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Effects of sodium-hyaluronate and glucosamine-chondroitin sulfate on remodeling stage of tenotomized superficial digital flexor tendon in rabbits: a clinical, histopathological, ultrastructural, and biomechanical study

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Abstract

This study was designed to evaluate the effects of sodium-hyaluronate (NaH) combined with glucosamine HCl-chondroitin sulfate (GlcN-CS) on the post-surgical repair of tendon rupture on day 84 post injury. Twenty white New Zealand female rabbits were divided randomly into two equal groups of injured treated and injured untreated. After tenotomy and surgical repair, using the modified Kessler technique and running pattern, the injured legs were casted for 14 days. NaH was injected subcutaneously over the lesion on days 3, 7, and 10 and was followed by daily oral administration of GlcN-CS on days 3 to 23 post injury. The control animals received normal saline injection and oral placebo similarly. The weight of the animals, tendon diameter, clinical manifestations, and radiographic and ultrasonographic evaluations were conducted for 12 weeks. The rabbits were euthanized 84 days post injury and the tendons were evaluated at macroscopic, histopathologic, and ultrastructural level and were assessed for biomechanical and percentage dry-weight parameters. Treatment significantly reduced the tendon diameter and ultimate and yield strain, and increased the echogenicity, dry-weight content, ultimate and yield strength, and stress and stiffness of the injured tendons compared to those of the untreated ones. Treatment also significantly enhanced the maturation rate of the tenoblasts, fibrillogenesis, the diameters of the collagen fibrils, and fibrillar density. These findings suggest that a combined treatment of NaH and GlcN-CS could be effective in restoring the morphological and biomechanical properties of injured superficial digital flexor tendon of rabbits and might be helpful for future clinical trial studies in tendon ruptures.

Keywords: tendon, healing, ultrastructure, histopathology, biomechanics, sodium-hyaluronate, glucosamine

INTRODUCTION

Tendon injuries are common orthopedic problems and their incidence is especially high in certain sections of the population, for example, athletes, but every human or animal, regardless of age or level of activity, is in some degree of tendinous injury during routine daily activities [1,2]. Adhesion formation after tendon injury poses a major clinical problem because the newly regenerated blood vessels, tenocytes, and collagen fibers from the surrounding tissues invade the injured site and result in adhesion formation [1]. An ideal method to restore the injury is one which is reinforced by administration of a lubricant which does not interfere with the healing of the tendon, but will allow low-resistance gliding [3].

From long before classical times, injured tendons have been subjected to all manner of treatments in the hope that the rate and quality of healing could be improved over natural repair. However, to date, no one method of treating acute or chronic tendon injuries has been generally acknowledged to increase the rate or improve the quality of healing. Studies on degenerative processes in articular cartilage indicate that local and general administration of a low concentration of polysulfated glycosaminoglycans (GAGs) such as hyaluronic acid (HA) and chondroitin sulfate (CS) slows down the rate of collagen degradation [4–7]. As some of the matrix constituents of tendon are similar to those found in cartilage, it was postulated that polysulfated GAGs might also prove beneficial in the treatment of tendon injuries.

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The tendinous ground substance, which surrounds the collagen fibrils, consists of proteoglycans (PG), GAGs, structural glycoproteins, and a wide variety of other small molecules. It is a hydrophilic gel and can vary in consistency depending on the relative proportions of HA and CS concentration in the ground substance [8]. The water-binding capacity of these macromolecules is considerable, and they are able to improve the biomechanical properties of a tendon against shear and compressive forces. They are also important for the stabilization of the whole collagenous system of connective tissues and for the maintenance of ionic homeostasis and collagen fibrillogenesis [8]. Physiologically, the most important GAGs of the ground substance of tendons include HA, CS, keratin, heparin, and heparin sulfate, which are the major components of joint cartilage, synovial fluid, tendon, and other soft connective tissues [4,9–11].

HA is a long polysaccharide chain of repeating disaccharide units of *N*-acetyl-glucosamine and glucuronic acid, occurring naturally in the synovial fluid and is responsible for its viscoelastic property [5]. It has been reported that the tendons that were treated with HA had less adhesions and better gliding properties than untreated ones [1,3,12]. In addition, it has been shown that HA enhances the synthesis of the synoviocyte at the earlier stages of cartilage healing, reduces the inflammatory stage, improves the chondrocyte density and metabolism, inhibits apoptosis, prevents degenerative injuries, and results in an improved repair process of the extracellular matrix [6,7]. It has been stated that one of the main characteristics of HA is related to its persistence in the injured area for several weeks or even months after intra-articular injection [13].

In addition, the beneficial effects of exogenous HA applied locally to the tympanic membrane rupture, cutaneous wounds, and cataract surgery have previously been reported [14,15]. Based on the clinical manifestations, it has also been demonstrated that HA has a promising curative effect on the degenerative tendon processes in the early acute and subacute periods [9,12]. However, these investigators suggested further biomechanical and morphological studies to show how useful this chemical agent is in treating tendon disorders [9,12].

On the other hand, CS is a polymeric carbohydrate which comprises a repeating disaccharide motif of glucuronic acid and *N*-acetyl-galactosamine [15]. The beneficial effects of CS on articular cartilage, including the inactivation of degradative enzymes, stimulating metabolic activities and fibrillogenesis, reducing the effects of IL-1 and prostaglandin E2, and inhibiting chondrocyte apoptosis, have previously been reported [7,15,16].

Glucosamine (GlcN), a derivative of cellular glucose metabolism, is also a component of GAGs and the PG of the cartilage matrix that covers the ends of normal bones. It has been reported that GlcN is effective for the treatment of osteoarthritis (OA) symptoms [17].

Therefore, this study was designed to investigate the effects of combined HA and GlcN-CS on superficial digital flexor tendon (SDFT) healing, 84 days after complete rupture and surgical repair, with the hope of having a role in tendon healing with a similar mechanism such as the joints. HA may inhibit adhesion and enhance the inflammatory and fibroplasia stages of tendon healing, whereas CS-GlcN may be effective on tissue maturation and improves tendon alignment and biomechanical properties.

MATERIALS AND METHODS

Experimental design (simple accidental interventional study)

Twenty skeletally mature (10–14 months) female white New Zealand rabbits of 1.72 ± 0.31 kg body weight were randomly divided into two equal injured treated and injured untreated groups. They were kept on the same standard rabbit diet and condition with no limitation of access to food or water. Each animal served as its own control, and the right SDFT was used as the normal control.

Injury induction

The animals were anesthetized by intramuscular injection of 6 mg/kg xylasin HCl as a premedication and 60 mg/kg ketamin HCl and 6 mg/kg diazepam for anesthesia. The left hind leg was designated as "experimental" and the skin over the SDFT was shaved and disinfected, using normal surgical aseptic technique. A 2-cm longitudinal incision was made through the skin and subcutaneous tissues, approximately 0.5 cm distal to the gastrocnemius muscle and 0.5 cm above the calcaneal tuberosity, and the common calcaneal complex was exposed. As is shown in Figure 1, an incision was made through the paratenon; the SDFT was exposed and carefully dissected and was completely incised transversely at the mid-part of the tendon exposure, approximately 1.5 cm distal to the gastrocnemius muscle and 1.5 cm above the calcaneal tuberosity. A scalpel blade number 10 was used to induce standard injury. Immediately after tenotomy, the tendon proper was sutured using the modified Kessler technique as a core suture with absorbable polyfilament polygalactin 910 (Vicryl) 0-4 suture material (coated Vicryl, taper cut needle, Ethicon, Johnson & Johnson, Trademark, USA). The edges of the tendon were sutured by running pattern using the same material number 0-6. The paratenon was sutured by simple interrupted pattern with the same material number 0-8. The skin was closed in a routine manner. Three throws in each knot in all patterns were applied for the treated and untreated animals. After surgery, a cast (Dyna cast 5 cm, An-Yang Co., Ltd., South Korea) was applied for 2 weeks. A rectangular window was created in the

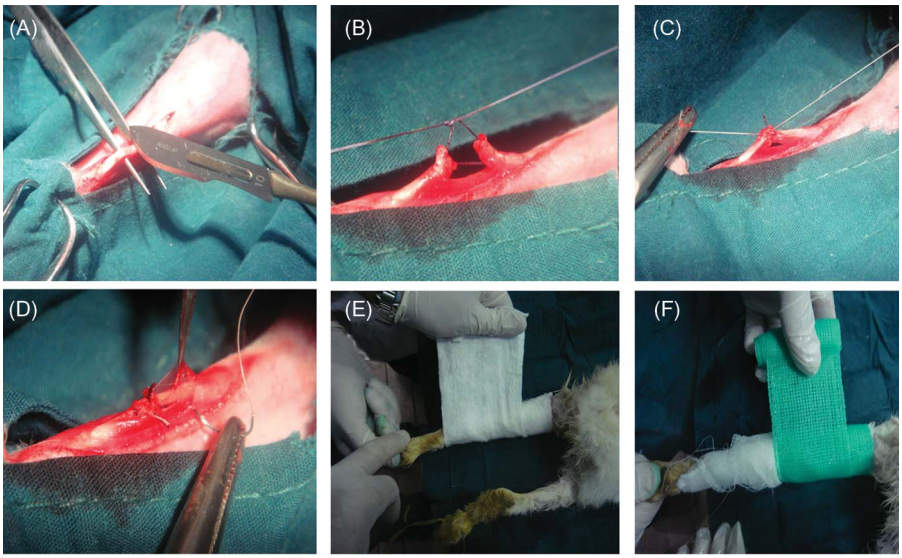

Figure 1. Surgical method: (A) injury induction; (B) modified Kessler core suture; (C) running pattern; (D) sheet closure; (E, F) immobilization technique.

cast at the site of injury to facilitate the injection of the reagent around the lesion.

Treatment program

About 2 mg/kg of high molecular weight (1.6 kDa) sodium-hyaluronate (NaH) (Hyalgan, 10 mg/mL, Fidia Farmaceutici S.p.A. Abano terme-PD, Italy) was injected subcutaneously at the site of the tendon injury through the window of the cast on days 3, 7, and 10 post injury. A dose of 100 mg/kg Preflex (500 mg GlcN HCl and 400 mg CS, oral capsules, Health Burst, Davie, FL, USA) was administered daily by oral route on days 3 to 23 post injury. In the control group, normal 0.9% saline (PBS) was injected on the site of the tendon injury and a placebo was administrated orally at the same time and volume as the treated animals.

Pre-euthanasia measurements

The animals were weighed and the right and left tendons and the covering skin diameter were blindly measured. The tendon and the covering skin diameter were measured around the injury site and in a comparable area of the uninjured contra-lateral tendon by using a micrometer measurement device (Samsung, Seocho-gu Seoul, South Korea) before injury, and then weekly, until the animals were euthanized. Each measurement was made three times to ensure that the repeatability of the measurements of the width was within 0.2 mm. From these, the average cross-sectional area of the tendon, together with the fascia and skin over it, was calculated.

For the clinical investigations, two blind observers determined the lameness and weight-bearing capacity of the rabbits. The walking activity of each animal in the cage was checked three times a day (8-hr intervals). The assessment was qualitative.

Radiographic and ultrasonographic observations were blindly evaluated by a radiologist at weekly intervals for 12 weeks. Lateral and dorsoventral position radiographs were provided from the whole body, using large film at 80 KVp and 6 MA. The animals were sonographed at longitudinal section with a 12 MHz linear probe (Simense SLR-400 device, Berlin, Germany; Echowave 3.23 software).

Ethics and euthanasia

This study was approved at the minimum of animals required for statistical analysis to prevent euthanasia of more animals. Eighty-four days after injury induction, the animals were euthanized by sodium-thiopental (50 mg/kg), xylazin (20 mg/kg), and ketamin HCl (300 mg/kg). The study was approved by the local ethics committee of our faculty, in accordance with the ethics standards of "Principles of Laboratory Animal Care."

Sample collection

Specimens from each of the injured and uninjured SDFT of five of the animals of each group were collected for light and electron microscopic studies and percentage dry-weight analysis. In the remaining five animals of each group, both injured and contra-lateral SDFT were carefully dissected from the surrounding tissues for biomechanical testing. The SDFT was cut and separated proximally to include 3 cm of the muscle belly and distally to the site of insertion of each phalangeal branch.

Light microscopy

After fixation in 10% neutral buffered formalin, the tendon samples were washed, dehydrated, cleared, embedded in ester wax, sectioned at 4–5 μm, stained with hematoxylin and eosin, and examined by a light

microscope (Olympus, Tokyo, Japan). The cells and vascular populations of each section were estimated using an eye-piece graticule. An average was then taken from five different microscopic fields for each cell type. Duplicate counts were carried out by double-blind method. In addition, using a digital camera (Sony T-700, Tokyo, Japan), the pictures from each slide were transferred to a computer for morphometric analysis. The maturity of the tenoblasts and tenocytes together with the density of the collagen fibers and blood vessels on the normal and inverted photomicrographs were determined using Adobe Photoshop CS3 10 final. The mesenchymal cells at the injury site were divided into three categories based on their diameter, cytoplasmic granules, and cell staining capacity. The largest elliptical cells with high granular and basophilic cytoplasm were determined as immature tenoblasts (fibroblasts). The long, cigar-shaped cells with less granulated but eosinophilic cytoplasm were estimated as tenocytes, whereas the medium-sized cells with neutral cytoplasm and medium amounts of cytoplasmic granules were determined as mature tenoblasts (fibroblast). Additionally, the crimp pattern, tissue maturation, alignment, and density, together with the types of degeneration and foreign body reactions on each sample, were qualitatively and semi-quantitatively analyzed and scored. The number of the vessels was evaluated in five fields of each histopathologic section with 200× magnification. The mean of the data for each animal and the mean of the five histopathologic sections were then determined for each group.

Electron microscopy

The samples were fixed in cold 4% glutaraldehyde, dehydrated, and embedded in Epon resin 97 for ultrastructuaral studies. Thin sections of 80–90 nm in diameter were cut and standard methods were employed for the production of transmission electron micrographs (Phillips CM 10 transmission electron microscope, Eindhoven, Netherlands). Ultramicrographs of different final magnifications (5200–158,000) were taken for studying the collagen and elastic fibrillar morphology, inflammatory cell constituents, and tenoblast maturity [18]. The diameter of the collagen fibrils of five different fields of the same magnification for each tissue section was measured. For fibrillar density, 10 pictures were captured from 10 horizontal and vertical fields; for each sample the surface area of the collagen fibrils regarding their category dependency were measured and analyzed. The number of collagen fibrils and their diameters in five different fields of each tissue section were measured. The collagen fibrils were divided, based on their diameter, into five different categories of 33–64, 65–102, 103–153, 154–256, and 257–307 nm, respectively. The number and diameter of the collagen fibrils were measured, and their mean diameter calculated by a computerized morphometric technique using Adobe Photoshop CS4. In addition, numbers of the elastic fibers of each field were counted and their maturity was qualitatively evaluated.

Biomechanics

After the application of standard preservation methods, biomechanical tests were performed using a tensile testing machine (Instron, London, UK) [19]. The specimens were mounted between two metal clamps and were 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. The complete method has previously been described [10,18,19]. The ultimate tensile strength, yield strength, ultimate strain, yield stain, stress, and stiffness were determined.

Percentage dry weight

The samples were weighed immediately after euthanasia and were freeze-dried (Helosicc, Ink, Co., London, UK) to a constant dry weight as previously described [18,19].

Statistical analysis

After application of the normality distribution test, the injured tendon of each animal was compared with the normal contra-lateral tendon of the same animal using paired sample *t*-test. The right and left tendons of the treated animals were compared with the right and left tendons of the untreated animals using the independent sample *t*-test. Nonparametric tests were applied to check the results again. Statistics were performed using the computer software SPSS version 17 for windows (SPSS Inc., Chicago, IL, USA). Differences of $p < 0.05$ were considered significant.

RESULTS

Weight

There were no significant differences in the weight of either group during the course of the experiment. However, the weight of the injured treated animals showed a significant increase at the end of the experiment compared to the beginning of the study ($p = 0.003$), although the increase in weight gain was not significant for the injured untreated group (Figure 2A).

Tendon diameter

There were no significant differences between the tendon diameters of either group before surgical operation; however, compared to the injured untreated animals, the treatment significantly decreased the diameter of the injured tendon from day 14 ($p = 0.005$) to 84 ($p = 0.001$) post-surgical operation (Figure 2B). Treatment decreased the diameter of the injured tendon so that at the end of the experiment, the tendon diameter of the injured treated tendons was approximated to those of their normal contra-lateral tendon

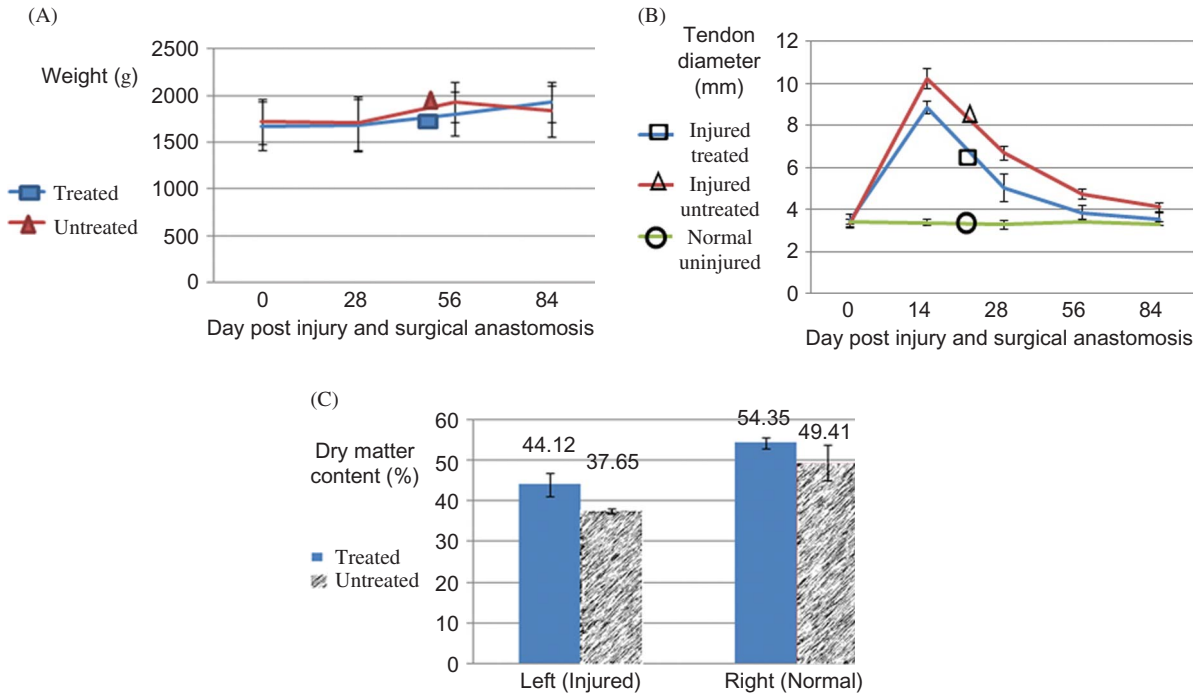

Figure 2(A). Weight of the injured treated and untreated animals during the course of the experiment is not significantly different. (B) The diameter of the injured treated tendons is significantly lower than those of the injured untreated ones from 14 to 84 DPI. (C) The dry-weight content of the injured treated tendons is significantly higher than the injured untreated ones. However, the dry-weight content of both injured treated and untreated tendons is still inferior to their normal contra-laterals.

diameter and also with the injured treated tendon diameter before injury induction and surgical repair, respectively ($p = 0.169$, $p = 0.081$).

Clinical observations

Treatment reduced tissue swelling and lameness. Although the treated animals showed better activity and weight-bearing capacity, and stood up and walked using all four of their feet without showing lameness on day 14 post operation, seven of the animals from the untreated group showed lameness and were unable to use their operated leg on the 14th day post injury (DPI). Also four and two rabbits were still unable to stand on the operated leg and showed lameness on days 21 and 28 post operation, respectively.

Radiography

No lesions such as soft tissue swelling and calcification, OA, and bone fracture were observed in either the treated or untreated lesions.

Ultrasonography

Treatment increased the echogenicity and homogenicity, but decreased the diameter of the injured tendon. No amputated view was observed in the treated lesions compared to those of the untreated ones (Figure 3).

Gross pathology

As is shown in Figure 4, treatment reduced the diameter of the injured tendon. However, the surrounding peritendinous tissues of the injured area of the

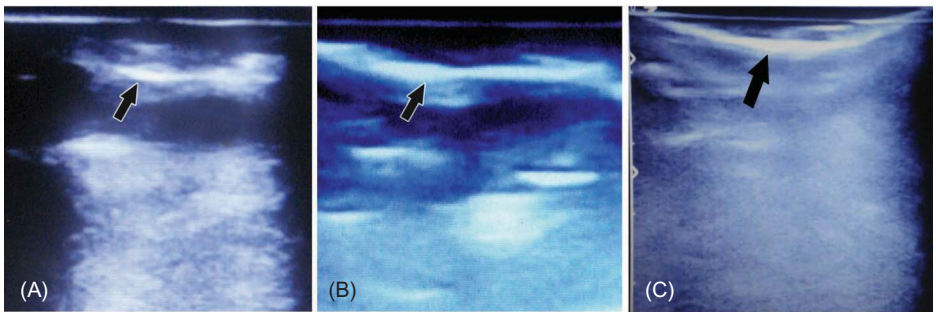

Figure 3. Ultrasonography: (A) injured untreated tendon. The diameter of the tendon is greatly increased; however, it is not uniform and shows an amputated view. Its echogenicity is not detectable from the surrounding fascia. (B) Injured treated tendon. The diameter of the tendon is almost similar to normal contra-lateral tendon. The tendon proper is detectable from its surrounding fascia. (C) Normal uninjured tendon. The diameter of the tendon is lower than (A) and (B). No amputated view is present and the echogenicity is smooth.

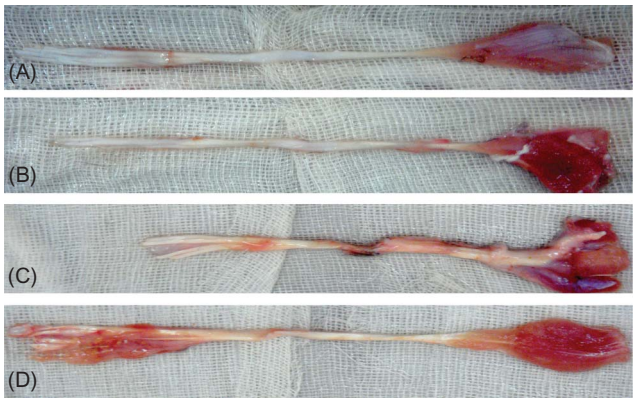

Figure 4. Gross pathology: (A) normal tendon of the treated group; (B) injured tendon of the treated group; (C) injured tendon of the untreated group; (D) normal tendon of the untreated group. The color of the injured treated tendon is almost similar to the normal tendon, whereas the color of injured untreated tendon is red to brown and the lesion is more hyperemic. The diameter of the injured treated tendon is comparable to the normal tendon. However, the diameter of the injured untreated tendon is large. Compared to the injured treated tendon, adhesion is more prominent in the periphery of the injured untreated one.

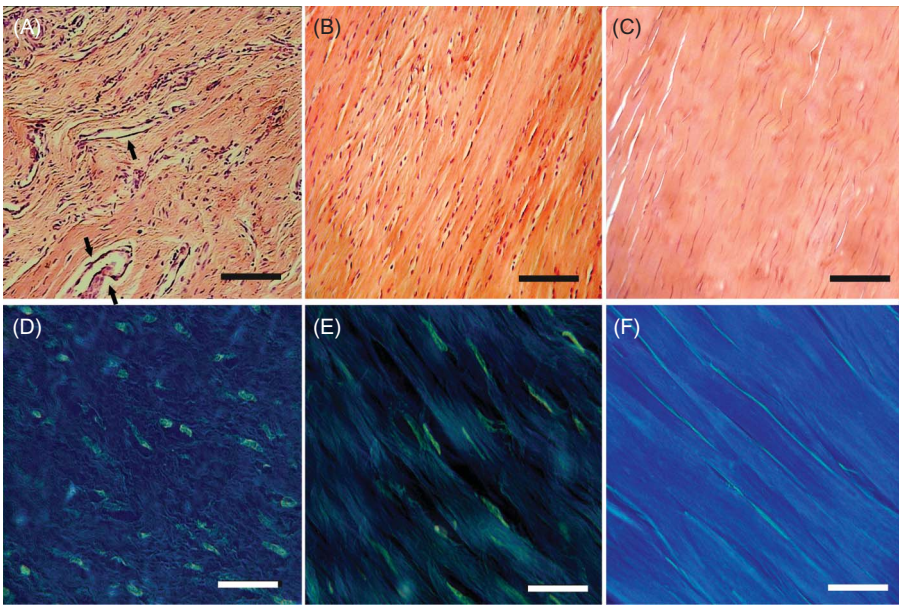

Figure 5. Histopathological findings: (A and D) The tissue section from the injured untreated tendon; the collagen fibers, blood vessels, and tenoblasts are disorganized. Numerous immature tenoblasts are seen in the tendon proper. The perivascular edema is marked with arrows. (B and E) Tissue sections from the injured treated animals; the tissue is aligned, the cells are mature, and the fiber density has been increased. (C and F) Normal tendon; the tissue is properly aligned and has very few tenocytes (scale bar for A–C = 50 μm and for D–F = 15 μm).

untreated tendon were fibrous in nature and adhesion between the tendon and peritendinous fascia was present and the tendon was still hyperemic. The treated lesions had a shiny glistening surface, a characteristic almost similar to those of the normal uninjured tendons, and the tendons showed no adhesion to the surrounding fascias.

Histopathology

As is shown in Figure 5, no area of necrosis, calcification, fibril lyses, or signs of acute inflammation were evident in both the treated and untreated lesions. However, compared to those of the injured untreated tendons, the treatment reduced edema, cellularity (Table 1), cell degeneration, and necrosis of the lesions. Also, very few neutrophils were seen in the lesions of one of the untreated animals, but other untreated and treated lesions were free of neutrophils and signs of acute inflammation. However, macrophages and lymphocytes infiltration in the untreated lesions were significantly higher than in the treated lesions ($p < 0.022$, $p < 0.040$) (Table 1). In addition, the maturity of the tenoblasts as well as the number of the tenocytes were enhanced by treatment and the immature tenoblasts of the treated lesions were significantly lower than those of the untreated ones ($p < 0.049$) (Table 1). Compared

Table 1. Cell differentiation and vascular analysis of injured treated and injured untreated tendons with their normal contra-lateral tendons after 84 days post injury and surgical intervention

	Injured treated tendons (left leg)	Injured untreated tendons (left leg)	*P*-value	Injured treated tendons (right leg)	Injured untreated tendons (right leg)	*P*-value
Fibroblast	139.25 ± 18.99	237.75 ± 26.58	0.001	10.75 ± 13.25	33.00 ± 49.65	0.420
Fibrocyte	42.25 ± 16.74	14.00 ± 7.43	0.022	33.50 ± 11.61	34.25 ± 12.03	0.931
Macrophage	0.50 ± 1.00	3.25 ± 1.50	0.022	Negative	Negative	
Lymphocyte	6.50 ± 2.38	12.25 ± 3.68	0.040	Negative	Negative	
Neutrophil	Negative	0.25 ± 0.50	0.356	Negative	Negative	
Mature fibroblast	15.75 ± 3.77	15.50 ± 3.00	0.921	Negative	Negative	
Immature fibroblast	7.75 ± 3.94	15.00 ± 4.83	0.049	Negative	Negative	
Vascularity	1.50 ± 1.91	3.75 ± 3.30	0.283	Negative	Negative	

Number of fields for each tendon = 5, $P < 0.05$ *was considered significant*, independent sample *t*-test was used for comparison between left–left and right–right tendons of injured treated and injured untreated groups, and paired sample *t*-test was used for comparison between left and right tendons of the same group. Microscopic field magnification for cell count = 200× and for cellular maturation analysis = 800×.

to the untreated lesions, the treated lesions showed enhanced alignment, a more developed crimp pattern, and higher tissue density than the untreated ones. Although the newly regenerated collagen fibers of the untreated lesions showed a disorganized pattern, they were aligned as parallel bundles along the longitudinal direction of the treated lesions. The density of the collagen fibers of the treated lesions was almost similar to those of their normal contra-lateral tendons. Compared to the injured untreated tendons, the treated lesions showed fewer blood vessels in their tendon proper. In addition, the treatment increased vasculogenesis in the tendon sheet.

Electron microscopy

Treatment enhanced the differentiation, maturation, and alignment of the collagen fibrils, and both the diameter and density of the collagen fibrils in the treated lesions were significantly higher than those of the untreated ones ($p < 0.001$, $p < 0.026$) (Figure 6, Table 2). Although the number of small-sized collagen fibrils (in the range) of 33–64 nm in diameter of the treated lesions was significantly less than the untreated lesions ($p < 0.001$), the treatment significantly increased the number and diameter of the collagen fibrils in the range of 65 to 102 nm ($p < 0.001$, $p < 0.001$) (Table 2). Although there was no significant difference in the number of elastic fibers of the treated with those of the untreated lesions, the treatment did increase the maturation of the elastic fibers.

Biomechanical properties

As is shown in Figure 7, treatment strongly improved the biomechanical properties of the injured tendons and the ultimate strength ($p = 0.001$), yield strength ($p = 0.001$), ultimate and yield strain ($p = 0.001$, $p = 0.005$), and stiffness ($p = 0.001$); the maximum stress ($p = 0.049$) of the injured treated tendons was significantly higher than that of the injured untreated ones. Except for the ultimate strength ($p = 0.049$) and stress ($p = 0.019$), which were significantly inferior to their normal contra-laterals, at this stage, other parameters were comparable with those of their normal contra-lateral tendons. In addition, there were no significant differences between the biomechanical properties of the normal tendons of the treated and untreated animals.

Dry matter

Treatment significantly increased the dry-matter content of the injured tendons compared to those of the untreated ones ($p = 0.004$) (Figure 2). However, at this stage the dry-weight content of the treated lesions was still significantly inferior to that of their normal contra-lateral tendons ($p = 0.012$). The contra-lateral tendons of both groups had no significant differences in the percentage dry-weight content (Figure 2C).

DISCUSSION

The results of the present study showed that treatment with HA–CS-GlcN enhanced the biomechanical properties so that most approximated the values of their normal contra-lateral tendons. Earlier improvement and better gliding movements in the treated group may be due to pain relief and the anti-degenerative effects of HA, as it has been shown that HA effectively reduces articular pain, slows down the degenerative process of OA, and improves the general activities of daily living and joint mobility [3,13]. This improvement could also be due to the pain relief property and structural role of CS, as it has been stated that the osmotic swelling resists compression and supports the weight-bearing capacity of the joints affected with OA [15,16]. In addition, it has been shown that exogenous GlcN stimulates the HA production of the injured joints through stimulation of the synovial membrane [20].

The ultimate strength of the injured untreated tendons was about 60%, whereas for the injured treated ones, it was close to 85% of their normal contra-laterals after 12 weeks post injury. Further, it has been reported

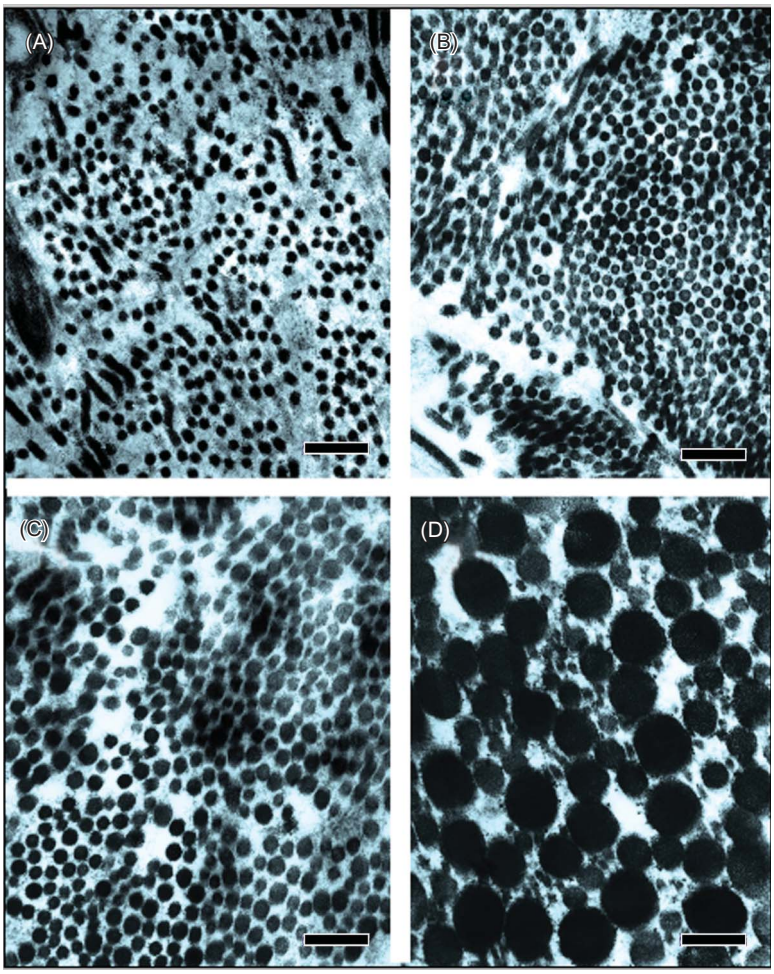

Figure 6. Ultrastructural findings: (A) injured untreated tendon. Large numbers of immature fibrils with low fibrillar density are seen in this ultramicrograph. (B and C) Injured treated tendon. Compared to the untreated lesions, the size and density of the collagen fibrils is higher and the fibrils show a bimodal distribution pattern. (D) Normal tendon. The collagen fibrils show a multimodal distribution pattern and their diameter ranges from 33 to 300 nm (scale bar = 360 nm).

that in the transected Achilles tendons of sheep that had spontaneous healing, without receiving any medication, the ultimate strength was 56.7% of their normal contralateral tendons at 12 months post injury [21]. The differences in the findings of this study with the biomechanical results of the present investigation may be due to species differences or because of the differences in the applied surgical techniques of these experiments.

Tendon repair has a great limitation in the form of pretendinous adhesions. The surgical area is frequently compromised by adhesions that restrict the gliding motion, decrease the gliding function, and may seriously impair the function of the limbs and lead to permanent deformation [3,9]. Despite careful anastomosis of the tendon sheet after surgical intervention in the present experiment, adhesion was inevitable in the lesions of the injured untreated animals. However, treatment with HA–CS-GlcN reduced the tendon diameter, the granulation tissue, and resulted in lower pretendinous adhesions. The decrease in the adhesion to the surrounding fascia could be a promising effect of HA on connective tissue wound healing. This finding is in agreement with earlier reports that showed a decrease in adhesion formation in the injured flexor tendons of rabbits and the Achilles tendon of rats after treatment with HA [1,12].

Histopathological observations also revealed the adherence of the peritendinous tissue of the injured untreated lesions with tendon proper. Development of adhesions between the healing tendon and the surrounding structures correlates with the intensity and duration of the inflammatory reaction and the degree of mobilization of the tendon during the healing period [3]. The adhesions of the injured untreated lesions to the surrounding fascia indicate that the granulation tissue and the proliferative fibrous tissue growth from the surrounding tissues are still active [3]. These adhesions limit the physical activity of the animals and elongate the maturation stage, and are therefore responsible for the inferior biomechanical properties of the injured untreated tendons.

Table 2. Ultrastructural morphometric analysis of the number, diameter (nm), and density (%) of the collagen fibrils at different ranges (nm), and number of elastic fibers: comparison between injured treated and injured untreated tendons after 84 days post injury and surgical intervention

Range diameter (nm)	Injured untreated tendons (left leg)	Uninjured normal tendon (left leg)	*P*-value
Number of collagen fibrils at different diameter ranges			
33–64	87.25 ± 47.34	378 ± 51.55	0.001
65–102	188.25 ± 21.41	88.00 ± 25.85	0.001
103–153	Negative	Negative	
154–256	Negative	Negative	
257–307	Negative	Negative	
Total	275.50 ± .56	466.00 ± 67.14	0.002
Collagen fibrils diameter at different ranges (nm)			
33–64	49.34 ± 8.45	46.79 ± 4.37	0.611
65–102	94.67 ± 1.98	70.51 ± 4.91	0.001
103–153	Negative	Negative	
154–256	Negative	Negative	
257–307	Negative	Negative	
Total	80.57 ± 8.19	51.12 ± 4.11	0.001
Elastic fibers	0.50 ± 1.00	0.50 ± 1.00	1.000
Density	93.12 ± 4.86	84.56 ± 3.16	0.026

Number of fields for each tendon = 5 ($P < 0.05$ *was considered significant*), *analysis magnification* = 39,000; independent sample *t*-test was used for comparison between left and left tendons between injured treated and injured untreated groups.

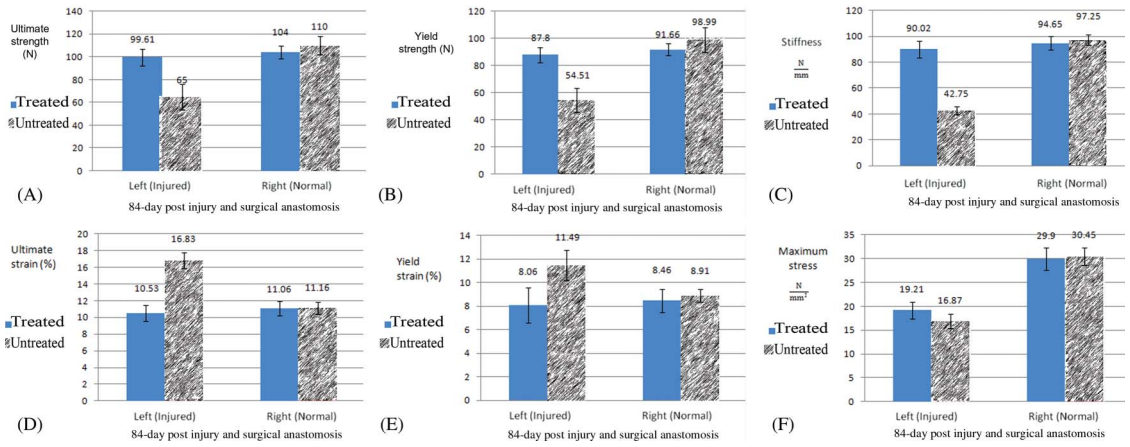

Figure 7. Biomechanical properties: treatment strongly improved the biomechanical properties of the injured tendons and the ultimate strength (A); yield strength (B); stiffness (C); ultimate strain (D); yield strain (E); and maximum stress (F) of the injured treated tendons showed significant improvement compared to those of the injured untreated ones. Except the ultimate strength (A) and maximum stress (F), which were significantly inferior to their normal contra-laterals, other parameters were comparable with those of their normal contra-lateral tendons.

In addition, compared to the untreated lesions, treatment significantly decreased the chronic inflammation and tissue swelling. It has been reported that HA, CS, and GlcN are potent anti-inflammatory reagents; however, each one exerts its anti-inflammatory effect through a different pathway. It has been stated that HA may bind to the inflammatory mediators and free radicals and remove them from the joint space via the lymphatics, resulting in reductions in damage to the cartilage [13]. On the other hand, it has been shown that GlcN reduces the IL-1-stimulated production of catabolic enzymes and inflammatory markers such as prostaglandin E2 by chondrocyte and synovial cells harvested from surgical specimens removed from patients with OA [3,12,17,22,23]. Finally, it is shown that CS exerts its anti-inflammatory response by binding to TNF-a, inhibiting its activity [15,16].

Swelling of the injured site at the earlier stages of healing of the tendon may be due to hematoma,

postoperative edema, and inflammatory cell infiltration. However, at the later stages of healing, the swelling could be due to the unorganized fibrous connective tissue and, particularly, the presence of haphazardly organized collagen fibers that adhere to the surrounding fascias and limit the normal gliding function [24]. The hematoma and edema restrict tendon gliding long before the collagenous adhesions are formed.

Treatment significantly decreased the cellularity of the injured tendon, and compared to the untreated lesions, the treated tendons contained fewer lymphocytes, macrophages, tenoblasts, and tenocytes. These findings are in contrast to those of Kawasaki et al. [25] and Kumar et al. [3]. Kawasaki et al. [25] showed that HA can promote the extracellular matrix synthesis and cell proliferation of the chondrocytes in tissue culture, whereas Kumar et al. [3] showed that after treatment of the tendon lesions of the rabbits with HA, the lesions showed hypercellularity together with more vasculogenesis, thickening of the epitenon, and enhanced collagen organization. This difference could be due to the nature of the lesions, behavior of the proliferating cells at in vivo and in vitro conditions, and the time of the studies after injury induction. On 84 DPI, normally the inflammatory and fibroplasia phases of the healing have finished and the remodeling phase is continuing. Therefore, it is expected that enhanced alignment and improved biomechanical properties of the injured treated lesions result in a decrease of the cellularity, but at the same time have improved the maturation of the tenoblasts and tissue organization. It is possible that CS had a role in the maturation of the tenoblasts and tissue alignment because it has been previously shown that CS is effective in enhancing the maturation of the young tenoblasts to mature tenoblasts and tenocytes [15].

At the ultrastructure level the collagen fibrils of the treated lesions were more differentiated, showed a higher mean diameter, and enhanced fibrillar density. Enlargement of the collagen fibrils may occur through deposition of more collagen by tenoblasts on the existing collagen fibrils, the aggregation of a number of newly regenerated small-sized collagen fibrils, or it could even be the outcome of the preservation of the original collagen fibrils from further degenerative changes [8,10,26,27]. It has been shown that the intra-articular injection of HA bounded to the free PGs in the cartilage matrix, inhibiting further release of PGs, thus protecting the cartilage matrix and cells from further degradation [13]. It has been reported that HA protected the cells and the structural integrity against mechanical overloading due to its viscoelastic characteristics. In addition, the administration of exogenous HA has been shown to stimulate its endogenous synthesis in the synovial membrane of the injured joints [27]. Parry et al. [11] reported that HA is the prominent GAG synthesized by fetal and regenerating connective tissue cells after wounding. They postulated that the increase in the diameter of the collagen fibrils is limited by the HA-rich matrix so that only small diameter unimodal collagen fibrils of less than 60 nm in diameter are formed initially. They reported that during maturation the HA content decreases and the concentration of CS and DS increases. The early inhibition of the lateral growth of fibrils imposed by the HA is later reversed by the increase in CS concentration, so that a number of small collagen fibrils aggregate to form a single large fibril. It is possible that the CS and GlcN used in the present study artificially removed the initial growth limitation on collagen fibrils induced by HA and, by enhancing the aggregation of the collagen fibrils, increased the fibrillar diameter. This finding was confirmed later by Volpi [16] who showed that GAGs can stimulate collagen fibrils aggregation and are effective in increasing their diameter and maturation.

Enhanced collagen fibrils maturation and density may also be due to CS effects, because it has been shown that CS can impact processes associated with cartilage degeneration, promote the synthesis of PG which are lost during cartilage degeneration, inhibit elastase and cathepsin G activities, reduce gene expression for a range of proteolytic enzymes, and, in combination with GlcN, reduce subchondral bone resorption [1,15,16]. Such mechanisms are likely to be involved in the preservation of collagen fibrils from further degradation and keep the tissue architecture and further restore the biomechanical properties of the healing tendon. Electron microscopic studies of the developing and mature rat tendon have shown that the GAGs are periodically arrayed on the collagen fibril. In addition, a correlation between the GAGs content of the tendon and the average diameter of the collagen fibrils in tendons from animals of different ages has previously been reported [16]. Higher fibrillar density, advanced differentiation and alignment of collagen fibrils, and improved tenoblasts maturation are all indexes of enhanced architectural development and result in higher dry-weight content, improved ultrasonography echogenicity, and lower adhesion to the surrounding tissues. These criteria result in better weight-bearing and effective physical activities and have a great role for better biomechanical properties that were present in the treated animals of the present study compared to those of the untreated ones and it seems that treatment with HA–GlcN-CS are responsible for those structural and biomechanical improvements.

Therefore, the results of the present study strongly suggest that the injection of HA, followed by the oral administration of GlcN-CS, has promising curative effects on the remodeling stage of healing processes and restores the structural and mechanical properties of the injured tendons. The findings of the present study may be encouraging for physicians and researchers who may consider investigating these agents in further experimental or clinical trials.

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REFERENCES

[1] Sharma, P., and Maffulli, N. (2005). Tendon injury and tendinopathy: Healing and repair. *J. Bone Joint Surg. Am.* 87: 187–202.

[2] Kannus, P., and Josza, L. (1991). Histopathological changes preceding spontaneous rupture of a tendon. *J. Bone Joint Surg.* 73A:1507–1525.

[3] Kumar, N., Kumar, N., Sharma, A.K., Maiti, S.K., Gangwar, A.K., Gupta, O.P., Kumar, S., and Singh, R. (2009). Modulation of peritendinous adhesions using hyaluronic acid and autogenous synovia: An experimental study in a rabbit model. *Trends Biomater. Artif. Organs* 23:34–45.

[4] Chang, X., Yamada, R., and Yamamoto, K. (2005). Inhibition of antithrombin by hyaluronic acid may be involved in the pathogenesis of rheumatoid arthritis. *Arthritis Res. Ther.* 7:268–273.

[5] Iwata, H. (1993). Pharmacologic and clinical aspects of intraarticular injection of hyaluronate. *Clin. Orthop.* 289: 285–291.

[6] Moreland, L.W. (2003). Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: Mechanisms of action. *Arthritis Res. Ther.* 5:54–67.

[7] Gonçalves, G., Melo, E.G., Gomes, M.G., Nunes, V.A., and Rezende, C.M.F. (2008). Effects of chondroitin sulfate and sodium hyaluronate on chondrocytes and extracellular matrix of articular cartilage in dogs with degenerative joint disease. *Arq. Bras. Med. Vet. Zootec.* 60:93–102.

[8] Kannus, P. (2000). Structure of the tendon connective tissue. *Scand. J. Med. Sci. Sports* 10:312–320.

[9] Thomas, S.C., Jones, L.C., and Hungerford, D.S. (1986). Hyaluronic acid and its effect of postoperative adhesions in rabbit flexor tendon. *Clin. Orthop.* 206:281–289.

[10] Oryan, A., Goodship, A.E., and Silver, I.A. (2008). Response of a collagenase-induced tendon injury to treatment with a polysulphated glycosaminoglycan (adequan). *Connect. Tissue Res.* 49:351–360.

[11] Parry, D.A.D., Flint, M.H., Gillard, G.C., and Craig, A.S. (1982). A role for glycosaminoglycans in the development of collagen fibrils. *FEBS Lett.* 149:1–7.

[12] Tatari, H., Özkal, S., Skiak, E., Destan, H., Ulukus, C., Ozer, E., and Satoglu, S. (2005). Comparison of early and late term effects of hylan G-F 20 in Achilles tendonitis: An experimental study. *Joint Dis. Rel. Surg.* 16:215–223.

[13] Jean, Y.H., Wen, Z.H., Chang, Y.C., Lee, H.S., Hsieh, S.P., Wu, C.T., Yeh, C.C., and Wong, C.S. (2006). Hyaluronic acid attenuates osteoarthritis development in the anterior cruciate ligament-transected knee: Association with excitatory amino acid release in the joint dialysate. *J. Orthop. Res.* 24: 1052–1061.

[14] Goa, K.L., and Benfield, P. (1994). Hyaluronic acid. A review of its pharmacology and use as a surgical aid in ophthalmology, and its therapeutic potential in joint disease and wound healing. *Drugs* 47:536–566.

[15] Lauder, R.M. (2009). Chondroitin sulphate: A complex molecule with potential impacts on a wide range of biological systems. *Complement. Ther. Med.* 17:56–62.

[16] Volpi, N. (2004). The pathobiology of osteoarthritis and the rationale for using the chondroitin sulfate for its treatment. *Curr. Drug Targets Immune Endocr. Metabol. Disord.* 4: 119–127.

[17] Fox, B.A., and Stephens, M.M. (2007). Glucosamine hydrochloride for the treatment of osteoarthritis symptoms. *Clin. Interv. Aging* 2:599–604.

[18] Oryan, A., Silver, I.A., and Goodship, A.E. (2009). Effects of serotonin S_2-receptor blocker on healing of acute and chronic tendon injuries. *J. Invest. Surg.* 22:246–255.

[19] Oryan, A., and Shoushtari, A.H. (2009). Biomechanical properties and dry weight content of the developing superficial digital flexor tendon. *Comp. Clin. Pathol.* 18:131–137.

[20] Uitterlinden, E.J., Koevoet, J.L.M., Verkoelen, C.F., Bierma-Zeinstra, S.M.A., Jahr, H., Weinans, H., Verhaar, J.A.N., and Van Osch, G.J.V.M. (2008). Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants. *BMC Musculoskelet. Disord.* 9:1–7.

[21] Bruns, J., Kampen, J., Kahrs, J., and Plitz, W. (2000). Achilles tendon rupture: Experimental results on spontaneous repair in a sheep-model. *Knee Surg. Sports Traumatol. Arthrosc.* 8:364–369.

[22] Nakamura, H., Shibakawa, A., and Tanaka, M. (2004). Effects of glucosamine hydrochloride on the production of prostaglandin E2, nitric oxide and metalloproteases by chondrocytes and synoviocytes in osteoarthritis. *Clin. Exp. Rheumatol.* 22:293–299.

[23] Uitterlinden, E.J., Jahr, H., and Koevoet, J.L. (2006). Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. *Osteoarthr. Cartil.* 14:250–257.

[24] Lane, J.M., Black, J., and Bora, W. (1976). Gliding function following flexor tendon injury. *J. Bone Joint Surg. Am.* 58A: 985–990.

[25] Kawasaki, K., Ochi, M., Uchio, Y., Adachi, N., and Matsusaki, M. (1999). Hyaluronic acid enhances proliferation and chondroitin sulfate synthesis in cultured chondrocytes embedded in collagen gels. *J. Cell Physiol.* 179:142–148.

[26] Oryan, A., Silver, I.A., and Goodship, A.E. (2010). Metrenperone enhances collagen turnover and remodeling in the early stages of healing of tendon injury in rabbit. *Arch. Orthop. Trauma Surg.*, in press, DOI: 10.1007/s00402-010-1057-8

[27] Roth, A., Mollenhauer, J., Wagner, A., Fuhrmann, R., Straub, A., Venbrocks, R.A., Petrow, P., Bröuer, R., Schubert, H., Ozegowski, J., Peschel, G., Müller, P.J., and Kinne, R.W. (2005). Intra-articular injections of high-molecular-weight hyaluronic acid have biphasic effects on joint inflammation and destruction in rat antigen-induced arthritis. *Arthritis Res. Ther.* 7: 677–686.