

Propolis has no effect on tendon healing: an experimental study

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Abstract

Tendon healing remains a slow process due to less blood supply compared to many other tissues. In previous studies, propolis was shown to modify collagen types I and III accumulation at wound healing site and positively affected wound healing however, no study has shown the effects of propolis on tendon healing. We aimed to investigate the effect of propolis on tendon healing. Twenty eight male rats were used. Achilles tendons of all rats were transected at midpoint and the proximal and distal ends were approximated to each other and repaired by modified Kessler technique with 6-0 ethylene-braided sutures. After surgical operation, the rats in control group received 0.9% SF orally during the next 28 days and the rats in study group received 100 mg/kg propolis extract orally during the next 28 days. Histological examination was made using the Cavalieri method which is an unbiased method for quantification of particles of variable size in a given volume. Compared to the control group, tendon and capillary volume were found to be increased in propolis treated group however the increase was not at a statistically significant level. Propolis may enhance tendon healing both by accumulating collagen type I and III and inducing extracellular matrix components at the injury site. Higher level of propolis dosage may be more beneficial to tendon healing.

Keywords: Propolis, tendon healing, collagen

Introduction

Propolis is a resinous substance collected from various plants by honeybees. It has broad spectrum of biological activities including antimicrobial, antioxidant, anti-inflammatory, and anticarcinogenic effects. Once it is collected, it is enriched with saliva and enzyme-containing secretions. Propolis is used in the construction and protection of hives. The components the propolis varies between geographic areas. Propolis contains polyphenols, steroids, terpenoids and amino acids [1-4].

Tendon healing has inflammatory, proliferative and remodeling phases. In the inflammatory phase, inflammatory cells migrate to the site of injury, chemotactic and vasoactive factors are released leading to increased vascular permeability, angiogenesis and also proliferation of tenocytes. In proliferative phase glycosaminoglycan concentrations and content of water are very high. And also there is a peak in the synthesis of the type-III collagen. Remodeling phase starts after proliferative phase. Remodeling phase is longest in duration; during this phase, fibrous tissue converts to

tendon tissue. The migrated fibroblasts begin to synthesize collagen. Initially, these collagen fibers are randomly orientated. The next five weeks collagen is continuously synthesized. During the 4th week, proliferation of fibroblasts of intrinsic origin is enhanced which is mainly from the endoneurion. These cells both synthesize and reabsorb collagen. The newly formed tissue starts to mature, and the collagen fibers start to be orientated along the direction of force through the tendon. This process continues for two months after the initial injury. Final stability is acquired during the remodeling which is induced by the use of the tendon. This further stimulates fibers to be oriented into the direction of force. Additionally, tensile strength of the tendon is increased by the cross-linking structure of the collagen fibrils. During the repair phase, the mechanically stronger type 1 collagen is produced more than type 3 collagen, which alters the initial ratio of these fibers and increases the strength of the repair [5-8].

Tendon healing remains a slow process due to less blood supply compared to many other tissues [9,10]. Tendon healing is classically considered to occur through extrinsic and intrinsic healing. The intrinsic model produces obliteration of the tendon and its tendon sheath. Healing of the defect involves an exudative and a formative phase, on the whole, which are very similar to those associated with wound healing [11]. Previous studies have shown that

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propolis has positive effects on wound healing. In the structure of the tendons, an abundant extracellular matrix (ECM) is found. ECM is composed of connective tissue in which collagen is the most abundant protein forming collagen fibers. Also proteoglycans (PGs), elastic fibers and non-collagenous proteins (NCPs) are found in the ECM. Type I collagen is predominantly found and provides tensile stiffness power to the tendons. Type III collagen is more flexible than the type I collagen molecule. So collagen type I and III together give elastic modality for the tendons. In previous studies propolis was shown to modify collagen types I and III accumulation in the matrix of burnt tissue [12]. Because of its complex structure and integrity, tendon healing is complex and very slow process. The healing process of the tendon lesions are a clinical issue due to the high incidence of recurrent rupture at the site of the injury. Therefore, new treatment modalities are investigated to improve tendon healing and remodeling. We hypothesized that propolis could enhance tendon healing via a mechanism similar to wound healing. Cavalieri estimation that we used in this present study is a kind of stereological method. It is an unbiased method for quantification of particles of variable size in a given volume. Hence, the stereological methods allow reliable and efficient estimation of the volume of several parameters such as capillaries and new-formed tendon [13].

Tendons have the highest tensile strength of all connective tissue because of a high proportion of collagen in the fibers and their closely packed parallel arrangement. The individual collagen fibrils are arranged into fascicles which contain blood vessels and nerve fibers [5]. Propolis may enhance tendon healing both by accumulating collagen types I and III and inducing extracellular matrix components at the injury site. The aim of the study is to explore the possible positive impact of propolis on tendon healing.

Material and Methods

Preparing Water Extract of Propolis

In the present study, water extract of propolis was used to avoid the effects of ethanol on the tendon healing process. The propolis that we used in our study was collected in the Trabzon province of Turkey. % 10 propolis-water extract was used and prepared as described by Barlak [14]. According to that description, 50 gr of crude propolis was dissolved in 200 ml water by continuous mixing for 24 hours. Propolis extracts were mixed by the processes of filtering and centrifuging. After that it was kept and stored at 4 °C. Finally 10% concentration of propolis extract was prepared by diluting water.

Animal Groups

Approval was obtained from the Animal Experimentation Ethics Committee of the Ondokuzmayis University. Twenty eight male, 250 to 300 gr. in weight, six month-old Wistar rats were used in our study. All of the rats were

kept in an air-conditioned room lighted 12 hours a day (12-hour, light-dark cycles). Temperature was kept constant at $22\pm1^{\circ}\text{C}$ and humidity at $55\pm10\%$. Rats were divided randomly into two groups as control and study groups. 14 rats were housed in each standard cage. All of the rats were obtained from "OMU Experimental Research and Application Center of Medical Sciences". They were all fed by standard food and water. Groups were as follows:

Group 1 (Control Group): After surgical operation, the rats in this group received only 0.9% SF orally during the next 28 days. 12 hours after the last administration of SF at the 28th day, all of the right achilles tendons of rats were removed histological evaluation.

Group 2 (Study Group): After surgical operation, the rats in this group were all received 100 mg/kg propolis extract orally during the next 28 days. 12 hours after the last administration of SF at the 28th day, all of the right achilles tendons of rats were removed histological evaluation.

Surgical procedure

All of the surgical procedures were performed under sterile conditions by the same surgeon. Rats were anesthetized by intraperitoneally administered 100 mg/kg ketamine and 0.75 mg/kg chlorpromazine. The right hind leg was shaved and then sterilized by using 70% alcohol, betadine, and 70% alcohol sequentially. After sharp dissection, the Achilles tendon was exposed. It was transected approximately at mid-point, the proximal and distal ends were approximated to each other and repaired by using a modified Kessler technique with 6-0 ethylene-braided sutures. Skin was closed by using 6-0 nylon sutures. None of the rats received antibiotic prophylaxis. Postoperatively no dressing or cast was applied. All of the animals were unrestricted and fed by a normal diet after surgery. After surgical procedure, each rat was housed individually. Left Achilles tendons were not operated. All right achilles tendons were removed at 28th day postoperatively by the same surgeon and put in %10 formaldehyde solution for histological evaluation. All animals were sacrificed by an overdose of pentobarbital inhalation.

Histological Examination

Tendon samples taken were fixed in 10% formaldehyde, dehydrated in a graded alcohol series, and cleared in xylol for light microscopic examination. After dehydration, specimens were embedded in fresh paraffin. Sections were cut using a microtome (Leica RM 2135; Leica Instruments, Nussloch, Germany). Each paraffin block was serially cut into 7- μm thickness [15]. For volumetric estimation procedure, every 20th section was selected through a set of consecutive paraffin sections from each tissue samples. Choosing the first section was done randomly. All of the sections were sampled from each tendon in a systematic random manner [16]. Selected sections were stained with tripple stain and photographed on the stereology analysis system (Stereoinvestigator 9.0, Microbrightfield, Williston,

VT, USA) using a light microscope (Leica M 4000 B, Germany) with a digital colour camera attachment (Microbrightfield, Williston, VT, USA). Unbiased Cavalieri method was applied to the light microscopic images for the quantitative estimation of volume of new-formed tendon and volume of the capillaries [17,18].

Estimation of sectioned area in the tendon samples was made by point counting test grids. These grids were used to estimate volume of new-formed tendon and volume of the capillaries. (Figure 1).

The point density of the point counting grids was designed to obtain an appropriate coefficient of error (CE) for interesting area in images of the serial sections [17]. CE and coefficient of variation (CV) were estimated according to Gundersen and Jensen' formula [19]. The test grid with systematic array of points was randomly placed on the screen of PC. The volume of each interesting area in all tendon sections was estimated with following formula:

$$\text{Volume} = t \times a/p \times \sum p$$

('t', section thickness; 'a/p', representing area of each point on the point counting grid; ' $\sum p$ ', total number of the points hitting the interesting area). [16].

The mean volume of tendon and capillaries were analyzed by analysis of variance. a/p value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (IBM SPSS Statistics 20 for Mac).

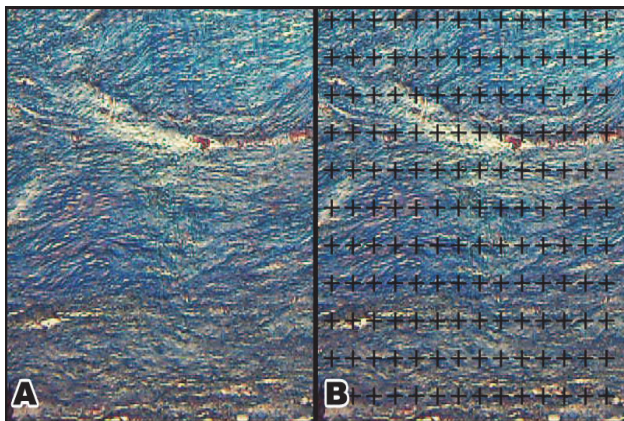


Figure 1: Estimation of sectioned area in the tendon samples was made by point counting test grids. These grids were used to estimate volume of new-formed tendon and volume of the capillaries shown on Figure 1.

Results

There is no significant difference between the groups in terms of both the new-formed tendon volume and the capillary volume (Fig. 2-3) ($p > 0.05$). Although it was observed that new tendon volume and capillary volume increased in propolis treated groups, these values were not statistically significant.

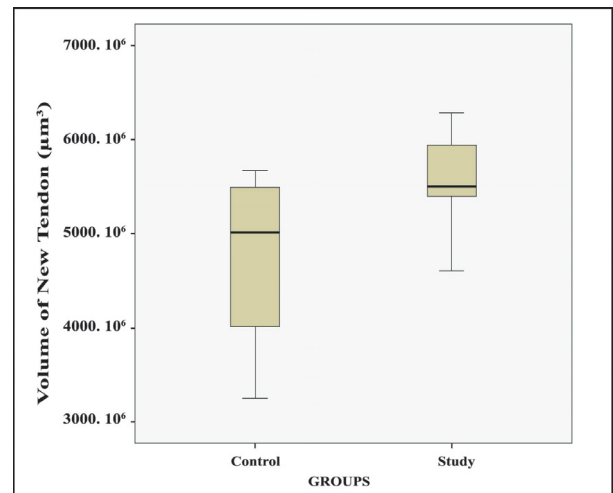


Figure 2: The volume of new-formed tendon is shown in Control and Study groups. Although there is a slight increase in Study group, there is no statistically significant difference between the groups.

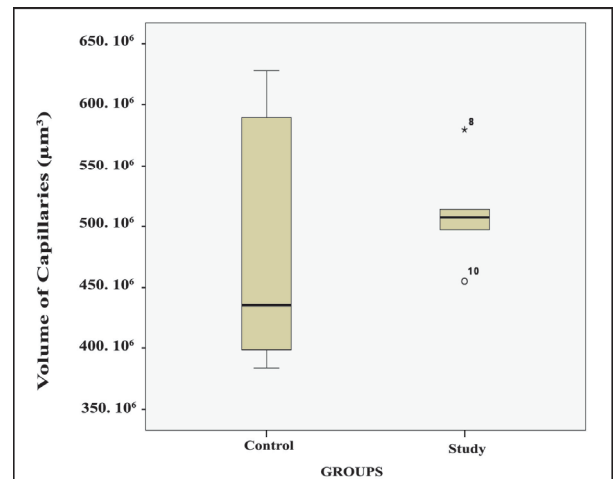


Figure 3: The volume of capillaries is shown in Control and Study groups. Although there is a slight increase in Study group, there is no statistically significant difference between the groups.

Discussion

Comparing the volume of new-formed tendon in both groups, a slight increase in study group was found; however a statistically significant difference was not detected among the groups. The comparison of volume of capillaries revealed that although there was a slight increase in study group, no statistically significant difference was found between both groups.

Tendon tissue contains abundant amount of extracellular matrix (ECM). In ECM, collagen structure is the key factor for biomechanical strength of this tissue. Collagen has been shown to stimulate epithelial migration and proliferation during repair process of the wound [2,20,21]. Numerous studies have shown that propolis potentiates the accumulation of collagen in tissue matrix of wounds [8] and some studies report wound healing properties of

propolis [12,22,23]. Wounds treated with propolis has been shown to extract more collagen type III influencing glucosaminoglycan synthesis [24,25]. Enhancement of healing of tendons has been studied in literature. Philip et al. stated that Amnion-derived multipotent progenitor cells improved Achilles tendon repair in rats compared with saline-treated rats [26].

In another study, Fernandez-Sarmiento et al histologically investigated the influence of plasma rich in growth factors (PRGF) and found that PRGF accelerated early healing process in repaired Achilles tendons in rats [27]. We have observed that use of propolis extract led to an increase in the amount of both collagen content and neovascularization during the healing process of the achilles tendon. But these increasing quantities did not have any statistically significant difference between the study and control groups. In one previous study by Temiz et al [28], they stated that exaggerated inflammation would lead collagenolysis and delay wound healing. Matrix Metalloproteinases (MMP's) have also collagenolytic effects [29]. Propolis has been shown to inhibit MMP's in experimental studies and has anti-inflammatory effects, via this mechanism. It is suggested to stimulate collagen accumulation at injury site [30].

Studies regarding the effect of propolis on angiogenesis are very few in number. In this study, using Cavalieri method volumetric data on an organ or tissue is obtained in vivo without any sampling bias. An increase in the amount of collagen was observed in the study group. Additionally, the increase in the volume of capillaries might also indicate the angiogenetic effect of propolis. Mc Lennan et al. in their animal study revealed that propolis did not have any effect on vessel number at days 6 and 12. In the present study, even though not statistically significant, an increase in the volume of capillaries was observed at 28th day indicating the angiogenetic effect of propolis. Stereological methods provide quantitative data on three-dimensional structures using two-dimensional microscopical sections. Cavalieri method, which is one of the stereological methods under the light microscopy was used in this study to estimate the volume of capillaries and new tendon.

The limitation of the study was that the animals could be fed parenterally instead of oral feeding, and that would probably prevent any bias.

In conclusion, use of propolis extract during the healing process of the achilles tendon led to an increase in the amount of both collagen content and capillary volume. But these increasing quantities were not at a statistically significant level. It might be related to dosage of propolis. Higher level of propolis dosage may be more beneficial to tendon healing and results of histological analysis may have statistical significance. Further studies may explain this point and to analyze the effect of propolis on the collagen bundles organization within the tendons during repair process.

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