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Original Article

Evaluating the Effect of Electrospun Polyvinyl Alcohol Nanofiber Containing *Eucalyptus globules* Extract on the Healing of Experimental Achilles Tendon Injury in Rat

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ABSTRACT

Injury and degeneration of tendons can be highly debilitating and can result in substantial pain, disability, and healthcare costs. Nano-sized fibers have a much wider surface area than conventionally produced fibers, which can hold composite materials more compactly and thus provide greater mechanical capabilities. In this study, the injured tendon was treated by electrospun PVA mats containing eucalyptus extract and histopathological results of healing were evaluated. For this study, 45 male Wistar rats were prepared and a partial thickness tenotomy was created on right hindlimbs. All rats were divided into three groups (n = 15) and three sub-groups (n = 5) including, eucalyptus extract-loaded nanofibers, PVA nanofibers, and without any treatment as a control group. Histological samples were taken on days 14, 28, and 42. The histological analysis on day 14 indicated no significant difference was observed between all groups ($p > 0.05$). While on days 28 and 42 post-rupture indicated a higher regenerating activity and capacity in the eucalyptus extract-loaded nanofibers than PVA nanofiber and control groups ($p \leq 0.05$). In summary, these results suggest that the eucalyptus extract-loaded nanofibers mats promoted the healing process of damaged Achilles tendon in rats.

Introduction

Dental Injuries to dense regular connective tissues, including tendons and ligaments, are of the leading limb injuries in humans and animals, especially during exercise. The tendon injuries are recognized as a common complication during show-jumping courses and classified as musculoskeletal injuries. The adverse effects caused by tendon injuries may persist for a long time despite therapeutic interventions.[1] Also,

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overburden on tendons may give rise to chronic tendon problems, accounting for over 30% of running injuries. The slow healing of these tissues, because of their special and inefficient blood supply, doubles the need to employ various approaches to improve the healing process. The healing process of tendon is very slow and it takes at least six weeks to six months or a year for the tendon to return to normal alignment, depending on the severity of the tendon injuries.[2] The tendon cell biology is not yet fully understood, and specialists continue to face the great challenge of treating tendon injuries. Deep tendon tissues receive the necessary nutrients, possibly through diffusion. The slow healing process of injured tendon tissue due to insufficient blood supply has led researchers in recent years to investigate the effect of various factors on the healing of tendon injuries and problems in different animal models and patients.[3,4]

A long history has been documented for use of medicinal plants in treatment of various diseases because mankind has empirically discovered the health-promoting effects of various plants, but the use of medicinal plants has gradually declined following the population growth, the prosperity of urban life and the advancement of science, and the fact that chemical substances and drugs have been replaced by plants in many cases. Studies in recent decades have clearly shown the complications of chemical drugs in addition to their beneficial effects. Adverse effects, high cost, complex process of synthetic drug production and the emergence of drug resistance once again diverted attention to the need for the employment of herbal medicines.[5] Eucalyptus is a plant belonging to the *Myrtaceae* family whose natural habitat in Australia, but it is currently widespread throughout the world, including Iran. The plant leaves owing to its various chemical compounds, have extensive bioactivities including analgesic, anti-inflammatory, antibacterial, antifungal and antioxidant properties.[6, 7]

Wound dressings with nanofiber membranes have unique properties compared to other coatings, because the nanofiber structures are very similar to the extracellular matrix, and are compatible with blood and other tissue fluids, thus facilitating wound healing and skin regeneration.[8] The fibers are synthesized through a variety of techniques, but some common methods for producing nanofibers include drawing-processing,[9] template-assisted synthesis,[10] phase separation,[11] self-assembly and electrospinning,[12,13] among them the electrospinning as the method of choice has attracted further attention due to simplicity, cost-effectiveness, high versatility, scalability and fiber arrangement.

Due to the great importance and high prevalence of tendon injuries and slow healing process in this tissue, it seems necessary to find an effective treatment solution; accordingly, the present study aimed to evaluate the effect of polyvinyl alcohol (PVA) nanofibers containing eucalyptus extract on the treatment of Achilles tendon injury in rats.

Materials and Methods

Animals

The present study was carried out on 45 male Wistar rats weighing about 300 g (about 4 months old). To avoid stress and adapt the animals to their environment, no experiments were performed on rats for one week and all animals were kept under the same environmental and nutritional conditions (temperature, humidity, light, type of ration, and frequency of meals). The rats were fed standard diet for laboratory animals. They also had free access to water. The study was conducted by the protocol developed by Ethics Committee for Research, Faculty of Veterinary Medicine, Semnan University, Iran (Approval number: E-96/01).

Extract Preparation

First, 500 grams of fresh eucalyptus leaves were obtained from agriculture and natural resources research center, Semnan, Iran. The leaves were dried in dark without heat and powdered using an electric grinder. A Soxhlet extractor was used for extraction. The prepared eucalyptus powder was soaked in a hydroalcoholic solvent (80% ethanol and distilled water at a ratio of 50:50) by a maceration method, wrapped in an extraction thimble and embedded in the Soxhlet apparatus, and then the extraction process was started. Subsequently, the obtained solution was transferred into a rotary evaporator (model STRIKE300, Wings Company, Italy) to discard the solvent and concentrate the extraction. The extract was stored in sterile glass containers at 4° C.

The Electrospinning Process

PVA powder (1 g) was dissolved in 10 ml of distilled water and stirred for 3 h at 80° C to obtain a uniform, clear solution (1% w/v). The electrospinning process was performed using the two-nozzle electrospinning device (NanoAzma Co., Iran) at an applied voltage of 15

kV, the needle-tip-to-collector distance of 18 cm and the feed rate of 2.5 ml/h using a G22 needle and a rotating cylinder collector at a speed of 300 rpm.

Scanning Electron Microscopy

A scanning electron microscope (SEM, Model XL-30, Philips, Netherlands) was used to evaluate the microstructure of the prepared nanofibers. Mean nanofibers diameter were obtained by ImageJ software (US, Bethesda, MD) (Figure 1).

Fourier Transform-Infrared Spectroscopy (FT-IR)

Infrared spectroscopy was performed to identify functional groups and to determine the type of reaction or bonds established between PVA and eucalyptus extract within the electrospun nanofibers. The absorbance peaks of all samples were obtained by Thermo Nicolet spectrometer (model 17DSX FT-IR, USA) at the wavelength range of 400-4000 cm^{-1} and with a resolution of 4 cm^{-1} (Figure 2).

X-Ray Diffraction (XRD)

XRD was recorded in the 2θ range of 20–90° using D8-Advance of Bruker (Germany) of CuKa radiation with the energy 8.04 keV and wave length 1.54 A°. The current was 25 mA and applied voltage was 40 kV. (Figure 3).

Experimental Protocol

Rats were anesthetized with ketamine hydrochloride (5%, 35 mg/kg) in combination with xylazine (2%, 5 mg/kg) intraperitoneally. Surgery was performed on the right hind limbs. The surgical area was prepared aseptically. A longitudinal skin incision was made over the Achilles tendon, and the paratenon was exposed and incised longitudinally as a separate layer. The three bundles of Achilles tendon were identified, and the central bundle was separated bluntly. A partial-thickness tenotomy (approximately 50% of tendon bundle width and 1 cm length) was created, at 5 mm to the proximal side of the Achilles tendon-calcaneus junction. The severed tendon was not sutured. The partial tenotomy allowed the rest of the tendon to act as an internal splint for the non-immobilized repair. The animals were divided into three groups, (i) control group, incisions without treatment, (ii) PVA group, incisions treated with PVA dressing, (iii) PVA-EO group, incisions treated with eucalyptus extract loaded nanofibers dressing. The incisions were sutured with 4-0 nylon to achieve primary closure. All animals received cefazolin (30 mg/kg twice a day for 3 days, IM) and flunixin meglumine (20 mg/kg once a day for 3 days, IM) postoperatively. The rats were then transferred to individual cages in the laboratory animal rooms.

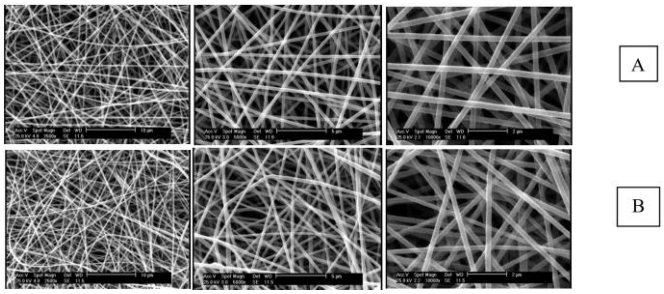

Figure 1. SEM image of **A**, PVA nanofiber and **B**, eucalyptus extract loaded nanofibers.

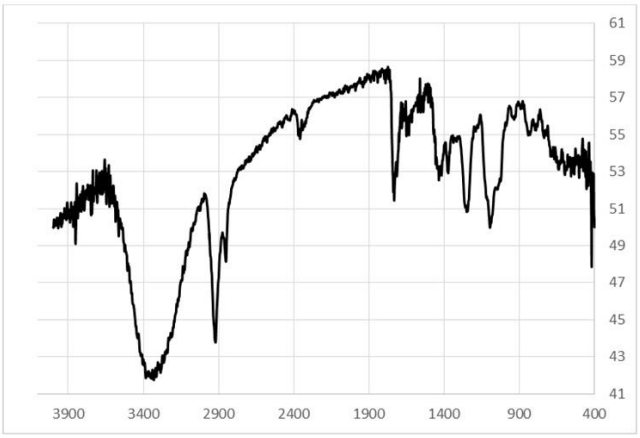

Figure 2. FTIR pattern of eucalyptus extract loaded nanofibers.

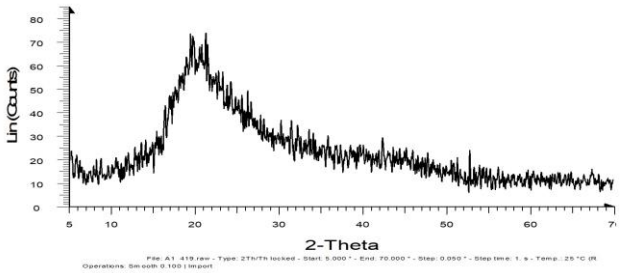

Figure 3. XRD pattern of eucalyptus extract loaded nanofibers.

Histopathological Studies

The animals were euthanized postoperatively at 14, 28, and 42 days with an overdose of thiopental sodium, then Achilles tendons were harvested by dissection. Specimens were fixed in 10% buffered formalin, dehydrated, and then embedded in paraffin. After 10 days, longitudinal sections of the tendon were stained with hematoxylin and eosin (H&E). Hematoxylin-eosin stained 5-µm sections were analyzed. Four parameters (fiber structure and fiber arrangement, inflammation,

increased vascularity, and cell density) were quantified using a 0-3 grading scale: 0 (normal), 1 (slightly abnormal), 2 (moderately abnormal) and 3 (maximally abnormal). In this grading system, a perfectly normal tendon would score 0 and a maximally abnormal tendon would score 12.[14] Three sections were randomly selected from each sample and were evaluated blindly by three independent assessors. The average score was used for comparison.

Data Analysis

Statistical analysis was performed using SPSS software v16.0 (SPSS Inc, USA) and the Kruskal-Wallis test. Data were expressed as mean ± standard deviation (SD). Differences were considered significant for $p < 0.05$.

Results

The results of histopathological studies is presented in Table 1. On day 14, histopathological findings in tendon healing, inflammatory cell infiltration, angiogenesis, fibroblast proliferation and the structure of collagen fibers were investigated (Figure 4). There was no significant difference either between the studied groups ($p > 0.05$). The results revealed a significant difference between the PVA-EO group and the PVA and control groups at days 28 and 42 ($p \leq 0.05$). Also, there was no significant difference between PVA and control groups ($p > 0.05$) (Figures 5 and 6).

Discussion

In the present study, the histopathological examinations were analyzed on days 14, 28 and 42 at the site of tendon injury. Accordingly, no statistically significant difference was observed on day 14 between the study groups, while the difference on days 28 and 42 between the PVA-EO group and PVA and control groups was significant. The difference occurred in the healing rate in the studied groups indicates the effect of using eucalyptus extract loaded nanofibers on the healing process.

Considering the mechanism of action and biology of free radicals and the beneficial effects of antioxidants in inhibiting the oxidative stress and thus increasing the healing process in the injured body tissues, the use of naturally occurring antioxidants has gradually elevated to suppress or reduce the progression of oxidative stress. Previous studies have shown the antioxidant effect of phenolic and flavonoid compounds present in

Table 1. The Mean ± SD of the histopathological changes in all groups.[14] There was no significant difference between the same letters in each column, but a significant difference was observed between the non-identical letters. The difference was significant ($p \leq 0.05$).

Days	Control	PVA	PVA-EO
14	10.5 ± 1.29	9 [a]	10 ± 0.81 [a]
28	9.5 ± 0.57	9.2 ± 0.44 [a]	7.8 ± 0.44 [a]
42	8 ± 0.7	9 ± 0.7 [b]	4.8 ± 0.83 [b]

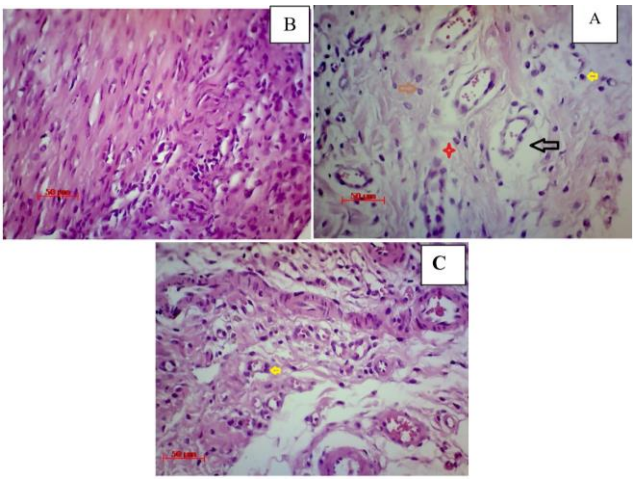

Figure 4. Histopathological changes in Achilles tendon images with H&E staining with 400× on the 14th day after treatment was shown new angiogenesis, presence lot of fibroblast, edema and no arrangement in thin collagen fibers in all groups. **A:** Eucalyptus extract loaded nanofibers group, **B:** PVA nanofiber group and **C:** control group (black arrow = angiogenesis, orange arrow = fibroblast, yellow arrow = inflammatory cell, and red star = edema).

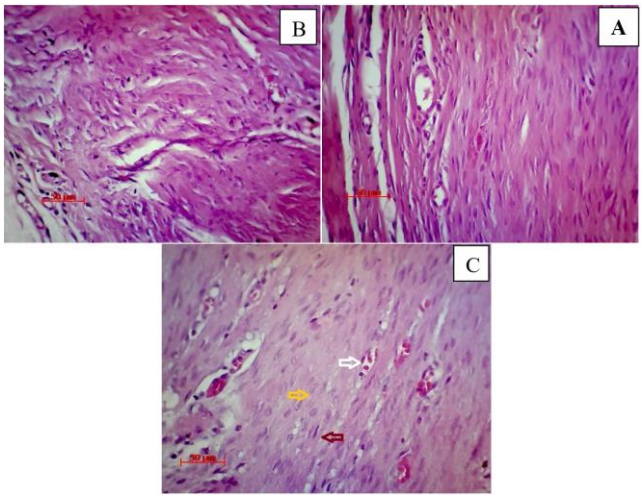

Figure 5. Histopathological changes in Achilles tendon images with H&E staining with 400× on the 28th day after treatment was shown a decrease of new angiogenesis, increase in the ratio of fibrocyte to fibroblast, decrease edema and low arrangement in thin collagen fibers in the eucalyptus extract loaded nanofibers. **A:** Eucalyptus extract loaded nanofibers, **B:** PVA nanofiber group and **C:** control group (white arrow = angiogenesis, orange arrow = fibroblast, and brown arrow = fibrocytes).

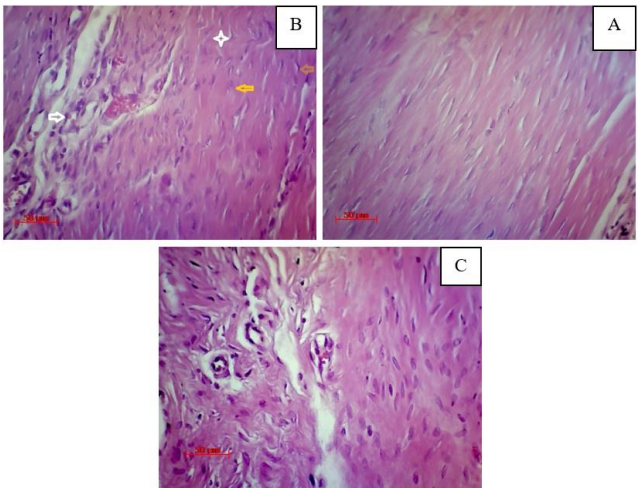

Figure 6. Histopathological changes in Achilles tendon images with H&E staining with 400× on the 42nd day after treatment was shown more decrease of new angiogenesis, a decrease of cell population fibroblast and fibrocyte, more decrease of edema and more arrangement in thick collagen fibers the eucalyptus extract loaded nanofibers. **A:** Eucalyptus extract loaded nanofibers, **B:** PVA nanofiber group and **C:** control group (white arrow = angiogenesis, orange arrow = fibroblast, brown arrow = fibrocytes, and white star = arrangement in thick collage fiber).

the structure of some herbs on the inhibition of oxidative stress.[15] The findings demonstrated that the phenolic compounds, among antioxidants, have stronger reducing properties. The herbal extracts can increase the antioxidant levels and thus protect the various tissues of organisms against the devastating effects of free radicals generated during tissue injuries.

The compounds present in eucalyptus seem to prevent the formation of free radicals and can accelerate the healing process of injured tendons due to the antioxidant and anti-inflammatory activities. Various studies have shown that the eucalyptus leaves are a rich source of phenolic and flavonoid compounds such as quercetin and metabolites such as tannins and saponins.[16] In the present study, the extract of this plant seems to be effective in preventing the development of damage caused by oxidative stress in tendon tissue. Nazari *et al*. reported high antioxidant capacity for the eucalyptus leaf extract.[17] The antioxidants are the main factors neutralizing the free radicals that are formed in response to environmental stresses. These compounds have free radical scavenging activity due to their hydroxyl groups. They can act as electron or hydrogen donors, stabilize the free radicals and thus stop the oxidation chain. Increasing total phenol compounds can promote antioxidant properties. High molecular weight phenolic compounds have a great ability to scavenge the free radicals, and this ability depends on the number of aromatic rings and the nature of the displaced hydroxyl groups.[17] The antioxidant properties of phenolic compounds are due to their reducing potential and chemical structure. This feature enables them to neutralize free radicals, form metal ion complexes and quench triplet oxygen molecules.[18]

Flavonoids are another compound found in eucalyptus, with the ability to absorb free radicals, like other phenolic compounds. In the oxidative stresses, the phenolic compounds, especially flavonoids, can bind to the polar heads of membrane phospholipids through hydrogen bonds. As a result, these compounds accumulate on the inner and outer surfaces of the cell membrane, thereby helping to maintain the fluidity and integrity of the cell membrane by preventing harmful molecules from entering the bipolar hydrophobic tails.[19] The flavonoids are of the nitric oxide synthase (NOS) inhibitors.[20] The flavonoids inhibit the N-methyl-D-aspartate receptor (NMDAR) activity and reduce intracellular calcium, thereby reducing the NOS and calcium-dependent phospholipase A_2 activity. As a result, they show their anti-inflammatory effects by reducing the levels of NO and prostaglandins.[21,22] The flavonoids can reportedly inhibit the conversion of arachidonic acid to prostaglandin E in response to inflammatory stimuli by inhibiting the cyclooxygenase. Since the prostaglandins, which are derived from arachidonic acid, affect the inflammation onset and the pain exacerbation, the flavonoids in eucalyptus may play an anti-inflammatory effect. A study attributed the anti-inflammatory effects of eucalyptus extract to the presence of compounds with anti-inflammatory properties, flavonoids or other plant substances such as quercetin and saponin.[23]

The quercetin is a plant-derived bioactive compound that has radical scavenging and antioxidant properties due to its polyphenol structure. The quercetin is one of the main members of the flavonoid family, with the highest antioxidant properties among other flavonoids, whose antioxidant properties are even approximately six times stronger than vitamin C. The results from the effect of quercetin on inflammation and the immune function introduced this compound as a long-lasting anti-inflammatory agent expressed in different types of cells in both animal and human models. The quercetin can alleviate inflammation and prevent oxidative damage by increasing superoxide dismutase activity and thus reducing malondialdehyde level.[24] Ruiz *et al*. showed

that quercetin attenuates inflammation by inhibiting the expression of inflammatory cytokines. In the present study, the flavonoids found in eucalyptus appeared to reduce the course of inflammation and accelerate the healing process of injured tendons in the treatment group, possibly due to their anti-inflammatory effects by inhibiting the relevant.[25] The saponins, another compound in eucalyptus extract, are high molecular weight glycosides that have a glycosidic linkage at triterpene or steroid aglycone. This unique structure of saponins establishes biological properties such as antioxidant, antimicrobial and anti-inflammatory activities.[26]

Therefore, due to the low cost, availability and significant effects of eucalyptus extract in nano-dimensions on the healing process of tendon injury owing to the antioxidant and anti-inflammatory properties of its compounds, this extract can be considered as a naturally occurring herbal medicinal product in the healing of tendon injuries.

Conflict of Interest

The authors declare no conflict of interest.

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