EFFECT OF LOW INTENSITY PULSED ULTRASOUND ON REPAIR OF TENOTOMIZED ACHILLES TENDON OF RABBIT

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ABSTRACT

Tenotomized Achilles tendon Rabbits were used to investigate the effect of low-intensity pulsed ultrasound (LPUS) to accelerate the repair of injured tendon. The medial Achilles tendons of 18 local breed male rabbits were transected of all animals without suture, and bandaging was performed to immobilization the injured limb. Nine animals were chosen randomly to receive low-intensity pulsed ultrasound, the other 9 animals served as control. pulsating ultrasound with intensities 500 mW/cm^2 and frequency 2.5 MHz were given over the healing tendons for 10 minute daily, using a gel as the coupling agent between the ultrasound probe and the skin. The healing tendons were evaluated clinically, macroscopically and microscopically for three periods at 7, 14 and 21 days respectively after tenotomy, three rabbits for each period. In the ultrasound-treated group at 14 and 21 days postoperatively, the macroscopic and histological studies demonstrated that the low-intensity pulsed ultrasound enhanced the healing of tendomize Achilles tendon. The ultrasound treated group showed more mature organization at all end points of injured tendons compared with controls.

INTRODUCTION

Primary healing of the tendon takes weeks to acquire the resistance needed to effectively transmit the force generated by a muscle (1). The healing process after a tendon injury is usually difficult and uncertain because the prognosis is influenced by extent of trauma, time elapsed between the trauma and its treatment and low tendon vascularization (2). However, there are drawbacks for this management as it requires a long duration for immobilization, thus risking atrophy and weakness of leg muscles. Researchers have studied non-pharmacological treatment modalities to accelerate tissue repair, including therapeutic ultrasound (US) and low-intensity laser therapy (3).

Recently, there have been an increasing number of reports suggesting that therapeutic ultrasound promotes tendon healing after open repair. (4). which demonstrated the stimulus from ultrasound in relation to angiogenesis, increased blood circulation, accelerated healing of ulcers, accelerated repair of fractures with

retarding of consolidation and pseudo-arthrosis, accelerated activation of macrophages and healing of tendons (1). Biophysical effects of ultrasound are traditionally separated into 2 types: a thermal effect from absorption and a non-thermal effect from scattering. The absorption of the ultrasonic sound energy leads to tissue heating. The scattering is thought to be that portion of ultrasonic energy that changes direction and leads to the non-thermal effects (5). Non-thermal effects have been divided by Ter Haar into cavitations and other mechanical effects such as acoustic streaming and micro streaming (6). US therapy has been used to treat musculoskeletal injuries, particularly in tissues with a high percentage of collagen fibers (7).

The dose-response of therapeutic US is influenced by many variables, including frequencies, intensities, irradiation times, application modes, type and coupling techniques, and early post-injury interventions (8). The US application time influences the amount of energy applied to the tissue, which is calculated by the following formula: Energy (Joules) = Power (Watts) * Time (Seconds) (9).Therefore the objective of the present study was to investigate the efficacy of pulsed therapeutic ultrasound on acceleration of tendon repair in animals subjected to partial tenotomy.

MATERIALS AND METHODS

Experimental Animals:

Eighteen healthy, adult local breed male rabbits (2-3 kg) were used. Animals underwent a preliminary adjustment period (10 days), when they received a balanced diet and water *ad libitum*; they were individually housed in standard room temperature (22 ± 3C˚) throughout the experiment.

Inducing of Partial Tenotomy:

After withholding food for six hours and water for three hours, animals received ketamine (35 mg/kg, intramuscularly), and xylazine (5 mg/kg, intramuscularly) as dissociative anesthesia (10).

The dorsal region of the leg around the Achilles tendon was shaved and prepared with povidine iodine solution. To create a partial tenotomy of the Achilles tendon, a longitudinal skin incision was made on the dorsal aspect above the Achilles tendon and the skin was retracted to expose the Achilles tendon. By blunt dissection, the medial and lateral portions of the Achilles tendon and the plantar tendon were identified and separated with a probe (fig.1). The medial Achilles tendon was then transected at the 1 cm above calcaneal insertion with the lateral Achilles tendon left intact in order to prevent retraction of the severed ends (4) (fig.2). The severed ends remained unsutured during healing, and skin suture were performed. After cleaning the surgical wound, a protective bandage was applied. The animals were kept in cages to limiting movement of animals.

Experimental Design:

The animals were randomly divided into 2 experimental main groups (treated and control) 9 animals for each group, the main group divided into 3 subgroup. Each subgroup corresponded to the day of evaluation after the ultrasound application: 7^{th}, 14^{th}, and 21^{th} days post-surgery. Animals underwent daily ultrasound therapy.

pulsating ultrasound with intensities **500** mW/cm^2 and frequency **2.5** MHz were given over the healing tendons for **10** minute daily, using a gel as the coupling agent between the ultrasound probe and the skin (fig.3).. The healing tendons were evaluated macroscopically and microscopically at **7**, **14** and **21** days after tenotomy.

Pathological Study:

Macroscopic and microscopic examinations of the healing tendons were performed on all rabbets at 7, 14, 21 days after treatment. Nine rabbets used for each group and three rabbets for each period.

On the day of biopsy the animals were subjected again to the anesthetic and surgical procedures as described before. Approximately 10 mm Specimens were harvested containing injured tendon. Macroscopic alterations of tissue repair were evaluated at the moment of tendon collection, considering stump coaptation, adhesion formation, and sliding.

The tendon specimen was fixed in 10% neutral buffered formalin for 48 hours, then washed, dehydrated in a serial graduated alcohol, cleared in xylol, embedded in paraffin wax, sectioned at 5 microns thickness and stained with Hematoxylin-Eosin (H and E) stain, and examined under light microscope (11). Each segment was morphologically analyzed for healing pattern

Several sections were cut from each paraffin-embedded portion, and the sections which appeared to have been least distorted by the preparation procedures were selected for cross-sectional area measurement, and each section was measured. Measurements were made using a planimeter on photomicrographs (12).

Statistical Analysis:

The results were expressed as Means and Standard Errors (M±SE). The data were analysis by one way Analysis of Variance (ANOVA). Differences between the mean treatment of each group, and at each time analyzed, using least significant differences (LSD).The P value <0.05 or 5% was considered statistically significant (13).

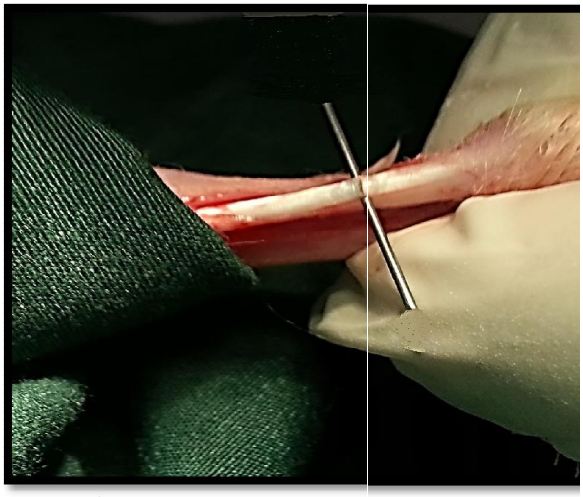


Fig.1- Medial and lateral portion of the Achilles tendon and the plantar tendon was identified and separated with a probe.

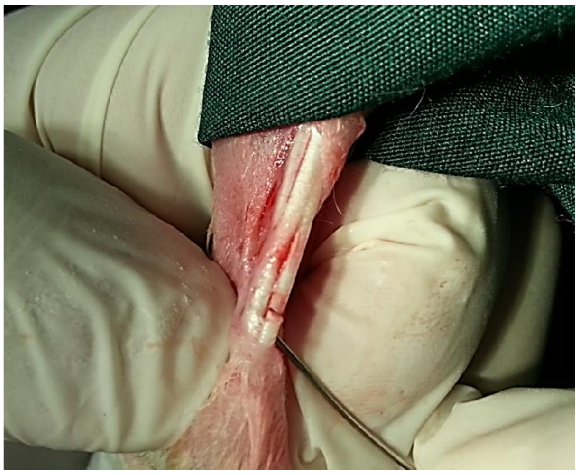


Fig.2- transecting of medial Achilles tendon at the 1 cm above calcaneal insertion.

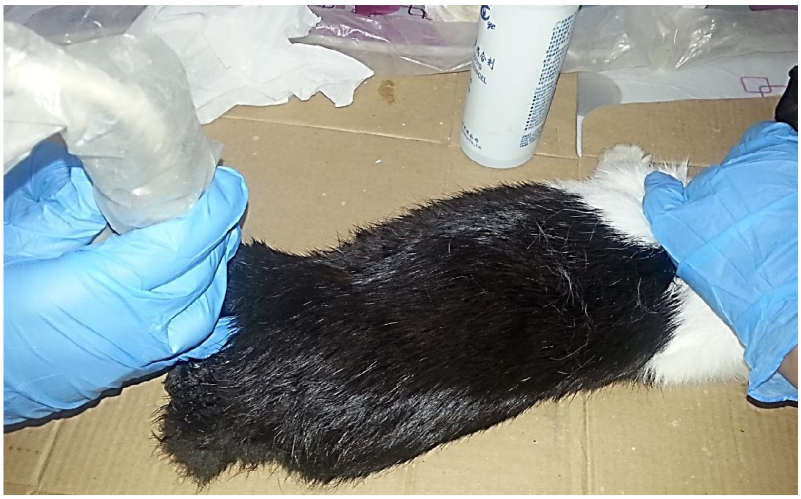


Fig.3- pulsating ultrasound was given over the healing tendons for 10 minute daily, using a gel as the coupling agent between the ultrasound probe and the skin.

RESULTS

Clinical Observations:

Initially the cardinal signs of inflammation (i.e. swelling, heat, pain and redness) were seen locally at the site of wound at some hours post induced wounding. It was more obvious and increased after 12 hours, due to increase the bacterial colonization. These signs were gradually subsided after treatment in all groups except one case of

wound necrosis in non-treated group. All animals after tenatomy become lame for 48-72 hours with exception of 3 animals in non-treated group extended for 10 days.

Macroscopic Evaluation:

Complete tendon separation occurs in 1 case of **7** days control subgroup (fig.4) and wound infection was observed in 1 rabbits of treated group at 7days period (fig.5). There was almost no macroscopic difference between two groups in terms of peritendinous adhesion and hypertrophy at 7days perod.

At 14 days, the laceration sites of the tendons appeared hypertrophic and semitranslucent, and the dense peritendinous adhesion was the most prominent in control groups (fig.6, fig.7).

At 21 days, the laceration sites were less hypertrophic and the peritendinous adhesion around the laceration sites in treated group than control group.

The cross-sectional dimensions of the laceration sites showed significant difference between treated and control groups at this time point ($p>0.05$) (Table 1).

Table (1). Effect of ultrasound application on cross sectional area (mm^2) of injured tendon compared with control group.

Group / Days	Control group	Treated group
0	3.15 ± 0.25	3.15 ± 0.25
7	12.14 ± 2.40*	10.55 ± 0.30*
14	11.11 ± 1.10	8.50 ± 1.40
21	8.25 ± 0.10*	4.65 ± 1.00**

Values are expressed as mean ± SE; N = 3 animals in each group; Test used: ANOVA, *** refer to a significant differences P<0.05,** within group, **** refer to a significant differences P<0.05**, between the control group and treated group.

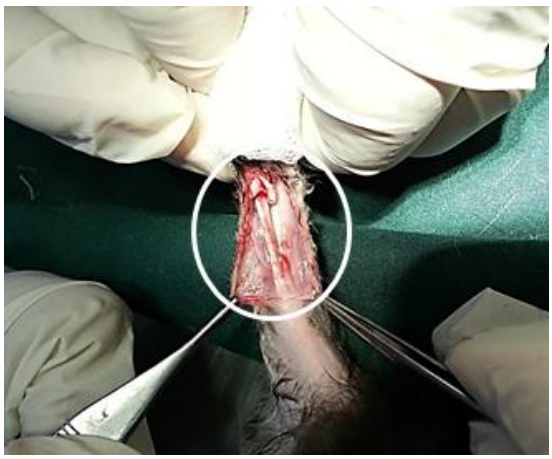


Fig.4– Achilles tendon, macroscopic feature of control Group, 7th day post operation show complete tendon separation.

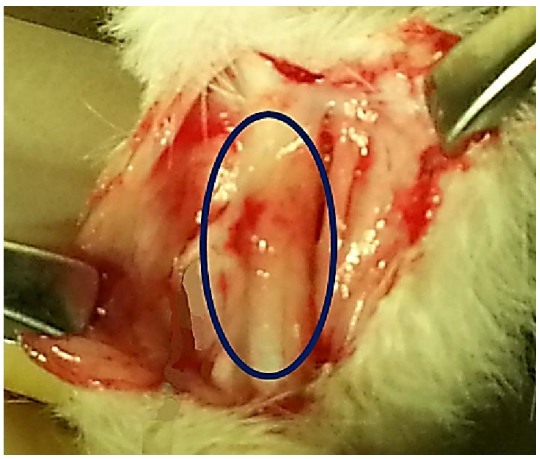


Fig.5– Achilles tendon, macroscopic feature of treated Group, 7th day post operation show tendonitis.

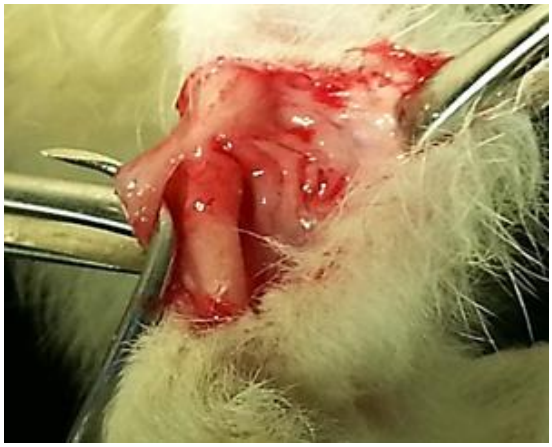


Fig.6– Achilles tendon, macroscopic feature of Control Group, 14th day post operation reveal's the laceration sites of the tendons and show hypertrophic and semitranslucent, and the dense peritendinous adhesion was the most prominent in control group.

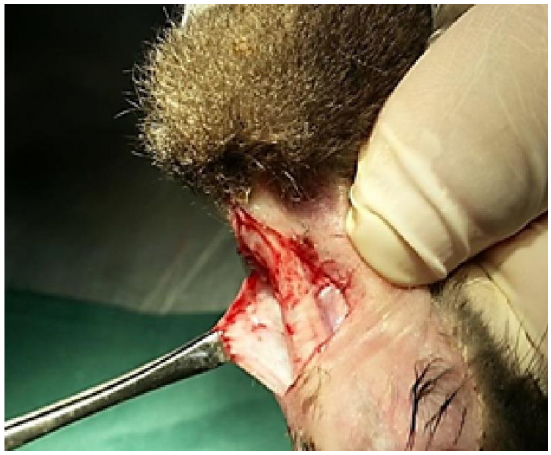


Fig.7– Achilles tendon, macroscopic feature of Treated Group, 14th day post operation reveal's the laceration sites of the tendons and show less hypertrophic and adhesion was less than control group.

Control Group:

Seven day post-injury the area between the cut ends was filled with loose connective tissue matrix, predominant active fibroblasts with large nuclei, neovascularization and infiltration of high population of mononuclear inflammatory cells(fig.8), the communication between the injured ends is very loose and easily stretched.(fig.9)

At fourteen days the gap of injured tendon filled with granulation tissue combined with abnormal hyalinaized collagen fibers (fig.10, fig.11).

Twenty one days period in relatively immature sections, the abnormality of collagen tissue was clearly appeared, with high number of fibroblasts, inflammatory cells and blood capillaries (fig.12, fig.13).

Treated Group:

Seven days period, the area between the cut ends was filled with loose connective tissue matrix, predominant active fibroblasts with large nuclei, neovascularization and infiltration of high population of mononuclear inflammatory cells (fig14), the communication between the injured ends is very loose and easily stretched(fig15).

Fourteen days period, the collagen tissue was of more density, some degree of organization of bundles with fewer fibroblasts and blood vessels without any collagen abnormalities (Fig.16), the injured ends was attached with wide point (Fig.17).

Twenty one days period, the hitopathological feature at this period is the complete replacement of injured tendon with relatively mature collagen fibers as regular orientation and the presence of extracellular matrix between collagen fibers with few blood capillaries (fig.18). Section revealed band of collagen fibers arranged in a wave-like fashion with few scattered fibroblasts (fig.19) which considered as an

indicative for organized healing, there is complete communication between the ends of injured tendon (fig.20).

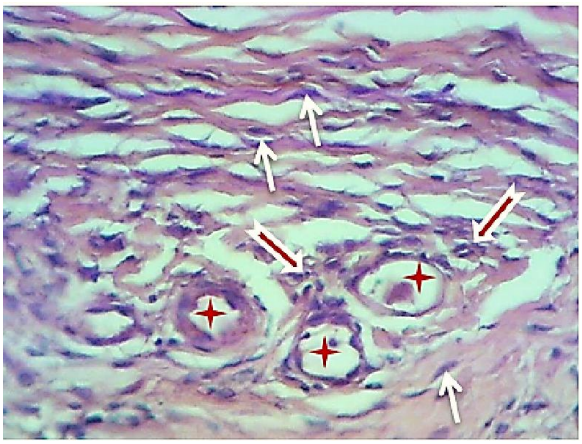


Fig. 8 - Achilles tendon, microscopical picture of control group, 7days post wounding, the area between the cut ends was filled with loose connective tissue matrix, predominant active fibroblasts with large nuclei(thin arrows), neovascularization (stares) and infiltration of high population of mononuclear inflammatory cells (thick arrows) (H&E 40X).

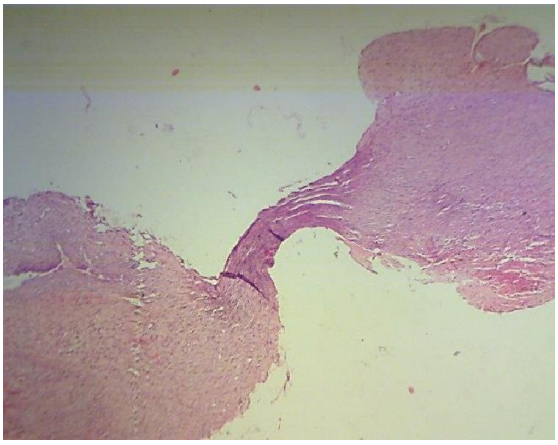


Fig. 9 - Achilles tendon, microscopical picture of control group, 7days post wounding, the area between the cut ends was filled with loose connective tissue matrix, and the communication between the injured ends is very loose and easily stretched. (H&E 5X).

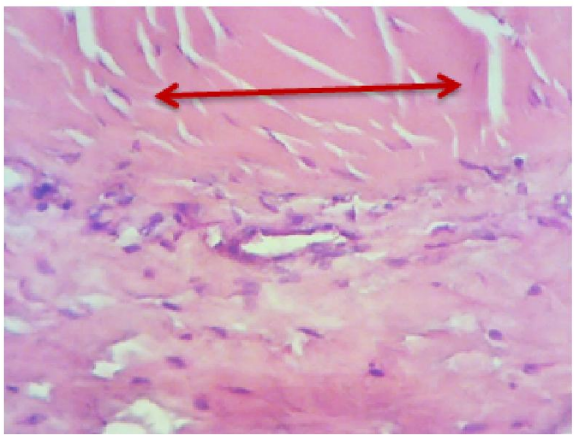


Fig. 10 - Achilles tendon, microscopical picture of control group, 14 days post wounding, area between the cut ends was filled with loose connective tissue matrix combined with abnormal hyalinaized collagen fibers (arrow) (H&E 40X).

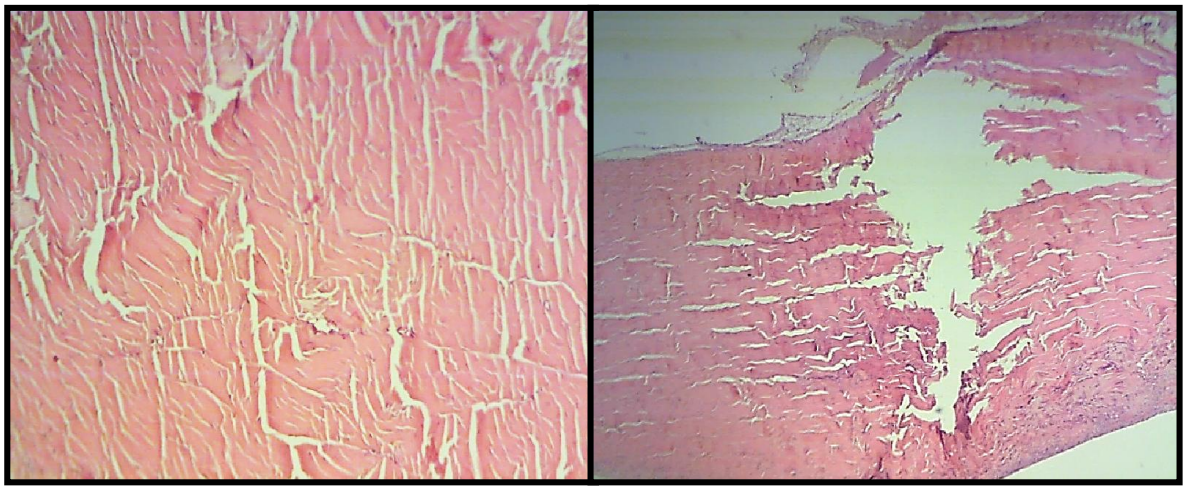


Fig. 11 - Achilles tendon, microscopic picture of control group, 14 days post wounding, the area between the cut ends was filled with hyalinized and thickened collagen bundles as well as their multidirectional growth (immature collagen fibers) (H&E 10X).

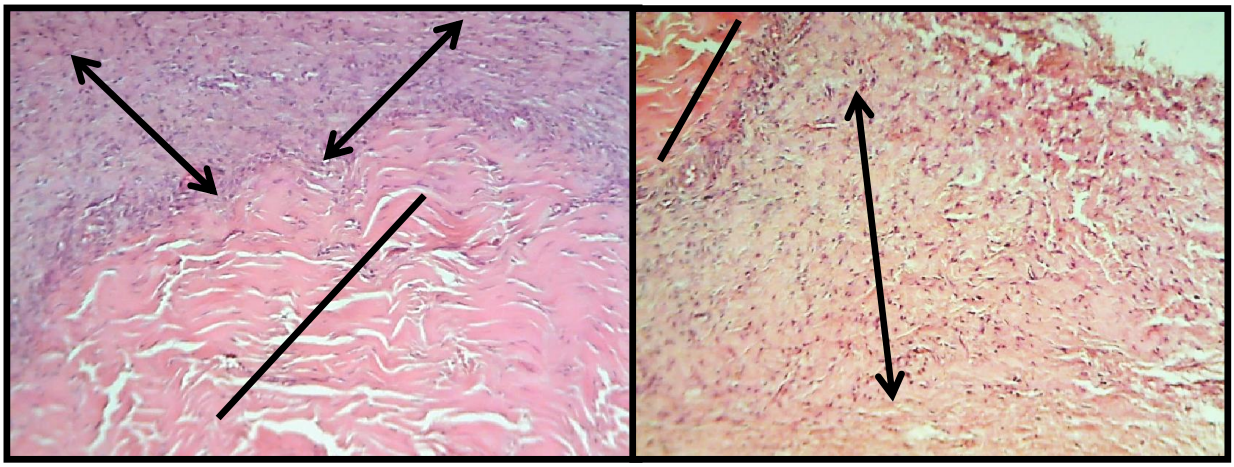


Fig. 12 - Achilles tendon, microscopic picture of control group, 21 days post wounding, the area between the cut ends was filled with relatively immature sections, the abnormality of collagen tissue was clearly appeared (line), with high number of fibroblasts, inflammatory cells and blood capillaries (arrows) (H&E 20X).

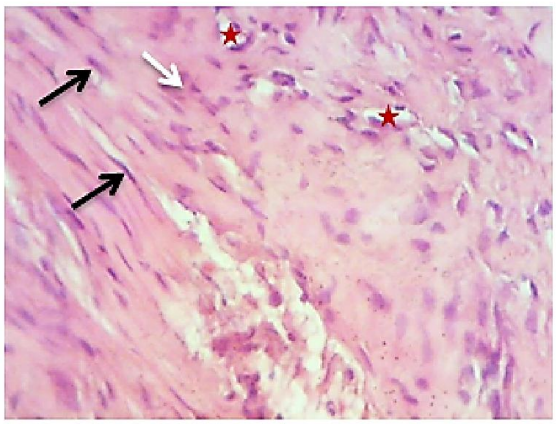


Fig. 13 - Achilles tendon, microscopic picture of control group, 21 days post wounding, show's infilling of the area between the cut ends with proliferation of cellular loose connective tissue infiltrated by large number of inflammatory cells (white arrow) and blood capillaries (stares) with fibroblasts (black arrows) (H&E 40X).

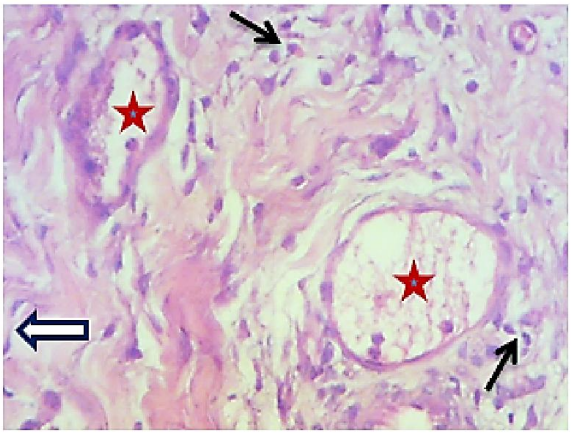


Fig. 14 - Achilles tendon, microscopic picture of treated group, 7 days post wounding, show's infilling of the area between the cut ends with proliferation of cellular loose connective tissue infiltrated by large number of inflammatory cells (white black arrow) and blood capillaries (stares) with fibroblasts (white arrows) (H&E 40X).

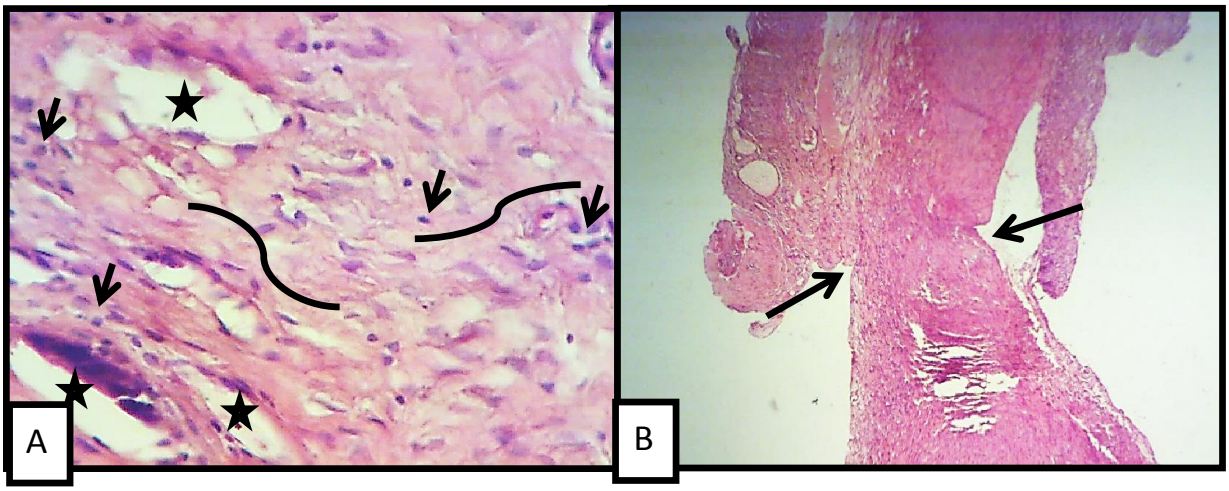
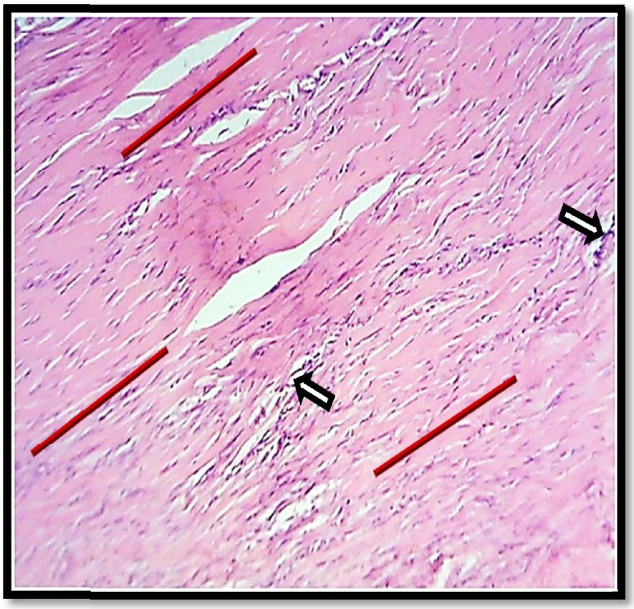


Fig. 15 - Achilles tendon, microscopic picture of treated group, 7 days post wounding, show's, A: infilling of the area between the cut ends with proliferation of cellular loose connective tissue which composed from blood capillaries (stars) , large cells population (arrows) and immature connective tissue(carve lines) , B: the communication between the injured ends is very loose (arrows) and easily stretched.(H&E A:40X, B:5X).



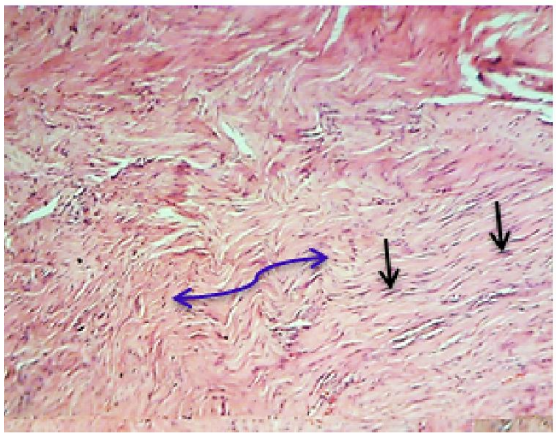


Fig. 19 - Achilles tendon, microscopic picture of treated group, 21 days post wounding, this Section shows bands of collagen fibers arranged in a wave-like fashion (carved arrow) with scattered fibroblasts (arrows) (H&E 20X).

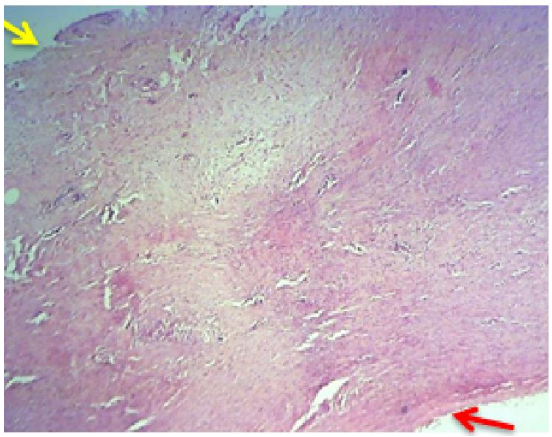


Fig. 20 - Achilles tendon, microscopic picture of treated group, 21 days post wounding, this Section shows complete communication between the ends of injured tendon (arrows) (H&E 10X).

DISCUSSION

Information in table (1) refers to the mean thickness of lacerated area was calculated on $7^{th,}$ 14^{th}. 21^{th}, day post tenotomy. On day "0" the area of wound in mm was 3.15 ± 0.25, in all groups.

The mean thickness in treated group on 21^{th} Day was 4.65 ± 1.00 mm showed significantly reduced ($p < 0.05$) tendon thickness area with respect to the control group 8.25 ± 0.10.

The initial collagen threads are reabsorbed and deposited thicker and organized along the stress lines. These changes are also accompanied by a wound with an increased tensile strength, indicating a positive correlation between collagen fiber thickness/orientation and tensile strength (14).

Study by Lu, *et.al.,* (2009)(15). At 2 weeks postoperatively, the mean maximal tensile strength in the ultrasound-treated group (30.676 N) was significantly stronger ($P<.05$) than the control group (17.924 N).

On the other hands, Larsen, *et. al*., (2005)(16) the extensibility of the healing tendons was greater after sonication at intensity of 2000 mW/cm2 than after 50 mW/cm2.

A study by GY, N. (2011)(17) reports for the *in vivo* tendon repair, in exploring the effects of therapeutic ultrasound and microamperage stimulation (MAS) on the biomechanical performance of repairing Achilles tendon in rats, ultrasound is considered to be more efficient than MAS for improving the strength of the repairing tendons.

In the present study and in the control group, the wound gap filled with highly cellular loss connective tissue and the wound still exhibited obvious inflammation and incomplete communication between the ends of injured tendon.

An Evident deterioration in the arrangement of collagen fibers and fibroblast progression into the incision area were seen. Throughout the tenotomy healing area, disorganized tendon structure and inflamed cells were evident. Neovascularization was conspicuous in all specimens.

Many sections in this group revealed abnormal hyalinized and thickened collagen bundles as well as their multidirectional growth.

Despite the fact that collagen synthesis continues at a higher rate, no further increase in scar mass occurs. At this point new collagen is created and old collagen is broken down in a balance fashion with the aid of an enzyme collagenase (18).

The collagen deposition at the 21^{th} day still remained in irregular direction in control group and this give an indication of immature connective tissue formation, this result was inaccordence with (19), who stated that collagen turnover allows the randomly deposited scar tissue to be arranged, in both linear and lateral orientation, while in treated group, collagen fibers impacted and assumed linear direction and appears as a wave-like fashion with disappear of blood capillaries, this means there is significant wound healing . This process continues until the remodeling phase end at months post injury. The research indicates that tensile strength is mainly dependent on the fiber orientation & thickness of laminated polymer composites (20**)**.

Study by Demir *et al*., (2004)(21). Indicated the duration of the inflammatory phase decreased with ultrasound treatments; with more significant results. The proliferation phase showed an increase in the level of hydroxyproline and the number of fibroblasts, as well as stimulation of the collagen synthesis and the composition.

The microscopic findings in the present study resembled the normal repair of wound which is divided into three stages that mentioned by several authors (22*,* 23). The first stage, is a period of cellular activity include autolysis, phogocytosis and

accelerated production of mucopolysaccharid, the second stage, fibroblastic stage, characterized by formation of granulation tissue, collagen production, and stage three differentiations which include maturation of collagen fibers and scar tissue formation.

In conclusion, the results obtained from this study reflected accelerated healing of the induced tenotomy by applying of ultrasound therapy which was highlighted by the clinical observation, macroscopic and microscopic evaluation. The enhanced capacity of tendon repair could be explained on the basis of Ultrasound stimulation of types I and III collagen expression of tendon cell and upregulation of transforming growth factor beta and stimulation of tendon cell migration that are documented in the literature.

تأثيرالامواج فوت الصوتية النبضية واطئة الشدة على أصلاح وتر العرقوب المقطوع في الارانب

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الخلاصة

أستخدمت في هذه الدراسة الارانب مقطوعة وتر العرقوب للبحث عن تأثير الامواج فوت الصوتية النبضية منخفضة الشدة على شفاء الاوتار المتضررة. تم قطع وتر العرقوب الانسي لثمانية عشر من ذكور الارانب المحلية البالغة بدون خياطة مع استعمال اللفاف لتثبيت الطرف المتضرر. تم اختيار تسع حيوانات بشكل عشوائي للعلاج بالامواج فوت الصوتية النبضية منخفظة الشدة، اما التسع الباقية من الحيوانات استخدمت كمجموعة سيطرة. تم علاج الاوتار المقطوعة بالامواج فوت الصوتية بشدة ٥٠٠مل واط/سم^{٢} وبتردد ٢,٥ميغا هرتز لمدة ١٠ دقائق يومياً، حيث استخدم الهلام كمادة موصلة بين جلد الحيوان ومقبض الامواج فوت الصوتية. شفاء الوتر المقطوع قييم سريريا، عيانيا ونسجيا على ثلاث فترات في اليوم ٧ و ١٤ و ٢١ على التوالي بعد قطع الوتر بواقع ثلاث حيوانات لكل فترة. حيث اظهرت الدراسة العيانية والمجهرية للمجموعة المعالجة وبالاخص خلال الفترة ١٤ و٢١ يوم بعد القطع ان الامواج فوت الصوتية سرعت من شفاء الاوتار المقطوعة، وان نهايات الاوتار المقطوعة في المجموعة المعالجة كانت اكثر نضجا وتخصصا مقارنتا بمجموعة السيطرة.

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