Fabrication of novel tubular scaffold for tendon repair from chitosan in combination with zinc oxide nanoparticles

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Abstract

Chitosan bears numerous properties, such as biocompatibility, biodegradability and non-toxicity making it suitable for use in different biomedical fields. Zinc (Zn) is required for fibroblasts proliferation and collagen synthesis as essential elements of wound healing. Its nanoparticles are well known for their capability to enhance wound healing by cell adhesion and migration improvement through growth factors-mediated mechanisms. Poor blood supply and unique histological characteristics of tendon make its regeneration always slow. Also, adhesion formation between tendon and its surrounding tissues is another problem for neotendon to return to its normal structure and functional activities. In this study, a novel tubular scaffold of zinc oxide (ZnO) nanoparticles loaded chitosan has been fabricated for tendon repair. Experimental complete tenotomy of deep digital flexor tendon in a rabbit model was done and scaffolds were placed in the transected area after two ends suturing. After four and eight weeks, adhesion formation around the tendons and tissue reaction to the scaffolds were evaluated macroscopically. Inflammation, angiogenesis and collagen fibers arrangement were also analyzed in histopathological evaluations. After eight weeks, the scaffolds were absorbed completely, adhesions around the tendon were decreased and there was no sign of significant tissue reaction and/or infection in histopathological analyses. The reduced adhesion formation, improved gliding function and better histopathological characteristics suggest this scaffold application as a potential therapy in treatment of tendon acute injuries.

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Introduction

Tendons transfer muscles-generated forces to bone and act as barriers through external forces absorption to limit muscular damages. Tendons exhibit high tensile strength, good flexibility and optimal levels of elasticity to perform their unique tasks. Tendon injuries can be acute or chronic and are caused by intrinsic or extrinsic trauma, either alone or in combination. In acute form, extrinsic factors predominate, whilst in chronic cases intrinsic factors also play roles. In chronic tendon disorders, interaction between intrinsic and extrinsic factors is common. Intrinsic factors such as alignment and biomechanical faults are claimed to play causative roles in two-thirds of athletes with Achilles tendon disorders. Tendon rupture is an acute injury in which extrinsic factors predominate, although intrinsic factors are also important. Oxygen consumption by tendons and ligaments is 7.50 times lower than skeletal muscles. Because of low metabolic rate and well-developed anaerobic energy generation capacity, tendons are able to carry loads and maintain tension for a long period of time, whilst avoiding the risk of ischemia and subsequent necrosis. However, tendons have a low metabolic rate resulting in slow post-injury healing. Tenoblasts and tenocytes situated between adhesion formations after intrasynovial tendon injury poses a major clinical problem. Synovial sheath disruptions in injury and/or during surgical approach allow granulation tissue formation and recruit tenocytes from surrounding tissue to the repair site. Exogenous cells predominate over endogenous tenocytes, allowing the surrounding tissues to attach to the repair site resulting in adhesion formation. Poor vascularity and cellularity of tendons are the other reasons for weakness and delay in tendon repair process. Despite remodeling, the biochemical and biomechanical properties of healed tendon never match those of intact tendon.

The tendon limited ability to self-repair and lacks of total efficiencies in current treatment regimens have accelerated the motivation to develop tissue engineering strategies for tissue repair. Use of different types of scaffold to help functional tendons and ligaments regenerations attracts a particular interest in recent years. It is critical to design and fabricate a suitable scaffold for use in specific tissue regeneration, as it directly contacts with the cells and provides structural support and regulation for subsequent tissue development. In this regard, more attention has been paid to the design of scaffolds for guiding cell behaviors and tissue regeneration and the design of scaffolds should be based on knowledge learned from native tissues including their anatomical structures, compositions and functions.

Tissue engineering is in need of effective biomaterials that can be employed as tissue scaffolds for a variety of applications. An optimal material would provide structural support and act as a reservoir for bioactive substances release. Chitosan was selected because of its biocompatibility, timely biodegradability and low toxicity and cost. Chitosan can form films that have been used as film formulation of film dosage forms and/or drug delivery systems. It has also hemostatic activity and has been suggested as a topical agent in tissue repair. It has also been shown that chitosan possesses antibacterial, fungistatic, anti-tumoral and anti-cholesteremic properties. Chitosan is a favorite option as a tissue support material because of the multiple paths by which its biological, physical and chemical properties can be controlled and engineered under mild conditions.

According to the description that was given about the benefits of nanotechnology, we decided to add zinc oxide (ZnO) nanoparticle to the scaffold. Re-epithelialization process augmentation, antibacterial activity and fibroblasts proliferation enhancement are also mentioned as ZnO nanoparticles properties. Furthermore, Zn as an essential micronutrient for metabolism catalyzes more than one hundred enzymes, facilitates protein folding and helps gene expression regulation. Reportedly, combination of chitosan and ZnO nanoparticle has been used successfully to make composite bandages for wound dressing and biofilms.

The purpose of this study was fabrication of a novel tubular scaffold for tendon repair and we fabricated a novel scaffold with chitosan and ZnO nanoparticles for use in experimentally induced deep digital flexor tendon (DDFT) injuries in rabbit and effects of this scaffold were evaluated alone and in combination with ZnO nanoparticles at the site of healing through histopathological and gross observations and compared with chitosan and Zn oxide scaffold.

Materials and Methods

All procedures in this study were carried out in accordance with the guidelines of the Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University and supervised by authority of Urmia University Research Council (3/T.TD/1360, Jan 6, 2016).

Animals. Forty-eight adult male white New Zealand rabbits, weighing 2.50-3.00 kg were included in this study. During the study, animals were housed individually in standard cages (60×55×40 cm) and fed commercial rabbit pellet (Azarkavian Co., Urmia, Iran) and water ad libitum.

Preparation of chitosan tubular scaffold. In this study, chitosan (85% deacetylated medium molecular weight) from Fluka (Taufkirchen, Germany), acetic acid and from Merck (Darmstadt, Germany) and glycerol from Sigma (St. Louis, USA), ZnO nanoparticles (80-200 nm) and Zn oxide alone from US Research Nanomaterials Inc. (Houston, USA) and Merck (Darmstadt, Germany) were purchased, respectively.
The aqueous solution (1.00% v/v) of glacial acetic acid was prepared, then, chitosan solution (2.00% w/v) was prepared by adding 2.00 g chitosan to 100 mL acetic acid (1.00% v/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (50 °C) for 1 hr. The resultant chitosan solution was filtered through a No. 3 filter paper (Whatman, Buckinghamshire, UK) to remove any undissolved particles. To overcome the fragility of chitosan, glycerol was added in amount of 30.00% of the total solid weight in solution.10 The prepared solution was divided into three equal parts. One part was considered as chitosan solution (CS), the other two parts were used for preparation of chitosan in combination with Zn oxide (CSZO) and ZnO nanoparticle (CSZN) solutions. For preparation of the later solutions, Zn oxide and ZnO nanoparticle in amount of 0.01% total solid weight of chitosan in each part were measured and separately added to these solutions.10 The solutions were rigorously stirred for 1 hr to get homogeneous mixture solutions.

This scaffold should be fabricated in a proper size and for this reason, diameter of DDFT was measured. Mean diameter of DDFT (3.50 ± 0.50 mm) was calculated by direct measurement of tendon (with caliper) under standard surgical exposure of eight rabbits DDFT. The molds with CS, CSZO and CSZN solutions were placed in a −80 °C freezer for 12 hr. The frozen molds were placed at room temperature and after 5 min; the outer layers of frozen molds were removed. The frozen solutions were dried in a freeze-dryer (model Alpha 1-4 LDplus; Martin Christ, Osterode, Germany). The main drying temperature was −40 °C and the main drying pressure was 12 Pa for 15 hr. Then, the scaffolds were immersed into 2.00% (w/v) sodium hydroxide solution (Merck) and equilibrated for 20 min to eliminate the remaining acetic acid. Scaffolds were rinsed several times using deionized water until the rinsing solution was neutral, and then equilibrated in 0.20 mol L−1 phosphate buffered saline (pH: 7.40) for 30 min and finally scaffolds were dried at room temperature for 6 hr.11,12

In this study, 0.20 mm thick and 3.50 ± 0.50 mm inner diameter of CS, CSZO and CSZN scaffolds were formed to wrap the tendons. All of the scaffolds were sterilized with formaldehyde tablets in airtight containers for 24 hr.13

Scanning electron microscope (SEM). Scaffolds were scanned with SEM (Mira II TE Scan, Tescan, Brno, Czech Republic) in Iranian Research Organization for Science and Technology (Tehran, Iran) to illustrate proper structure of scaffolds and correct size of the nanoparticles.

Induction of experimental tendon injury and placement of scaffolds. Rabbits were randomized into four groups (n = 12). Anesthesia was induced using 40 mg kg−1 ketamine hydrochloride (Alfasan, Woerden, Netherlands) and 10 mg kg−1 xylazine hydrochloride (Alfasan), intramuscularly. All rabbits received a single dose of cefazolin (20 mg kg−1; Loghman Pharmaceuticals, Tehran, Iran) as prophylaxis at the time of anesthetic induction. One hind limb of each rabbit was randomly selected and prepared for aseptic surgery. Plantar skin was incised longitudinally and DDFT was exposed. The injury model was a sharp complete transection through the middle of the tendon. In control group, tendon stumps were sutured with 3-0 monofilament nylon (Ethicon Inc., Somerville, USA) using modified Kessler pattern. In CS, CSZO and CSZN groups, after suturing the stumps, 1 cm of CS, CSZO and CSZN scaffolds were wrapped around the injury site, respectively. Skin was closed routinely and a short cast was applied to the limb for two weeks.

Clinical evaluation. At the end of the 4th and 8th weeks, the animals were euthanized by IV injection of 2.50% sodium thiopental (Biochemie, Kundl, Austria) overdose. The surgical site was re-opened and the tendons were exposed with minimal manipulation. Clinical evaluation in terms of adhesion formation was done according to Tang Scoring System as Absent = 0, Inferior = 1-2, Medium = 3-4 and Severe = 5-6.14,15

Histopathological evaluation. The harvested tendon samples in 4th and 8th weeks from all four groups were fixed by immersion in 10.00% formalin solution. Samples were dehydrated and embedded in paraffin wax and longitudinal sections of 5.00 μm from the tenotomy site were prepared for hematoxylin and eosin (H & E) staining. The sections were studied under the light microscope (HumaScope Light LED; Human Diagnostics, Wiesbaden, Germany). Inflammation, angiogenesis and collagen fibrils arrangement were evaluated in the sections.16

Statistical analysis. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney U tests in SPSS (version 20; SPSS Inc., Chicago, USA). A p-value less than 0.05 was considered statistically significant.

Results

The chitosan solutions were kept at room temperature and after more than two months, there was no precipitation and/or denaturation. Ultra-micrographs obtained from SEM are shown in Figure 1.

Macroscopic observations revealed no signs of local infection or purulent discharge around the tendons and in the site of healing in any group and also following histopathological observations no signs of infection characterized by severe infiltration of inflammatory cells or presence of pus at the site of healing were found. Severe adhesion was noted mostly in control group. However, there was a mild adhesion formation around the neotendons in the other groups (Fig. 2).

In terms of adhesion formation, statistical analysis at the 4th week showed significant differences between CSZO and CSZN groups compared to control group (p < 0.05) and there was no significant difference between other groups. At the eighth week, there was a significant
difference between CSZO and CSZN groups in comparison with control group \( (p < 0.05) \). However, there was no significant difference between treatment groups; CS, CSZO and CSZN \( (p > 0.05) \), \(^{(Figs. \ 3 \ and \ 4)}\). In both intervals of evaluation, there was no significant difference between CS and the other two treatment groups \( (p > 0.05) \).

Histopathological observations demonstrated much better healing process following these novel scaffolds application. In terms of angiogenesis, statistical analysis at the 4\(^{th}\) week showed significant differences between CSZO and CSZN groups compared to control group \( (p < 0.05) \) and also there was a significant difference between CSZO and CSZN groups compared to CS group \( (p < 0.05) \). After eight weeks, there were significant differences between CSZO and CS groups and between CSZO and CSZN groups among the treatment groups \( (p < 0.05) \). Regarding collagen fibrils arrangement, at the 4\(^{th}\) week there was significant differences between CSZO and control groups \( (p < 0.05) \). Among the treatment groups, statistical analysis showed two significant differences, between CSZO and CS groups and between CSZO and CSZN groups. After eight weeks, there were significant differences between CSZO and CSZN groups compared to control group and also between CSZO and CS groups among the treatment groups \( (p < 0.05) \).

In terms of inflammation, at fourth week statistical analysis showed just significant difference, between CSZO and CS groups. After eight weeks, statistical analysis showed significant differences between CSZO and CSZN groups compared to control group and also there was a significant difference between CSZO and CSZN groups compared to CS group \( (p < 0.05) \), \(^{(Figs. \ 5)}\).
Discussion

There are few records about the combination of ZnO nanoparticles with chitosan. Jayasuriya et al. combined above-mentioned materials to develop chitosan-Zn oxide nanocomposite films for biomedical purposes. Their films were fabricated with different percentages of Zn oxide nanoparticle incorporated with chitosan. They found out that the films microhardness, nanohardness and their corresponding elastic modulus are increased with the increase in Zn oxide nanoparticle percentage in the chitosan films. However, the ductility of films was decreased as the percentage of Zn oxide nanoparticle was increased. Cell attachment and cytotoxicity of the prepared films were evaluated using osteoblasts. They observed that osteoblast viability is decreased in films with Zn oxide nanoparticle higher than 5.00%. This result suggested that although Zn oxide nanoparticle can improve the mechanical properties of pure chitosan films, only a low percentage of Zn oxide nanoparticle can be applied for biomedical and bioengineering applications due to cytotoxic effects of these particles. Kumar et al. have developed composite bandages via incorporation of Zn oxide nanoparticles with chitosan hydrogel and swelling, degradation, blood clotting, antibacterial properties, cytocompatibility and cell attachment to the material as well as cell infiltration into the composite bandages were evaluated. They have fabricated chitosan hydrogel-nano Zn oxide composite bandages with different percentages of ZnO nanoparticle and following evaluations, the most plausible results were obtained from chitosan with 0.01% ZnO nanoparticle composite. Accordingly, 0.01% ZnO nanoparticles was used to fabricate the chitosan nanocomposite scaffold in the present study.
Lack of precipitation or denaturation in the solutions can be attributed to the correct combination of materials and formation of homogeneous and stable solutions.

Substantial progresses in the understanding of flexor tendon repair and reconstruction have been made since the early 1970s, when reports first have indicated that flexor tendon lacerations within the fibro-osseous digital sheath can be repaired primarily and recovered successfully without early tendon excision and delayed intrasynovial grafting. The sense of adhesion-free or primary tendon healing has been validated experimentally and clinically in other studies. Recent intentions to improve the results of flexor tendon repair have focused on gliding surface restoration, early postoperative repair site biomechanical strength augmentation and early postoperative tendon healing molecular biology explanation. The goals of surgical treatment in patients with flexor tendon lacerations remain unchanged ends position and are achieving a primary tendon repair with sufficient tensile strength. This plane should inhibit the formation of adhesions and restore the gliding surface while facilitating repair site healing. With regard to these considerations we design our protocols for DDFT.

Few days after injury, inflammation in tendon decreases and fibroblasts proliferation and extracellular matrix and collagen (mostly type III) syntheses are occurred on day 5. The newly formed collagen fibrils are appeared randomly in the extracellular matrix and then started to aggregate as organized bundles after 3-4 weeks. Decrease in collagen type III contents and increase in collagen type I synthesis are the main characteristics of tendon healing remodeling phase beginning 6-8 weeks after injury. Despite immature and weak nature of collagen type III fibers and their random orientation (compared to collagen type I fibers), they are responsible for neotendon stability. On the other hand, high expression of type I collagens and longitudinal organization of these fibers are seemed to be essential to reach the maximum tensile strength and faster tendons healing. In fact, early increase in collagen type I fibers following any treatment would provide the benefit of early increase in wound tensile strength during the time in which the tendon would be at the risk of re-injury. Therefore, the neotendons were evaluated in 4th and 8th weeks.

During eight weeks, scaffolds were absorbed completely at the repair site which was in agreement with some properties of components used for scaffolds fabrication including assimilability, biocompatibility and biodegradability.

Prevention of tendon adhesion and re-rupture after surgical repair remains a clinical challenge. Ni et al. have developed an approach by combining standard suture and the photo bonded electrospun silk nanofiber wrap, which can provide a stronger and adhesion resistant repair to a tendon injury in adult female White New Zealand rabbits. Biological cues were also incorporated with nanofiber scaffolds for tendon adhesion prevention and simultaneous improvement of tendon healing. Liu et al. have invented a bilayer sheath membrane consisting of hyaluronic acid-loaded polycaprolactone (PCL) fiber membranes as the inner layer and PCL fiber membranes as the outer layer for mimicking the tendon sheath and have checked its performance in peritendinous adhesion prevention in a chicken model. It was found that the outer PCL layer is able to reproduce the anti-adhesive act of the outer fibrotic layer to reduce peritendinous adhesions, while the inner hydroxyapatite-loaded PCL layer can mimic the biological function of hydroxyapatite secretion to promote tendon healing and gliding. Recently, the same group encapsulated basic fibroblast growth factor bFGF-loaded dextran nanoparticles in poly-L-lactic acid nanofiber membranes and examined their capability in tendon healing and adhesion prevention in male Sprague-Dawley rats, demonstrating that such membranes can protect the bioactivity of bFGF in a sustained manner for the promotion of tendon healing and simultaneous adhesion prevention. Two intrinsic and extrinsic patterns were introduced for tendon repair. In the present study, in both time points, adhesion formation around the neotendon was reduced in treatment groups compared to control group. Prevention from extrinsic repair pattern, inhibiting exogenous reparative cells entry and creating a barrier between neotendon and its adjacent tissues are seemed to be the main reasons for lower adhesion formation observed in the treatment groups.

Although extrinsic tendon repair pattern and proliferative cells entrance were approximately inhibited after these scaffolds application in the first week of healing process but according to mentioned contents of scaffolds and their properties such as anti-inflammatory and fibroblast growth enhancing activities, with lapse of healing procedure, histopathological evaluations showed much better conditions in repairing tendons.

Nowadays, nanotechnology has abundant applications in biology, medical science, industry and biomedical engineering including using as a vehicle to deliver therapeutic agents, as scaffolds for engineering various tissues and serving as an integrated part of biomedical implants. The ZnO nanoparticles with a size scale of < 100 nm were prepared with centrifuged Zn oxide, washed several times with distilled water to remove byproducts and dried at −80 °C. There were no significant differences between scaffolds made from chitosan and Zn oxide and chitosan and ZnO nanoparticles during preparation of solutions, fabrication of scaffolds and also during its implementation in the surgical site. Furthermore, no significant difference was found in terms of adhesion formation between these two scaffolds, however, the severity of adhesion was milder in CSZN group. The histopathological evaluations also revealed better conditions.
(well-organized connective tissue) in the healing process in neotendons wrapped by CSZN scaffold. However more investigations should be carried out for this deduction.

According to macroscopic and histopathological evaluations, no signs of infection and inflammatory reactions were found at the repair site of tendons. These findings were expected due to antibacterial and hemostatic activities as well as biocompatibility, timely biodegradability and low toxicity of materials used in scaffolds. In this study, a novel tubular scaffold was designed and fabricated to use around the injured tendon. The final structure of this scaffold was examined with SEM. Porous structure of chitosan and being coated with ZnO nanoparticle were confirmed in SEM ultramicrographs confirming previous reports and the sizes of ZnO nanoparticle were also evaluated in SEM ultramicrographs.

In conclusion, the biocompatibility and biodegradability of the construct, reduced adhesion formation and therefore predictable improved gliding function of the neotendons resulted from CSZN scaffold suggest its application in acute tendon repair protocol. However, further investigations should be carried out to examine its effectiveness in terms of biochemical and biomechanical properties of the repairs.

**Conflict of interest**

The authors declare that there are no conflicts of interest related to this article.

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