

# Effects of basic fibroblast growth factor (bFGF) on early stages of tendon healing

## A rat patellar tendon model

Barbara P Chan<sup>1</sup>, Sai-chuen Fu<sup>1</sup>, Ling Qin<sup>1</sup>, Kwong-man Lee<sup>2</sup>, Chriter G Rolf<sup>1</sup> and Kai-ming Chan<sup>1</sup>

<sup>1</sup>Department of Orthopedics & Traumatology, The Chinese University of Hong Kong, Room 74029, 5/F., Clinical Science Building, Prince of Wales Hospital, Shatin, N.T. Hong Kong. Tel +852 2646 1477. E-mail: bpchan@netvigator.com; <sup>2</sup>Lee Hysan Clinical Research Laboratories, the Chinese University of Hong Kong, Shatin, N.T. Hong Kong  
Submitted 99-05-18. Accepted 00-06-19

**ABSTRACT** – We studied the effects of basic fibroblast growth factor (bFGF) on cell proliferation, type III collagen expression, ultimate stress and the pyridinoline content in the early stages of healing in rat patellar tendon.

96 male Sprague Dawley rats were injected with increasing doses of basic fibroblast growth factor (bFGF) at 3 days after a “window defect” was induced in the mid-part of the patellar tendon. They were killed at 7 and 14 days after the injury. A dose-dependent increase in the number of proliferating cells and the level of expression of type III collagen was demonstrated at only 7 days post-injury. On the other hand, we found no effects of bFGF on ultimate stress and the pyridinoline content of healing tendons. Only time significantly affected both strength-associated parameters.

We showed that *in vivo* supplementation with bFGF affected the initial events of healing such as cell proliferation and type III collagen expression.

In general, the tendon heals at a relatively slower rate than other connective tissue—e.g., bone (Hefli and Stoll 1995)—even though they usually undergo similar stages during healing. Initial tendon healing includes inflammatory infiltration, cell proliferation and alteration in collagen phenotypes. The tendon then enters a synthetic phase, in which the deposition of extracellular matrix starts. This is accompanied by the remodeling and maturation phases, in which the tensile strength of the injured tendons is regained. The restoration of me-

chanical strength is the goal of tendon healing. The ultimate stress and pyridinoline content are parameters closely associated with mechanical strength (Frank et al. 1995, Woo et al. 1997). In the present study, 1 and 2 weeks after the injury were selected for measuring these parameters because they had not yet reached the optimal level (Chan et al. 1998a) and the effects of the growth factor could therefore be studied.

The literature frequently describes attempts to identify modulating agents in the hope of affecting tendon healing positively. Growth factors, a major group of candidates of modulating agents, defined by their primary growth-stimulating functions, have proved to be involved in almost every stage of the healing process (Chan et al. 1998b). Of various growth factors, bFGF is one of the most studied. It is angiogenic (Gabra et al. 1994) and has mitogenic effects on many mesenchymal cells such as ligament fibroblasts (Kobayashi et al. 1995, Lee et al. 1995). In the present study, bFGF was selected because of its mitogenic effects on rat patellar tendon fibroblasts (Chan et al. 1997). Upregulated expression of bFGF (Lee et al. 1998) and its receptors (Panossian et al. 1997) in healing of the medial collateral ligament also indicates the large role of bFGF in soft tissue healing.

We hypothesized that bFGF affects tendon healing during an early phase of this process. We injected bFGF into the injured rat patellar tendon and evaluated healing with respect to cell proliferation, type III collagen expression, pyridinoline content and ultimate stress at 7 and 14 days after injury.

## Methodology

### *Patellar tendon gap wound healing model*

All procedures in the animal experiments were approved by the Animal Research Ethics Committee, the Chinese University of Hong Kong.

We used 96 male Sprague Dawley rats, aged 8 weeks, and weighting 250–300 g. They were anesthetized with an abdominal injection of 2.5% (w/v) pentobarbital solution in a dose of 4.5 mg/kg body weight. After shaving of both hind limbs, the skin and the patellar tendon sheath were opened with a surgical blade to expose the tendon. On the experimental side, a window defect of 1 × 4 mm was created in the mid-part of the rat patellar tendon (Chan et al. 1998a). The tendon sheath and skin were closed with absorbable sutures (Dexon II, 6-0, Davis+Geck, Cyanamid Medical Device Co. Inc, Anyang, Korea). The contralateral knee served as the sham control. The rats were allowed to run free in the cage immediately after the injury.

### *Injection of growth factors*

Animals were randomly divided into 4 groups for injections of 0, 10, 100 or 1000 ng basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN) dissolved in 100 µL sterile distilled water at 3 days postinjury. An airtight microsyringe (Alltech, Deerfield, IL) for injecting 50–200 µL solution was used for a single injection. On injection, the skin of the lower limbs and the needle were cleaned with hibitane in alcohol. The site of injection was mid-way between the lower pole of the patella and the tibiae tuberosity. The needle was held perpendicular to the longitudinal axis of the patellar tendon and the solution was injected steadily after inserting the needle of the syringe into the center of the old scar at a depth of approximately 0.3 cm. The syringe was washed thoroughly with sterile distilled water 3 times between successive injections.

### *Sample harvesting*

Half of the rats were killed with an overdose of anesthetic at 7 days, and the other half 14 days after the injury. At each time-point, patellar tendons dissected from 4 animals in each dosage group were fixed and paraffinized for immunohis-

tochemical analysis of proliferating cell nuclear antigen (PCNA) and type III collagen. In the remaining 8 rats in the group, the whole leg was harvested and kept moist with saline in a -70 °C freezer for subsequent mechanical testing for ultimate stress and biochemical analysis of the pyridinoline crosslink.

### *Immunohistochemical staining*

*Proliferating cell nuclear antigen (PCNA).* Immunohistochemical staining of proliferating cell nuclear antigen (PCNA), or cyclin (Ogata et al. 1987) was done to determine the proliferative response at the wound site. After quenching of the endogenous peroxidase and blocking the non-specific binding sites with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO), the sections were directly incubated with a mouse anti-PCNA antibody (Serotec Ltd. Oxford, England) in a dilution of 1:10. The sections were then incubated with an anti-mouse secondary antibody (DAKO, Glostrup, Denmark) for 30 min. Freshly prepared ABC reagent (Vectastain, Amersham, U.K.) was then used to amplify the signal for 1 hour at room temperature. Finally, staining was achieved by incubation with freshly-prepared substrate, diaminobenzidine tetrahydrochloride (DAB), for 15 min and supplemented with 0.3% hydrogen peroxide and 0.3% cobalt chloride (Sigma). Eosin counterstaining was done before mounting.

An image analyzer, Nikon Eclipse TE300 (Nikon, Japan), with 3CCD camera (Dage, USA), was used to analyze PCNA immunohistochemistry. The MetaMorph Imaging System (Universal Imaging Corporation, West Chester, PA) was used for the measurements and calculations. The slides were blinded and analyzed in a random order. The number of positively stained cells was counted by an experienced technician in 6–8 fields along the central gap wound in each slide. The average number of proliferating cells per unit area in each section was then calculated.

*Expression of type III collagen.* Immunohistochemistry of type III collagen phenotype was done using a monoclonal rabbit anti-rat type III collagen antibody (Monosan, cat#PS066) in a working dilution of 1:100. Brief protease digestion with 0.1% trypsin was performed for better exposure of the epitopes recognizing the antibody.

Hematoxylin was used to counterstain the nuclei. The same procedures for image analysis were performed. The level of expression was measured as the percentage of positive signal per unit area of the section. In each section, 6–8 fields along the central gap wound were measured while the average percentage was calculated.

### **Tensile testing**

The patellar-patellar tendon-tibia (PPT) composite was prepared from the hind limb, as in our previous study (Chan et al. 1998a). In brief, the central one third strip of the patellar tendon, including the gap wound, was isolated by removing the medial and lateral one third of the patellar tendon after measuring the width of the patellar tendon with a precision caliper. In the subsequent procedures, the composite was kept moist by saline irrigation to avoid dehydration. The measurement of the patellar tendon, the preloading conditions and the tensile testing set-up have been described elsewhere. The ultimate stress (Nm) of the central one third patellar tendon was obtained by dividing the force at failure (Newton) by its cross-sectional area.

### **Pyridinoline crosslink**

The same specimens were used to measure of the pyridinoline content after tensile testing. The patellar tendon mid-substance was excised sharply from the bony ends and lyophilized in a freeze-drier immediately after the mechanical tests. The dried tissue was hydrolyzed in concentrated hydrochloric acid. The pyridinoline content was measured by a method modified from Eyre's study (Eyre et al. 1984) with a high performance liquid chromatography (HPLC) system (Beckman, Fullerton, California) described by Chan (Chan et al. 1998a). The peak area of the sample was measured against an external pyridinoline standard (Metra Biosystem, Mountain View, CA).

### **Statistics**

The non-parametric Kruskal-Wallis test was used to determine the difference in the number of proliferating cells and the expression of type III collagen in various bFGF groups. A non-parametric post-test trend analysis was done to analyze further the relationship between the dosage of bFGF

and each of the immunohistochemical parameters. Two-way ANOVA was used to detect the effects of bFGF dosages on the ultimate stress and the pyridinoline content, measured at different times after injury. Non-parametric trend analysis of the effects of increasing dosage of bFGF on the mean ultimate stress was also done. The statistical significance was set at  $p < 0.05$ . SPSS 9.0 was used for the statistical analysis.

## **Results**

### **Effect of bFGF on cell proliferation**

Proliferating cells were detected in the wound in tendons injected with bFGF at 1 week (Figure 1). The number of proliferating cells was plotted against the dosage of bFGF (Figure 2). There was a difference in the number of proliferating cells among the various treatment groups (Kruskal Wallis  $p = 0.03$ ). A non-parametric trend analysis also showed that there was a positive association between the number of proliferating cells and the dosage of bFGF ( $p = 0.006$ ). No corresponding change in proliferating cells was found 2 weeks after the injury.

### **Effect of bFGF on type III collagen expression**

The effects of bFGF on type III collagen expression in the patellar tendon were also detected at only 1 week postinjury. The level of expression of type III collagen was greatest in patellar tendons injected with 100 or 1000 ng bFGF, but not in other groups (Figure 3). The level of expression was plotted against the dosage of bFGF (Figure 4). We found no difference in the level of expression of type III collagen among the various treatment groups (Kruskal Wallis  $p = 0.09$ ). However, there was a positive association between the level of expression and the dosage of bFGF using non-parametric trend analysis ( $p = 0.01$ ).

### **Effect of bFGF on ultimate stress**

The mean ultimate stress of the patellar tendons in various treatment groups ranged from 18% to 25% at 1 week and from 33% to 46% at 2 weeks postinjury. There was a difference in mean ultimate stress associated with time (ANOVA  $p < 0.001$ ), but not with the dosage group ( $p =$

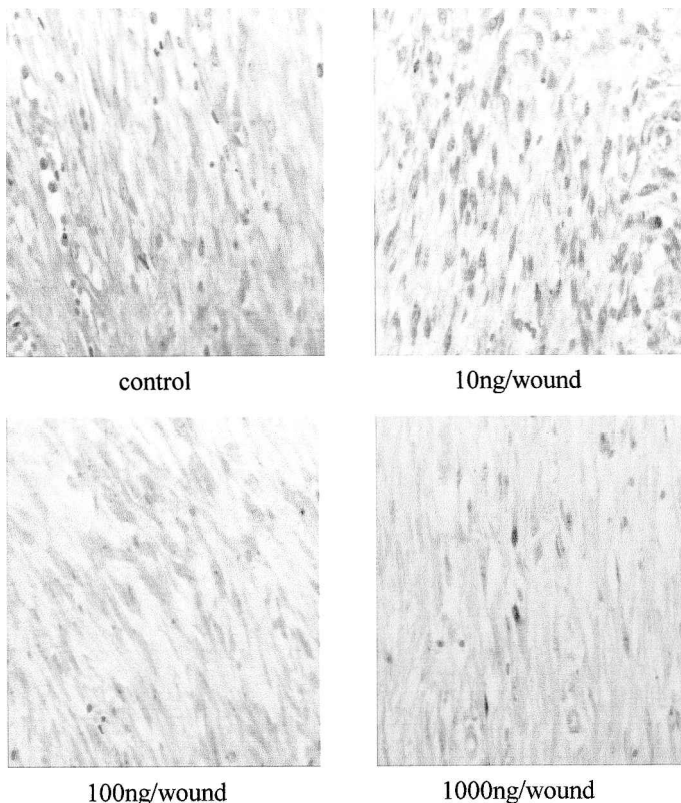


Figure 1. Fibroblast proliferation at 1 week after injury with bFGF. Immunohistochemical staining of proliferating cell nuclei was performed using a monoclonal anti-PCNA antibody in a dilution of 1:10. Eosin was used as the counterstain ( $\times 400$ ).

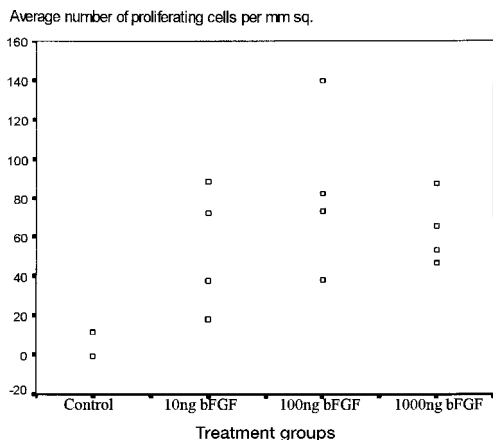


Figure 2. Effect of bFGF on fibroblast proliferation at 1 week postinjury. Significant differences were found between treatment groups (Kruskal Wallis  $p = 0.03$ ) (n 4). The non-parametric test for trend across ordered groups showed an association between the number of proliferating cells and the increasing order of bFGF concentration ( $p = 0.006$ ).

0.8). Trend analysis of the data at 1 week showed no significant trend with increasing bFGF dosage on the mean ultimate stress ( $p > 0.05$ ) (Table).

**Effect of bFGF on pyridinoline crosslink**

At 1 week postinjury, the mean pyridinoline contents of the patellar tendons in various treatment groups ranged from 58% to 70% (Table). At 2 weeks postinjury, the mean contents had all returned to more than 90%. There were no significant differences in mean pyridinoline contents between the various treatment groups (ANOVA  $p = 0.1$ ) although the time factor had a significant effect ( $p = 0.01$ ).

The mean ultimate stress (Nm) and the mean pyridinoline concentration (nmol/ $\mu$ g), with 95% CI of the healing patellar tendon at 1 week postinjury (n 8)

Dosage	Mean ultimate stress % of control (SD)		Mean pyridinoline cont. % of control (SD)	
	1 week	2 weeks	1 week	2 weeks
Control	18 (10)	46 (11)	71 (18)	96 (37)
10 ng bFGF	20 (10)	41 (16)	79 (42)	100 (40)
100 ng bFGF	24 (17)	33 (10)	71 (22)	93 (37)
1000 ng bFGF	25 (11)	38 (10)	59 (40)	101 (22)

**Discussion**

Our findings suggest that bFGF may modulate cell proliferation and type III collagen expression in the early stage of tendon healing.

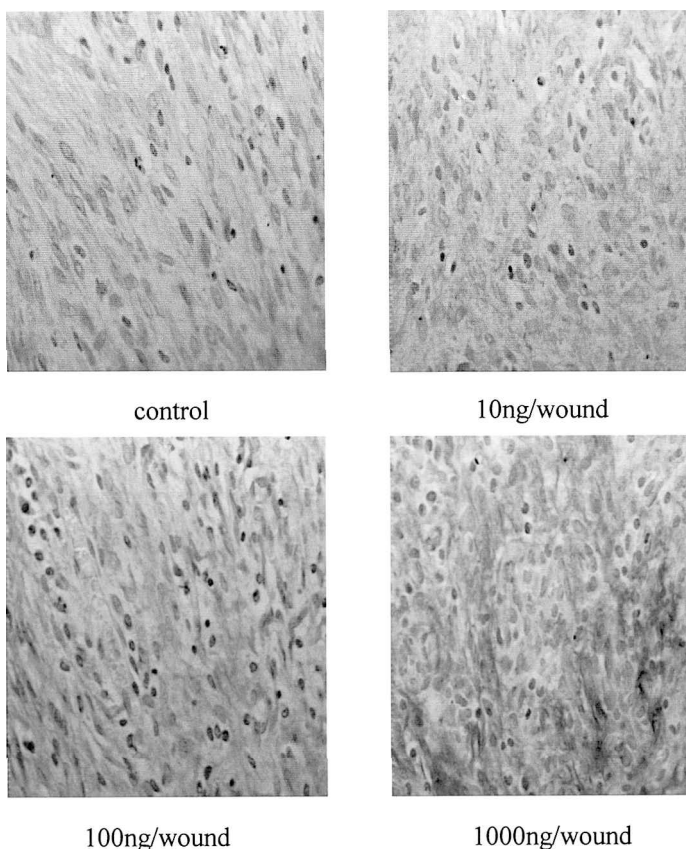


Figure 3. Expression of collagen type III in the gap wound of the healing patellar tendon at 1 week postinjury after bFGF treatment. Immunohistochemical staining using a monoclonal rabbit anti-rat collagen type III antibody in a dilution of 1:100 was performed. Hematoxylin was used as a counterstain ( $\times 400$ ).

Using *in vitro* models, bFGF has been shown to increase proliferation of rat tail tendon-derived fibroblasts (Stein 1985) and rat patellar tendon-derived fibroblasts (Chan et al. 1997). The mitogenic effect of this growth factor suggests that it can modify the tendon-healing process *in vivo* because the provision of newly synthesized cells is crucial to the subsequent synthetic phase. Cell proliferation occurs during the early phase of healing in the first week after injury (Chan 1998). In the present study, the mitogenic effect of bFGF suggests that it may benefit tendon healing by increasing the number of cells for later synthesis of the extracellular matrix.

Type III collagen is highly expressed during development and predominates in the early phase of tendon healing (Birk et al. 1997, Chan 1998). It is

expressed at a low level in adult tendons (Liu et al. 1997). The significance of type III collagen expression during the early phase of tendon healing is not fully understood. Type III collagen is advantageous to tendon healing, since it stabilizes the repair process (Liu et al. 1995). In the present study, there is a positive trend of the level of type III expression with the increase in the dosage of bFGF. This suggests that bFGF may be related to extracellular matrix deposition, a critical step during healing.

We could not show that bFGF supplementation significantly increased the ultimate stress and pyridinoline content, even though a large increase in ultimate stress in the bFGF-treated groups was observed. The mean ultimate stress accounts for about a 38% difference. This indicates the presence of a large type II error. Apart from sample size planning, the ability of the mechanical test to detect such a treatment effect may also contribute to the error. One important factor is the accuracy of the estimation of the

irregular cross-sectional area of the patellar tendon as a rectangular shape in the present model. This may introduce a large variation in the ultimate stress, which is calculated from the cross-sectional area, and may limit the ability of the mechanical test to detect any difference other than a large treatment effect. Therefore, a more accurate estimate of the cross-sectional area, using advanced technology such as laser (Woo et al. 1990), may be necessary to increase the power of the mechanical test.

Another limitation of the present study is the utilization of a simple microsyringe device to inject the growth factor. It has the advantage that the growth factor can be given in a known concentration. On the other hand, the growth factor delivered will be removed from the wound site quickly

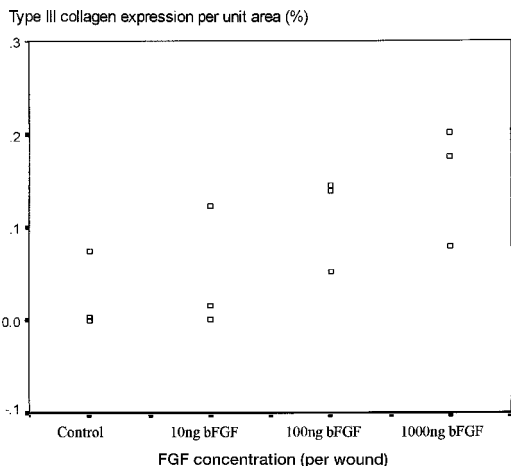


Figure 4. The expression of type III collagen per unit area at 1 week postinjury. No significant differences were found among the various treatment groups (Kruskal Wallis  $p = 0.09$ ) at  $\alpha = 0.05$ . The non-parametric test for trend across ordered groups showed an association between the expression of type III collagen and the increasing order of bFGF concentration ( $p = 0.01$ ).

without sustained release. As a result, the effects of bFGF at a later stage of healing—e.g., 1 month postinjury—could not be measured. Therefore, the effects at a later stage of healing should be studied, using delivery systems able to localize and retain growth factors for a longer period at the wound site.

The present study was supported by the Earmarked Grant CUHK4256/98M, Direct Grant 2040629, the University Grant Committee, the Chinese University of Hong Kong.

Birk D E, Mayne R. Localization of collagen types I, III and V during tendon development. Changes in collagen types I and III are correlated with changes in fibril diameter. *Eur J Cell Biol* 1997; 72 (4): 352-61.

Chan B P. Effects of basic fibroblast growth factor (bFGF) and platelet-derived growth factor isoform B (PDGFBB) in tendon healing. In vitro and in vivo models in rat patellar tendon. Thesis. The Chinese University of Hong Kong, Hong Kong 1998.

Chan B P, Chan K M, Maffulli N, Webb S, Lee K K H. Effect of basic fibroblastic growth factor. An in vitro study of tendon healing. *Clin Orthop* 1997; 342: 239-47.

Chan B P, Fu S C, Qin L, Rolf C, Chan K M. Pyridinoline in relation to ultimate stress of the patellar tendon during healing. An animal study. *J Orthop Res* 1998a; 16: 597-603.

Chan K M, Liu S, Maffulli N. ACL reconstruction with autogenous patellar tendon graft. Donor site consideration and potential for reharvest. In: *Controversies of Orthopaedic Sports Medicine*. (Eds. Chan K M, Fu F, Maffulli N, Rolf C, Kurosaka M, Liu S H). Williams and Wilkins Asia-Pacific Ltd., Hong Kong 1998b: 38-45.

Eyre D R, Koob T J, Van Ness K P. Quantitation of hydroxypyridinium crosslinks in collagen by High-Performance Liquid Chromatography. *Anal Biochem* 1984; 137: 380-8.

Frank C, McDonald D, Wilson J, Eyre D, Shrive N. Rabbit medial collateral ligament scar weakness is associated with decreased collagen pyridinoline crosslink density. *J Orthop Res* 1995; 13 (2): 157-65.

Gabra N, Khiat A, Calabres P. Detection of elevated basic fibroblast growth factor during early hours of in vitro angiogenesis using a fast ELISA immunoassay. *Biochem Biophys Res Commun* 1994; 205 (2): 1423-30.

Hefli F, Stoll T M. Healing of ligaments and tendons. *Orthopade* 1995; 24 (3): 237-45.

Kobayashi D, Kurosaka M, Yoshiya S, Hashimoto J, Saura R, Akamatu T, Mizuno K. The effect of basic fibroblast growth factor on primary healing of the defect in canine anterior cruciate ligament. 41st Annual Meeting, Orthopaedic Research Society, Feb 13-16, 1995. Orlando, Florida.

Lee J, Green M H, Amiel D. Synergistic effect of growth factors on cell outgrowth from explants of rabbit anterior cruciate and medial collateral ligaments. *J Orthop Res* 1995; 13 (3): 435-41.

Lee J, Harwood F L, Akeson W H, Amiel D. Growth factor expression in healing rabbit medial collateral and anterior cruciate ligaments. *Iowa Orthop J* 1998; 18: 19-25.

Liu S H, Yang R S, al-Shaikh R, Lane J M. Collagen in tendon, ligament, and bone healing. A current review. *Clin Orthop* 1995; 318: 265-78.

Liu S H, Panossian V, al-Shaikh R, Tomin E, Shepherd E, Finerman G A, Lane J M. Morphology and matrix composition during early tendon to bone healing. *Clin Orthop* 1997; 339: 253-60.

Ogata K, Kurki P, Celis J, Nakamura R, Tan E M. Monoclonal antibodies to a nuclear protein (PCNA/cyclin) associated with DNA replication. *Exp Cell Res* 1987; 168 (2): 457-86.

Panossian V, Liu S H, Lane J M, Finerman G A. Fibroblast growth factor and epidermal growth factor receptors in ligament healing. *Clin Orthop* 1997; 342: 173-80.

Stein L E. Effects of serum, fibroblast growth factor, and platelet-derived growth factor on explants of rat tail tendon: a morphological study. *Acta Anat (Basel)* 1985; 123 (4): 247-52.

Woo S L Y, Danto M I, Ohland K J, Lee T Q, Newton P O J. The use of a laser micrometer system to determine the cross-sectional shape and area of ligaments: a comparative study with two existing methods. *Biomech Eng* 1990; 112 (4): 426-31.

Woo S L Y, Niyibizi C, Matyas J, Kavalkovich K, Green C W, Fox R J. Medial collateral knee ligament healing - Combined medial collateral and anterior cruciate ligament injuries studied in rabbits. *Acta Orthop Scand* 1997; 68 (2): 142-8.