Alcoholic Extract of Tarantula Cubensis Improves Sharp Ruptured Tendon Healing After Primary Repair in Rabbits

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Abstract

This study was designed to investigate the effects of tarantula cubensis (TC) on the superficial digital flexor tendon (SDFT) rupture after surgical anastomosis, on day 84-postinjury (DPI) in rabbits. Forty white New Zealand, mature, male rabbits were randomly and evenly divided into treated and control groups. After tenotomy and primary repair, the injured legs were immobilized for 2 weeks. TC was injected subcutaneously over the lesion on 3, 7, and 10 DPI. The control animals received subcutaneous injections of normal saline similarly. Animal's weight, tendon diameter, clinical status, radiographic and ultrasonographic evaluations were recorded at weekly intervals. The animals were euthanized on 84 DPI and the injured tendons and their normal contralaterals were evaluated for histopathologic, histomorphometric, ultrastructural, biomechanical, and percentage dry weight parameters.

Treatment significantly improved the clinical performance, cell, collagen and tissue maturation, tissue alignment and remodeling, ultimate strength, stiffness, maximum stress, and dry weight content and decreased the tendon diameter, inflammation, adhesions and degeneration of the injured treated tendons compared to the injured control ones.

The present findings showed that TC is effective on sharp ruptured SDFT in rabbits and it could be one of the novel therapeutic options in clinical trial studies.

Sharp rupture of the Achilles, gastrocnemius, superficial, and deep digital flexor tendons has been shown to be very prevalent in middle ages and sport activities in humans and animals.[1-4] Sharp rupture of the flexor tendons could accidentally occur during orthopedic procedures, movement of the fractured bones, car accidents, and gunshot fractures in humans and animals.[4] The healing ability of the sharp ruptured tendons would be a concern for orthopedic surgeons. Sharp ruptured tendons heal slowly and often result in formation of scar tissue or fibrous adhesion which increases the risk of reinjury at the repair site.[2,4,5]

Various techniques have been described that provide robust tendon anastomosis with minimal gap formation, limited adhesion, and preservation of the tendon's intrinsic blood supply.[2,3,6] However, after surgical reconstruction, the tendons do not properly heal[2,7] and therapeutic agents may have some values to stimulate tendon healing.[4]

Tarantula cubensis (TC) has been used in homeopathy to treat mixed mammary tumors, pododermatitis, abscesses with burning pains, gangrene, septicemia, and toxemia by forming a demarcation line around the lesions.[8-11] In addition, TC reduced inflammation and tarsal bursitis volume, stimulated epithelialization in the full thickness cutaneous wounds,[11,12-14] improved the uterine involution, and treated the genital microbial diseases,[15] oral ulcers,[14] and cutaneous papillomatosis of various species of animals.[16]

The present study sought to investigate the effects of TC on the remodeling phase of tendon healing after surgical anastomosis of the tenotomized superficial digital flexor tendon (SDFT) in rabbits. This experiment was designed on the basis that TC may have a role on lyses of the postsurgical tissue necrosis, reducing the inflammatory processes, and inhibiting migration of the collagenolytic enzymes from the necrotic tissue to the surrounding area.

Materials and Methods

Forty skeletally-mature male white New Zealand rabbits of approximately 12±2 months age and 1.98±0.19 kg body weight were randomly divided into 2 groups: injured animals treated with TC, and injured controls treated with saline. The left SDFT of each animal was determined as the injured tendon and the right one (normal contra-lateral tendon) was determined as an index of normal tendon for each group.

For injury induction and primary repair, the animals were anesthetized by intramuscular injection of 1 mg/kg Xylazine 2% as premedication and 60 mg/kg Ketamin-HCl-10%. The left hind leg of each animal was designated as experimental. A 2 cm longitudinal incision was made through the skin and subcutaneous

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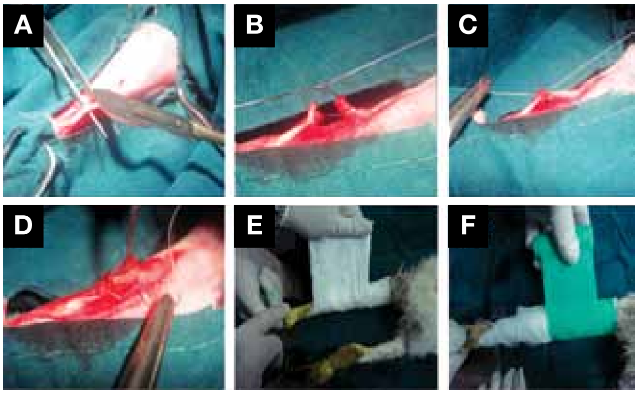

Figure 1. Surgical methods: (A) Injury induction, (B) modified Kessler core suture, (C) running pattern anastomosis, (D) paratenon closure, (E, F) immobilization technique.

tissue, approximately 0.5 cm distal to the gastrocnemius muscle and 0.5 cm above the calcaneal tuberosity (CT), and the Common Calcaneal Tendon (CCT) complex was exposed. An incision was made in the paratenon; the SDFT was exposed and carefully dissected. The SDFT was completely incised transversely at the mid part of the tendon, approximately 1.5 cm distal to the gastrocnemius muscle and 1.5 cm above the CT (Figure 1A). Immediately, the tendon was sutured using the modified Kessler technique with absorbable polyfilament polygalactin 910, 4-0 sutures (Vicryl plus, Ethicon Inc, Somerville, New Jersey, USA) (Figure 1B). The edges of the tendon were sutured by running pattern, with the same material #0-6 (Figure 1C). The paratenon and the skin were sutured in a routine manner (Figure 1D). After surgery, a cast (Dyna-cast 5CM, Anyang, Korea Co, LTD) was applied for 2 weeks (Figure 1E, 1F). A rectangular window was created in the cast at the site of injury to facilitate the injection of the reagent around the lesion.

TC (Theranekron, Richter-Pharma AG, Wels, Austria) at a dose of 1 μg/kg was injected subcutaneously at the site of injury[8-11,14] through the window cast, on days 3, 7, and 10 postinjury. Normal 0.9% sterile saline was similarly injected subcutaneously at the site of injury in the injured control group at the same time, viscosity, and volume as the injured treated animals.

Before injury induction, the animals were weighed and the right (intact) and left (injured) tendons and the covering skin diameter as an index of tendon swelling and postsurgical inflammation were measured using a micrometer measurement device (Samsung, Seocho-gu, Seoul, Korea), before injury, and then weekly, until the animals were euthanized.[17-19] The routine clinical examination and lameness scoring were performed at weekly intervals.

The radiographic and ultrasonographic observations were evaluated at weekly intervals for 12 weeks to determine if the tendon injury could alter the joints and bony structures of the hind paw. The cross section echo texture of the SDFT of the rabbits due to their low diameter and view was not diagnostic; therefore, the animals were sonographed at longitudinal section with a 12 MHz linear probe (SLR-400, Siemens Deutschland, Berlin, Germany).[4,7,20]

Eighty-four days after injury induction, the animals were euthanized by administering sodium thiopental (50 mg/kg) to induce coma, pancuronium (Pavulon, Organon Inc, Roseland, New Jersey, USA) 1 mg/kg was delivered in order to stop breathing to perform a comfortable euthanasia. The study was approved by the local ethics committee of our faculty, in accordance with the ethics standards of Principles of Laboratory Animal Care.

Specimens from each of the injured and uninjured SDFT of 10 animals of each group were collected for light and electron microscopic studies together with percentage dry weight analysis. In the remaining 10 animals of each group, both injured and contralateral SDFT were used for biomechanical testing.

For gross-pathology observation, each injured or normal contralateral tendon was carefully evaluated for pathologic appearance and it was analyzed with computerized morphometric technique for determination of the hyperemia, peritendinous adhesions, and tendon diameter.[4,7]

For histopathology and histomorphometric analysis, after routine preparation, the samples were stained with hematoxylin and eosin. The sections were studied using a light microscope (Olympus, Tokyo, Japan) and were photographed at x200, x400, and x800 magnification, and the figures were transferred to Adobe-Photoshop (Adobe Systems Inc, San Jose, California) and ImageJ (National Institutes of Health, Bethesda, Maryland) software for histopathologic and histomorphometric evaluations.[4,17] The total cellularity, cell differentiation, collagen, vascular density, and vascular diameter were counted and measured. The maturity of the tendon cells and vessels, tissue alignment, crimp pattern, tissue maturity and amount of the suture material was analyzed too.

For ultrastructural analysis, after routine preparation of the ultrathin sections, the sections were transferred to transmission electron micrographs (Philips CM10 transmission electron microscope, Eindhoven, Netherlands). Ultra-micrographs of different magnifications (x5,200-158,000) were used for studying the morphology and morphometric analysis of the collagen fibrils and elastic fibers, constituents of the inflammatory cell and maturity of the tenoblasts.[17-19]

Using the standard preservation methods the samples were biomechanically tested using a tensile testing machine (INSTRON, Norwood, Massachusetts).[17] The specimens were mounted between the 2 metal clamps and were then subjected to tensile deformation at a strain rate of 10 mm/min and the load deformation and stress-strain curves were recorded by a personal computer.[4,18,20]

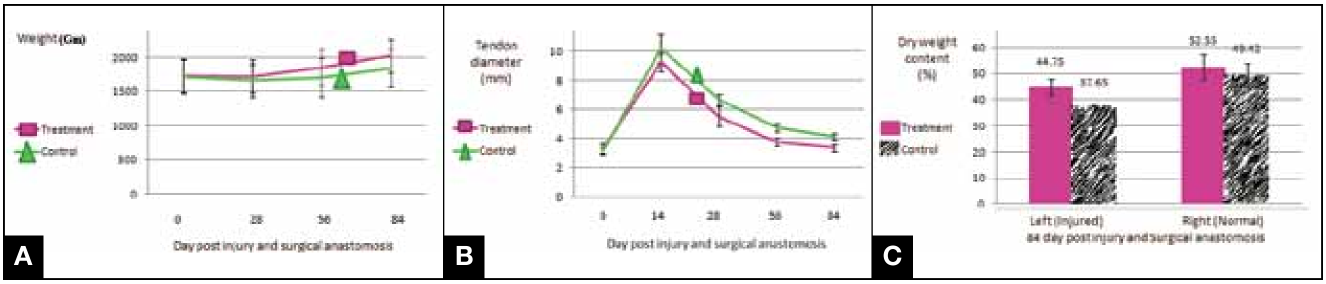

Figure 2. (A) Weight of the animals during the course of the experiment. (B) The tendon diameter of the injured treated tendons is significantly lower than those of the injured untreated ones from 14 to 84 days postinjury. (C) Dry weight content of the injured and normal tendon of both groups.

For percentage dry weight analysis, the samples were weighed immediately after euthanasia and were freeze-dried (Modulyo freeze-dryer; Edwards, Crawley, New Jersey, USA) to a constant dry weight as previously described.[17,18]

For statistical analysis, the pair sample *t*-Test and independent sample *t*-Test was performed to analyze the significant differences ($P<.05$) between variables within and between groups, respectively.[4,17,18,20]

Results

The animals of both groups had increase in body weight during the course of the experiment but only the injured treated rabbits were significantly heavier at the end of the study, compared with the beginning of the study ($P = .001$) (Figure 2A).

Compared to the injured control animals, treatment significantly decreased the diameter of injured tendon from day 14 after cast opening ($P = .027$) to day 84 ($P = .001$) postsurgical operation (Figure 2B), so that the diameter of the injured treated tendons was almost similar to those of their normal contralateral tendons at the end of the experiment.

In clinical observations, treatment reduced pain (75%), temperature (90%), and swelling (100%) of the injured area of the animals and no lameness was observed from day 21 postinjury up to the end of the experiment in 90% of these animals. Compared with the injured control animals, movement in 85% of the treated animals was almost normal from 28 DPI to the end of the study. More physical activity in the cages was observed in the 80% of the injured treated animals, compared with the injured control animals.

There was no radiographic diagnostic abnormality in both legs of the animals of both groups. At ultrasonography level, compared with day 0 postinjury, treatment significantly increased the echogenicity and homogenicity scoring number in 85% of the injured tendons on day 84 after injury ($P = .014$) while 70% increase in the same factors was recorded in the injured control tendons ($P = .049$).

Compared with injured control tendons, treatment with TC decreased the diameter of 95% of the injured treated tendons at weekly intervals, from day 14 to 84 after injury. No amputated view was observed in 85% of the injured treated tendons, compared with the injured control tendons on day 84 after injury (Figure 3).

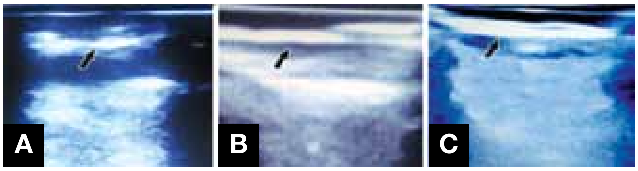

Figure 3. Ultrasonography: longitudinal section is diagnostic, (A) injured control tendon, (B) injured treated tendon, (C) normal uninjured tendon.

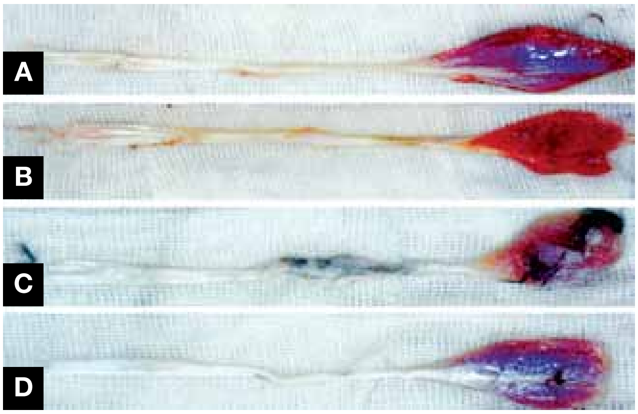

Figure 4. Gross pathology: (A) Normal tendon of the injured treated group, (B) injured tendon of the injured treated group, (C) injured tendon of the injured control group, (D) normal tendon of the injured control group.

At gross pathology, 95% and 90% of the treated lesions had smaller diameter and less fibrous connective tissue adhesions to the surrounding areas, respectively, compared with the injured control tendons, The surrounding peritendinous tissues of the 90% of the injured control tendons were fibrous in nature, and adhesion between the tendon and the peritendinous fascia was evident in 85% of them. While 65% of the injured control tendons were red in color due to their hyperemic blood vessels, 100% of the injured treated tendons had shiny glistening surfaces, a characteristic almost similar to those of the normal uninjured tendons, and 75% of the treated tendons showed no adhesion to the surrounding fascias (Figure 4).

Compared with the injured control tendons, treatment significantly reduced edema and cellularity of the lesions in 80% of the samples ($P = .003$). Lymphocyte

Table I. Histopathologic Cell Differentiation Analysis: Cell Types of Injured Treated Tendons With TC Versus Injured Control Tendons[a], Day 84 Postinjury and Surgical Anastomosis

Variable	Injured treated tendons	Injured untreated tendons	*P* value
Fibroblast	150.50+22.82	237.75+26.58	.003
Fibrocyte	46.00+9.41	14.00+7.43	.002
Macrophage	2.75+3.59	3.25+1.50	.806
Lymphocyte	7.00+1.05	12.25+3.68	.049
Neutrophil	negative	negative	--
Mature fibroblast	21.50+5.71	15.50+3.00	.139
Immature fibroblast	7.50+1.25	15.00+4.83	.049
Vascularity	1.75+1.71	3.75+3.30	.323
	Normal contralateral tendon of the treated group	Normal contralateral tendon of the untreated group	
Fibroblast	7.25+13.20	33.00+49.65	.355
Fibrocyte	41.50+12.01	34.25+12.03	.427
	Injured treated tendon	Normal contralateral tendon	
Fibroblast	150.50+22.82	7.25+13.20	.004
Fibrocyte	46.00+9.41	41.50+12.01	.683

[a]Number of fields for each tendon (5), *P*<.05 was considered significant, independent sample *t*-test was used for comparison between left-left tendons of injured treated and injured control groups. Microscopic field magnification for cell count (x200) and for cellular maturation analysis (x800).

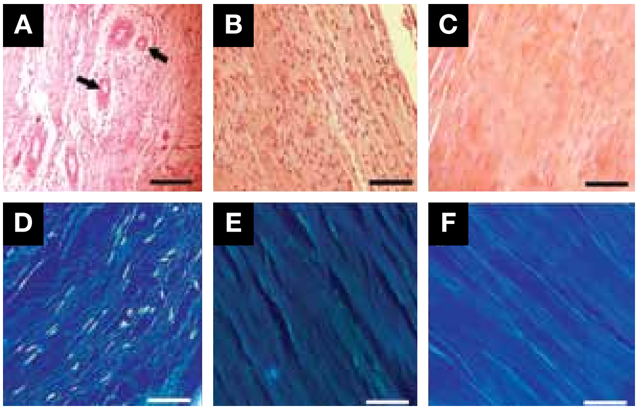

Figure 5. Histopathological findings (normal and inverted figures): (A) and (D) Injured control tendon. Numerous new regenerated blood vessels with prevascular edema (arrows) are seen in the section. (B) and (E) Injured treated tendon. (C) and (F) Normal tendon. Note the parallel array and crimp pattern of collagen fibers and tenocytes. The tissue is less cellular and the cells have cigar shaped nuclei. (*H & E*, scale bar for A, B, C = 170 µm and for D, E, and F = 42 µm).

infiltration in the injured control tendons was significantly higher than the injured treated tendons (*P* = .049). Treatment also enhanced maturity of the tenoblasts and also the number of tenocytes of the injured treated tendons so that mature tenoblasts and tenocytes were significantly greater than those of the injured control ones (*P* = .002). However, the immature tenoblasts of the injured treated tendons were significantly fewer than the injured control tendons (*P* = .049) (Table I). The injured treated tendons showed better alignment (90%), enhanced crimp pattern (70%), and higher tissue density (100%) than the injured control tendons. The newly regenerated collagen fibers of 90% of the injured control tendons showed a disorganized and haphazardly distributed pattern, while in the 85% of the injured treated tendons, they were organized in a proper order as parallel bundles along the longitudinal axis of the tendon. The fiber density of 60% of the injured treated tendons was comparable to their normal contralateral tendons. While immature blood vessels were evident in the tendon proper of 80% of the injured control tendons, no vascular abnormality was seen in the lesion of the injured treated ones. Treatment also reduced degeneration, ineffective fibrous tissue and suture material content in 90%, 70%, 90% of the samples, compare with injured control tendons (Figure 5).

Treatment enhanced orientation, alignment, differentiation, and maturation of the collagen fibrils at ultrastructural level. Both the diameter and density of the collagen fibrils of the injured treated tendons were significantly higher than those of the injured control tendons respectively (*P* = .001*, P* = .016) (Figure 6, Table II). While there were significantly fewer fibrils in the range of 33 to 64 nm in the injured treated tendons than the injured control tendons (*P* = .001)*,* treatment significantly enhanced the fibrillogenesis and collagen fibrils differentiation in the range of 65 to 102 nm (*P* = .021) (Table II). The elastic fibers of 90% of the injured treated tendons were more mature than the injured control ones (Figure 6).

Treatment strongly improved the biomechanical properties of the injured tendons so that the ultimate strength (94.50±4.12 N; *P* = .001), yield strength (85.00±7.34 N;

Table II. Ultrastructural Morphometric Analysis Between Injured Treated With TC and Injured Control Tendons[a], Day 84 Postinjury

	Variable	Injured treated tendons	Injured untreated tendons	*P* Value
Number of collagen fibrils at different ranges	33-64 (nm)	106.50+52.32	378.00+51.55	.001
	65-102 (nm)	175.75+50.24	88.00+25.88	.021
Total		282.25+57.31	466.00+67.14	.006
Collagen fibril/area (%)	Collagen density	92.58+3.70	84.56+3.16	.016
Diameter of collagen fibril at different range	33-64 (nm)	53.84+2.95	46.79+4.37	.037
	65-102 (nm)	96.22+5.65	70.50+4.91	.001
Total		80.60+8.10	51.12+4.11	.001
Number elastic fibers		0.50+1.00	0.50+1.00	>.99

[a]Number of fields for each tendon (5), $P<.05$ was considered significant, independent sample *t*-test was used for comparison between left-left tendons of injured treated and injured control groups. Microscopic field magnification for cell count (x200) and for cellular maturation analysis (x600).

P =.001), ultimate strain (11.33±1.35%; P = .001) and yield strain (8.79±0.961%; P = .006), stiffness (89.20±7.69 N/mm, P = .001), and maximum stress (19.83±1.43 N/mm^2; P = .014) of the injured treated tendons were significantly improved, compared with those of the injured control tendons (65.00±11.33 N, 54.50±8.55 N, 16.83±0.98%, 11.49±1.29%, 42.75±3.11 N/mm, 16.87±1.54 N/mm^2, respectively). However, except yield strain (P = .159), these parameters were still inferior to the ultimate strength (107.60±7.73 N; P = .015), yield strength (98.50±0.01 N, P = .018), stiffness (96.80±6.57 N/mm, P = .009), ultimate strain (9.99+1.43%, P = .041) and maximum stress (32.85+2.62 N/mm^2, P = .001) of their normal contralateral tendons. In addition, there were no significant differences between the biomechanical properties of the normal contralateral tendons of both groups (Figure 7).

Treatment enhanced the percentage dry weight content and this parameter was significantly higher in the injured treated tendons compared to the injured control ones (P = .005). However, at this stage the dry weight content of the injured treated tendons were still significantly inferior to their normal contralateral tendons (P = .026). The normal contralateral tendons of both groups had no significant difference in the percentage dry weight (Figure 2C).

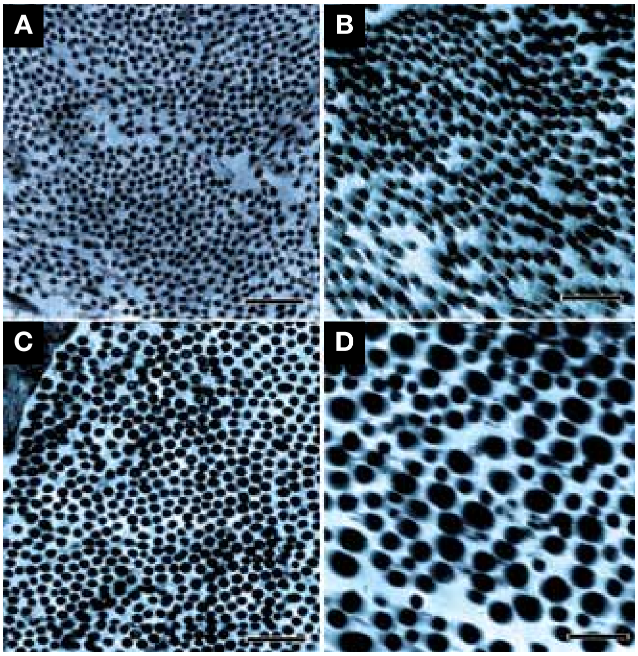

Figure 6. Ultrastructural findings: (A) injured control tendon, (B, C) injured treated tendon, (D) normal tendon, electron-micrograph from cross-sectional area of a normal tendon; the collagen fibrils are distributed in a multimodal pattern and ranges of the collagen fibrils from 33 to 300 nm are seen in this ultra-micrograph (Scale bar, 360 nm).

Discussion

Treatment with TC could significantly increase the biomechanical properties of the tenotomized SDFT so that at 84 DPI most biomechanical parameters were close to their normal contralateral tendons. This beneficial effect may initially be due to the inhibitory effects of TC on postsurgical necrosis, edema, fibrin clot, and further tissue reaction in the lesions.[9] It has been stated that TC provides a demarcation line around the necrotic tissue early after surgical repair and inhibit the inflammatory processes and the catabolic enzymes to migrate from the necrotic tissue to the surrounding areas and therefore could preserve the connective tissue structure from further degradation.[8,10,14,21] The findings of the present study showed that TC strongly preserved the old collaged fibrils from further degradation but all the large and medium sized collagen fibrils of the injured control tendons were degraded and disappeared and only the newly-regenerated, small-sized, unimodally distributed collagen fibrils were present in their lesions. In addition, the present study showed the anti-inflammatory effects of TC on the tendon lesion so that the number of the inflammatory cells and the amounts of tissue swelling in the injured treated tendon was significantly lower than the injured control tendons.

The beneficial effects of TC were possibly effective in reducing pain in the lesion and therefore, it initiated

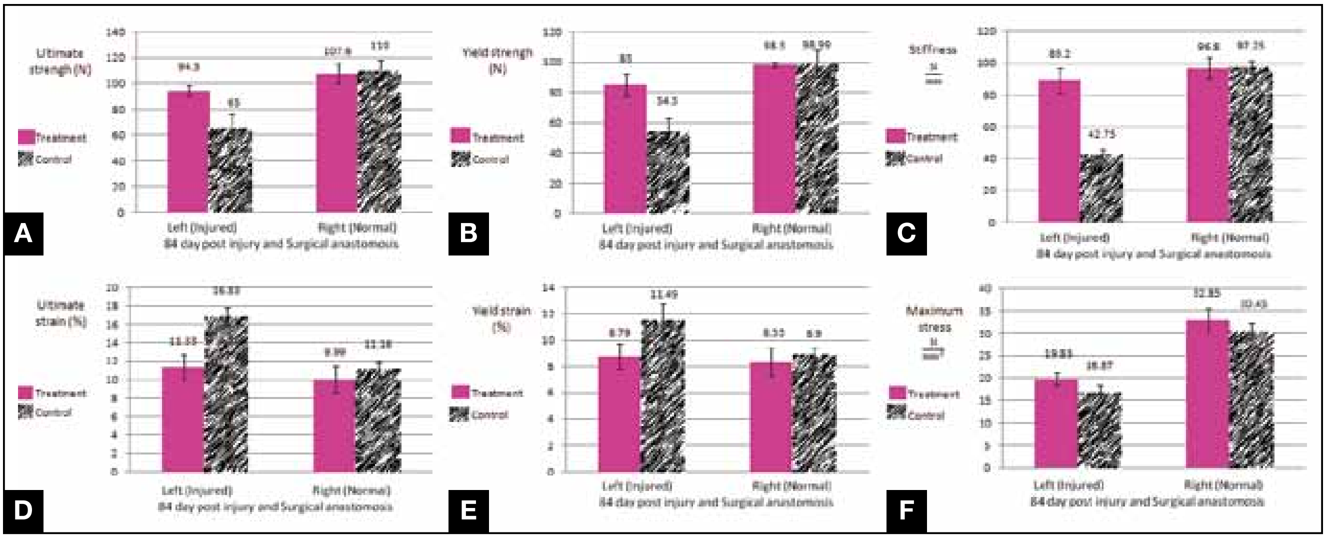

Figure 7. Biomechanical properties: (A-F) different biomechanical factors of the force displacement and stress-strain curves of the injured and their normal contra-lateral tendons of both groups.

more physical activity in the animals so that they used their injured hind limbs more effectively than those of the injured control group. Improved weight bearing and physical activity was the main reason for less peritendinous adhesion formation and advanced tissue alignment along the longitudinal axis of the tendon in the injured treated tendons. Adhesion is a part of the healing process and almost inevitably produces functional disability following the biological response of the tendon to injury.[22]

Presence of higher number of matured tenoblasts and tenocytes together with the higher amount of collagen fiber mass density and matured collagen fibrils in the injured treated tendons, indicate that TC restored the structural performance of the injured tendon in a more efficient way compared to the injured control tendons. The structural improvement was evident at histological and ultrastructural levels of the treated lesions so that the newly formed collagen fibers and fibrils were aligned along the longitudinal axis of the tendon and showed significantly greater diameter than those of the injured control area respectively.[4,5,23]

The mature tenoblasts and tenocytes of the treated lesions were properly arranged in rows along the longitudinal axis of the tendon fibers and this specialized arrangement was related to their function, because the mature tenoblast and tenocytes synthesize both fibrillar and nonfibrillar components of the extracellular matrix, and are able to reabsorb the degraded collagen fibrils.[5,6]

Higher potential of fibrillogenesis and improved fibrillar diameter and alignment, which are the main reasons of improved fiber density and higher dry weight content and ultra sonographical echogenicity, were the most important reasons for enhanced biomechanical performance of the injured treated tendons.[4,5,7] A group of these individual collagen fibrils arrange into fibers, fiber bundles, and finally fascicles.[5,7] Specialized tenoblasts and tenocytes lie within these fascicles and exhibit high structural organization.[4,24] Final stability is acquired during the remodeling induced by the normal physiological use of the tendon.[5,20] This further orientates the fibers into the direction of force.[7] In addition, cross linking between the collagen fibrils further enhance the tendon tensile strength.[5,24] These improvements could be due to the debridement criteria and edema relief properties of the TC together with its effect in reducing the acute inflammatory cell infiltration at the beginning of healing and increase in the rate of fibroblasts proliferation and maturation at the proliferative and remodeling stages of tendon healing.[14]

Therefore, the inhibitory effects of TC on inflammatory processes together with its collagen preservation criteria in the injured tendons, seems promising in the quest to produce a new tendon that is almost clinically, biomechanically, morphologically, and physiologically similar to that of the normal tendon after 12 weeks post rupture. We suggest designing similar experiments in the first few weeks after injury and biochemically analyzing the amounts of matrix metalloproteinases, tissue glycosaminoglycans, and types of the collagenic materials. It is possible that TC may be applicable as a drug in the future human clinical trial studies on tendon healing.

Authors' Disclosure & Acknowledgements

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