EFFECTS OF ASCORBIC ACID AND α-TOCOPHEROL ON COLLAGEN FIBRIL STEREORELOGICAL PARAMETERS IN RABBITS

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Abstract

Twenty adult (8 to 10 months old) male New Zealand White rabbits were divided randomly into four equal groups. After preparation of each rabbit for surgical procedure, the right deep digital flexor tendon was crushed in a standard method. Then the limb was fixed with external coaptation for 7 d. The post-operative treatment in each group included: no treatment (CN), ascorbic acid, 100 mg.kg⁻¹, i.p. (AA), α-tocopherol, 20 mg.kg⁻¹, i.m. (AT), and both vitamins simultaneously (CM) for nine consecutive days. At the 13th d after surgery each rabbit was euthanized and tissues from the crushed tendon were prepared for ultrastructural evaluation by transmission electron microscopy. The median values of collagen fibril diameter in the AT group were the highest; group AA was more than the CN and CM groups; and group CM was the least. The results obtained indicated a significant positive effect of ascorbic acid and α-tocopherol on collagen fibril structural properties and tendon healing. However, combination of both vitamins had no synergistic effect on tendon healing and even significantly decreased the effect in comparison to each individual vitamin (P<0.05).

Key words: rabbit, ascorbic acid, α-tocopherol, tendon, collagen, healing.

Tendons are soft connective tissues consisting of the parallel collagen fibrils as a part of the extracellular matrix that are embedded in an inter-fibrillar non-collagenous matrix mainly consisting of proteoglycans. The main function of tendon is to transmit load and maintain the stability of the musculoskeletal system (2, 30). The organised structure of tendons allows withstanding and transmitting large forces between muscle and bone. These structures are subjected to repeated motion and degeneration over time they are prone to both acute and chronic injuries.

The use of various drugs e.g. non-steroidal anti-inflammatory drugs, phenytoin, ascorbic acid, vitamin A, and α-tocopherol - and strategies - e.g. physical modalities, cytokines, and growth factors, gene therapy, tissue engineering with mesenchymal stem cells, prevention of adhesions, mobilisation and mechanical loading are helpful to promote tendon healing (10, 23-25). Unfortunately, excellent results are not yet attained universally following the treatment (12).

Since the late 1970's, studies on the morphological profiles of collagen fibrils have been performed using electron microscopy and image analysis techniques (1, 19). Generally speaking, the collagen fibril diameter has been regarded as the most important factor related to the biomechanical characteristics of the connective tissues, particularly in tendons and ligaments (1, 17, 18, 29). It has been suggested that the distribution of collagen fibril diameters may be of high importance in determining the tensile strength of a tendon. The measurement of the fibril size can be used as an indication of the extent of change in tendons during normal development, aging or healing (28). The form of the collagen fibril diameter distribution can be directly related to the mechanical properties of the tissue (17).

Ascorbic acid is required as a co-factor for prolyl hydroxylase and lysyl hydroxylase essential for collagen synthesis. When ascorbic acid is deficient, collagen is malformed (8). Ascorbic acid plays a vital role as a pre-eminent water-soluble antioxidant, whereas
α-tocopherol is considered as a very important fat-soluble antioxidant. However, the effect of α-tocopherol on surgical wounds is inconclusive. Ascorbic acid, as one of the non-enzymatic aqueous antioxidants, has been shown to have a positive effect on mesenchymal tissues healing, and to increase α-tocopherol levels by reducing its metabolism (7).

The main objective of the presented study was to determine the attributes of collagen fibrils during tendon healing in rabbits supplemented with α-tocopherol and/or ascorbic acid compared to unsupplemented rabbits.

Material and Methods

Twenty adult (8 to 10 months old) male New Zealand White rabbits weighing 1,900 g (±414 g), were obtained from Urmia University animal house. The rabbits were kept in a controlled environment (room temperature - 18-21°C; humidity – 55%-65%), fed a standard commercial pellet diet (Niro Sahand Co., Tabriz, Iran) to meet all necessary mineral and vitamin requirements of rabbits (including vitamins C and E) and allowed ad libitum access to tap water. The rabbits were quarantined and acclimated to environmental conditions for 4 d before the experiment. The rabbits were randomly assigned into four equal groups: not treated (CN), ascorbic acid treated (AA), α-tocopherol treated (AT), and treated with the combination of ascorbic acid and α-tocopherol (CM).

The rabbits were anaesthetised with a mixture of ketamine (ketamine 10%, Alfasan, Woerden, Holland), 120 mg.kg⁻¹, and xylazine (xylazine 2%, Alfasan, Woerden, Holland), 10 mg.kg⁻¹, and prepared for aseptic surgery. The depth of anaesthesia was assessed by tickling the inside of ear pinna and pedal withdrawal reflex.

The deep digital flexor tendon in each rabbit was exposed through a 3-cm mid plantar incision on the right paw. Standardised crushing, as previously described by Constantinescu et al. (6), was induced in flexor tendons over a length of 1 cm. The skin incision was then sutured in simple interrupted pattern with 3-0 monofilament polyamide (Supalon®, Supa Medical Devices, Iran). The limb was immobilised with Plaster of Paris (D-Cast®, Bandhaye Pezeshki Iran) for 7 d. Neither prophylactic, nor post-operative antibiotics were used.

The post-operative treatment in each group included: CN - no treatment; AA - ascorbic acid (vitamin C 500 mg, Osveh Pharmaceutical Co., Iran), 100 mg.kg⁻¹, i.p.; AT - α-tocopherol (vitamin E acetate 100 mg, Weimer Pharma, Germany), 20 mg.kg⁻¹, i.m.; CM - combination of both vitamins in the mentioned doses. The treatments were administered for nine consecutive days.

At day 13, each rabbit was euthanised and specimens from the crushed tendons were taken and prepared for ultrastructural study according to the standard methodology (5). The fibril diameters were measured manually on electron-micrographs. All fibrils within each scanned field were analysed. According to Lavagnino et al. (11) to eliminate any potential error due to non-perpendicular cuts, the collagen fibril minor diameter was used to represent actual collagen fibril diameter. The fibrils were then pooled into three groups according to their thicknesses based on Södersten et al. (26) as small - <60 nm, medium - 60-150 nm, and thick - >150 nm fibrils.

The following parameters were determined for each animal: (1) frequency of distribution (% of collagen fibril diameter, (2) median value of collagen fibril diameter (nm), (3) collagen fibril area fraction (average percentage of cross-sectional area taken up by collagen fibrils), and (4) number of fibrils per unit area (number of fibrils per square mm) (4, 15). Five hundred to 1,600 fibrils were examined and analysed per rabbit.

Statistical analysis. Data analysis was carried out using SigmaStat software (version 3 for Windows, SPSS Inc, USA). To evaluate whether data were normally distributed, a Kolmogorov-Smirnov test was carried out. Due to the lack of normal Gaussian distribution, the Kruskal–Wallis one way analysis of variance on ranks was used to compare the dependent variables (frequency distribution of collagen fibril diameter, median value of collagen fibril diameter, collagen fibril area fraction, and number of fibrils per unit area) between the four groups of treatment. When the test was found significant, Dunn’s multiple comparison test was applied to examine all possible pairwise comparisons. Results are expressed as median and interquartile range (25% to 75%). Significance was set at P<0.05 for all comparisons.

Results

The frequency distribution of collagen fibril diameter and other stereological parameters of collagen fibril (i.e. median value of collagen fibril diameter, collagen fibril area fraction, and number of fibrils per unit area) are shown in Tables 1 and 2, respectively. Figs 1 to 4 represent electron microscopic images of collagen fibrils from the deep digital flexor tendon (DDFT) of CN, AA, AT, and CM groups, respectively.

A significant difference in the median value of fibril size distribution was detected between the control group compared with the AT, AA, and CM groups (P<0.05) (Table 1).
Discussion

Using ultrastructural analyses on healing tendons from 8-10 months old male New Zealand White rabbits, it was found that α-tocopherol and ascorbic acid had a statistically significant, possibly positive effect on the healing process of the tendons, whilst their combination exhibited a possible negative effect, suggesting that both compounds actually antagonised each other. Nonetheless, the CM group had the second highest fiber density.

In this study, the fibril diameter distributions in all groups were bimodal and the majority of fibrils were in range of small and medium sized fibrils. This is in contrast to the finding of Sarrafzadeh-Rezaei (23), where the distribution of collagen fibrils in a normal and intact deep digital flexor tendon of rabbit were trimodal with the percentage of small, medium, and thick fibrils of 7%, 33%, and 60%, respectively. We assume that during the short period of the study, i.e. 13 d, the maturation of the healed tendon tissue (formation and existence of type I collagen fibrils), and therefore detection of the trimodal pattern were not achievable.

α-tocopherol, one of the fat-soluble vitamins, has an antioxidative function. Mori et al. (16) have reported that the stimulatory effect of tretinoin tocoferil on the gene expression of many extracellular matrix components might be one of the mechanisms of its promotion of wound healing. Galeano et al. (9) found that raxofelast, a synthetic analogue of α-tocopherol, a hydrophilic molecule with antioxidant properties, was able to reverse the effects of diabetes on wound healing by reducing lipid peroxidation, neutrophil infiltration, oedema, and stimulating re-epithelialisation, neovascularisation, proliferation of fibroblasts, and collagen and extracellular matrix synthesis and maturation.
Our results present structural endpoints but not functional, nor biochemical endpoints. A number of reports with biochemical or structural endpoints present alternative conclusions on the effect of α-tocopherol on collagen fibril morphology. Card et al. (3) and Mazzotta (14) reported that α-tocopherol can inhibit collagen synthesis and also can decrease hydroxyproline content. Likewise, Greenwald et al. (10) concluded that supplemental dietary α-tocopherol decreases the breaking strength of composite tendon wounds. These findings point that α-tocopherol may have various and sometimes contradictory effects on connective tissues healing.

Ascorbic acid is an essential factor in collagen synthesis (13). Ascorbic acid acts as a cofactor in the enzymes prolyl and lysyl hydroxylase, which catalyse the hydroxylation of proline and lysine in the procollagen molecule and consequently the synthesis of high tensile strength collagen (14, 27). Ramirez et al. (20) reported a greater than 200% increase in the rate of collagen synthesis by Achilles tendon fibroblasts, when ascorbic acid was added to the basic culture media. A deficiency in ascorbic acid is associated with poor collagen formation and delayed wound healing.

According to the results of this study, combination of vitamins E and C had a significant negative effect on tendon fiber diameter in comparison with the control group. Combined administration of the both compounds failed to promote an increase in collagen fibril diameter. Sarisozen et al. (22) reported that both vitamins had no synergistic impact on fracture healing.

It is well known that the mechanical properties of tendon are essential for its proper function. The organisational properties, such as collagen fibril diameter and collagen fibril area fraction, and compositional properties, such as collagen and glycosaminoglycan (GAG) content, likely significantly influence biomechanical properties of tendons. According to Robinson et al. (21), the most prevalent predictors of mechanical properties of tendons were collagen fibril area fraction (average percentage of cross-sectional area taken up by collagen fibrils) and GAG contents. Mean fibril diameter was correlated with total collagen content, total GAG content, and GAG

### Table 1

Median (interquartile range) values for frequency distribution of collagen fibril diameter (%) in rabbits treated with vitamin C (AA), α-tocopherol (AT), combination of both vitamins (CM), and in non-treated control group (CN)

<table>
<thead>
<tr>
<th>Group</th>
<th>Median collagen fibril diameter (nm)</th>
<th>Collagen fibril area fraction (%)</th>
<th>Number of fibrils per unit area (×10⁶/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>38.9† (28.3-49.4)</td>
<td>58.5 (55.4-60.5)</td>
<td>155.0† (70.0-177.5)</td>
</tr>
<tr>
<td>AA</td>
<td>46.2‡ (29.7-66.7)</td>
<td>70.4 (57.7-75.0)</td>
<td>260.0‡ (213.8-272.5)</td>
</tr>
<tr>
<td>AT</td>
<td>52.6§ (34.2-70.0)</td>
<td>45.6 (34.6-62.3)</td>
<td>160.0‡ (90.0-198.8)</td>
</tr>
<tr>
<td>CM</td>
<td>21.2* (13.2-56.3)</td>
<td>61.7 (51.6-69.5)</td>
<td>245.0‡ (200.0-525.0)</td>
</tr>
</tbody>
</table>

Values with different symbols (†, ‡, § and *) indicate significant difference between groups at P<0.05.

### Table 2

Median and interquartile range (25% to 75%) of collagen fibril stereological parameters in rabbits treated with vitamin C (AA), α-tocopherol (AT), combination of both vitamins (CM), and in non-treated control group (CN)

<table>
<thead>
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<td>245.0‡ (200.0-525.0)</td>
</tr>
</tbody>
</table>

For each animal 500-1,600 fibrils were analysed.
In each row values with different symbols (†, ‡, § and *) indicate significant difference between groups at P<0.05.
content/dry weight. Robinson et al. (21) showed that as more volume of fascicles was taken up by GAG, there was a corresponding decrease in collagen. In addition, instead of increasing fascicle volume to maintain a constant collagen content, the amount of collagen in the tissues was reduced, which was associated with smaller fibrils. Alternatively, in fascicles with more collagen and larger fibrils, less surface area existed for the binding of proteoglycans, leading to fewer GAG per unit mass in tendons. Robinson et al. (21) concluded that collagen fibril area fraction was a significant predictor of mechanical properties of tendons including failure load and failure stress. The effects of α-tocopherol and ascorbic acid on mechanical properties and composition of tendons apparently have not been adequately evaluated. Thus, it is essential to examine these parameters in further studies.

In summary, the presented study demonstrates that the tendon structural properties might be enhanced in healing rabbit tendons by either ascorbic acid or α-tocopherol treatment alone, but not in combination. All three supplemented groups developed significant structural differences from controls. Further investigations correlating structural and functional indices are necessary to resolve conflicting conclusions on the effects of supplementation. Eventually a definitive clinical study should be conducted prior to providing specific vitamin supplementation guidance for patients with tendon disorders. Our results support the potential to significantly modulated healing by supplementation with vitamins E and C.

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