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ORIGINAL ARTICLE

Histopathological and Biomechanical Survey on Effect of CoQ10 in Combination with Chitosan Conduit on Deep Digital Flexor Tendon Healing in Rabbits

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Abstract

Objective- Chitosan is of great interest in regenerative medicine because of its plentiful properties, like biocompatibility, biodegradability and non-toxicity. The objective of the present study was histopathological and biomechanical survey on effect of CoQ10 in combination with chitosan conduit on deep digital flexor tendon (DDFT) healing in rabbits.

Design- Experimental Study

Animals- Eighteen healthy male white New Zealand rabbits

Procedures- The animals were randomized into three groups of 6 animals each. In Control group the DDF tenotomy was performed and the stumps were sutured. In Chitosan group the DDF tenotomy was performed and the stumps were sutured and chitosan conduit was wrapped around the damaged area. In Chit-CoQ10 group the procedure was the same as Chitosan group as well as local administration of 100 µL CoQ10 (100 µg/rabbit) into the Chitosan conduit. The histopathological assessments including inflammation, angiogenesis and collagen fibers arrangement, and biomechanical assessments were performed after 8 weeks.

Results- Histopathological observations showed that the conduit was absorbed and adhesion around the tendon was deceased in Chitosan and Chit-CoQ10 groups. The biomechanical parameters showed significant improvement in Chit-CoQ10 group ($p < 0.05$). There were no noticeable signs of infection and tissue reaction in the granulation tissue in Chit-CoQ10 group compared to other groups ($p < 0.05$).

Conclusion and Clinical Relevance- Local administration of CoQ10 in combination with chitosan conduit could accelerate deep digital flexor tendon healing via decrease in adhesion around the tendon with no signs of excessive tissue reaction or infection in rabbits.

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1. Introduction

Tendons are the functional link between the dynamic and the static part of the musculoskeletal system transferring muscle contraction to the skeletal system and thus leading to motion. Consequently, function and motion are impaired in the case of tendon injury varying from acute traumatic ruptures to chronic overuse and degenerative tendinopathy. But even with improved therapeutic strategies (non-surgical, surgical, and rehabilitation techniques), outcomes remain inconsistently: Repaired tendon tissue rarely achieves functionality equal to that of the pre-injured state.[1,2] Tendon injuries produce considerable morbidity, and the disability that they cause may last for several months despite what is considered appropriate management.[3]

Tendon injuries can be acute or chronic, and are caused by intrinsic or extrinsic insults, either alone or in combination. In acute trauma, extrinsic factors predominate, whilst in chronic cases intrinsic factors also play a role. In chronic tendon disorders, interaction between intrinsic and extrinsic factors is common. Intrinsic factors such as alignment and biomechanical faults are claimed to play a causative role in two-thirds of athletes with Achilles tendon disorders or for example tendon rupture is an acute injury in which extrinsic factors predominate, although intrinsic factors are also important.[3]

The limited ability of tendon to self-repair and the general inefficiencies of current treatment regimens have accelerated the motivation to develop tissue engineering strategies for tissue repair. One of the particular interest in recent years has been the use of different types of scaffold to regenerate functional tendons and ligaments. It is critical to design and fabricate a suitable scaffold for use in specific tissue regeneration, as it directly comes into contact with tissue cells, and provides structural support and regulation for subsequent tissue development. Towards this, 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, such as their anatomic structures, compositions and functions.[4]

Chitosan, a linear polysaccharide, is associated with scarless healing of soft tissues and prevention of adhesion formation both intraperitoneally and during tendon healing after surgery.[5,6] Chitosan tends to precipitate in physiologic pH, thereby mitigating its potency. Fortunately, a chitosan solution that does not precipitate in physiologic conditions was recently developed.[7] The solution's lack of precipitation, coupled with its in situ gelling, allows it to adhere to the repair site long enough to take effect. These characteristics could allow for intimate contact between gel and tendon, facilitating guided-tissue regeneration and preventing adhesion of the rotator cuff to surrounding tissue. By contrast, other biological agents (eg, platelet-rich plasma) are administered as fluid rather than gel and are therefore more susceptible to diffusing from the repair site, mitigating their effects. Thus, chitosan is fairly unique among agents.[8]

The process of inflammation normally leads to the release of biologically active mediators to attract neutrophils, leucocytes and monocytes to the wound area to attack foreign debris and microorganisms through phagocytosis. This process leads to the production of oxygen-free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl anion, which excess of them, causes tissue damage in humans or animals if they overwhelm the natural antioxidant enzymes of the host such as catalase, superoxide dismutase, and glutathione peroxidase. Therefore, antioxidants prevent the activity of the free radicals and thereby prevent the damage of cells and tissues, provide protection to human and animal subjects, and also enhance healing of infected and non-infected wounds.[9,10]

Coenzyme Q10 is a vitamin-like, oil-soluble molecule. Its reduced form is an effective fat-soluble antioxidant and an essential element of the mitochondrial respiratory chain.[11,12] Therefore, CoQ10 may have healing effects on

wound tissues by decreasing oxidative stress and improved mitochondrial efficiency. A previous study demonstrated that oral administration of CoQ10 induced synthesis of collagen on injured skin tissue, and had positive effects on cutaneous healing in mice.[13] It has been demonstrated that CoQ10 improves inflammation and collagen density.[14,15]

The objective of the present study was to histopathologically and biomechanically investigate effect of CoQ10 in combination with chitosan conduit on deep digital flexor tendon healing in rabbits. The assessments were based on macroscopic, histopathological and biomechanical criteria.

2. Materials and Methods

Preparation and Fabrication of Chitosan Conduit

Chitosan (85% deacetylated medium molecular weight) was purchased from Fluka, Sigma-Aldrich (USA). Acetic acid and glycerol were purchased from Merck (Germany) and Sigma Chemical Co. (USA). The aqueous solution (1% v/v) of glacial acetic acid was prepared at first, then chitosan solution (2% w/v) was prepared by adding 2 g chitosan to 100 ml acetic acid (1% v/v) while stirring on a magnetic stirrer-hot plate, The solution was stirred with low heat (at 50 °C) for 1 hour. The resultant chitosan solution was filtered through a Whatman No. 3 filter paper to remove any un-dissolved particles and to overcome the fragility of chitosan, glycerol was added in amount of 30% of the total solid weight in solution.[16,17]

The conduit was fabricated based on a method described by others.[18] The mold with chitosan solution was placed in a –80 ˚C freezer for 12 h. 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 hours. Then, the scaffolds were immersed into 2.00% (w/v) sodium hydroxide solution (Merck) and equilibrated for 20 minutes 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 minutes and finally scaffolds were dried at room temperature for 6 hours.[19,20] The fabricated conduit was 0.20 mm thick and 3.50 ± 0.50 mm in inner diameter. All of the conduits were sterilized with formaldehyde tablets in airtight containers for 24 hours.

Animal Grouping and Procedures

Eighteen male New Zealand white rabbits, weighing 2.5-3.0 kg were used. The animals were housed in standard cages and fed with commercial rabbit pellet and water ad libitum. All procedures were carried out based on the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The research project received the confirmation of the ethical code from Islamic Azad University Research Committee under the number of 8672. The animals were randomized into three groups of 6 animals each. The animals were intramuscularly anesthetized using xylazine hydrochloride 10 mg/kg (Alfasan, The Netherlands) and ketamine hydrochloride 40 mg/kg (Ketaset, Germany). The right hind limb of each rabbit was prepped and plantar skin incision was made longitudinally and DDF tenotomy form middle part equidistant from origin and insertion was performed and tendon stumps were sutured in modified Kessler pattern using 3-0 monofilament nylon (Ethilon, Ethicon, Inc., Somerville, USA). In Control group, the DDF tenotomy was performed and the stumps were sutured. In Chitosan group the DDF tenotomy was performed and the stumps were sutured and chitosan conduit was wrapped around the damaged area. In Chit-CoQ10 group the procedure was the same as Chitosan group as well as local administration of 100 µL CoQ10 (100 µg/rabbit) into the chitosan conduit. Despite variation in weight of animals, the same concentration were used.

Macroscopic Assessments

Following 8 weeks post operation, the animals were euthanized using overdose of anesthetic agent (Thiopental 1.0 gr, Biochemie, Austria). Macroscopic assessments including gliding performance of tendon and formation of adhesions were performed based on a scoring system described by others (Table 1).[21]

Histopathological Assessments

Following 8 weeks post operation and after macroscopic assessments the tendon samples were taken (three samples from each group) and fixed in 10% formalin solution. They were then dehydrated and embedded in paraffin wax, sectioned at 5 µm and stained with and stained with hematoxylin and eosin (H&E) stains. Photomicrographs were obtained under light microscope to assess inflammation, angiogenesis and collagen fibrils arrangement.

Table 1. Macroscopic evaluation criteria for adhesion based on Tang *et al.*

	Points	Adhesion appearance
Length	0	*No adhesion*
	1	*Localized, <10 mm longitudinal*
	2	*10-15 mm*
	3	*Intense, >15 mm*
Characteristics	0	*No adhesion*
	1	*Loose, elastic and mobile*
	2	*Average thickness and mobile*
	3	*Thick, hard and immobile*
Classification	0	*No adhesion*
	1	*Mild adhesion*
	2	*Moderate adhesion*
	3	*Advanced stage adhesion*

Biomechanical Testing

The DDF tendons (three samples from each group) were taken and wrapped in saline-soaked gauze. They were immediately stored at -20 ºC until day of biomechanical testing. The suture materials were removed before initiation if testing process and the samples were thawed at room temperature. The TA.XTPlus Texture Analyzer mechanical test device was used for the assessment (Stable Micro Systems, Surrey GU7 1YL, UK). The samples were attached on mechanical testing machine jaws. The initial length was set to 10 mm. Each sample was stretched at a constant rate of 60 mm/min. The load and displacement were sampled 5 times per second. Each sample was stretched to complete tensile failure. Samples were kept wet moist during testing using a drop of normal saline solution on tendon segments.

Statistical Analyses

Differences among groups were evaluated by Kruskal–Wallis variance analysis. When the *p*-value from the Kruskal–Wallis test statistics was statistically significant, multiple comparison tests were used to know differences. SPSS version 18 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A p-value < 0.05 was considered as statistical significance.

3. Results

Macroscopic Findings

There were no signs of local infection around tendons in all experimental groups. The conduit was absorbed in the Chit-CoQ10 and Chitosan groups. Remarkable peritendinous adhesions were found in the Control group that needed sharp dissection for detachments. The adhesion scores in Chit-CoQ10 and Chitosan groups were significantly lower than that of the Control group (p = 0.001) (Table 2).

Table 2. Results of criteria for macroscopic evaluation for adhesion formation. The data are expressed as Mean ± SD.

Experimental groups	Criteria scores for adhesion formation
Control	6.2 ± 2.3
Chitosan	3.1 ± 1.2
Chit-CoQ10	1.5 ± 0.7*

* p < 0.001, indicates significant diminution in adhesion formation in Chit-CoQ10 in comparison with the Control and Chitosan groups.

Biomechanical Findings

In tensile test, rupture at the repair site of samples was selected as the mode of the failure. The mechanical testing demonstrate that biomechanical indices including stress, strain, stiffness and ultimate load were significantly increased in Chit-C0Q10 group compared to those of other groups ($p = 0.001$) (Figure 1).

Histopathological Findings

The histopathological findings of the present study demonstrated that number of ovoid-shaped tenoblasts, inflammatory cells and newly formed blood vessels were significantly decreased in number in Chit-CoQ10 group compared to those of other groups ($p < 0.05$). In H&E staining, there were fibroblasts bearing hyperchromatic and elongated nuclei between collagen fibers. Higher collagen bundles densities were observed in Chit-CoQ10 group compared to those of other groups ($p = 0.001$) (Figures 2-4).

4. Discussion

Results of tendon treatment in daily practice are inconsistent. There is a lot of new knowledge about the healing mechanisms but only very little has been established in clinical practice as far as the current investigations on tendon tissue engineering are mainly preclinical studies. The aim in treatment of acute or chronic tendon pathologies must be, to come as close as possible to a normal tendon with comparable properties. A great challenge lies in degenerated tissues.[22]

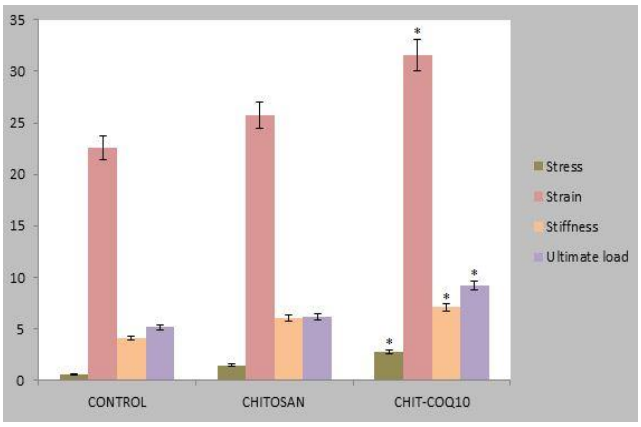

Figure 1. Biomechanical characteristics of repaired tendons in experimental animals are shown in bar graphs. Data are expressed as Mean ± SD. *$p < 0.05$ *vs*. Control and Chitosan groups.

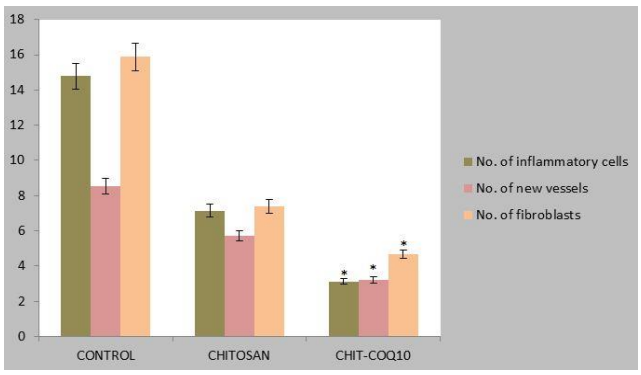

Figure 2. Number of inflammatory cells, new vessels and fibroblast in repaired tendons in experimental animals are shown in bar graphs. Data are expressed as Mean ± SD. *$p < 0.05$ *vs*. Control and Chitosan groups.

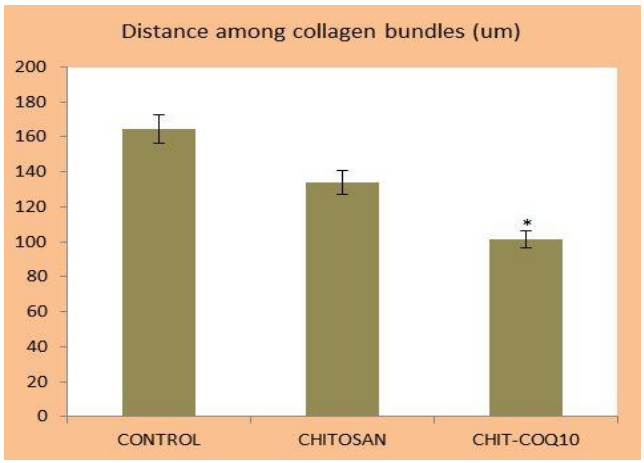

Figure 3. Distance among collagen bundles in repaired tendons in experimental animals are shown in bar graphs. Data are expressed as Mean ± SD. *$p < 0.05$ *vs*. Control and Chitosan groups.

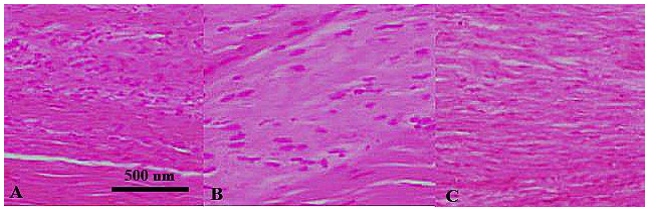

Figure 4. Representative micrographs of tendon tissue eight weeks post operation in experimental groups stained with H & E (400×). A) Control group showing loose connective tissue with a lot of tenoblasts, B) Chitosan group showing fewer tenoblasts and no polymorphonuclear cells infiltration and C) Chit-CoQ10 group showing more dense and organized connective tissue.

Few days after injury, inflammation in tendon declines, and fibroblasts proliferation, extracellular matrix and mostly collagen type III syntheses after five day occur. The newly synthetized collagen fibrils are arranged in the extracellular matrix in a random fashion and after 3-4 weeks are aggregated in organized bundles. Diminution in collagen type III contents and escalation in collagen type I synthesis are considered as a main characteristics of tendon healing remodeling phase starting within two month post injury. In spite of immature and weak nature of collagen type III fibers and their random orientation, they are responsible for neotendon stability. Furthermore, high expression of type I collagens and longitudinal organization of these fibers are thought to be indispensable to get to the maximum tensile strength and accelerated healing of tendons. 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.[23,24] Therefore, the neotendons were evaluated within two months. It has been approved that extreme inflammatory response will interfere with the proliferative phase of healing and the tensile strength of the wound repair will decline as a result of scar formation.[25]

Based on histopathological findings of the present study significant reduction in inflammatory cells was observed in Chit-CoQ10 group, indicating beneficial effect of CoQ10 in combination with chitosan in the healing process of the tendon.

Chitosan, a natural polymer from deacetylation of chitin (poly-N-acetylglucosamine), has been widely applied to topical dressing in wound healing due to its stypticity, antimicrobi-al and nontoxic, biocompatible and biodegradable properties.[26] At the end of the study period, the conduits were totally absorbed in Chit-CoQ10 and Chitosan groups indicating biocompatibility and biodegradability of the conduit.

Despite many efforts, adhesion formation after tendon trauma remains a clinical problem, with no ideal method of prevention. With advances in the understanding of the mechanisms involved in adhesion formation, it may be possible to formulate improved strategies of prevention.[27] The most important factor implicated in adhesion formation is trauma.[28] Tenocytes and tenoblasts are key cells in tendon healing. The isoform α-smooth muscle actin has been identified in tendons and ligaments.[29] Tenocytes that express α-smooth muscle actin are known as myofibroblasts. There are three essential morphological elements that define myofibroblasts: stress fibers (actin microfilaments), well-developed cell-stroma attachment sites (fibronexus), and intercellular gap junctions.[30] The fibronexus is presumed to transfer tensile forces to the extracellular matrix network.[31] Myofibroblasts are thought to play a role in extracellular matrix network homeostasis in tendons and ligaments, and they may well be responsible for the formation of tendon adhesions.[32] Many attempts have been made to reduce adhesion formation by using materials acting as mechanical barriers such as polyethylene or silicone or by using pharmacological agents such as indomethacin and ibuprofen, but no simple method is widely used.[33-35]

Others demonstrated that chitosan inhibits tendon sheath cells proliferation and collagen production.[36] Chitosan inhibits fibroblasts survival, which could be one of the reasons for the increase of tendon gliding. The adhesion formation inhibits the gliding function of tendon and therefore, limits the range of motion of affected limb.[37] In our findings there was no peritendinous adhesions in Chit-CoQ10 and Chitosan groups indicating that tendon gliding was achieved in the injured tendons.

The collagen fibers are considered as the leading structural components of tendon in charge of its mechanical strength.[38,39] The findings of biomechanical indices in the present study demonstrated higher tensile loads compared to Control group. Significant augmentation in stress, strain, stiffness and ultimate load in Chit-CoQ10 showed that CoQ10 administration ended up additional collagen deposition and remodeling.

In conclusion, local administration of CoQ10 in combination with chitosan conduit improved tendon healing in rabbits. It could be concluded that use of the chitosan conduit could be of clinical benefit due to reduced peritendinous adhesion formation around injured site of tendon during repairing period and also the conduit could be used as a carrier for drug delivery to improve and accelerate tendon healing.

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Conflict of Interests

None.

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