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Curcumin improves tendon healing in rats: a histological, biochemical, and functional evaluation

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ABSTRACT

Curcumin, a compound extracted from the roots of Zingiberaceae, has been proposed as a treatment for tissue injury but studies are yet to be done on its effect on tendon healing. Therefore, we performed a series of experiments to test our hypothesis that curcumin has positive effects on tendon repair. Patellar tendon window defect was created in Sprague–Dawley rats and these were divided into two groups: (i) control and (ii) curcumin-treated. Curcumin (100 mg/kg body weight) was applied by oral gavage. Its potential for promoting tendon healing was assessed by histological evaluation, mRNA expression of tenocyte-related genes, malondialdehyde (MDA) levels, manganese-dependent superoxide dismutase (MnSOD) activity, quantification of hydroxyproline (HOPro), and biomechanical testing. In this tendon injury model, curcumin significantly improved the healing properties as evidenced by extensive deposition of well-organized collagen fibers, decreased MDA levels, and increase in the biomechanical properties and MnSOD activity of the regenerated tendon tissues. The current study showed that curcumin can improve the quality of tendon rupture healing, and thus represents a promising strategy in the management of injured tendon tissue.

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Introduction

Tendons mediate normal joint movement and stability, and injuries frequently occur in both occupational and athletic activities. It has been shown that 44% of tendon ruptures occurred during athletic activities (1). This represents a serious health concern for society in general, which always results in restricted activity or life-long disability. There are several distinct but overlapping phases during the healing process of tendon tissue: inflammatory, proliferative, and remodeling. Tendon healing is a complex process and often results in the formation of fibrotic scar tissue, which has poor tissue quality and inferior mechanical properties. Although many strategies for tendon injuries, such as growth factors (2,3), stem cells (4), and tissue engineering (5,6), have been improved over the past decades, the outcome of tendon injury repair remains unsatisfactory. The recovery and regeneration of tendon injury has become a significant challenge in orthopedics. So, a more in-depth investigation of new strategies for promoting tendon regeneration may provide novel opportunities for such patients.

Treatment of tissue injury using plant products is becoming more widespread (7–9). Curcumin (1,7-

bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a naturally occurring compound extracted from the roots of Zingiberaceae, exhibits a variety of pharmacologic properties that contribute to reduction of tissue damage and promotion of tissue repair. It shows promise of being an efficacious treatment for tissue injuries in dermis and nerve fibers, due to its physiological functions such as wound healing and cell migration (10–12). Several other studies have provided scientific evidence regarding the antioxidant, anti-inflammatory, and anti-infectious properties of curcumin (13–15). Moreover, curcumin downregulates NF- κ B-regulated gene products involved in apoptosis, matrix degradation, and inflammation in human tenocytes *in vitro* (16). It has been suggested that decreasing the inflammatory response in the early stages of tendon wound healing could enhance the tissue quality of the tendon (17). Also, tendon injuries have been shown to benefit from antioxidant therapy (18).

Based on these facts, we hypothesized that the addition of curcumin would induce the promotion of regeneration and functional recovery after tendon injury. Rat patellar tendon window defect is among the more common experimental models for tendon

healing studies because of its accessibility and healing properties (5). The present study was designed to investigate the efficacy of curcumin on the healing of injured tendons using a rat patellar tendon injury model.

Methods

Animals

Sixty-four male Sprague–Dawley rats (weight 270–300 g), purchased from the Experimental Animal Center of Weitonglihua (Beijing, China) were used in this study. The animals had free access to sterile food and water. Animal experiments were carried out under the Rules and Regulations of the Animal Care and Use Committee at Shanghai Jiao Tong University School of Medicine (protocol number C0062014003).

Drugs and chemical reagents

Curcumin, xylazine, and ketamine were obtained from Sigma Chemical Co. (St. Louis, MO). Hematoxylin-eosin and Masson trichrome staining kits were obtained from Beyotime Co. (Haimen, China).

Surgical procedure, animal grouping, and drug administration

On the day of surgery, the rats were anesthetized with xylazine and ketamine (8.7 mg/100 g BW, IM). Using a sterile technique, a 1.5 cm skin incision was made over the right knee. Then, the central one-third of the patellar tendon was removed from the distal apex of patella to the insertion of the tibial tuberosity to create a tendon defect, as reported in earlier studies (5,19). After surgery, the skin incision was closed using 5-0 nylon. The left knee of all rats was left intact. The animals were placed in recovery cages on heating pads until they recovered from the anesthesia. Over the first 24 h of recovery, the animals were housed in individual cages and were provided with softened rat chow. After 24 h, the animals were inspected and weighed daily. Following surgery, those rats with tendon injury were randomly divided into two groups. In group I, the rats were administered 10 ml of saline by oral gavage, and these comprised the non-treatment control group. In group II, the animals were administered curcumin (100 mg/kg BW) by oral gavage. The required amount of curcumin was added to saline immediately before use. The once-daily administration of curcumin and saline was started immediately after tendon injury for a period of 14 days. The dosage of curcumin was based on

previous similar studies (11,20) which indicated that curcumin can promote tissue regeneration after peripheral nerve injury and dermal excision.

Histological examination

At 14 days post-treatment, eight rats in each group were euthanized. The patellar tendon tissues were harvested and fixed using a 4% formaldehyde solution. Samples were sectioned longitudinally to a thickness of 5 μ m. The sections were then processed for histological examination using hematoxylin-eosin (H&E) and Masson trichrome staining. Organization of fibrous connective tissue at the healing site was evaluated using the fiber alignment score: 3 = 75–100% parallel fiber alignment; 2 = 50–75% parallel fiber alignment; 1 = 25–50% parallel fiber alignment; and 0 = 0–25% parallel fiber alignment.

Immunohistochemical staining with anti-rat type I collagen antibody (1:300; Santa Cruz Biotechnology, Dallas, TX, #sc-25974) was also performed to evaluate tendon healing. Cy3-conjugated goat anti-rabbit IgG (1:500; Santa Cruz Biotechnology, #sc-3752) was used as a secondary antibody. Nuclei were counterstained with Hoechst fluorochrome 33342 (1 mg/ml; Sigma, St. Louis, MO, #B2261).

Gene expression

Rats were anesthetized with isoflurane on day 14 after surgery. Eight healing patellar tendon tissues in each group were stored at -80°C to be processed for mRNA extraction for real-time PCR. Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using the First-strand kit (Invitrogen, Carlsbad, CA). qRT-PCR was carried out with the QuantiTect SYBR Green RT-PCR kit (Qiagen); 2 μ l of the total cDNA of each sample were amplified in a 50 μ l reaction mixture. Cycling conditions were: held at 65°C for 5 min, snap cooling at 4°C for 1 min, 42°C for 50 min, and 72°C for 15 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Relative gene expression levels were calculated using the $2^{\Delta\text{CT}}$ formula. Gene expression levels were normalized with respect to controls.

Rat-specific primers were used for collagen type I, tenomodulin, collagen type III, and GAPDH as follows: 5'-AGAAAGGATCTCCTGGTGC-3' (forward) and 5'-ACGTTCCACTTGCTCCA-3' (reverse) for collagen type I; 5'-CCATGCTGGATGAGAGAGGTTAC-3' (forward) and 5'-CACAGACCCTGCGGCAGTA-3' (reverse) for tenomodulin; 5'-TGCACCTGGCAAAAACGG-3' (forward) and 5'-TTCCATTTTCTCCTGGAGG-3'

(reverse) for collagen type III; and 5'-CGAGCTGAACGGGAAAC-3' (forward) and 5'-CCTGGTCCTCGGTGTAG-3' (reverse) for GAPDH.

Biochemical assay

Eight healing patellar tendon tissues in each group were weighed, minced, and homogenized. Malondialdehyde (MDA) concentrations in tissue homogenate supernatant were measured by colorimetric assay. The assay was performed at 532 nm by measuring the presence of thiobarbituric acid reactive substances. Manganese-dependent superoxide dismutase (MnSOD) activity was measured in the presence of potassium cyanide as a reduction in the rate of cytochrome-c by 50%. MnSOD activity was expressed as U/mg protein.

Quantification of hydroxyproline (HOPro) was also performed. Fragments of the healing tendon tissues were hydrolyzed in 6 N HCl, then the lysate was treated according to the method previously described (21). The absorbance was measured at 550 nm.

Mechanical examination

At week 4, eight rats in each group were sacrificed and patella–patellar tendon–tibia complexes were harvested for biomechanical testing. The regenerated tissue in the window defect was isolated according to the procedure described in previous studies (5,6). The patella–patellar tendon–tibia complex was mounted in a biomaterial testing machine (Z10, Zwick, Germany). Samples were distracted at a testing speed of 0.6 mm/s. A preload of 0.1 N was applied to the samples. Ultimate force and Young's modulus were chosen for mechanical examination. Ultimate force represents the maximum load that will result in fracture. Young's modulus is used to describe the material stiffness of tissues as they are loaded. From the load–elongation curve, the ultimate force was determined for each test specimen.

Young's modulus was obtained from the stress–strain curve as the slope of the linear portion. The tendon cross-sectional area at the rupture site was measured by the animal ultrasound system.

Statistical analysis

The data are presented as mean \pm standard deviation (SD). Unpaired *t*-testing was performed to evaluate differences between the two groups. Values of $p < 0.05$ were accepted as statistically significant.

Results

Histology of regenerated tendon tissue

In order to investigate the effect of curcumin on tendon regeneration, the healing tendon samples were collected for H&E staining and Masson's trichrome staining at 14 days. The healing tendon cells in the control group were round and randomly oriented (Figures 1 and 2). However, the cells became more elongated and longitudinally arranged along with parallel collagen fibers in the curcumin group. Further, more extracellular matrix primarily collagen was produced in the curcumin group compared with the control group (Figures 1 and 2), and remodeling of fiber alignment was improved following the use of curcumin (Figures 1 and 2). Fiber alignment scores were significantly higher in the groups treated with curcumin than in the control groups (Figure 3; $p < 0.05$).

Immunohistochemical staining for collagen type I was also performed to demonstrate regeneration of tendon tissues. Higher expression of type I collagen was observed in the curcumin group than the control group. The alignment of type I collagen fibers was also enhanced in the curcumin group (Figure 4).

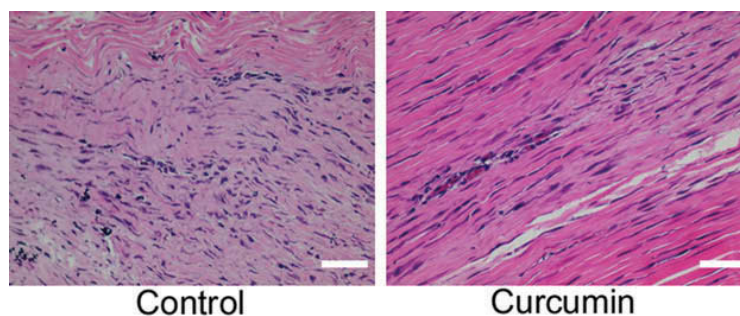


Figure 1. H&E staining of window defect in patellar tendon 2 weeks after repair. Magnification: 400 \times , scale bar: 50 μ m.

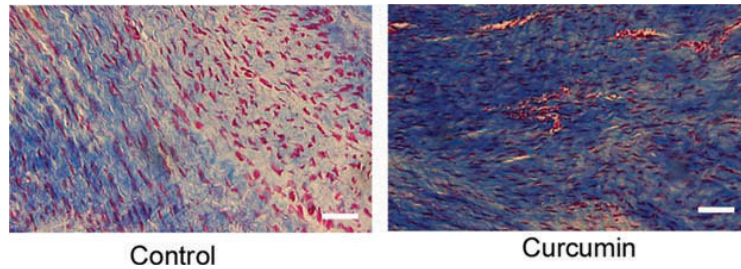


Figure 2. Masson's trichrome staining of window defect in patellar tendon 2 weeks after repair. Remodeling of fiber alignment was improved in the curcumin group. Magnification: 400 \times , scale bar: 50 μ m.

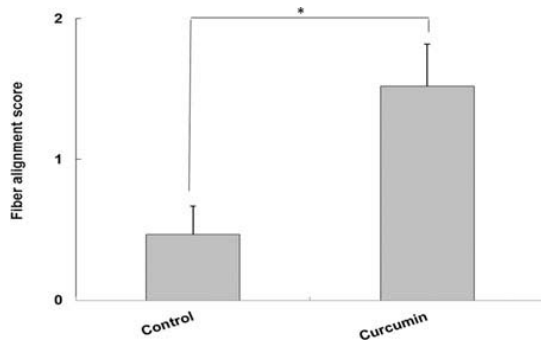


Figure 3. Fiber alignment score for each group. Fiber alignment score was highest in the group treated with curcumin. * $p < 0.05$.

Tenocyte-related gene mRNA expression during tendon healing

We examined the expression of the tenocyte-related genes collagen type I, tenomodulin, and collagen type III at week 2. It was found that collagen type III, a key fibrillar collagen synthesized during the healing process, was significantly higher in the curcumin group compared with the control group (Figure 5; $p < 0.05$). Additionally, the expression of collagen I was also higher in the curcumin group.

There was no significant difference in tenomodulin mRNA levels among treatment groups.

Biochemical results

The results of MDA levels in all groups are shown in Figure 6. Treatment of rats with curcumin significantly decreased tissue MDA levels. MnSOD activity in the curcumin group was higher than in the control rats (Figure 7; $p < 0.05$). This result indicates that curcumin treatment may be useful in scavenging oxygen-derived free radicals.

The quantification of HOPro (Figure 8) revealed that there was a significant increase in the concentration of hydroxyproline in the curcumin group when compared with the control group ($p < 0.05$).

Mechanical properties of regenerated tendon tissue

The mean ultimate stress and Young's modulus for regenerated tendon tissue are shown in Figure 8. Both parameters were significantly lower in the control group than in the curcumin group (Figure 9; $p < 0.05$), suggesting that curcumin significantly improves the biomechanical function of injured tendon tissue.

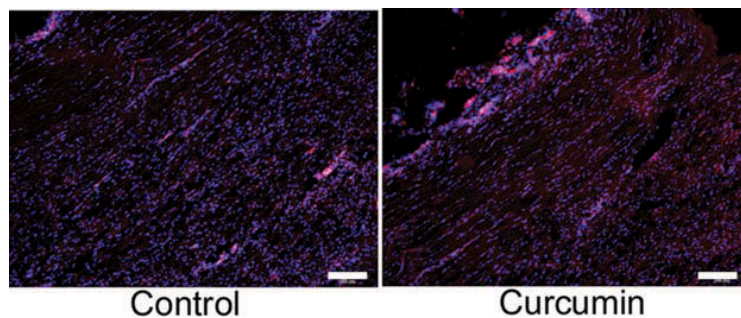


Figure 4. Immunostaining for type I collagen of window defect in patellar tendon 2 weeks after repair. Extensive expression of collagen type I was found in the curcumin group. Magnification: 100 \times , scale bar: 100 μ m.

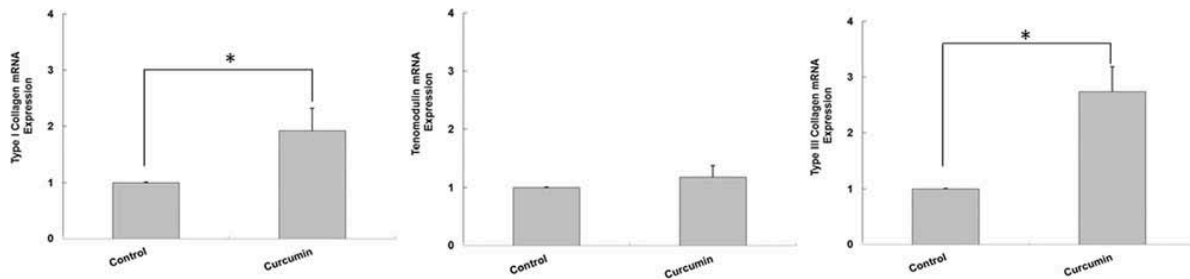


Figure 5. The expression levels of tenocyte-related genes were evaluated by qRT-PCR. The expression of collagen types I and III was significantly upregulated after treatment with curcumin. $*p < 0.05$. The expression level was normalized to that of GAPDH.

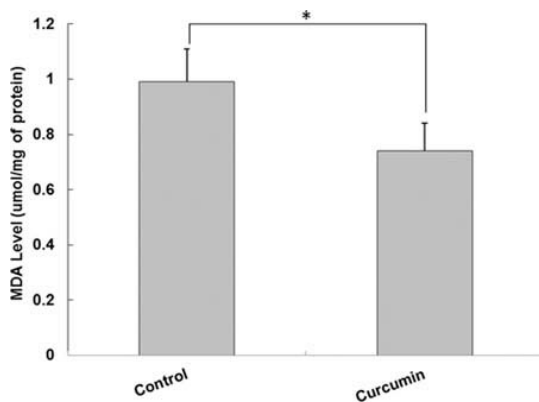


Figure 6. Levels of MDA in healing tissue of the control and curcumin-treated rats 2 weeks after injury ($*p < 0.05$).

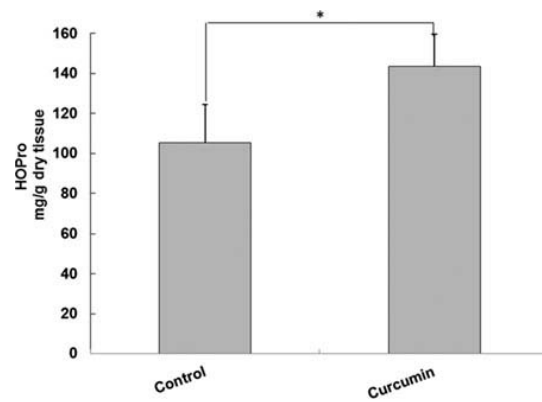


Figure 8. Concentration of HOPro in healing tissue of the control and curcumin-treated rats 2 weeks after injury ($*p < 0.05$).

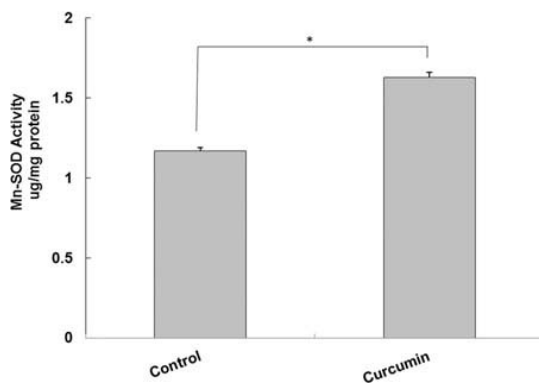


Figure 7. Mn-SOD activity in healing tissue of the control and curcumin-treated rats 2 weeks after injury ($*p < 0.05$).

Discussion

Tendon injuries account for a significant percentage of sport-related injury and the enormous associated costs. Although methods of treating tendon injuries have been improved over the past decades, the clinical treatment of tendon loss or wounds continues to be a major problem in surgical procedures. A therapeutic agent selected for the treatment of

tendon injury should improve the phases of healing without producing deleterious side effects. Moreover, to combat increasing health care costs, it is important to understand the role of natural products in the healing of injured tendons. Previous studies found that curcumin could elevate the synthesis of collagen, promote angiogenesis, decrease reactive oxygen species, and enhance the healing processes in skin and nerve tissue wounds (11,22–26). However, the effects of curcumin on the properties of the healing tendons remain to be elucidated, which invokes the research question of whether curcumin could improve the process of tendon healing.

In this study, we evaluated the effects of curcumin on tendon healing. Based on observed changes in the expression of genes involved in tendon healing, our results suggest that curcumin had positive effects on healing. An increase in collagen I and III mRNA level expression was noted in healing tendon tissues treated with curcumin compared with those treated with only saline. Collagen I is the predominant genetic type of collagen in normal tendon tissue. The early production of type III collagen is also important in the initial

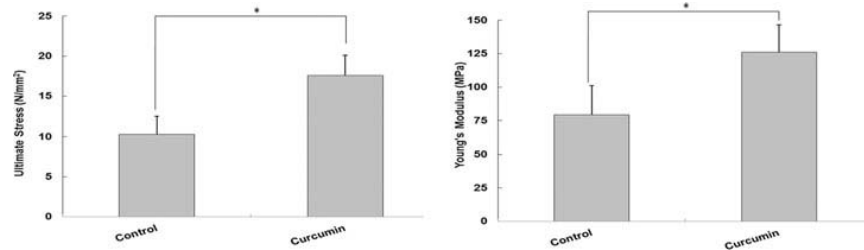


Figure 9. Mean ultimate stress and Young's modulus of healing tendon tissue 4 weeks after repair. * $p < 0.05$.

wound structure during the healing process (27). Increased collagen I and III expression suggests improved regeneration, healing, and recovery. Our findings suggest that curcumin may have a key role in the healing and development of ligaments by regulating the synthesis of collagen I and III. An optimized collagen type I/III ratio might account for the quality of matrix organization in tendon healing (28). However, the gene expression mRNA levels of collagen I and III was normalized with respect to controls in this study, so the ratio of collagen type III to type I was not measured. Future studies will need to be conducted to clarify this issue. Based on the results from histological evaluation in our patellar tendon injury model, our data also suggest that curcumin improved tendon healing via well-organized collagen fibers. This is in accordance with an earlier finding that curcumin application results in more rapid wound closure and greater collagen deposition in the healing wound (29). Hydroxyproline content is a measure of synthesis of newly formed collagen. The determination of hydroxyproline also showed markedly increased levels in the rats that received curcumin treatment. We chose also to measure the expression of tenomodulin, which is a marker of tenocyte differentiation during the repair process (30). However, no alteration in the level of tenomodulin mRNA was observed in the curcumin-treated specimens, which might indicate that the healing tendon tissue was not in an anabolic state.

Free radicals and oxidative reaction products result in tissue damage and play a major role in tissue injury. It was found that oxidative stress can cause damage leading to tendon tissue degeneration (31). Tendon injuries have also been shown to benefit from antioxidant therapy (18). MDA is a by-product of lipid peroxidation and is widely used as a reliable marker of tissue damage. In our study, we observed that treatment by curcumin significantly decreased MDA concentration in healing tendon tissue. Moreover, SOD is a key component in cell growth and protection. MnSOD is known to be a key antioxidant enzyme in the prevention of reactive oxygen species-induced tissue damage. It also plays an important role

in the breakdown of tendon matrix due to the matrix metalloproteinase (32). MnSOD activities were maintained by the treatment of rats with curcumin in this study. These results demonstrate that curcumin possesses significant antioxidant activity, which helps to prevent oxidative damage and promote the healing process. Additionally, the improved tissue quality with curcumin treatment was further supported by the biomechanical results in the present study, showing that curcumin significantly increased the ultimate stress and Young's modulus of the regenerated tendon tissues compared with the control group.

One potential limitation of this study is that other cytokines or growth factors that regulate expression of the extracellular matrix were not evaluated. Moreover, the detailed mechanisms underlying curcumin-enhanced healing potential of injured tendon tissue need further investigation.

Conclusions

In conclusion, our study shows that curcumin enhanced tendon regeneration through well-organized collagen fibers, extensive deposition of collagen, decreased MDA levels, and increase in the biomechanical properties of the regenerated tendon tissues. Curcumin could become an additional and novel therapeutic agent in the management of injured tendon tissue.

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Declaration of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the article.

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