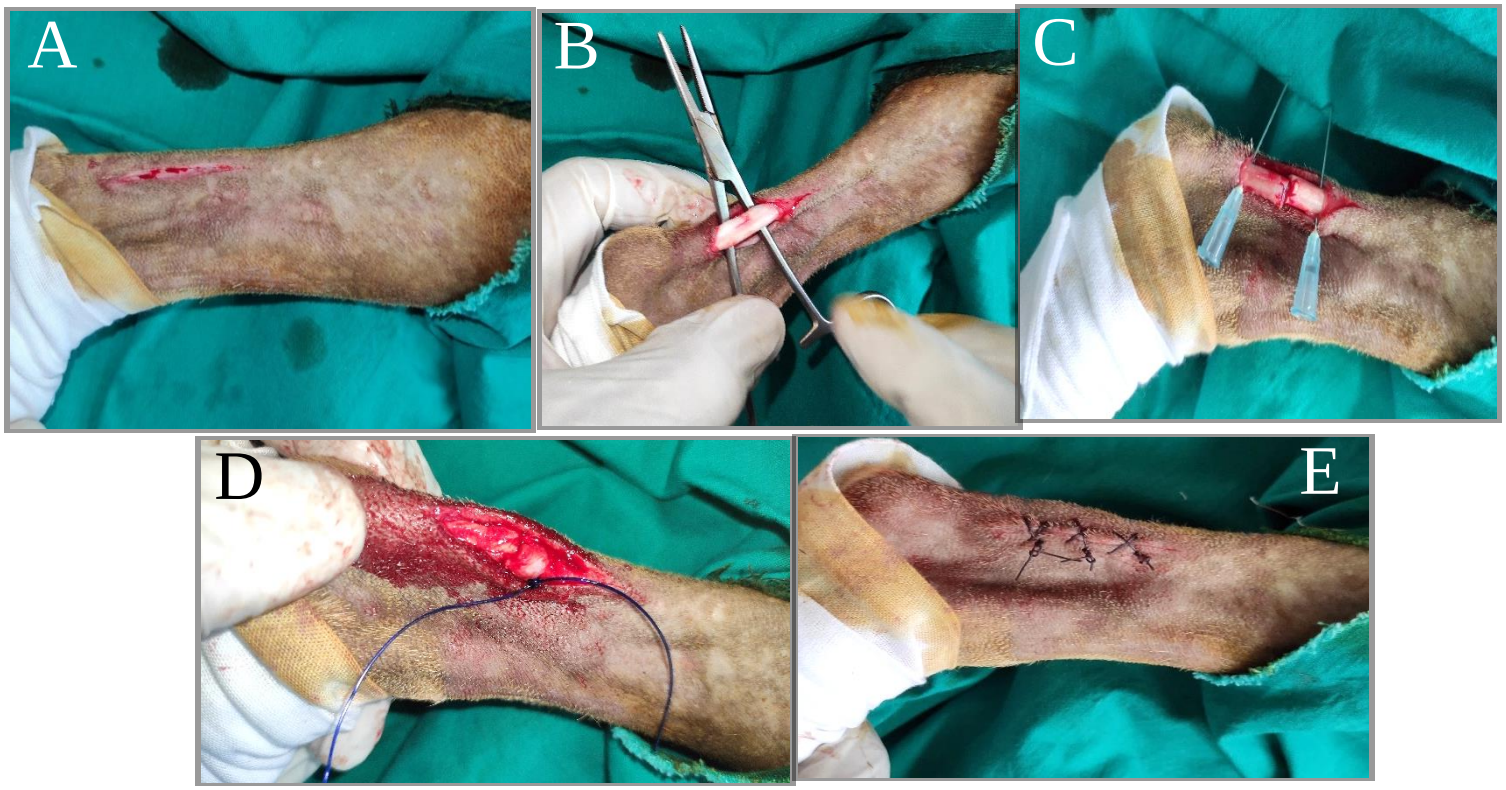

Figure (3): Surgical procedure for Induction and repair of AT rupture in dog. **(A)** Longitudinal skin incision, **(B)** Separation and exposure of the Achilles tendon, **(C)** Complete severing of the AT, **(D)** Repair of the tendon with modified kessler tenorrhaphy, **(E)** Closure of the skin with cross mattress suture pattern.

Evaluation and Assessment:

Clinical Evaluation:

Clinical observation based onthe weight bearing capacity on the treated limb, the degree of lameness during walk as well asthe physical examination through palpation and measurement of the thickness of the thickness of the tendon.

Palpation was used to monitor the integrity of the tendon at the cut site and the presence of inflammatory signs (pain and swelling)while the tendon thickness was measured using digital caliper after removal of the cast at 3rd, 6th, 8th, 10th and 12thweek after surgery in all animals of both groups and all the results were recorded and statistically analyzed.

The weight bearing capacity and the lameness score were done by 2 different assistances blinded of the groups and the results were recorded as means and standard deviations and statistically analyzed.

The lameness scoring was done after removal of the cast at 3rd, 6th, 8th, 10th and 12th week after the surgery using the scoring system as reported by(Saini et al. 2002)where Score 1 was recorded for Animals that did not put any weight on the operated limb and kept it off the ground or touched the limb to the ground without weight bearing, plantigrade stance on affected pelvic limb while

Score 2 wasrecorded when the dog placed the affected limb on the ground and put some weight on it and occasionally kept the limb off the ground with moderate lameness during walk and Score 3 meant that the dog always kept the affected limb on the ground and started bearing weight, but was still slightly lame during walk and finally Score 4 was given to the animal that started putting equal weight on the limbs and apparently was not lame during walk. i.e., normal weight bearing

Histopathological Evaluation:

At the 6th and 12th week, the operated tendons were harvested just below the musculotendinous junction and at its calcaneal insertion of the animals of both groupsand the tendon at the same level of the intact no operated limb was also harvested then all the specimens were washed with physiological buffer, fixed in buffered formalin 10% for 24 hrs., dehydrated and cleared in xylene then embedded in paraffin. After solidification of paraffin thin longitudinal sections 4-5 µm thickness from the center (site of the cut) and the periphery and stained with H&E and MTC.

Histological Tendon repair assessment score was evaluated on six parameters with grading score 0-3; fiber structure, fiber arrangement, rounding of the nuclei, inflammation, angiogenesis and cell density (Chen et al. 2014); Score 0 was assigned when the fiber structure was continuous and log with compacted parallel fiber arrangement and replacement of the rounded nucleated cells with long spindle shape cells with less than 10% inflammatory cell infilteration and less than 10% Neovascularization with normal cell density,Score 1 was assigned when the fiber structure was slightly fragmented with slightly loose and wavy fiber arrangement and presence of slightly rounded nucleated cells with 10-20% inflammatory cell infilteration and 10-20% Neovascularization with slightly increased cell density, Score 2 was assigned when the fiber structure was moderately fragmented with moderately loose and wavy fiber arrangement and presence of moderately rounded nucleated cells with 20-30% inflammatory cell infiltrationand 20-30% Neovascularization with moderately increased cell density and Score 3 was assigned when the fiber structure was severly fragmented with no identifiable fiber arrangement and presence of severely rounded nucleated cells with over 30% inflammatory cell infilteration and over 30% Neovascularization with highly increased cell density

Statistical analysis:

All the results were reported as mean, standard deviations. The statistical analyses were performed using one-way ANOVA. P-value was considered as statistically significant when $P \leq 0.05$. All the value were analyzed using the SPSS software (version 20.0; IBM, America).

RESULTS

The results of this study demonstrate the clinical and histological findings of the effect of the single use of A-PRF membranes in comparison with the triple administration of I-PRF on the healing of surgically repaired an experimentally induced AT rupture in dogs.

Clinical Evaluation:

Physical palpation of the tendon at the site of the operation revealed that all the animals of both groups showed intact and continuous tendon throughout the observation period with mild inflammatory signs in both groups at the 3rd week observationand subsided at the 6th week observation in the I-PRF group while continued to the 8th week in the A-PRF group then subsided in both group at the end of the observation period.The tendon thickness and lameness score were recorded and statistically analyzed inTable (1,2); Figures (3,4).

Table (1): Showing the Average and Standard deviation of thickness of the Achilles tendon at the site of the operation of both groups in different examination times:

Group	Surgery day	Week3	Week6	Week8	Week10	Week12	P-value
A-PRF	9.72 ± 1.32^{a}	$14.89 \pm 2.11^{b*}$	$15.86 \pm 4.24^{bc*}$	$15.61 \pm 0.49^{c*}$	$14.89 \pm 0.97^{bc*}$	$12.99 \pm 0.39^{b*}$	**0.000**
I-PRF	8.41 ± 0.51^{a}	$14.85 \pm 0.76^{b*}$	$14.09 \pm 0.47^{bc*}$	$13.45 \pm 0.55^{c**}$	$12.27 \pm 0.59^{cd**}$	$10.97 \pm 0.48^{d**}$	**0.000**
P- Value		**0.941**	**0.532**	**0.010**	**0.022**	**0.010**	

*, **: Means and standard deviations with different asterisks superscripts in the same column are significantly different at P <0.05.

A, b, c, d: Means and standard deviations with different small superscripts letters in the same row are significantly different at P <0.05.

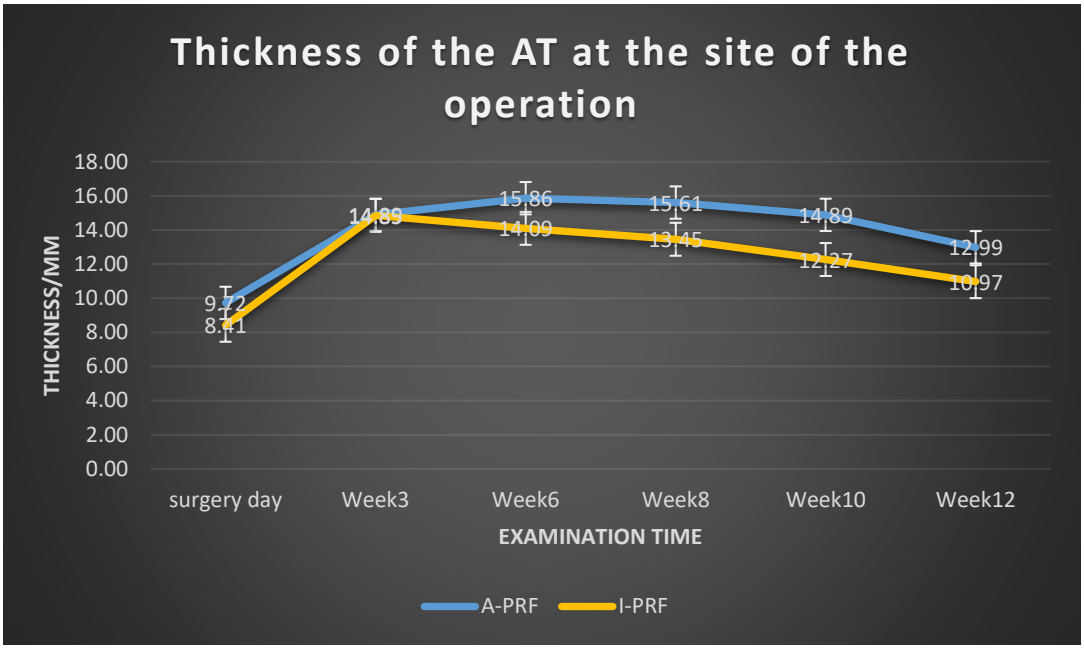

Figure (4): Illustrates the difference of the average thickness of the of the tendon at the site of the operation of both groups throughout the observation period and the chart shows the significant increase in the thickness of the tendon in both groups than the surgery day with the peakof the A-PRF group at the 6th weekand at the 3rd week in the I-PRF group while there was significant decrease in the thickness in the I-PRF group than the A-PRF group from the 8th week till the end of the study.

The statistical analyses of the previous data revealed that, the tendon thickness at the site of the operation significantly increased in both groups at the 3rd week observation than the preoperative thickness (P= 0.001) and continued to increase until the 6th week in A-PRF group then started to decrease until the end of the study while the thickness of the tendon of the animals of I-PRF group started to decrease directly after the 3rd week observation. However, the thickness of the tendon was lower in the in the I-PRF group than A-PRF without significant difference between them at the 3rd and 6th week observations (P= 0.941, 0.532), the I-PRF group had significantly lower thickness of the tendon from the 8th week until the end of the study (P= 0.010-0.022).

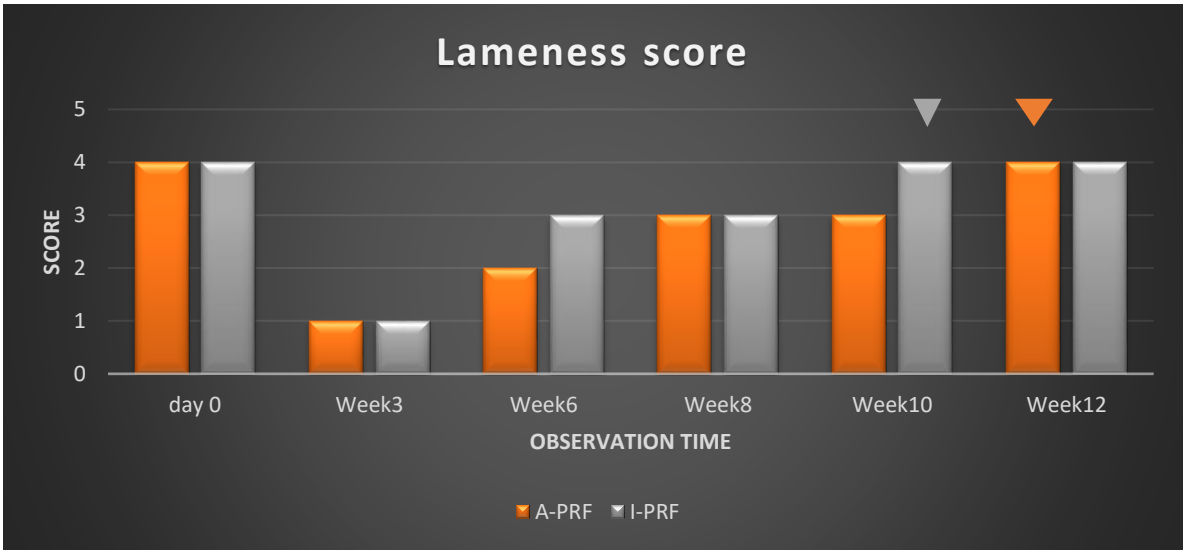

Figure (5): Illustrates the difference of the average Lameness Score of both A-PRF and I-PRF groups in different examination times and the chart shows the significant increase of the lameness score in both groups throughout the observation period with the same time point where the animals of both groups returned to normal weight bearing with no obvious lameness, score 4 (colored Arrow head).

From the previous data statistical analysis revealed that there was significant improvement in the weight bearing capacity and lameness score in both groups throughout the observation period (P= 0.000) with higher increase in the I-PRF group but without significant difference between the animals of the 2 groups in different examination times (P= 0.141- 1.000).

Table (2): Median lameness score and its ranges of A-PRF and I-PRF groups at different examination periods:

Group	Day 0	Week3	Week6	Week8	Week10	Week12	P-Value
A-PRF	4.0 (4.0-4.0)	1.0 (1.0-2.0)*^{a}	2.0 (2.0-3.0)*'**^{b}	3.0 (3.0-3.0)*'**^{bc}	3.0 (3.0-4.0)*'**^{cd}	4.0 (4.0-4.0)*^{d}	**0.000**
I-PRF	4.0 (4.0-4.0)	1.0 (1.0-2.0)*^{a}	3.0 (2.0-3.0)*'**^{b}	3.0 (3.0-4.0)**^{bc}	4.0 (3.0-4.0)*'**^{c}	4.0 (4.0-4.0)*^{c}	**0.001**
P-Value	**1.000**	**0.487**	**0.077**	**0.055**	**0.077**	**0.441**	

*, **: Median and Ranges with different asterisks superscripts in the same column are significantly different at P ≤0.05.

A, b, c, d: Median and Ranges with different small superscripts letters in the same row are significantly different at P ≤0.05.

Histopathological Evaluation:

Histological evaluation of tendon defects healing with H&E stain showedvariation in repair parameters, where I-PRF treated group showed significant improvement compared to A-PRF group; At the 6thweek evaluation,I-PRF treated group was better in most of the parameters with higher cellularity(cell migration and density) and angiogenesis than the A-PRFgroup also, the fiber structure and arrangement was more condensed, regular and parallel with spindle nucleated cells but wavier.At 12thweek observation, the fiber structure was more condensed and expressed more collagen fibers and decrease in nucleated cell number compared to the A-PRF group(Figure 8).

MTC stain showed nearly similar degree of collagen deposition in both groups at the 6th week observation but with higher condensation of the fiber arrangement of the I-PRF group than the A-PRF group while at the 12th week observation the fiber arrangement and collagen deposition were higher in the I-PRF treated group than the A-PRF group (Figure 9).

Six parameters were evaluated in Histological Tendon repair assessment score with grading score 0-3; fiber structure, fiber arrangement, rounding of the nuclei, inflammation, angiogenesis and cell density. In this score, the normal or ideal tendon will score 0 and the severely affected tendon will score 18. The results listed in (Table 3,4) and illustrated in (Figures 5,6).

Table (3): Histological assessment score of tendon repair of A-PRF and I-PRF groups at the 6th week observation represented as median and ranges:

Parameters	A-PRF Group	I-PRF Group	P-Value
Fiber structure	2.0 (2.0-2.0)**	2.0 (1.0-2.0)**	0.000
Fiber arrangement	3.0 (2.0-3.0)*'**	2.0 (2.0-2.0)**'***	0.005
Rounding of nuclei	2.0 (2.0-2.0)*'**	2.0 (1.0-2.0)**	0.055
Inflammation	3.0 (2.0-3.0)*	2.0 (1.0-2.0)**	0.001
Vascularity	2.0 (2.0-2.0)*'**	2.0 (2.0-3.0)*'**	0.163
Cell density	2.0 (1.0-2.0)*	2.0 (2.0-3.0)*	0.163

Total Score	14.0 **(11.0-14.0)	12.0 **'***(11.0-12.0)	0.001

*'**'***: Median and Ranges with different asterisks superscripts in the same row are significantly different at P <0.05.

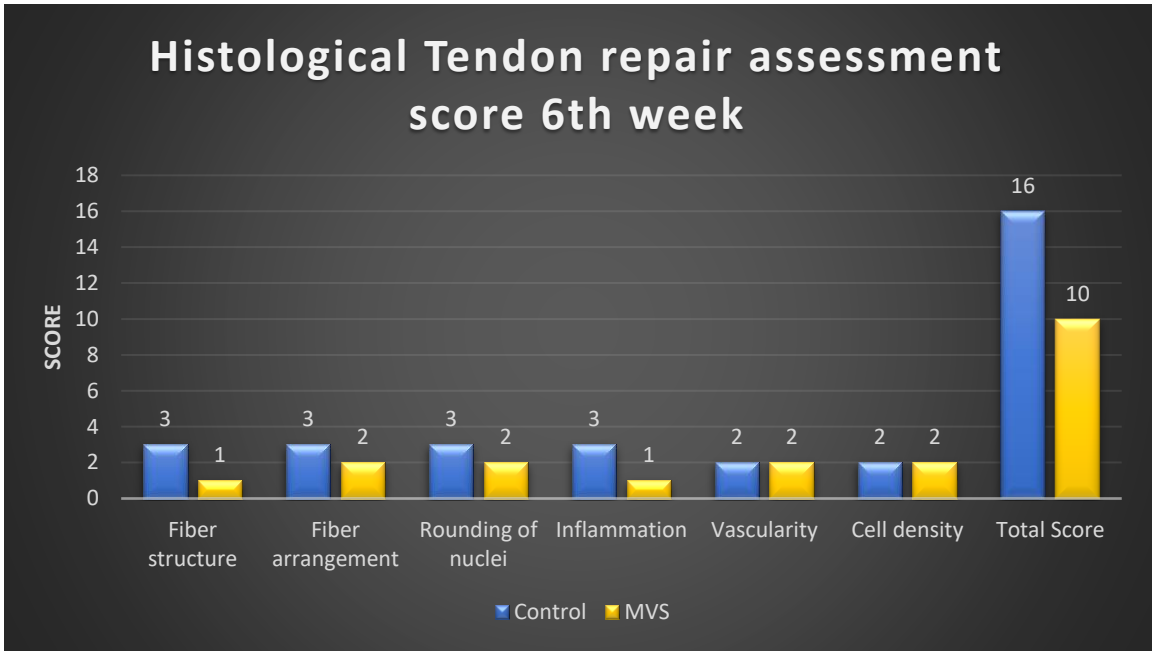

Figure (6): Chart illustrating the differences in the histological assessment score of tendon repair of the 4 treated groups at the 6th week observation.

Table (4): Histological assessment score of tendon repair of A-PRF and I-PRF groups at the 12th week observation represented as median and ranges:

Parameters	A-PRF Group	I-PRF Group	P-Value
Fiber structure	1.0 (1.0-2.0)**	0.0 (0.0-1.0)***	0.001
Fiber arrangement	2.0 (1.0-2.0)*'**	1.0 (1.0-2.0)**'***	0.008
Rounding of nuclei	1.0 (1.0-2.0)*	0.0 (0.0-1.0)**	0.001
Inflammation	1.0 (1.0-1.0)*	1.0 (0.0-1.0)*'**	0.021
Vascularity	2.0 (1.0-2.0)*	2.0 (1.0-2.0)*	0.802
Cell density	2.0 (1.0-2.0)*	1.0 (1.0-1.0)*	0.163
Total Score	9.0 (8.0-9.0)**	5.0 (5.0-6.0)***	0.000

*'**'***: Median and Ranges with different asterisks superscripts in the same row are significantly different at P <0.05.

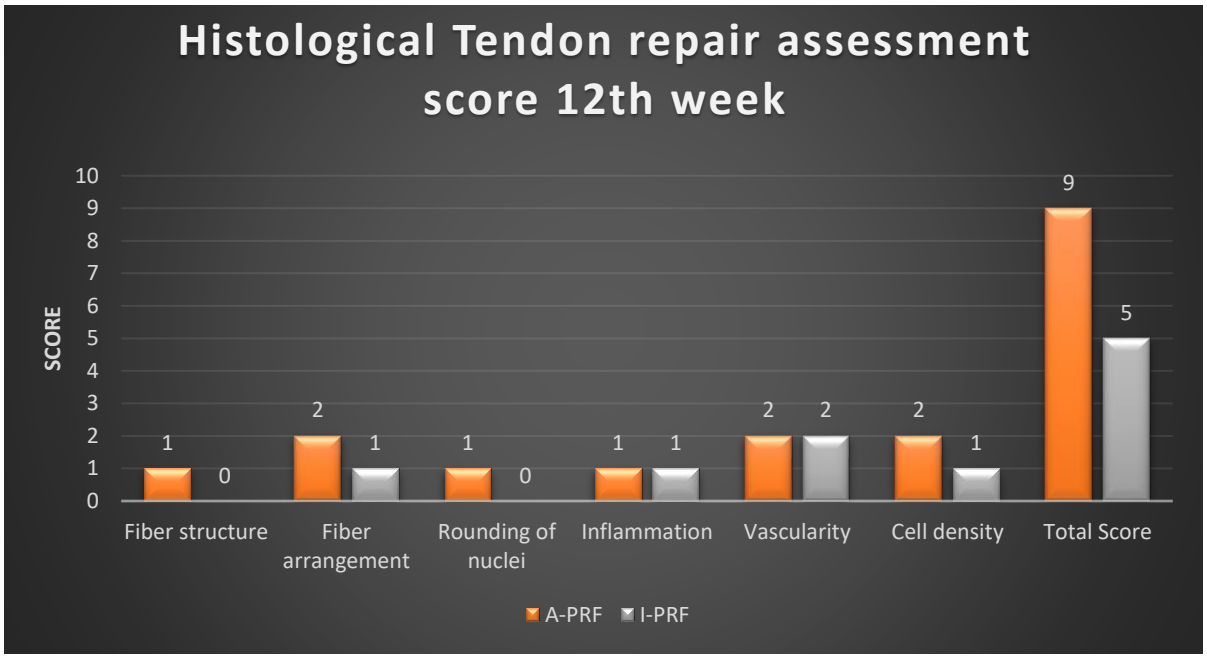

Figure (7): Chart illustrating the differences in the histological assessment score of tendon repair of the A-PRF and I-PRF groups at the end of the study (12th week).

The data of the 6th week observationrevealed that,the histological score of I-PRF was insignificant with the A-PRF group (P=0.105).Thecell density, fiber structure and arrangement had better score in the I-PRF group compared to the A-PRF groupwhile both groups had the same score regarding to the degree of inflammation and neo angiogenesis.

The data of the 12th week observation revealed that, the I-PRF group had significantly better Histological score than A-PRF group (P=0.000).The fiber structure and the number of the rounded nuclear cells was significantly better in the I-PRF group compared to A-PRF group (P=0.000-0.017). there was insignificant difference between both groups regarding the degree of neovascularization and cell density (P=0.141-1.000) but the type of cells contributed had great difference between both groups. Fibroblasts were the common cell type in I-PRF group while rounded nuclear cells were the common cell type in A-PRF group.

In summary, histopathological examination of both groups showed tendon fiber condensation and arrangement with new collagen deposition in all samples of both groups which was higher in the I-PRF treated group compared to the A-PRF treated group.

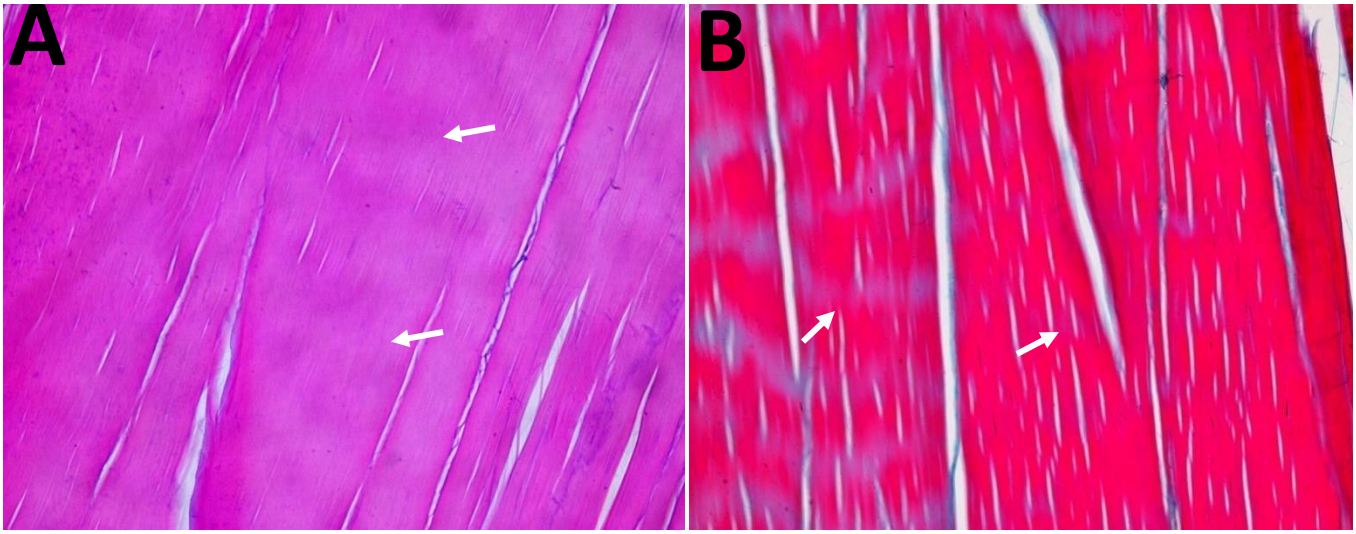

Figure (8): Photomicrograph of normal tendon fibers showing arrangement of highly condensed fibers with very low cellularity. H&E stain 20X **(A)** and Photomicrograph showing normal tendon fibers condensly arranged with high production of collagen fibers marked in Reddish pink colour. MTC Stain 20X**(B)**.

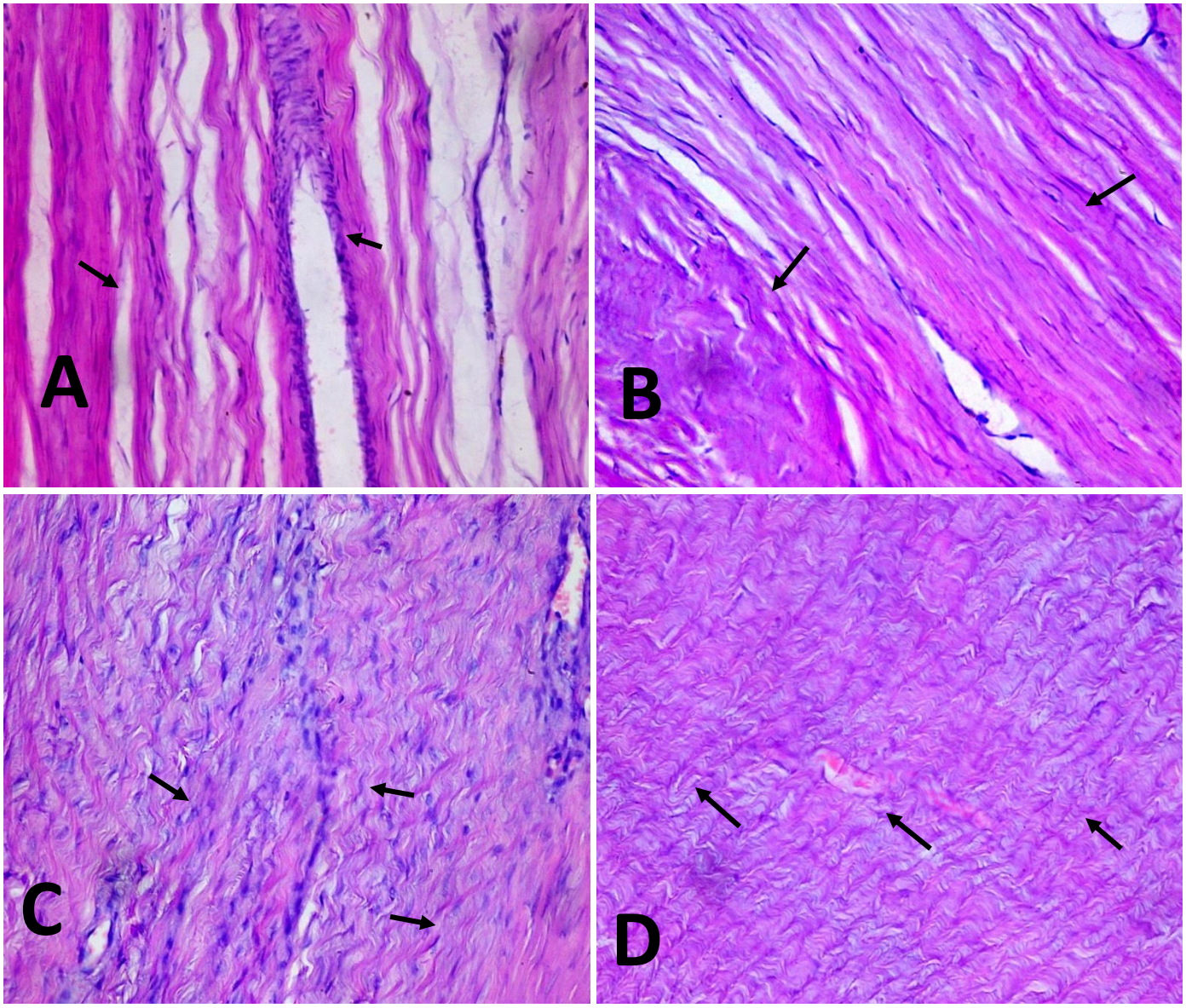

Figure (9):

1. Photomicrograph of tendon defect healing of the A-PRF group after 6 weeks showing arrangement of loose fibers (Black arrows) with high cellularity around blood vessels (white arrows). H&E stain 20X.
2. Photomicrograph of tendon defect healing of the A-PRF group after 12 weeks showing arrangement of loose fibers (Black arrows) with moderate cellularity around blood vessels (white arrows). H&E stain 20X.
3. Photomicrograph of tendon defect healingof the I-PRF group after 6 weeks showing arrangement of dense fibers with high cellularity (white arrows) and newly formed blood vessels (Black arrows). H&E stain 20X.
4. Photomicrograph of tendon defect healing of the I-PRF group after 12 weeks showing arrangement of dense wavy fibers vessels (Black arrows) with low cellularity and darker stained collagen fibers. H&E stain 10X.

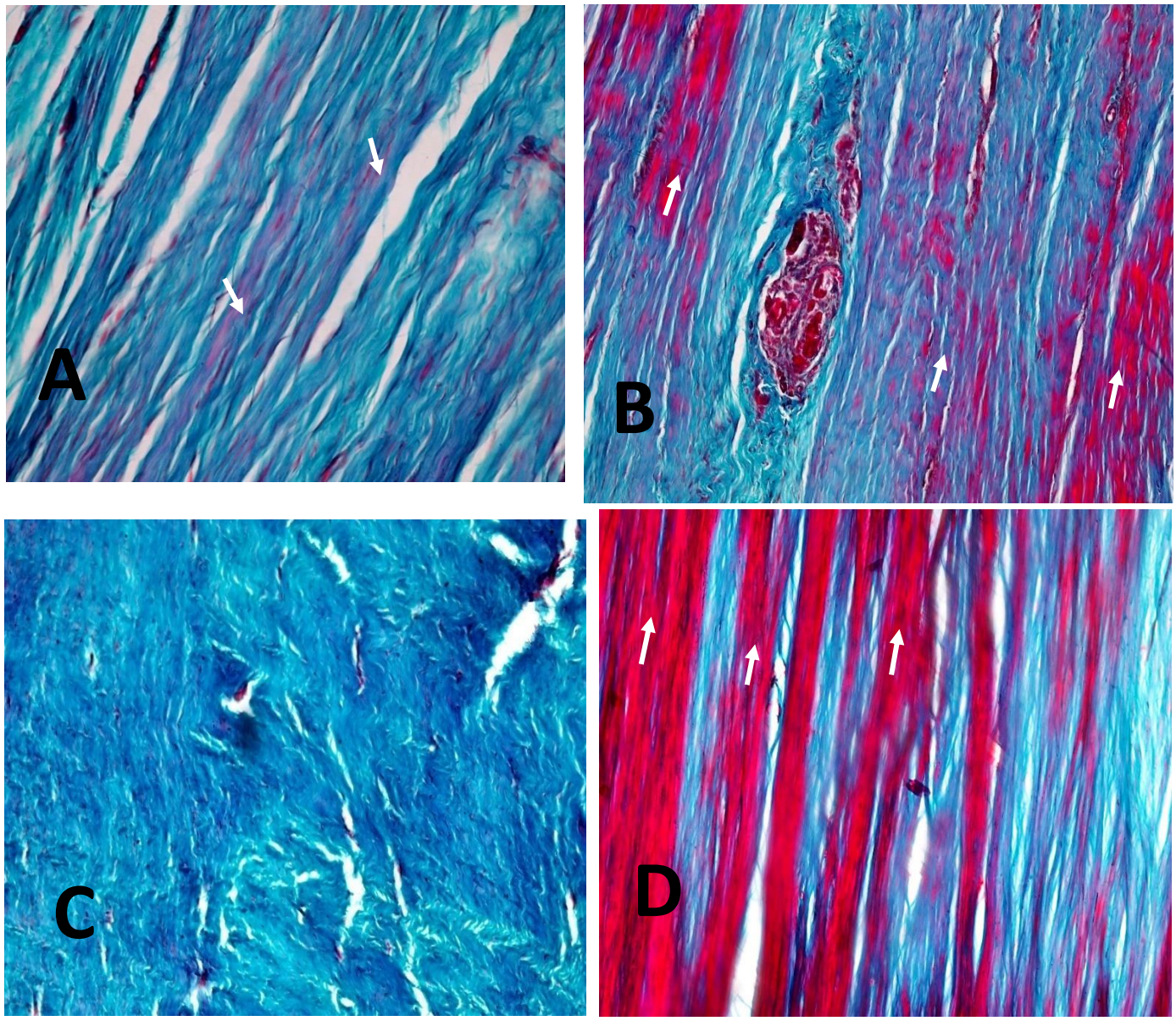

Figure (10):

3. Photomicrograph of tendon defect healing using A-PRF after 6 weeks showing arrangement of condensed hypercellular fibers with mild expression of collagen (white arrows). MTC stain 20X.
4. Photomicrograph of tendon defect healing using A-PRF after 12 weeks showing arrangement of condensed hypercellular fibers with moderate expression of collagen (white arrows). MTC stain 20X.
5. Photomicrograph of tendon defect healing using I-PRF after 6 weeks showing arrangement of highly condensed fiber networks without expression of collagen. MTC stain 10X.
6. Photomicrograph of tendon defect healing using I-PRF after 12 weeks showing arrangement of condensed hypercellular fibers with moderate expression of collagen (white arrows). MTC stain 20X.

DISCUSSION

Several studies reported the superiority of PRF over PRP in the healing process in different tissues becauseit doesn't need anticoagulant during preparation and their prolonged release of GFs over 2-4 weeks after its administration. Moreover, the activation of the platelets in the PRF products occurs by endogenous coagulation cascade (i.e. no need for external activators as calcium or thrombin) as the

autologous thrombin contributes to the direct polymerization of fibrinogen into fibrin, resulting in a 3D flexible fibrin network, in which platelets and WBC are entrapped (Isobe et al. 2017) that's why 2 different versions of PRF (A-PRF and I-PRF) were used in this study to test their efficacy to enhance the healing of the Achilles tendon.

However, many studies reported greater efficacy of PRF over the PRP in the healing process, its preparation as a membrane limits its use in the orthopedic field as it can't be injected intraarticular or in the tendon sheath to get its benefits so it can be applied only once during the surgery that's why a novel method for preparation of I-PRF ready to be injected multiple times was evolved by(Fujioka-Kobayashi et al. 2017). Also, it was confirmed in many studies that, the multiple injections of PRP had greater efficacy on the healing process of different tissues over the single injection (Yurtbay et al. 2021).

Recently, Abd El Raouf, Wang et al. (2019) reported that, the preparation of I-PRF based on the low-speed centrifugation conceptthat allows the formation of I-PRF ready for injection in about 10 minutes that will coagulate after the injection forming the fibrin network inside the injected tissue (that mimics the A-PRF membrane) releasing the GFs, cytokines and inflammatory mediators that have an important role in the healing process. So, in thisstudy, A-PRF membranes were applied once during the surgery in the A-PRF group while the I-PRF was injected during the surgery followed by 2 booster injections with 3 weeks intervals in the I-PRF group to evaluate the efficacy of both of them to enhance the healingand suppress the inflammatory process in the early stage of healing and compare the long-term outcome of the single use of the A-PRF versus the multiple injection of I-PRF on the healing process of the tendon.

The results of this study revealed the ability of both groups to enhance the healing process of the tendonat the early stages of the healing process wherethe clinical observation revealed that,both groups showed mild inflammatory signs at the 3rd week observation also, the thickness of the tendon at the site of the operation was nearly similar at the 3rd week in both groups without significant difference between both groups ($P > 0.05$) and that indicated that both groups had similar effects at the early stage of the healing process. That was supposed to be due to similar amount of the GFs and cytokines released from the first administration of both groups that helped for support/control the inflammatory process, angiogenesis and cellular proliferation (Dietrich et al. 2015).

After 6 weeks, the I-PRF group had lower inflammatory signs and tendon thickness than A-PRF group but with significant decrease in the I-PRF group from the 8th week till the end of the study ($P < 0.05$)and that indicated better support/control of the inflammatory and proliferative process occurred during the healing process in the I-PRF group than the A-PRF group. We postulated that this may be due to higher and continuous release of the GFs in the I-PRF group because of the multiple injection times that acted as booster release of GFs and that agreed with (Yurtbay et al. 2021) when they compared single to multiple injections of PRP in osteoarthritis and reported that the single use enhanced the healing till certain period and its effect started to decrease after that while the multiple injection had continued enhancement of the healing process.

Histological evaluation revealed that the multiple use of I-PRF promoted better healing of the tendon. At the 6th week observation, histological evaluation revealed better cell migration, proliferation and density as well as better arrangement and condensation of the fibers in the I-PRF group than A-PRF group while at the 12th week observation showed higher condensation and arrangement of the fiber content with higher degree of collagen expression and lower cellularity indicating faster healing and regeneration of I-PRF group than A-PRF group and that was theoretically due to the continuous release of the GFs, cytokines and presence of leukocytes that was evident in the I-PRF because of the low-speed centrifugation that prevented the push of these cells and permitted their presence in the I-PRF liquid during its preparation and that agreed with (Ghanaati et al. 2014, Shevchenko and Rublenko 2020).

CONCLUSION

The results of this study revealed the ability of both A-PRF and I-PRF of them to enhance the

healing and suppress the inflammatory process during the healing of Achilles tendon rupture after surgical repair. Also, the study proved that multiple injection of I-PRF had higher efficacy on the long-term outcome on the healing process of the tendon thanthe single use of the A-PRF. I-PRF can be used as a powerful additional therapy to enhance the heeling of tendon affections both in human and veterinary practice. Further future studies are required for longer observation periodeto confirm the ability of I-PRF to improve the healing process and remolding of the tendon to reach its normal structural, mechanical and functional characters of the tendon.

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