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Effect of curcumin-loaded polycaprolactone scaffold on Achilles tendon repair in rats

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Abstract

Scaffolds play a crucial role in tendon healing by providing structural support, promoting cell infiltration, and guiding tissue regeneration. Polycaprolactone (PCL) has been used as a polymer in biological scaffolds for several tissue engineering studies. This study aimed to investigate the effects of curcumin-loaded PCL scaffold on Achilles tendon using a tenotomy model in rats. Twenty adult male Wistar rats were randomized into two groups. In control group, tenotomy and suture placement were performed. The identical intervention followed by the implantation of curcumin-loaded PCL scaffold around the tendon stumps was performed in the treatment group. The nanofibrous PCL scaffold containing 5.00% curcumin was fabricated by electrospinning. Walking track analysis was performed weekly. Then, after 6 weeks, histopathological examination and tendon mechanical tests were performed. The weekly walking track analysis revealed a significant improvement in Achilles functional index in scaffold-treated rats from week three to six. The rate of functional improvement was remarkably slower in the control group. Histopathological examination revealed aseptic inflammation and enhanced neovascularization in the treatment group. Also, collagen arrangement and density were significantly improved in this group compared to the control samples including less regular orientation and loose organization of collagen fibers. Significant increase in mechanical properties, except for strain, was observed in the treatment group. The present study demonstrated that implantation of curcumin-loaded PCL scaffold resulted in increased fibrillar architecture, as well as improved mechanical properties and Achilles functional index in rats. To reduce the biodegradation-induced inflammation, an anti-inflammatory treatment is recommended.

Introduction

Achilles tendon is the strongest tendon in the body; however, it is more prone to tears and its rupture often occurs during sports activities or due to a sharp trauma.[1] Surgical intervention with the purpose of suture placement is the only treatment of choice to reconstruct the tendon which, of course, is not associated with complete success.[2] The loss of tensile strength of the tendon following the surgery is a common post-operative complication,[3] reducing its functionality and also increasing the possibility of re-rupture.[4]

Scaffolds are a crucial component in tissue engineering, providing a three-dimensional framework for cells to attach, grow, and differentiate into functional tissue. They can be categorized into biological, synthetic, and hybrid types, each with its own advantages and applications, such as mimicking the extra-cellular matrix, providing specific mechanical and biological cues, and combining elements of both natural and synthetic materials. The choice of scaffold material and fabrication technique depends on the specific tissue engineering application and desired properties of the scaffold, such as mechanical strength, biocompatibility, and biological cues.[5]

Scaffolds offer a promising solution to the challenges associated with tendon tissue engineering. In fact, scaffolds play a crucial role in providing mechanical support and creating a conducive environment for tendon repair and regeneration.[5]

Due to the complicated manufacturing process of biological scaffolds, including decellularization and sterilization, as well as their poor mechanical properties, the use of

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synthetic ones is progressively interested. Synthetic scaffolds may lack some of the intrinsic biochemical cues found in natural extra-cellular matrices, necessitating the incorporation of biologically active molecules to achieve optimal performance.[6]

Among the different types of biodegradable synthetic polyesters, polycaprolactone (PCL) has been widely used in medical studies, especially in the field of wound healing and tissue engineering.[7] The products of its enzymatic breakdown are easily metabolized and excreted by the body.[8] The PCL nanofibers in the form of a sheet, in addition to act as a physical barrier, have also the ability to deliver pharmaceuticals; thus, they can play a more crucial role in improving the wound healing process.[7] In previous studies, the combination of this polymeric scaffold with essential oils, growth factors, antibiotics, nanoparticles, and bioactive compounds has been investigated to accelerate wound healing.[9-13] Among these compounds, curcumin has become a source of significant interest due to its anti-oxidant, anti-inflammatory, anti-bacterial, and cyto-protective properties.[14-16] A previous study indicated the beneficial effects of curcumin on different stages of the healing process through increasing granulation tissue formation, collagen deposition, and angiogenesis, collagen fibers rearrangement, and elevated wound contraction.[17] Reportedly, curcumin accelerated wound healing by decreasing the production of reactive oxygen species and increasing cellular proliferation.[18]

This study aimed to investigate the potential use of curcumin-loaded PCL scaffold on the healing of experimentally tenotomized Achilles tendon injury in rats.

Materials and Methods

Study design. In this experimental animal study, 20 adult male Wistar rats weighing 200.00 ± 25.00 g were used. The rats were kept in pairs in plastic cages under standard environmental conditions of temperature (22.00 - 26.00 ˚C), relative humidity (50.00 - 60.00%), and natural dark/light cycle. They were fed with standard commercial rodent chow and water *ad libitum*. After a week of adaptation, the animals were randomly divided into two equal groups (n = 10). All the experimental protocols used in the current study were approved by Urmia University Ethics Committee, Urmia, Iran (No. IR-UU-AEC-3/65).

Fabrication of curcumin-loaded PCL scaffold. The preparation of nanofibers was carried out in the Shaya Standard Nano Technologies Company (National ID: 14009867750, Registration No. 52983) located in East Azarbaijan Science and Technology Park (Tabriz, Iran). For this purpose, PCL granules (Sigma-Aldrich; St. Louis, USA) with a molecular weight of 80,000 g mol^{-1} were dissolved in a two-solvent system of acetic acid and formic acid (Sigma-Aldrich) in a 70:30 ratio and then, gradually curcumin (Sigma-Aldrich) was added to the prepared polymer solution at a ratio of 5.00% (w/w).[19] In the next step, the polymer solution was loaded into a 5.00 mL syringe and driven through a metallic needle at a constant feed rate by a syringe pump. The parameters of the electrospinning machine were set in the following conditions: Tip-to-collector distance = 210 mm, voltage = 19.00 kV, nozzle diameter = 16-G, mass flow rate = 0.90 mL *per* hr, and collector rotation speed = 440 rpm. The electrospinning process was carried out at room temperature, and after the electrospinning process was completed, the foil containing the electrospun nanofibers was placed in an oven at a temperature of 37.00 ˚C for 48 to 72 hr to evaporate the solvent.[20] A scanning electron microscope (Tescan, Brno, Czech Republic) was used to observe the morphology and structure of scaffolds. A small piece of the scaffold was covered with a thin layer of gold and then, imaged on the electron microscope stage under vacuum and a potential difference of 15.00 kV with a magnification of 2,500×. The average fiber diameter was estimated using Digimizer image analysis software (MedCalc Software bvba, Ostend, Belgium). For this purpose, the diameter of 50 fibers was measured in the image of the nanofiber network.

Surgical procedure. General anesthesia was induced by intra-peritoneal injection of 75.00 mg kg^{-1} ketamine (Alfasan, Woerden, The Netherlands) and 5.00 mg kg^{-1} xylazine (Kela Lab, Hoogstraten, Belgium). The left hind limb of each rat was shaved and aseptically prepared. In the control group, the Achilles tendon was exteriorized through a 1.00 cm surgical incision on the posterior aspect of the leg. The Achilles tendon was carefully dissected free from the surrounding connective tissues and completely transected using a scalpel blade at its midpoint. The tendon stumps were immediately sutured with a modified Kessler pattern using 4-0 nylon (Supa, Tehran, Iran). Finally, the area was irrigated with normal saline solution, and the skin was sutured in a simple interrupted pattern using 3.0 monofilament nylon (Supa). In the treatment group, the two ends of the cut tendon were sutured and then, curcumin-loaded PCL scaffold was wrapped around the tendon before the skin closure.

Walking track analysis. The Achilles functional index (AFI) was recorded at the end of each week after surgery for 6 weeks based on the method of walking track analysis proposed by Murrell *et al.*[21] For this purpose, the hind paws of rats (n = 5) were wet with ink and they crossed a paper walkway (100 cm length and 10.00 cm width) to record the paw prints. The AFI was then determined using the Murrell's formula as follows:

$$AFI = 74(PLF) + 161(TSF) + 48(ITF) – 5$$

where, PLF is the print length factor, TSF is the toe spread factor, and ITF is an intermediate toe factor representing the difference between the experimental and contralateral print measurements.

Sampling. The rats were kept for 6 weeks after surgery and then, euthanized by anesthetic over-dose using intra-peritoneal injection of 300 mg kg^{-1} ketamine and 30.00 mg kg^{-1} xylazine. Then, the skin was opened and the tendons were harvested for further studies. For biomechanical evaluation, the tendons were harvested with the calcaneal bone and gastrocnemius muscle (n = 5). The samples were kept in a sterile gauze soaked with normal saline in – 20.00 ˚C until the test. Five samples from each group were collected for histopathological evaluation.

Histopathological examinations. Tendon samples (n = 5) in 10.00% formaldehyde solution underwent routine histological processing. Five-micron thick sections were longitudinally cut in the direction of the tendon fibers and then, stained with Hematoxylin and Eosin for histo-pathological analysis by light microscopy (Nikon, Tokyo, Japan). The slides were examined through a modified semi-quantitative scoring using Curtis and Delee's grading system. The degree of inflammation, neovascularization, and collagen fiber sequencing parameters were used in this grading system.[22] The degree of inflammation was rated as follows: None (0), mild (1), moderate (2), and pronounced (3). Neo-vascularization was rated based on the number of capillaries *per* high-power magnification field. Less than five was rated as mild (1), 5 to 10 rated as moderate (2), and greater than 10 rated as pronounced (3). Collagen fiber sequencing was rated as follows: Scattered (1), slightly regular (2), and regular (3).

Biomechanical evaluation. The biomechanical properties of the samples (n = 5) were evaluated by tensile testing (STM-5; Santam, Tehran, Iran) using a 20.00 kg load cell. The samples were thawed at room temperature before testing. Then, suture materials were removed, and samples were mounted on a tensile device. The constant velocity of 60.00 mm *per* min was used for the test.[23] A stress-strain curve was recorded during the test, and the following parameters were recorded: Maximum load (N), load at yield point (N), maximum stress (MPa), maximum strain (%), energy absorption (J), and stiffness (MPa).

Statistical analysis. The results of the biomechanical evaluation were analyzed using one-way analysis of variance (ANOVA) and Tukey *post hoc* test. Two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test was used for the AFI in SPSS Software (version 21.0; IBM Corp., Armonk, USA). Scores of histopathological assessments were analyzed with Mann-Whitney test in Minitab Software (version 16.0; Minitab Inc., Boston, USA). A significance level of 0.05 was considered as a statistically significant difference.

Results

Scaffold characteristics. The structural properties and orientation of nanofibers are shown in Figure 1A. Based on the analysis of electron micrographs, the mean diameter of the fibers was 230 ± 50.00 nm, and the minimum and maximum diameters of the fibers were measured as 127 and 390 nm, respectively (Fig. 1B).

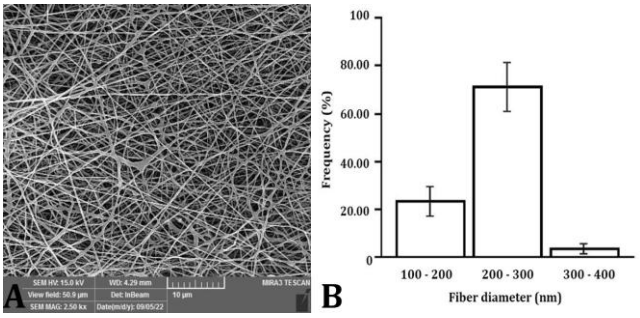

Fig. 1. Characterization of curcumin-loaded polycaprolactone scaffold. **A)** Scanning electron micrograph of electrospun polycaprolactone nanofibers containing 5.00% curcumin. **B)** Diameter distribution of nanofibers in the scaffold. The average diameter of the fibers was 230 ± 50.00 nm (minimum: 127 and maximum: 390 nm).

Achilles functional index. As shown in Figure 2A, the injured hind paw prints of rats in the control group were markedly longer and narrower than those in the treatment rats during post-operative assessments. A partial recovery in the 3rd week was observed in the rats of the treatment group, evidenced by raising their heel and weight bearing on their toes, resulting in wider and shorter paw prints. According to the repeated measure ANOVA, no significant improvement was observed in the first 2 weeks between the studied groups ($p > 0.05$). At the end of the 3rd week, a significant increase in AFI value was observed in the treatment group; being continued to the end of the study ($p < 0.05$). There was a significant change between weeks two and three ($p < 0.05$) in the treatment group. This indicates that there was a noticeable improvement in the tendon repair at the end of the 3rd week. According to AFI analysis, the improvement rate of motor function was remarkably slower in the control group until the 5th week (Fig. 2B). All AFI values increased gradually with the extension of the healing time; the trend of increase was more evident in the treatment groups. At 6 weeks postoperatively, the highest AFI value was obtained in the treatment group (−12.73 ± 2.05 *versus* −37.59 ± 13.52 in control group) and approached the near normal level, indicating favorable repair performance.

Histopathology. Figure 3 shows the histopathological features of the repairs after 6 weeks. Infiltration of inflammatory cells was observed in both groups with an increased level in the treatment group (mean scores: 2.40 *versus* 2.00, for treatment and control groups, respectively; $p < 0.05$). A few necrotic foci were observed within the granulation tissue in the control group. In contrast, there were metaplastic areas, including hyaline and bone tissues within the intact areas in some treatment samples. Regarding neovascularization, a higher number of newly

formed vessels was observed in the treatment group (mean scores: 2.00 *versus* 1.40, for treatment and control groups, respectively). The collagen fibers in the treatment group were regular and neatly arranged (mean score: 2.40); however, in the control group, slightly regular and loosely organized fibers were observed (mean score: 2.00). Statistical analysis showed significant improvement in collagen alignment in the treatment group ($p < 0.05$).

Biomechanical properties. The biomechanical tensile test indicated increased mechanical properties of healing tendons in terms of ultimate load, yield load, stress, energy absorption, and stiffness in the treatment group compared to the control samples ($p < 0.05$). Statistical analysis indicated no significant differences regarding strain between the groups ($p > 0.05$). The results of mechanical evaluations are presented in Table 1.

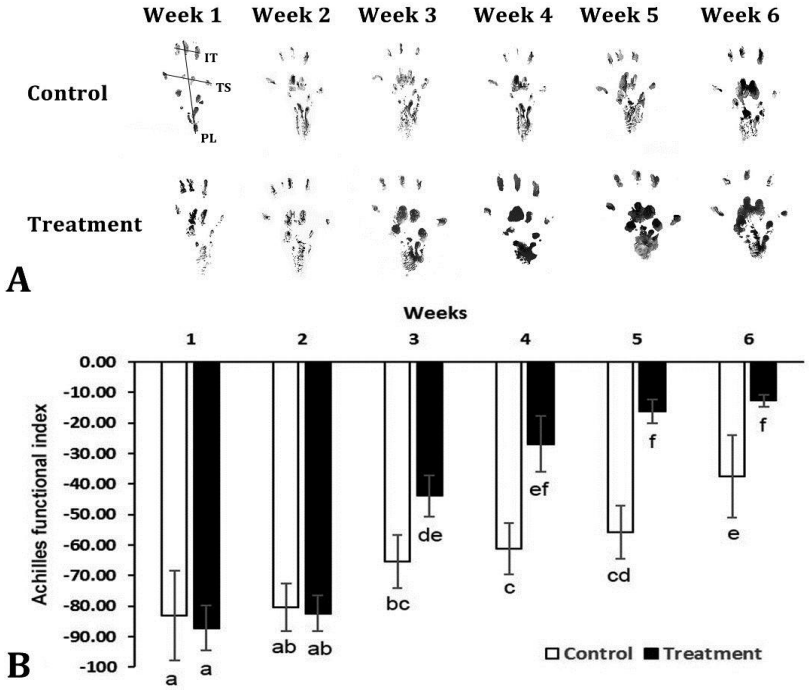

Fig. 2. Walking track analysis. **A)** Footprints of control and treatment rats at 1 - 6 weeks postoperatively. PL: Print length; IT: Intermediate toe; TS: Toe spread. **B)** Achilles functional index (AFI) value was obtained weekly after surgery by analysis of paw prints. In general, AFI oscillates around 0 for normal tendon and a more negative AFI value represents more serious hypomotility. [a-f] Different letters indicate significant differences between the control and treatment groups ($p < 0.05$).

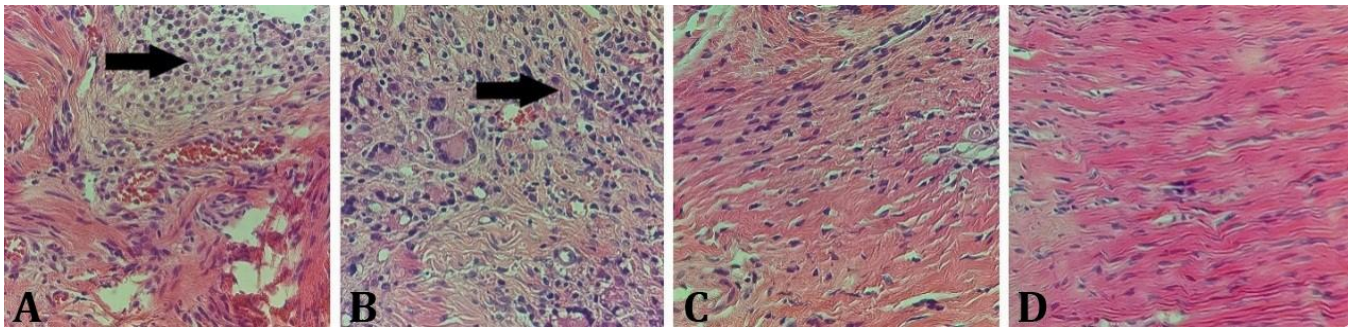

Fig. 3. Photomicrographs of the Achilles tendon after 6 weeks. **A** and **B)** Arrows indicate inflammatory cells infiltration in control and treatment groups, respectively. Increased number of inflammatory cells was observed in treatment group *versus* control group. **C** and **D)** Collagen fibers organization in granulation tissue can be seen in control and treatment groups, respectively. In comparison, densely packed and parallel collagen fibers are observed in the treatment group. No significant changes were observed between the groups (Hematoxylin and Eosin, 100×).

Table 1. Mechanical properties of Achilles tendons after 6 weeks. Data are presented as mean ± standard deviation.

Groups	Ultimate load (N)	Yield load (N)	Stress (MPa)	Strain (%)	Energy (J)	Stiffness (MPa)
Control	13.23 ± 3.60	11.50 ± 3.63	2.81 ± 1.41	8.18 ± 2.00	21.28 ± 9.22	2.80 ± 1.57
Treatment	43.36 ± 9.59*	42.37 ± 14.62*	6.07 ± 1.62*	9.42 ± 1.81	62.6 ± 26.6*	7.03 ± 3.56*

* indicates significant differences between the groups ($p < 0.05$).

Discussion

Reportedly, oral administration of curcumin after experimentally induced acute injury in the rat Achilles tendon could strengthen the biomechanical properties, as well as increase the functional index of this tendon and improve the quality of the tissue structure, including increasing the number of tenocytes and amount of accumulation. The extra-cellular matrix and collagen fibers were found in the Achilles tendon healing site.[24,25] Although these studies have reported the positive effect of oral administration of curcumin on the healing of tendon, due to its low solubility in water, poor tissue absorption, rapid metabolism, and short plasma half-life,[26] systemic administration of curcumin is a topic of debate among researchers. In this regard, various studies investigated the local administration of this bioactive compound for the repair of various wound models and favorable results have been obtained.[27,28] Zhang *et al*.'s study on the topical administration of curcumin using gold nanofibers indicated an increase in mechanical strength in rats.[29]

Based on the above findings, it seems that combination of PCL as a physical barrier and curcumin as a healing agent may exhibit potentiated healing effects. In the present study, a mixture of PCL and curcumin was used to synthesize a hybrid scaffold. The structural characteristics of the scaffold, such as nanofibers diameter and porosity can be tailored to enhance its biological properties and suitability for tissue engineering applications.

Previously, such a combination has been used as a dermal wound dressing, which led to an increase in the healing speed and contraction of diabetic wounds in mice. In another study, the same composite was used as a scaffold of chondrocytes to repair the experimental lesion of rabbit tracheal cartilage, and the results of the investigations showed that this compound leads to an increase in the viability of chondrocytes and prevents overgrowth of granulation tissue at the repair site.[30]

The use of PCL in tendon tissue engineering has shown mixed effects on inflammation. Some studies have demonstrated that PCL-based scaffolds do not elicit a systemic inflammatory response and are well-tolerated *in vivo*, making them a promising option for tendon repair.[31,32] However, other studies showed that implantation of PCL materials may provoke aseptic inflammation while degrading, which can facilitate the formation of fibrotic tissue and metaplastic changes.[33,34]

The presence of metaplastic areas in some treatment samples may be related to the physical manipulations and soft tissue injury during scaffold implantation[35] which may disrupt the peritendinous circulation and extrinsic arterial supply of the Achilles tendon,[36] inducing hypoxia and metaplasia. In this regard, a careful implantation of a not outsized scaffold is recommended to avoid the peritendinous vascular compromise.

The selection of scaffold material and its interaction with the inflammatory response should be carefully considered to minimize potential adverse effects. In the present study, significant inflammation was observed in some treatment samples compared to the controls. This finding suggests that while using a PCL scaffold, an appropriate anti-inflammatory treatment may help reduce the risk of inflammation.

Angiogenesis is an essential process in healing since the proliferation depends on an adequate supply of oxygen and nutrients and removal of waste products, thus promoting neo-tissue formation. In this study, the increased number of new vessels in the treatment group was considered a positive finding which led to a promoted healing process, being verified by other analyses in this study.[37]

Collagen fibers orientation plays a crucial role in determining the mechanical properties of healing tendons. Tendons are composed of collagen fibers being highly oriented, contributing to their high tensile strength. Under mechanical load, the crimps in the collagen fibers and molecules are stretched out, and the crimps store a part of the energy generated by the load. As the tensile force gradually increases, the collagen fibers become straighter, and the stored energy is released, leading to an increase in the tendon's stiffness and strength. The orientation of the collagen fibers also affects the distribution of stress within the tendon, with aligned fibers leading to a more uniform distribution of stress. Therefore, the orientation of collagen fibers is a critical factor in determining the mechanical properties of healing tendons.[38] In this study, more longitudinally oriented collagen fibers were observed in the treatment group.

The higher ultimate load and stress found in the treatment group suggest that the treated tendon can withstand greater force and tension before failing, indicating improved structural integrity. Additionally, the increased energy absorption implies that the treated tendon can better dissipate and absorb energy, being crucial for withstanding repetitive loading and reducing the risk of injury. The greater stiffness of the treated tendon indicates enhanced resistance to deformation under tensile forces, contributing to its overall mechanical stability and function.[39] These findings are consistent with the potential benefits of the treatment on tendon repair and mechanical properties, suggesting improved healing and functional outcomes.

The *ex vivo* assessments of repairs, such as histo-pathological and biomechanical analyses, provide important data regarding healing in terms of structural and tensile properties. However, these assessments do not demonstrate clinical evidence for tissue repair or indicate functional recovery and patient experience during convalescence. Therefore, the AFI is a more practical and appropriate approach to evaluate the clinical outcomes of

any treatment recruited for Achilles tendon repair. This index is known as the most powerful indicator of Achilles tendon function recovery after injury.[40] It is a widely used method for evaluating the functional performance of the Achilles tendon in rats, particularly during the healing process after injury or repair. The AFI measures various parameters related to the rat's gait, such as print length, toe spread length, and intermediary toe spread length, as well as ankle joint angles. These parameters provide insights into the animal's ability to move and bear weight on the injured tendon, allowing researchers to assess the functional recovery and healing process.[41] Here, early changes were observed in the treatment group which may reflect some potentially positive effects of the scaffold application. Also, curcumin has been shown to accelerate tendon healing following its oral administration in a previous study.[25]

In conclusion, the use of a scaffold around the tenotomized Achilles tendon may have provided a mechanical and conducive environment for tendon healing, leading to increased mechanical properties and improved AFI. The scaffold may have influenced the mechanobiology of the healing process, promoting the expression of collagen and enhancing tendon structure and function.

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Conflict of interest

The authors declare no conflict of interest.

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